Published in final edited form as:

Macromol Biosci. 2010 July 7; 10(7): 703–713. doi:10.1002/mabi.201000069.

Analysis of PKR Activation Using Analytical Ultracentrifugation

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Abstract

PKR is a central component of the interferon antiviral defense pathway. Upon binding to dsRNA, PKR undergoes autophosphorylation reactions that activate the kinase, resulting in the inhibition of protein synthesis in virally-infected cells. We have used analytical ultracentrifugation and related biophysical methods to quantitatively characterize the stoichiometries, affinities and free energy couplings that govern the assembly of the macromolecular complexes in the PKR activation pathway. These studies demonstrate that PKR dimerization play a key role in enzymatic activation and support a model where the role of dsRNA is to bring two or more PKR monomers in close proximity to enhance dimerization.

Keywords

centrifugation; proteins; RNA; sedimentation equilibrium; sedimentation velocity

Introduction

Protein kinase R (PKR) is an interferon-induced kinase that plays a key role in the innate immunity response to viral infection.[1,2] PKR is induced in a latent form that is activated by binding dsRNA to undergo autophosphorylation and subsequently phosphorylate cellular substrates. The most well characterized substrate of PKR is the alpha subunit of eukaryotic initiation factor 2 (eIF2 α). Phosphorylation of eIF2 α at serine 51 blocks the recycling of eIF2 between GTP- and GDP-bound states, thereby inhibiting initiation of protein synthesis. Thus, production of dsRNA during viral infection[3] results in PKR activation and inhibition of viral and host protein synthesis.

PKR contains an N-terminal dsRNA binding domain (dsRBD), consisting of two tandem copies of the dsRNA binding motif (dsRBM),[4] and a C-terminal kinase, with a ~90 amino acid linker lying between these domains (Figure 1). Each of the dsRBMs has the typical $\alpha\beta\beta\alpha$ fold[5] with a short, unstructured region between the folded regions. Crystallographic and NMR studies indicate that the dsRBM binds to one face of the dsRNA helix, spanning ~16 bp.[4] The interaction is not sequence-specific; however, there are some reports of specific binding to RNA secondary structural features.[6-9] In the crystal structure of a complex of the PKR kinase domain with an N-terminal fragment of eIF2 α , the catalytic domain adopts a bilobal structure typical of protein kinases.[10] The kinase forms a dimer mediated by interactions between residues within the C-terminal lobes (Figure 1). Helix α C in the N-lobe of the kinase domain forms part of the dimer interface[10] and conformational changes in this helix have been demonstrated to modulate activity in other protein kinases, [11] suggesting that dimerization may allosterically modulate PKR.

The regulation of PKR by dsRNA and other effectors has fascinated researchers for over 30 years. However, we lack a detailed molecular picture of how binding of dsRNA to the N-terminal dsRBD results in kinase activation. Evidence has accumulated in support of an autoinhibition model in which the latent form of PKR exists in a closed conformation where the dsRBD interacts with the kinase and blocks substrate binding.[12] In this model, dsRNA activates PKR by binding to the dsRBD, thereby releasing it from the kinase. Other models emphasize the role of dimerization in PKR activation.[1,12-14] Fusion of a heterologous dimerization domain with the PKR kinase domain enhances autophosphorylation.[15,16] A defining feature of PKR is the “bell-shaped” curve for activation where low concentrations of dsRNA activate but higher concentrations are inhibitory.[17,18] These results can be rationalized in a model where low concentrations of dsRNA favor assembly of multiple proteins – possibly assembling as dimers – on a single dsRNA whereas higher dsRNA concentrations dilute PKR monomers onto separate molecules of dsRNA.[19] Hybrid autoinhibition/dimerization models have also been proposed where dsRNA binding induces a conformation change in PKR that leads to protein dimerization and activation.[14,20]

PKR is regulated by a network of protein-protein and protein-RNA interactions and the broad objective of our research program has been to quantitatively characterize the stoichiometries, affinities and free energy couplings that govern the assembly of the macromolecular complexes in the PKR activation pathway. In order to obtain reliable results it is critical that macromolecular interactions are measured at equilibrium using rigorous biophysical methods. Nonspecific protein-nucleic acid interactions are particularly labile and can be perturbed by the separation of free and bound forms that may occur when using conventional gel-shift and filter binding assays.[21] We have found analytical ultracentrifugation to be especially useful and below we highlight several applications of sedimentation equilibrium and sedimentation velocity in our ongoing studies of PKR self-association, domain interactions and binding to dsRNA and complex natural RNAs.

PKR Self-Association

A large body of early *in vitro* and *in vivo* data implied that PKR is capable of dimerizing, but quantitative methods are required to define the relationships between dimerization and enzymatic activation. Initial evidence for dimerization came from studies indicating that the activation rate displays a second-order dependence on PKR concentration.[19] Several studies have demonstrated RNA-independent dimerization of PKR[22-27] or the isolated dsRBD[23,28-30] using a variety of assays. Dimerization is believed to be mediated by interactions involving the dsRBD[23,26,29,31] and a second dimerization motif lying between residues 244-296.[30]

In sedimentation velocity studies of purified full length PKR at 0.7 mg /ml, a single species is detected at $s = 3.35$ S in $c(s)$ distributions (Figure 2A).[32] The apparent sedimentation coefficient increases slightly with protein concentration from ~ 3.3 to ~ 3.4 S over a range of 0.1-1.6 mg /ml. The slight increase in s with concentration indicates that PKR undergoes rapidly reversible and low affinity self-association. Sedimentation coefficients were extrapolated to zero protein concentration and buffer corrected to calculate a monomer sedimentation coefficient of $s_{20,w}^0 = 3.50$ S and a frictional ratio of $f/f_0 = 1.58$. Thus, PKR is highly asymmetric. It is not convenient to characterize weak self-association by sedimentation velocity because of the marked contribution of hydrodynamic nonideality at the high protein concentrations required to substantially populate the dimeric species complicates the analysis. Therefore, we employed sedimentation equilibrium measurements (Figure 2B).[32] A good global fit to sedimentation equilibrium is obtained for a monomer-dimer model, with $K_d = 446$ (340, 580) μ M. Although dimerization of unactivated PKR is quite weak and thus would not occur at concentrations attained in the cell, it has mechanistic

significance. PKR undergoes dsRNA-independent autophosphorylation upon incubation at higher protein concentrations in the presence of ATP.[32] These data indicate that dimerization is sufficient to activate PKR. Remarkably, PKR dimer stability is enhanced[27] by ~500-fold upon autophosphorylation.[32] PKR autophosphorylation reduces the binding affinity for dsRNA.[14, 33, 34] These observations led us to propose a chain reaction model for PKR activation where dimerization of latent enzyme followed by intermolecular phosphorylation serves as the initiation step.[13, 32] Subsequent chain propagation steps likely involve phosphorylation of latent PKR monomers by newly activated enzyme, possibly via formation of mixed heterodimers of phosphorylated and latent enzyme. However, we have recently detected an additional phosphorylated form of PKR where K_d is the same as latent enzyme, suggesting that both monomeric and dimeric forms of phosphorylated PKR may participate in the interferon antiviral pathway.[34]

It has been reported that the dsRBD comprised of two tandem dsRBMs as well as the isolated dsRBMs self associate.[23,28-30] In sedimentation velocity measurements dsRBD exhibits a single feature at $s = 1.8$ S (Figure 2C). The sedimentation coefficients decrease slightly with increasing protein concentration due to slight hydrodynamic nonideality; however, there is no evidence for concentration dependent self-association and sedimentation equilibrium data fit to a single ideal species model with molecular weight corresponding to monomer.[35] Similarly, dsRBM1 is also monomeric over a broad concentration range.[35] Thus, analytical ultracentrifugation clearly demonstrates that the dsRBD does not constitute a dimerization interface. These results are supported by NMR measurements.[36] Extensive mutagenesis data[37] are consistent with a dimerization interface located on the N-lobe of the kinase catalytic domain as observed in the crystal structure.[10]

Interaction of PKR with dsRNA

PKR interactions with dsRNA have been investigated using a wide variety of experimental approaches. Early studies have employed nitrocellulose filter retention assays,[19,38,39] gel mobility shift,[18,40-42] crosslinking,[39,43] nuclease protection,[44] and affinity cleavage.[8] Later studies have employed equilibrium biophysical methods, including fluorescence,[25,45] small angle neutron scattering,[25] circular dichroism,[21] isothermal titration calorimetry[9,14,46-49] and dynamic light scattering.[14] In our studies we have emphasized the use of equilibrium[21,35,45] and velocity[34,45,50] analytical ultracentrifugation. We find that these approaches are particularly powerful in the context of nonspecific protein-nucleic acid interactions where it is necessary to resolve the energetics of sequential binding events. These methods are also capable of differentiating among alternative assembly mechanisms.

A useful first step in studies of nonspecific protein-nucleic acid binding is to define the stoichiometry of association prior to more detailed studies required to measure equilibrium constants. It is convenient to perform these studies by sedimentation equilibrium measurements at moderate rotor speeds using short solution columns (< 3 mm) such that relatively shallow concentration gradients are achieved.[21,51] Under these conditions, the radial dependence of the solution composition is small, and we can treat the sample as being characterized by an average molecular weight.[52] When using absorption optics, a “signal average” molecular weight is obtained where the contribution of each species in solution is weighted according to its molar concentration and extinction coefficient. We typically monitor the gradients at 260 nm where the absorbance of the nucleic acid predominates. The buoyant, signal-average molecular weight at 260 nm (M^*_{260}) is given by

$$M_{260}^* = \frac{\epsilon_{P,260} [P] M_P^* + \sum_{i=0}^S (\epsilon_{R,260} + i \epsilon_{P,260}) [RP_i] (M_R^* + i M_P^*)}{\epsilon_{P,260} [P] + \sum_{i=0}^S (\epsilon_{R,260} + i \epsilon_{P,260}) [RP_i]} \quad (1)$$

where $\epsilon_{P,260}$ is the extinction coefficient of the protein monomer P at 260 nm, [P] is the molar concentration of P, M_P^* is the buoyant mass of P, S is the maximal number of protein monomers that bind to the nucleic acid R, and $[RP_i]$ is the molar concentration of the complex contain one molecule of R and i molecules of P. The buoyant mass is defined by

$$M^* = M(1 - \bar{v}\rho) \quad (2)$$

where M is the mass (P or R), $\bar{v}$ is the partial specific volume and ρ is the solvent density. Often, the absorption contribution of the nucleic acid is much greater than the protein ($\epsilon_{R,260} \gg \epsilon_{P,260}$) and the saturating stoichiometry of binding can be assessed by simply measuring the increase in the buoyant mass of the oligonucleotide in the presence of a saturating concentration of protein ligand,

$$S = \frac{M_{260}^*(\text{max}) - M_{R,260}^*}{M_{P,260}^*} \quad (3)$$

where $M_{260}^*(\text{max})$ is the maximal value of M_{260}^* in the presence of saturating protein.

In our initial studies of the interaction of the dsRBD of PKR with dsRNAs we measured a binding stoichiometry of three dsRBD/ 20bp dsRNA.[21] This stoichiometry implies an unreasonably small site size of 6-7 bp that is not compatible with structures of dsRBM-RNA complexes. This discrepancy led us to propose an overlapping ligand binding model where PKR binding occurs on multiple faces of the dsRNA duplex, and bound proteins overlap along the helical axis. An analogous model where bound proteins overlap along the nucleic acid contour has been proposed for the interaction of O6-alkylguanine-DNA alkyltransferase with single stranded DNA.[53,54] The overlapping ligand binding model accounts for the stoichiometries for binding of the PKR dsRBD to dsRNA oligomers ranging from 15 to 30 bp with a site size (N) of 12 bp and a minimal overlap along the helical axis (Δ) of 4 bp (Figure 3A). Similar behavior is observed for a construct containing only dsRBM1.[35] However, in the case of dsRBD, longer dsRNA sequences show lower stoichiometries than expected, indicating a change in binding mode. NMR analysis support a model where dsRBM1 plays the dominant role in binding short dsRNA sequences and dsRBM2 makes additional interactions with the longer sequences, thereby increasing the binding site size. [35]

Sedimentation equilibrium is also a powerful method to characterize the affinities of protein-nucleic acid interactions. Owing to the greater number of adjustable parameters, analysis of heterogeneous interactions is considerably more difficult than analysis of self-associating systems.[55-59] In the case of protein-nucleic acid interactions, where the two reactants have different absorption maxima, it is extremely useful to collect data at multiple wavelengths to constrain the fitting process by accurately defining the concentration of each component.[60-63] Using global analysis of sedimentation equilibrium data obtained at 230,

260 and 280 nm, we defined the energetics for binding of dsRBD to a 20 bp RNA (Figure 3B).[21] The data fit well to model in which three dsRBD bind sequentially. The first dsRBD binds strongly ($K_d = 11$ nM) and the affinity decreases with successive ligands with K_d values of 210 nM and 780 nM for the second and third dsRBD, respectively. A decrease in binding affinity with successive ligand binding events is predicted from statistical effects. The stepwise macroscopic constants are equal to the product of the intrinsic constant and a statistical factor determined by the number of microscopic states constituting each level of saturation of the nucleic acid with ligand. The combinatorial expression for the number microscopic configurations for lattice containing x bound ligands is

$$C_x = \frac{(M - N - (x - 1)\Delta + x)!}{(M - N - (x - 1)\Delta)!x!} \quad (4)$$

where M is the length of the lattice, N is the ligand site size and Δ is the minimal overlap. [21] In the context of the overlapping ligand model, the sedimentation data fit almost equally well (RMS = 0.00448) to a model where the relative values of the binding constants are constrained to the expected statistical factors predicted for $N=12$ and $\Delta=4$. These fits reveal a value of the intrinsic binding constant of $K_d = 83$ -110 nM. The relative dissociation constants for the sequential binding of dsRBM1 to the same 20 bp dsRNA are consistent with the same statistical model. However, the absence of the second dsRBM1 reduces the intrinsic affinity by a factor of about 30-fold.[35]

Detailed analysis of the binding stoichiometries and affinities of full length PKR for dsRNA oligonucleotides has proven more challenging due to the tendency of the complexes to precipitate. Although the solubility of the PKR-dsRNA complexes is improved at higher salt, the solubility decreases with increasing length of dsRNA. Sedimentation velocity experiments are less affected by slow precipitation and have been often been used to characterize associating systems that are not sufficiently stable for equilibrium analysis. Typically, weight average sedimentation coefficients are obtained by integrating differential sedimentation coefficient distributions and are fit to alternative association models.[64] More recently, it has become feasibly to directly fit sedimentation velocity to Lamm equation solutions incorporating reversible chemical reactions to obtain association constants for self-associating[65] and heteroassociation[66,67] systems.

We recently applied these new sedimentation velocity analysis methods to characterize the affinity of full length PKR binding to dsRNA.[45] These studies were performed in buffers containing 200 mM NaCl to enhance solubility of the protein-RNA complexes. Two dsRNA oligoribonucleotides were chosen: 20 bp, which does not activate PKR and 30 bp, which is the minimal dsRNA capable of activating.[18,68] Initially, the data were analyzed using the time derivative method[69] to define the binding stoichiometries. Figure 4A show the normalized $g(s^*)$ distributions for a titration of the 20 bp dsRNA with increasing PKR. Upon addition of PKR, the 2.5 S peak associated with the free dsRNA decreases in amplitude and a new feature develops at higher S , consistent with formation of a dsRNA-protein complex. The peak maximum of the new feature shifts continuously with increasing concentration of PKR, indicating rapid exchange on the timescale of the sedimentation velocity experiment. The magnitude of the shift is consistent with binding of a single PKR monomer. Model – dependent analysis was performed by subtracting the sedimentation velocity traces in pairs for each sample to remove systematic noise and fitting the resulting difference curves to association models using the program SEDANAL.[67] Figure 4B shows a fit of the same data depicted in Figure 4A (plus an additional 6:1 ratio sample) to a simple 1:1 binding model assuming a rapid equilibrium. The data fit well to this model with randomly

distributed residuals. The best fit K_d of 859 nM (Table 1) is relatively weak. For a 30 bp dsRNA two PKR monomers sequentially bind. The dissociation constant for binding the first PKR is about 10-fold lower than for the 20 bp dsRNA, indicating that PKR binds more strongly to the longer dsRNA (Table 1). A decrease in K_{d1} is qualitatively consistent with the expected statistical effects in the overlapping ligand binding model. In parallel enzyme assays performed under the same conditions as the sedimentation velocity experiments, 30 bp is the smallest dsRNA that elicits autophosphorylation activity.[45] Thus, the ability of dsRNAs to function as PKR activators is correlated with binding of two or more PKR monomers. These results support an activation mechanism where the role of the dsRNA is to bring two or more PKR monomers in close proximity to enhance dimerization via the kinase domain (Figure 5A).

PKR binding to the short 20 bp dsRNA could also be analyzed at lower salt concentration to facilitate comparison with the studies of dsRBD –dsRNA interactions performed in 75 mM NaCl.[35]

Interesting, we observed that two PKR monomers bind to the shorter dsRNA at this lower salt concentration. The value of K_{d1} of 15 nM is about 60-fold lower at 75 mM than at 200 mM NaCl, indicating a very strong salt dependence. The binding affinities of PKR and dsRBD are similar, consistent with a similar binding interface, but the presence of the kinase domain and linker region results in steric hindrance which prevents a third PKR from binding and increases the minimal overlap. These observations appear to contradict the model depicted Figure 5A: if two PKR monomers are capable of binding to the short 20 bp dsRNA, why is a minimum of 30 bp required for activation? The likely answer lies in avidity effects. Figure 6 shows a simulation of the fraction of PKR present in active RNA-protein complexes containing two bound PKRs as function of RNA concentration over a range of binding affinities. As is observed in activation assays,[18, 45, 68] the simulated curves exhibit a maximum at intermediate dsRNA concentrations corresponding to the stoichiometric equivalence point where $[dsRNA] = [PKR]/2$. Significantly, the peak amplitudes decrease strongly with reduced binding affinity. Thus, the 20 bp RNA fails to activate because it binds too weakly to PKR to populate a significant fraction of the active dimer form. This model also explains why longer RNAs are more potent activators: the higher binding affinities result in greater population of active complexes containing multiple bound PKRs.

In vivo, PKR is regulated by large RNAs with complex secondary and tertiary structures. However, the structural features that distinguish activators of PKR from those that fail to activate are not yet well understood. Adenovirus produces a large amount of the highly structured VAI RNA which binds to PKR and serve as an in vivo inhibitor of activation by dsRNA.[70] In contrast, the interferon- γ mRNA[71] and the 3'-UTR regions of several cytoskeletal muscle mRNAs[72] function as activators. The HIV transactivation-responsive region (TAR) RNA consists of a stem-loop interrupted by three bulges (Figure 7A) and has served as a model system to investigate regulation of PKR by complex RNAs. Although this interaction has been extensively studied,[9,14,73-80] the PKR binding and activation properties of TAR remain controversial. The TAR RNA hairpin is known to dimerize,[81] which complicates analysis of protein-RNA interactions. Therefore, we prepared and isolated homogeneous TAR monomer and dimer and compared their PKR binding and activation properties.[50] Enzymatic probing experiments were used to verify the secondary structure of the TAR monomer and to define the TAR dimer secondary structure (Figure 7A). The PKR binding stoichiometries and affinities for TAR monomer and dimer were determined by sedimentation velocity experiments.[50] Figure 7B shows the normalized $g(s^*)$ distributions for PKR binding to the TAR monomer and dimer. The monotonic increase in the peak position with increasing PKR concentration is consistent with PKR

binding in rapid exchange on the timescale of the sedimentation velocity experiment. The magnitude of the increase in the sedimentation coefficient is consistent with a single PKR binding to TAR monomer. Global analysis using SEDANAL reveals that K_d is similar to that observed for the simple 20 bp dsRNA (Table 2). PKR binds with similar affinity to two TAR monomer mutants (A34U and U37A) that have slightly different secondary structures. However, PKR binds about four-fold more strongly to a self-complementary TAR variant (scTAR) in which all of the bulges were removed and three mutations were incorporated into the loop to make it self-complementary and to facilitate formation of a defect-free helix (Figure 7A).

Titration of TAR dimer with PKR produces a more complex pattern in the overlay of the $g(s^*)$ distributions (Figure 7B). A small shift from about $s=4.3$ S to 5.1 S occurs upon addition of 1-2 equivalents of PKR and a more dramatic upward shift occurs at higher PKR concentrations. This pattern is consistent with sequential binding of two PKRs to the TAR dimer. Global analysis confirms that two PKR bind both the wild-type and A34U:U37A TAR dimers with similar dissociation constants (Table 2). We also considered an alternative binding model that has been proposed for activation of PKR activation TAR.[14, 47] In this case, binding of a molecule of PKR to TAR induces dimerization of a 1:1 PKR-TAR, resulting in formation of a $(\text{PKR-TAR})_2$ complex. This model is inconsistent with the observed 1:1 binding stoichiometries for the monomeric TAR constructs and also does not fit the sedimentation velocity data for PKR binding to dimeric TAR constructs. For example, the fit of the A34U:U37A dimer data to this alternative model gives higher RMS deviations relative to the sequential binding model and unreasonable parameters (Table 2).

Analysis of PKR activation by wild-type TAR monomer and dimer reveals that the former is a weak activator that only slightly enhances PKR autophosphorylation relative to background levels (Figure 7C). The dimer activates 23-fold more effectively than monomer and approaches the potency of a 79 bp dsRNA standard. Similarly, the A34U and U37A TAR monomers were not effective PKR activators whereas the A34U:U37A dimer showed appreciable activation. The self-complementary TAR dimer is a particularly potent PKR activator and gave nearly a 100-fold increase in activation as compared to scTAR monomer (data not shown).

Our analysis of PKR interactions with the TAR hairpin is fully consistent with the dimerization model for activation of PKR (Figure 5B). The TAR monomer does not activate because it binds only a single PKR. However, TAR dimerization effectively doubles the length of dsRNA allowing it to bind two PKRs and support functional dimerization. The secondary structure defects in the TAR RNA stem function as antideterminants to PKR binding and activation. Thus, PKR does not specifically recognize the bulges present in TAR as previously reported[9] and prefers to bind to a regular dsRNA lattice. In conjunction with the secondary structures depicted in figure 7A, the model also explains the relative affinities for PKR binding to the bulged monomers and dimers. There is only a slight decrease in K_d from 600-900 nM for the bulged monomers to 300-400 nM for the dimers and there is about a five-fold increase in K_d for the second PKR binding to the dimer relative to the first (Table 2). Assuming a simple model of a pair of noninteracting, identical sites in the dimer, these relative affinities agree with the expected statistical effects on macroscopic binding constants. The binding affinity for the dimer RNA is predicted to be twice that for the monomer and the second PKR is expected to bind 4-fold weaker than the first to the dimer. Larger statistical factors are observed for PKR binding to regular dsRNAs because of the larger number of microstates accessible for nonspecific binding to an uninterrupted finite lattice.[21, 35]

Conclusion

Analysis of PKR by analytical ultracentrifugation has helped to define the self-association reactions of this enzyme and the mechanism of PKR assembly on simple dsRNA lattices as well as on a more complex natural RNA hairpin. These studies indicate that PKR dimerization plays a key role in the activation process and support a mechanism where the role of RNA activators is to provide a scaffold for binding multiple PKR monomers, leading to dimerization via the kinase domain. Analysis of PKR –RNA interactions by sedimentation velocity has proven particularly valuable in defining the stepwise assembly of PKR monomers on the RNA lattice. We have found that the model editor in the program SEDANAL[67] is very useful to discriminate among alternative assembly models. However, our ability to study PKR binding to longer RNAs has been limited by poor solubility of the complexes and by the detection limits imposed by the absorbance optics. We are currently using fluorescence detection and labeled RNAs to perform sedimentation velocity experiments at lower concentrations to avoid sample precipitation and to measure higher affinity interactions.

Acknowledgments

This research was supported by grant AI-53615 from the National Institutes of Health. I thank the students, postdocs, research associates and collaborators for their contributions to the work I have highlighted in this review.

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Biography

James Cole received his Ph.D. in Biophysical Chemistry at the University of California, Berkeley in 1987. After postdoctoral work at Stanford University, he joined Merck Research Laboratories. In 2001 he moved to the University of Connecticut where he is now Associate Professor in the Department of Molecular and Cell Biology and Department of Chemistry. His research interests include the analysis of macromolecular interactions by analytical ultracentrifugation and related biophysical techniques and the innate immunity response to viral infection.

Figure 1.

PKR domain organization and structure. The N-terminal regulatory domain is comprised of two dsRNA binding motifs, dsRBM1 and dsRBM2 connected by an unstructured linker. Each of these motifs adopts the canonical $\alpha\beta\beta\alpha$ fold in the NMR structure of dsRNA binding domain (PDB: 1QU6). In the crystal structure of a complex of the PKR kinase domain and eIF2 α (PDB: 2A1A), the kinase domain has the typical bilobal structure observed in other eukaryotic protein kinases and dimerizes via the N-terminal lobe (note, the substrate eIF2 α was omitted from the figure for clarity)

Figure 2.

Characterization of PKR by analytical ultracentrifugation. A) Sedimentation velocity analysis.[32] Continuous sedimentation coefficient distribution analysis of PKR. Conditions: sample concentration, 0.7 mg/ml; rotor speed, 50,000 RPM; temperature, 20°C; interference optics. B) Sedimentation equilibrium analysis. [32] Conditions: sample concentrations, 0.3, 0.6, 1.0, 1.5 and 2.0 mg/ml; rotor speeds, 18,000 (●) and 24,000 (□) RPM; temperature, 20°C; interference optics. The dataset was globally fit to monomer-dimer model yielding $K_d = 446 \mu\text{M}$. The solid lines show the best-fit model and the inset shows the residuals. C) Sedimentation velocity analysis of PKR dsRNA binding domain constructs.[35] Continuous sedimentation coefficient distribution analysis of dsRBD (—) and dsRBM 1 (.....).

Conditions: sample concentration, 0.5 mg/ml (dsRBD) and 0.6 mg/ml (dsRBM 1); Rotor speed, 50,000 RPM; temperature, 20°C; interference optics.

Figure 3.

Analysis of the PKR dsRNA binding domain binding to dsRNA. A) Dependence of dsRBD binding stoichiometry on dsRNA length.[35] Stoichiometries were measured by sedimentation equilibrium (black symbols) and CD spectroscopy (red symbols). The dashed line represents the prediction from the overlapping ligand model with $N=12$ and $\Delta=4$. B) Multiwavelength sedimentation equilibrium analysis of dsRBD binding to a 20 mer dsRNA. [21] Conditions: rotor speed, 23,000 RPM; temperature, 20°C; RNA concentration, 0.5 μ M and protein concentrations of 0.5 μ M (black), 1 μ M (red) and 2 μ M (blue) in 75 mM NaCl, 20 mM HEPES, 5 mM MgCl₂, 0.1 mM EDTA, pH 7.5. Detection wavelengths are: 230 nm (○), 260 nm (□) and 280 nm (△). Solid lines are a global fit of the data to an unconstrained

model of three ligands binding to the 20 mer RNA yielding $K_{d1} = 11$ nM, $K_{d2} = 210$ nM and $K_{d3} = 780$ nM and an RMSD = 0.00437 OD. Inset: residuals. Traces have been vertically offset for clarity.

Figure 4.

Sedimentation velocity analysis of PKR binding to a 20 bp dsRNA.[45] A) Normalized $g(s^*)$ distributions of 1 μ M dsRNA (black), dsRNA + 0.5 eq. PKR (blue), dsRNA + 1 eq. PKR (red), dsRNA + 2 eq. PKR (green). The distributions are normalized by area. B) Global analysis of sedimentation velocity difference curves. The data were subtracted in pairs and four data sets at the indicated ratios of PKR: dsRNA were fit to 1:1 binding stoichiometry model. The top panels show the data (points) and fit (solid lines) and the bottom panels show the residuals (points). The best fit parameters are shown in Table 1. For clarity, only every 4th difference curve is shown. Conditions: rotor speed, 50,000 RPM; temperature, 20°C; detection: absorption optics at 260 nm; scan interval, 6 minutes.

Figure 5.

PKR activation models. A) Dimerization model for PKR activation by dsRNA. Binding to dsRNA induces PKR dimerization via the kinase domain, resulting in enzymatic activation. B) Model for PKR activation by TAR RNA dimer. The monomer RNA hairpin (~ 23 bp) binds a PKR monomer and fails to activate. In contrast, two PKR monomers can bind to the TAR dimer RNA (~ 46 bp) which leads to PKR dimerization and activation.

Figure 6. Simulation of PKR activation by dsRNA. Fraction of PKR present in the RNA-PKR₂ species was simulated using a model where two PKR monomers sequentially bind a dsRNA. The binding equilibria were numerically simulated using IGOR Pro (Wavemetrics Inc.) for [PKR] = 200 nM. A ratio of $K_{d2}/K_{d1} = 10$ was assumed with the following values of K_{d1} : 1 nM (purple), 3 nM (tan), 10 nM (black), 30 nM (green), 100 nM (blue), 300 nM (red), 1 μ M (aqua).

Figure 7.

Sedimentation velocity analysis of PKR binding to HIV TAR RNA.[50] A) Secondary structures for TAR variants. Wild-type TAR monomer (wtTAR, left), with changes (red) used to make the single mutants A34U and U37A, and the double mutant A34U:U37A. Wild-type TAR dimer secondary structure (middle) was determined by enzymatic probing experiments.[50] Self-complementary TAR (scTAR, right). This variant deletes the three bulges present in TAR, while retaining the two GU wobbles at positions 6 and 9; in addition, it is mutated on the 3'-side of its loop to add perfect self-complementarity. B) Sedimentation velocity analysis of PKR binding to wtTAR. Top: plot of normalized $g(s^*)$ distributions for 1 μ M wtTAR monomer (red), 1 μ M wtTAR monomer plus 0.5 μ M PKR (green), 1 μ M PKR (blue) or 4 μ M PKR (black). Conditions: temperature, 20°C; rotor speed, 40,000 RPM; detection: absorption optics at 260 nm. Bottom: plot of normalized $g(s^*)$ distributions for 0.25 μ M wtTAR dimer (red), 0.25 μ M wtTAR dimer plus 0.25 μ M PKR (green), 0.5 μ M

PKR (blue) or 1.5 μ M PKR (black). Conditions: temperature, 20°C; rotor speed, 40,000 RPM; detection: absorption optics at 260 nm. C) PKR activation assays carried out using native gel-purified wtTAR monomer and dimer. Autophosphorylation reactions were performed at various concentrations of RNA in the presence of 5 μ M PKR and were analyzed by SDS-PAGE.

Table 1 a

RNA	PKR ^b	[NaCl] mM	Model ^c	K _{d1}	K _{d2}	s(RP) ^d	s(RP ₂) ^d	RMS ^e
20 bp	wt ^b	200	1	859 (746, 987) nM		5.14 (5.03, 5.25)	-	0.00961
30 bp	wt	200	2	87 (49, 148) nM	970 (690, 1450) nM	4.85 (4.68, 5.09)	6.88 (6.70, 7.13)	0.00772
30 bp	R262D	200	2	93 (51, 157) nM	1070 (620, 1730) nM	4.94 (4.77, 5.14)	7.32 (6.97, 7.76)	0.00887
20 bp	wt	75	2	15.0 (8.5, 25.3) nM	228 (148, 351) nM	4.48 (4.40, 4.57)	6.60 (6.46, 6.78)	0.00967
20 bp	R262D	75	2	44.3 (20.3, 125) nM	386 (204, 1021) nM	4.41 (4.19, 4.84)	6.78 (6.52, 7.38)	0.00960

^aParameters were obtained by global nonlinear least squares analysis of sedimentation velocity experiments. The values in parentheses represent the 95% joint confidence intervals.

^bWT is wild type, R262D is a nondimerizing PKR mutant.

^cModel 1 is R+P=RP; Model 2 is R+P=RP, RP+P=RP₂

^dUncorrected sedimentation coefficient (Svedbergs).

^eRoot mean square deviation of the fit in absorbance units.

^fFor this model, the dimeric species is R₂P₂ not RP₂.

Table 2a

RNA ^b	Model ^c	K _{d1} (nM)	K _{d2} (nM)	s(RP) ^d	s (RP) ^d	RMS ^e
Monomers						
wtTAR	1	667 (589,752)	-	5.10 (5.04,5.16)	-	0.00699
A34U	1	752 (641,900)	-	5.01 (4.93,5.09)	-	0.00729
U37A	1	926 (746,1150)	-	4.92 (4.84,5.00)	-	0.00637
scTAR	1	188 (148, 236)	-	4.97 (4.88,5.07)	-	0.00595
Dimers						
wtTAR	2	404 (257, 648)	2760 (1170, 4540)	5.51 (5.02,5.95)	7.70 (6.97,8.60)	0.00794
A34U:U37A	2	331 (226,490)	1470 (910,2430)	5.29 (5.13,5.47)	7.18 (6.86,7.66)	0.00581
A34U:U37A	3	980 nM	2.58×10 ⁶	6.75	11.85 ^f	0.00785

^aParameters were obtained by global nonlinear least squares analysis of sedimentation velocity experiments. The values in parentheses represent the 95% joint confidence intervals. Experiments were performed in a buffer containing 200 mM NaCl.

^bwtTAR is wild type TAR, U37A and U37A are TAR mutants, and scTAR is a TAR mutant where the bulges have deleted and the loop residues have been mutated to produce a form capable of forming a fully self-complementary dimer.

^cModel 1 is R+P=RP; Model 2 is R+P=RP, RP+P=RP₂; Model 3 is R+P=RP; 2RP=(RP)₂.

^dUncorrected sedimentation coefficient (Svedbergs).

^eRoot mean square deviation of the fit in absorbance units.

^fFor this model, the dimeric species is R₂P₂ not RP₂.