Characterization of the Solution Complex between the Interferon-induced, Double-stranded RNA-activated Protein Kinase and HIV-I Trans-activating Region RNA*

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The antiviral activity of the interferon-induced, double-stranded RNA (dsRNA)-activated protein kinase (PKR) is mediated through dsRNA binding leading to PKR autophosphorylation and subsequent inhibition of protein synthesis. Previous biochemical studies have suggested that autophosphorylation of PKR occurs via a protein-protein interaction and that PKR can form dimers in vitro. Using four independent biophysical and biochemical methods, we have characterized the solution complex formed between PKR and trans-activating region (TAR) RNA, a 57-nucleotide RNA species with double-stranded secondary structure derived from the human immunodeficiency virus type I genome. Chemical cross-linking and gel filtration analyses of PKR-TAR RNA complexes reveals that TAR RNA addition increases PKR dimerization and results in the formation of a solution complex with a molecular weight of approximately 150,000. Addition of TAR RNA to PKR results in a quenching of tryptophan fluorescence, indicative of a conformational shift. Through small angle neutron scattering analysis, we show that PKR exists in solution predominantly as a dimer, and has an elongated solution structure. Addition of TAR RNA to PKR causes a significant conformational shift in the protein at a 2:1 stoichiometric ratio of protein to RNA. Taken together, these data indicate that the PKR activation complex consists of a protein dimer bound cooperatively to one dsRNA molecule.

The human double-stranded RNA (dsRNA)-1-activated protein kinase (PKR) is a 551-amino acid, 62-kDa protein product of an interferon-inducible gene (1). PKR functions in the cellular antiviral response through inhibition of eukaryotic initiation factor 2 via phosphorylation of its α-subunit (2). More recently, PKR has been shown to activate the transcription factor NFκB through phosphorylation and release of its inhibitor, IκB (3). The antiviral and signal transducing activities of PKR are mediated by the interaction of PKR with dsRNA, leading to PKR autophosphorylation on serine and threonine residues (3–5). Binding of dsRNA is necessary to expose the ATP binding site on PKR and initiate the autophosphorylation reaction (6, 7). The catalytic domain of PKR is located in the C-terminal region of the protein, while the N-terminal region of PKR contains two nearly identical dsRNA binding motifs (2, 8–12). The dsRNA binding sites comprise two consensus sequences of 20–21 amino acids that share a high degree of homology with corresponding domains in rat and murine PKR, as well as with RNA binding domains of a number of other proteins (13). The two sites have different affinities for dsRNA, with the higher affinity site (residues 55–75) being absolutely required for dsRNA binding, and the lower affinity site (residues 145–166) not absolutely required (10). Point mutations within the higher affinity site have been shown to decrease or eliminate dsRNA binding in vitro (9, 10, 12, 14) and to inhibit the growth-suppressive effect of PKR in vivo (9, 14). In addition to dsRNA, PKR can be activated by polyanionic molecules such as heparin (15), although the binding sites for these agents may be different from the dsRNA binding sites (16). Recently, the solution structures of two peptides corresponding, respectively, to the dsRNA binding domains of Escherichia coli RNase III (17) and Drosophila staufen protein (18) have been solved by nuclear magnetic resonance spectroscopy. In both studies, molecular modeling predicted that the dsRNA makes contact with one end of a helical region containing amino acids that correspond to conserved sites in the homologous PKR dsRNA binding domain. The binding site in each case was modeled to interact with dsRNA of approximately 20 base pairs in length (21, 22). One may consider this model in light of one study in which it was found that dsRNA molecules of at least 30 base pairs in length were required to bind to and activate PKR (19). Moreover, synthetic TAR RNA, a 57-nucleotide RNA strand derived from the genome of HIV-1 and containing 22 base pairs of putative double-stranded secondary structure, is able to bind to and activate PKR at low concentrations (20–22), while inhibiting PKR at higher concentrations (21, 23). Despite these findings, the mechanism of dsRNA activation/inhibition of PKR and the mechanism of PKR autophosphorylation and substrate phosphorylation remain poorly understood. PKR activation may involve an intermolecular phosphorylation event (24, 25). Furthermore, there is evidence that PKR can dimerize with itself (25–28) as well as chemical cross-linking experiments, which show that the N-terminal dsRNA binding domain of PKR tends to dimerize in solution, even in the absence of dsRNA (27). Thus, it is possible that a protein-protein interaction plays an important role in PKR activation. In this study, we examine the interaction between highly purified, recombinant PKR and TAR RNA in solution using four independent biophysical and biochemical methods. Through the use of...
chemical cross-linking and gel filtration chromatography, we show that addition of TAR RNA increases PKR dimer formation and also causes a chromatographic shift in RNA absorption to a position corresponding to a $M_r$ 150,000 protein. Using small angle neutron scattering and tryptophan fluorescence quenching experiments, we show that PKR undergoes a conformational change upon binding to TAR RNA. Neutron scattering experiments also reveal that PKR exists as a dimer in solution and has an elongated structure. We conclude that the PKR-TAR RNA activation complex consists of a protein dimer together with one dsRNA molecule, and present a model for PKR-TAR RNA cooperativity.

**EXPERIMENTAL PROCEDURES**

**Preparation of PKR and TAR RNA**—The enzymatically inactive mutant form of human PKR used in this study, PKR(Lys296→Arg) (2), was cloned into the vector pET15b (Novagen) and used to transform *E. coli* BL21(DE3) cells as described previously (14). The cells were grown in 1.5-liter Luria broth culture containing 50 mg/ml carbencillin at 37 °C to an $A_{600}$ of 0.6–1.0, treated with 2 mM isopropyl-1-thio-β-D-galactopyranoside, incubated for a further 3.5 h at 37 °C, and centrifuged at 5000 $g$ for 20 min at 4 °C, and the pellets were washed and stored at −80 °C. Cell pellets were resuspended in ice-cold lysis buffer (50 mM NaCl, 50 mM Tris-SCl, pH 8.0, 1 mM Nonidet P-40), sonicated 4 × 30 s at full power on ice, and centrifuged at 20,000 × $g$ for 20 min at 4 °C. The supernatant was passed over a NTA-agarose metal affinity column (His-Bind, Novagen), and the histidine-tagged protein was eluted according to the manufacturer’s instructions. PKR was further purified by gel filtration chromatography on a Superdex-75 column (Pharmacia Biotech Inc.) as described elsewhere (14) and concentrated using either an Ultrafree-CL 30,000 MWCO (Millipore) or a Centricon-10 ultrafiltration device (Amicon). The purity and concentration of the final purified protein was determined by SDS-PAGE with Coomassie staining, UV spectrometry, and amino acid analysis. The 57-nucleotide form of HIV-1 TAR RNA (TAR-57) was prepared synthetically and purified from a 12% polyacrylamide, 8M urea sequencing gel as described previously (21). The TAR RNA concentrations were determined spectrophotometrically.

**Chemical Cross-linking of PKR**—PKR at a concentration of 1 μM was incubated on ice in 10 μl of binding buffer (100 mM NaCl, 50 mM HEPES, pH 7.2, 5 mM MgCl$_2$, 1 mM EDTA) either alone or in the presence of TAR RNA at concentrations ranging from 0.01 to 10 μM. After 2 h, 5 μl of a 24 mM mg/ml solution of dimethyl suberimidate (DMS; Pierce) (29) in 0.4 μl triethanolamine hydrochloride, pH 8.5, was added to each sample, and the incubation was continued for 2 h at room temperature. Each sample was treated with 15 μl of 2% (v/v) SDS, 2% (v/v) 2-mercaptoethanol, incubated at 37 °C for 2 h, and 30 μl of 80% (v/v) glycerol, 0.01% (v/v) bromphenol blue added to each tube, and the samples were electrophoresed on an 8% SDS-PAGE gel. After electrophoresis, the gel was transferred onto a polyvinylidene fluoride membrane (Immobilon-P, Millipore), Western blotted using a 1:1000 dilution of anti-PKR rabbit antiserum, and visualized using the ECL detection reagent (Amersham Corp.).

**Gel Filtration Chromatography of PKR-TAR RNA Complexes**—PKR (4.2 μg) was combined with TAR RNA (8.5 μg) in a total volume of 350 μl of binding buffer, incubated on ice and at 45, 90, and 150 min and gel filtration chromatography resulted in a final product containing to a position corresponding to a $M_r$ 50,000. Incubation under these conditions also causes a chromatographic shift in RNA absorbance at 280 nm according to the formula $F_r = F_{blue} \times$ antilog (A$_{260}$/2) (30).

**Trycotton Fluorescence Quenching**—Fluorescence spectroscopy was performed with a Perkin-Elmer LS-5B luminescence spectrometer using excitation and emission wavelengths of 280 and 340 nm, respectively. PKR at a concentration of 300 nM in an initial volume of 270 μl was scanned in a quartz cuvette following stepwise addition of aliquots of TAR RNA from a 30 μM stock solution. TAR RNA was added to a maximum of 4.5 μM, a 15-fold excess over PKR. The emission intensities at 340 nm were recorded and corrected for dilution factor and for intrinsic quenching due to RNA absorbance at 280 nm according to the formula

$$I(Q) = I(0)\exp(-Q^2R_g^2/3)$$

(Eq. 1)

where $I(Q)$ is the Raman scattering from water was used to calculate the molecular weight, and hence the stoichiometry, from the forward scatterer formula (32).

$$I(Q) = I(Q)_{lo,inc} \exp(-R_{sc}Q^2/2)$$

(Eq. 2)

where $I(Q)$ is the wavelength-dependent correction factor for the anisotropy in the incoherent scattering of water; $I(Q)_{lo,inc}$ are the incoherent scattering and transmission of water respectively; $c$, $t$, and $T$ are, respectively, the concentration, thickness, and transmission of the sample; $N_r$ is the Avogadro number; $M_r$ is the molecular weight; $S$ is the sum of the scattering lengths, $Q$ is the solvent scattering length density; and $V$ is the volume of the particle. For long extended particles, the scattered intensity follows a similar relation

$$I(Q) = Q(I(Q)_{lo,inc} \exp(-R_{sc}Q^2/2))$$

(Eq. 3)

In this equation, $R_{sc}$ is the cross-sectional radius of gyration and is related to the diameter of the rodlike particle.

**RESULTS**

**Design and Preparation of PKR**—Due to the growth-suppressive properties of PKR (Fig. 1) and the requirement for large amounts of purified recombinant protein for structural studies, we elected to express a mutant form of PKR, termed PKR(Lys296→Arg). This mutant is enzymatically inactive in terms of both auto- and substrate phosphorylation but retains the TAR RNA-binding properties of wild type PKR (2, 8). Overexpression of histidine-tagged mutant PKR in *E. coli* and subsequent purification by metal chelation affinity chromatography and gel filtration chromatography resulted in a final product with greater than 99% purity, as assessed by densitometric analysis of Coomassie-stained SDS-PAGE gels and amino acid analysis.

**Chemical Cross-linking of PKR**—Solutions of PKR in the absence and presence of increasing amounts of TAR RNA were chemically cross-linked with DMS and detected by Western blotting with anti-PKR polyclonal antiserum. PKR is present in both its monomeric and dimeric forms, even in the absence of TAR RNA (Fig. 2), possibly indicating an equilibrium between the two forms. Addition of TAR RNA significantly increases the degree of PKR dimerization, while reducing the relative levels of monomer (Fig. 2). Addition of TAR RNA also indicates the formation of high molecular weight aggregates of PKR, which are visible at the top of the gel (Fig. 2). Densitometry analysis of the autoradiogram depicted in Fig. 2 indicated that, in the absence of TAR RNA, the percentage ratio of PKR monomer to dimer was 71 to 29% (Fig. 2). It was 72 to 28% in the presence of 0.01 μM TAR RNA, 74 to 26% with 0.1 μM TAR RNA, 57 to 50% with 1 μM TAR RNA.
43% with 1.0 μM TAR RNA, and 58 to 42% with 10 μM TAR RNA (Fig. 2). Thus, TAR RNA increases PKR dimerization, either through a shift of the equilibrium toward the dimeric state, or through stabilization of the dimeric complex.

**Gel Filtration Analysis of a PKR TAR RNA Solution Complex**—When injected on a Superdex-75 gel filtration FPLC column, TAR RNA migrates with a retention time corresponding to a protein of molecular mass of approximately 20 kDa (Fig. 3A). However, when PKR (4.2 μM) and TAR RNA (8.5 μM) are incubated together for 45 min on ice and injected, a second absorbance peak is seen which corresponds to 150 kDa (Fig. 3B). Since the absorbance of the RNA species completely overwhelms that of the protein, Western blotting of aliquots from the gel filtration run depicted in chromatogram B by Western blot. Positions of the molecular mass standards are indicated.

Thus, under these conditions, PKR and TAR RNA form a solution complex composed of 2 molecules of PKR together with 1 molecule of TAR RNA.

**TAR RNA Quenching of PKR Tryptophan Fluorescence**—PKR contains three tryptophan residues, all of which fall within the C-terminal half of the protein. Thus, any quenching of internal tryptophan fluorescence can be attributed to a change in the tryptophan aromatic ring stacking due to a conformational change in the C terminus of PKR. Addition of increasing amounts of TAR RNA to 300 nM PKR resulted in a biphasic quenching of fluorescence (Fig. 4). The initial steep phase of the quenching occurs between TAR RNA concentrations of 0–900 nM to a quenching level of 18%. The second phase, from 1.5 to 4.5 μM TAR RNA, is much flatter, with the maximum level of quenching reaching 26% (Fig. 4). Thus, addition of TAR RNA to PKR at stoichiometric ratios of dsRNA/protein between 1:2 and 3:1 results in a significant conformational change in the C-terminal half of PKR. Addition of RNA beyond this level results in only a marginal change.

**Neutron Scattering Studies and Determination of Particle Molecular Weight, Radius of Gyration, and Effects of TAR RNA Addition on the Radius of Gyration of PKR**—Measurement of the coherent neutron scattering intensity of a polymer of known concentration in solution allows the determination of the molecular weight of the polymer or oligomer in the case of complex formation (32). To determine the molecular weight and radius of gyration of PKR, we performed a set of experiments on PKR solutions at concentrations of approximately 2 mg/ml.
The Solution Complex between PKR and TAR RNA

in H₂O and D₂O buffers, respectively. Guinier analysis of the I(0)/c(T value measured for PKR in H₂O solution gave a molecular weight for the particle of 119,000 (Fig. 5). Given that the Mₙ of the histidine-tagged fusion protein is approximately 64,000 (determined from the amino acid composition), these data suggest that PKR is a dimer in solution. In a separate experiment, PKR (2 mg/ml) in D₂O buffer solution was analyzed by small angle scattering to determine the radius of gyration of the particle. A Guinier plot of the scattering data of the PKR solution is shown in Fig. 6. From these data, the radius of gyration was measured at 48.7 Å. This value of R_G is much larger than the 26 Å predicted for PKR on the basis of comparison with standard globular proteins. The radius of gyration was also determined for each titration point when PKR (2 mg/ml) in D₂O buffer solution was titrated with TAR RNA. When the TAR RNA was added, the R_G of PKR increased from 48.7 Å to a plateau of about 57 Å, an increase of more than 20% (Fig. 7). Moreover, the R_G reached its plateau value at a TAR RNA/PKR molar ratio of 1:2 and remained steady near this value throughout the remainder of the titration (Fig. 7). Such a large change in R_G could be accounted for either by the addition of the TAR RNA to the protein, or by a conformational change in the protein itself, or by a combination of both. To address this question, we fitted the data in Fig. 7 to an additive combination of two sizes; one originating from the R_G of the PKR dimer, and the other due to the R_G of the of the PKR dimer-TAR RNA complex. The result is the continuous line in Fig. 7 computed using the equation

\[ R_G^2 = (\text{[PKR]} b_1 R_{1G}^2 + [\text{PKR} - \text{TAR}] b_2 R_{2G}^2 + [\text{TAR}] b_3 R_{3G}^2) \]

where \([\text{PKR}]\), \([\text{PKR} - \text{TAR}]\), and \([\text{TAR}]\) are the concentrations of the PKR dimer, PKR dimer-TAR RNA complex, and TAR RNA, respectively; \(R_{1G}\) is the total radius of gyration of the heterogeneous mixture; \(R_{2G}\) and \(R_{3G}\), and \(b_1\), \(b_2\), and \(b_3\), are the radii of gyration and total scattering lengths for the PKR dimer, PKR dimer-TAR RNA complex, and TAR RNA, respectively. The profile of the TAR RNA/PKR titration (Fig. 7) is characteristic of the tight binding case; each TAR RNA molecule added will be bound, until the PKR is saturated. Beyond the break point (i.e. the inflection point in Fig. 7), although more molecules of TAR RNA are added, they will not bind. Since the concentration of the complex remains constant after the break point, very little change in R_G is observed. In the analysis based on the above equation, for all data points leading up to the break point, the concentration of free TAR RNA is zero \([\text{TAR}] = 0\), the concentration of the complex formed is equal to that of the TAR RNA added \([\text{[PKR} - \text{TAR}] = [\text{TAR}]\) added), and the remaining concentration of free PKR dimer is given by \([\text{[PKR}_2] = [\text{PKR}]_{\text{total}} - [\text{TAR}]\). R_G1 and R_G2 are 48.7 and 57 Å, respectively. The scattering lengths of PKR dimer and PKR dimer-TAR RNA complex are 5184 \(\times 10^{-12}\) and 5869 \(\times 10^{-12}\) cm, respectively. For data points after the break point, the concentration of free PKR is now zero \([\text{[PKR}_3] = 0\), the concentration of the complex remains constant and is equal to the total concentration of PKR in solution \([\text{[PKR} - \text{TAR}] - [\text{PKR}]_{\text{total}}\) and free TAR RNA is given by \([\text{TAR}] = [\text{PKR}]_{\text{total}} - [\text{PKR} - \text{TAR}]\). The total scattering length of the TAR RNA in this model is 685 \(\times 10^{-12}\) cm. Since an R_G for TAR RNA was not determined, we have calculated one based on a simple cylindrical model with diameter 20 Å and height 85 Å; the resulting R_G is 26 Å. TAR RNA contributes very little to the overall scattering, owing to its small size and the fact that its scattering contribution in D₂O is significantly reduced in comparison to the PKR dimer-TAR RNA complex. Nevertheless, the scattering is sufficient to change the slope of the plateau (Fig. 7) when present in high concentrations. To unambiguously deter-

![Fig. 4. Quenching of PKR tryptophan fluorescence by TAR RNA addition.](image)

![Fig. 5. Neutron scattering analysis of the molecular weight of the PKR particle in solution.](image)

![Fig. 6. Guinier plot for the determination of the radius of gyration of PKR in solution by small angle neutron scattering.](image)
DISCUSSION

While it is well established that dsRNA, as well as polyanions such as heparin, are activators of PKR, the molecular details of the interaction that gives rise to PKR autophosphorylation are largely unknown. The important role of PKR in the interferon-mediated cellular antiviral response and as a signal transducer argues for an understanding of the biophysical basis of PKR activation and inhibition. To determine the importance of PKR/dsRNA stoichiometry, PKR dimerization, and conformational changes within PKR to its activation, we utilized four independent biochemical and biophysical methods to study the in vitro interaction between PKR and TAR RNA.

Preincubation with TAR RNA increases the level of PKR protein-protein cross-linking (Fig. 2). Our results show that PKR exists both as a monomer and a dimer in the absence of dsRNA and that addition of TAR RNA increases the degree of PKR dimerization (Fig. 2). Thus, it is likely that, under these experimental conditions, PKR is in equilibrium between the monomeric and dimeric forms. Addition of TAR RNA may serve to shift the equilibrium toward the dimeric state or to stabilize the dimer. Taken together with the previous findings, that PKR may be in equilibrium between its monomeric and dimeric forms and thus would be expected to be sensitive to experimental conditions, the presence of ligands, and other factors.

PKR contains two nearly identical dsRNA binding sites within its N-terminal dsRNA binding domain. Moreover, PKR has been shown to bind to dsRNAs ranging in size from 30 to hundreds of base pairs. Thus, the potential exists for two dsRNA molecules to bind to one PKR molecule, as well as for a number of proteins to bind to one dsRNA strand of sufficient length. To further investigate this we determined the approximate size of a complex of PKR bound to TAR RNA, a relatively small oligonucleotide putatively containing 22 base pairs of double-stranded secondary structure (Fig. 1A). PKR forms a complex with TAR RNA that migrates on a gel filtration column with a retention time corresponding to a protein of approximately \( M_r 150,000 \) in size (Fig. 3B). Since TAR RNA migrates at \( M_r 20,000 \) (Fig. 3A), this retention time corresponds to that of a complex consisting of two PKR molecules bound to one TAR RNA. It is interesting to note that PKR, although readily capable of dimerization, generally migrates as a monomer under the conditions we use for gel filtration.3

However, Western blot analysis of the fractions from the chromatographic run of the PKR-TAR RNA mixture reveals that PKR co-elutes with the 150-kDa TAR RNA peak (Fig. 3C). Thus, the 150-kDa species is a protein-RNA complex. A possible model for the organization of this complex might be a single TAR RNA molecule extending between or across a PKR homodimer.

Tryptophan fluorescence quenching is indicative of a change in the stacking environment of the aromatic side chains of tryptophan residues within the protein. The three tryptophans of PKR all fall within the C-terminal half of the protein; that is, within the catalytic domain and outside of the N-terminal dsRNA binding domain. Therefore, the fluorescence quenching that is observed in PKR upon TAR RNA addition (Fig. 4) is indicative of a conformational change within the catalytic domain. This conformational shift may be responsible for inducing the PKR autophosphorylation event leading to kinase activation. The biphasic shape of the fluorescence quenching curve (Fig. 4) indicates that TAR RNA exerts its maximal quenching effect at an RNA/protein molar ratio of approximately 2:1. This ratio corresponds to a saturation of the two dsRNA binding sites per PKR molecule. Thus, the conformational change in the catalytic domain of PKR proceeds until saturation of the binding sites with TAR RNA.

To determine this size and shape of the PKR molecule in solution, and to examine the effects of dsRNA binding on these characteristics, we employed the technique of small angle neutron scattering. We measured the molecular weight and radius of gyration of PKR in \( H_2O \) and \( D_2O \) solution, respectively (31, 32). The \( R_G \) value of 48.7 Å determined for PKR is too large to correspond to that of a monomeric subunit, and suggests that the particle is highly elongated or is present as a multimeric subunit in solution, or a combination of the two. The determined value for the molecular weight of PKR of 119,000 suggests that PKR is present predominantly as a dimer in solution. Thus, PKR exists under these conditions as a protein dimer...
with an elongated ellipsoid or domain structure.

Our finding that addition of TAR RNA to PKR in solution causes a large increase in the protein radius of gyration (Fig. 7) indicates that dsRNA binding to PKR results in a major conformational change in the protein, although we cannot rule out the possibility that the change is due solely to the addition of the TAR RNA molecule to the protein complex. This change is likely to be at least partially distinct from the shift within the C terminus which was observed by tryptophan fluorescence (Fig. 4). That the $R_g$ increases rather than decreases suggests that initial binding of TAR RNA to the first N-terminal dsRNA binding site of PKR causes the protein to elongate, which may serve to expose or otherwise facilitate the attachment of the other binding site to dsRNA. This model is supported by the shape of the curve which reaches a plateau at a dsRNA/protein ratio of 1:2. This would correspond to a single TAR RNA molecule binding to a PKR dimer, possibly interacting with one dsRNA binding site on each of the protein molecules. The observed increase in $R_g$ is consistent with an extended conformation of the polypeptide chain in the region spanning the two motifs. In this model, occupancy of the initial, high-affinity site would bring about a conformational change in the protein dimer, facilitating the attachment of the second site, either to the same dsRNA, or possibly to a second molecule. Additional conformational changes in PKR may follow full occupancy of the dsRNA binding sites, leading to autophosphorylation and catalytic activation of PKR. Indeed, as shown by tryptophan fluorescence quenching (Fig. 4), the conformational changes within the catalytic domain of PKR are manifest up to approximately a 2:1 dsRNA/protein ratio, corresponding to saturation of the two dsRNA binding sites per protein molecule. Thus, the attachment of PKR to dsRNA via the high-affinity binding site, along with the accompanying conformational shift, may serve to anchor the protein to the dsRNA, facilitating the remaining conformational changes necessary to bring about kinase activation. This view is supported by mutagenesis studies which have found that mutations within motif 1 are far more detrimental to PKR dsRNA binding and biological activity than those within motif 2 (9, 10, 12, 14), although the presence of both sites is required for activity (10). It is possible that structural features outside of the two dsRNA binding motifs contribute to the stability of the protein-dsRNA interaction. Amino acid mutations N-terminal of motif 1 in PKR (14) and N-terminal of the corresponding domain in Drosophila staufen protein (18) have been shown to affect dsRNA binding. Moreover, the homology between the amino acid sequences of PKR from human, mouse and rat is higher in the region N-terminal of motif 1 than in the corresponding region near motif 2, possibly underlining the importance of amino acids or structural features within this region. Deletion mutants of PKR lacking 24 amino acids or more from the N terminus have been shown to completely lack dsRNA binding activity (16). The region at the extreme N-terminal end of PKR might be expected to have somewhat more flexibility than the regions more toward the C terminus, thus allowing the portions of the polypeptide chain about motif 1 the flexibility to serve as a molecular anchor for dsRNA. Mutagenesis studies on residues within the dsRNA binding domain of PKR which fall outside of the two motifs have been limited to a single set of random mutagenesis experiments (14), and would be an interesting focus of future research.

Of equal interest is the size and molecular organization of the PKR-dsRNA complex itself. Gel filtration analysis (Fig. 3B) indicates that PKR and TAR RNA form a complex consisting of two protein molecules together with one dsRNA molecule. Neutron scattering data indicating that PKR preferentially forms dimers in solution, and that the shift in $R_g$ is maximal at a dsRNA/protein ratio of 1:2 (Fig. 7), also support this model for the complex. Although there is some question in the literature as to whether PKR can dimerize independently of dsRNA (25–28), it is clear from these data that dsRNA is required for the conformational shifts in PKR, manifest across multiple domains, which presumably give rise to its enzymatically active state. There is also the question of how the stoichiometries of PKR and dsRNA within the complex relate to activation and/or inhibition of PKR. Since PKR has two dsRNA binding sites and the possibility exists that multiple PKR molecules could bind to one dsRNA (either through dimerization or along the length of a large dsRNA molecule), one can appreciate that highly complex stoichiometries between the two species are possible. The possibility that large protein-RNA complexes could form in the presence of excess dsRNA may represent a possible mechanism for the inhibition of PKR which has been observed at high concentrations of dsRNA, including TAR RNA (21, 23). In fact, we have observed fibrous precipitation bodies within PKR-TAR RNA mixtures at high dsRNA/protein ratios.2 In addition, titration of PKR with TAR RNA results not only in increased dimer formation, but in formation of high molecular weight cross-linked protein species (Fig. 2), which may represent extensive protein-dsRNA complexes. It would be interesting to determine how the conformational changes observed in PKR by fluorescence quenching at values around the dsRNA saturation level relate to the activation/inhibition states of PKR. Experiments aimed at further characterization of the PKR-TAR RNA complex and its organization are in progress.

In summary, TAR RNA stabilizes the dimeric form of PKR and forms a protein-dsRNA complex of approximately $M_t$ 150,000. TAR RNA causes a conformational change in the C terminus of PKR up to a dsRNA/protein ratio of 2:1. PKR has an elongated, dimeric solution structure, and undergoes a separate conformational shift which is maximal at a dsRNA/protein ratio of 1:2. This shift may serve to anchor the protein to the dsRNA molecule and facilitate further conformational changes leading to kinase activation.

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