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Analysis of high affinity binding of PKR to dsRNA

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Abstract

Protein kinase R (PKR) is an interferon-induced kinase which plays a pivotal role in the innate immunity response to viral infection. PKR is activated upon binding to dsRNA. Our previous analysis of PKR binding to dsRNAs ranging from 20–40 bp supports a dimerization model for activation where 30 bp represents the minimal length required to bind two PKR monomers and activate PKR via autophosphorylation. These studies were complicated by the formation of protein-RNA aggregates, particularly at low salt concentrations using longer dsRNAs. Here, we have taken advantage of the enhanced sensitivity afforded using fluorescence-detected analytical ultracentrifugation to reduce the RNA concentrations from micromolar to nanomolar. Under these conditions, we are able to characterize high affinity binding of PKR to longer dsRNAs in 75 mM NaCl. The PKR binding stoichiometries are increased at lower salt but remain lower than those previously obtained for the dsRNA binding domain. The dependence of the limiting PKR binding stoichiometries on dsRNA length does not conform to standard models for nonspecific binding and suggests that binding to longer sequences occurs via a different binding mode with a larger site size. Although dimerization plays a key role in the PKR activation mechanism, the ability of shorter dsRNAs to bind two PKR monomers is not sufficient to induce autophosphorylation. We propose that activation of PKR by longer RNAs is correlated with an alternative binding mode where both of the dsRNA binding motifs contact the RNA, inducing PKR to dimerize via a direct interaction of the kinase domains.

Protein kinase R (PKR) is an important component of the interferon-induced innate immune pathway (1). PKR is synthesized in the cell in a latent, unphosphorylated form and becomes activated and undergoes autophosphorylation upon binding to certain host or pathogen associated RNAs or upon interacting with the protein PACT. Activated PKR phosphorylates eukaryotic Initiation Factor 2 α (eIF2 α), causing an inhibition of protein synthesis. Many viruses have developed mechanisms to inhibit PKR (2).

PKR contains an N-terminal dsRNA binding domain and a C-terminal kinase domain, separated by a flexible linker region. The N-terminal dsRNA binding domain (dsRBD)

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Supporting Information

Supporting Information Available. Table S1: Hydrodynamic parameters for dsRNAs and dsRNA-PKR complexes. This material is available free of charge via the Internet at <http://pubs.acs.org>.

corresponds to the regulatory domain which consists of two tandem copies of the dsRNA binding motif, dsRBM1 and dsRBM2. This motif binds duplex RNA in a non-sequence specific manner but discriminates against ssRNA and dsDNA (3). Each motif adopts the conserved $\alpha\beta\beta\beta\alpha$ fold (4) and they are separated by an unstructured linker of ~20 amino acids. The crystal structure of a dsRNA binding domain complexed with dsRNA reveals a site size of 16 bp (5). The C-terminal kinase domain is the catalytic center. The crystal structure of the PKR kinase domain in complex with eIF2 α reveals that the catalytic center has the canonical protein kinase fold. The complex forms a dimer where the interface is in the N-terminal lobe of the kinase domain (6).

PKR is regulated by a variety of cellular and viral RNAs that contain duplex regions (7). The most well characterized activators of PKR are simple dsRNAs. There is strong evidence that dimerization plays a crucial role in the mechanism of PKR activation by dsRNA (8, 9). Latent PKR dimerizes weakly in solution but this reaction is sufficient to activate PKR in the absence of RNA (10). An allosteric pathway has been identified that couples the dimer interface with the kinase active site and dimer interface mutations can modulate PKR activation (6, 11). A hallmark of PKR activation by dsRNA is the “bell-shaped” curve where low RNA concentrations activate but higher concentrations are inhibitory (12, 13). These observations support a model in which multiple PKRs assemble on a single dsRNA and high dsRNA concentrations dilute PKR monomers onto separate molecules of dsRNA (14). In the context of regular duplex RNA, a minimum of 30-33 bp of dsRNA are required to activate PKR autophosphorylation and the maximal level of activation increases with duplex length (13, 15).

The mechanism of assembly of PKR on duplex RNAs has been extensively investigated. PKR can bind to duplexes as short as 16-18 bp (16, 17). As expected for a non-sequence specific interaction, the binding stoichiometry increases with dsRNA length (13, 15-18). The binding stoichiometries for the dsRBM1 and for a construct containing dsRBM1 and dsRBM2 are greater than predicted from the site size (17, 19). Based on these results, we proposed an overlapping ligand binding model where the motif binds to multiple faces of the dsRNA duplex and overlaps along the helical axis. The increase in binding affinity with increasing dsRNA length can be understood as a statistical consequence of the greater number of configurations available on the longer lattice. The intrinsic binding affinities of a dsRBM1-dsRBM2 construct and full length PKR for a 20 bp RNA are similar (15, 17) but the stoichiometries are lower for the latter. A minimum of 30 bp of dsRNA are required to bind two full length PKR monomers with high affinity, which correlates with the minimal dsRNA capable of activating through autophosphorylation. Binding affinities increase dramatically upon reducing the salt concentration from 200 to 75 mM NaCl and a second PKR can bind to 20 bp duplex at lower salt (15).

The non-sequence specific interactions of PKR with RNAs are labile and it is important to use solution biophysical methods to accurately measure the binding parameters at thermodynamic equilibrium. Our previous analyses of PKR binding to dsRNA by sedimentation velocity analytical ultracentrifugation (10, 15) were limited by formation of nonspecific aggregates, even at the minimal RNA concentrations required for detection using absorption optics. Aggregation is enhanced for longer RNAs and at lower salt concentrations. In addition, it is difficult to quantify high affinity binding constants due to the limited sensitivity of the absorbance optics. Here, we have used fluorescence detection of labeled dsRNAs to enhance the sensitivity of sedimentation velocity measurements and directly characterize the interaction of PKR with duplex RNAs ranging from 20-40 bp and at multiple salt concentrations. The binding affinities increase at lower salt concentration and with longer dsRNA length, and in some cases the stoichiometries of binding of PKR to dsRNA increase at 75 mM NaCl. The dependence of the binding stoichiometries on dsRNA

length does not conform to standard statistical models for nonspecific binding. We propose a model where the activation of PKR by longer RNAs is correlated with an alternative binding mode that allows for direct interaction of the kinase domains.

Experimental Procedures

All reagents purchased were of reagent grade. Wild type PKR was expressed and purified as described previously (10) with the dsRNA affinity column replaced by a hydroxyapatite column (20). Just before use, PKR was chromatographed on a Superdex 200 size exclusion column equilibrated in AU200 buffer (20mM HEPES, 200 mM NaCl, 0.1 mM EDTA, 0.1 mM TCEP, pH 7.5). When required, the protein was buffer exchanged into AU75 (20 mM HEPES, 75 mM NaCl, 0.1 mM EDTA, 0.1 mM TCEP) using spin columns packed with Bio-gel P-6 (Biorad). The oligoribonucleotides were obtained from Dharmacon (Lafayette, CO) with one strand containing a 5' fluorescein label. The complementary strands were annealed at 80°C for 5 minutes in either AU200 or AU75 and slowly cooled to room temperature before use. Sedimentation velocity analysis was used to ensure that the dsRNAs were homogeneous (see below).

Quantitative phosphorylation assays were carried out as previously described (10) by incubating 200 nM PKR with varying concentrations of dsRNA in AU75 buffer containing 5 mM MgCl₂ and 0.1mM TCEP at 32 °C for 20 minutes. The phosphorylation reactions were initiated with 0.4 mM ATP containing 4 μCi [γ -³²P] ATP in each reaction mixture and were quenched after 20 minutes by adding SDS sample loading buffer. The reaction mixtures were then heated to 90 °C for 3 minutes and were analyzed using SDS PAGE electrophoresis and phosphor imaging (Typhoon, GE Healthcare).

Sedimentation velocity experiments were performed using a Beckman-Coulter XL-I analytical ultracentrifuge equipped with an AU-FDS Fluorescence detector (AVIV Biomedical, Lakewood, NJ) at 20°C and 40,000 rpm. Samples contained 20-30 nM dsRNA and varying concentrations of PKR in either AU200 or AU75 buffer were loaded into double sector cells equipped with 1.2 cm Spin60 charcoal-epon centerpieces (Spin Analytical, Berwick, ME) and quartz windows. During a pre-run at 5,000 RPM the photomultiplier voltage and gain multiplier settings were adjusted to give ~ 3000 counts and the focus depth was set to give a maximum signal. Absorbance intensity scans were recorded for each channel following the sedimentation velocity experiment and the meniscus position was determined from a characteristic downward intensity spike. Although it has recently been asserted that fluorescence detector produces nonlinear responses and underestimates sedimentation coefficients (21), we have found that the detector produces linear responses up to 500 nM and the sedimentation coefficients agree with those determined by absorbance detection (Lary, J.W., Husain, B. and Cole, J. L, manuscript in preparation).

The protein partial specific volume and the buffer densities and viscosities were calculated using SEDNTERP (22). The partial specific volumes of the dsRNAs were fixed at 0.55 ml g⁻¹. The sedimentation velocity data for the dsRNAs were first analyzed using a c(s) distribution model with Sedfit (23) to ensure that the preparations were homogenous and free of contaminants. The data were then fit to a single species model to obtain the sedimentation coefficients and the buoyant molar masses that we were then fixed in subsequent global analyses of PKR binding. Sedimentation velocity analyses of PKR-dsRNA interactions were performed as previously described (24), with some modifications to accommodate fluorescence data as described below. Initially, the data were analyzed using the time derivative method (25) with DCDT+ (26) to obtain g(s*) distributions to define the interaction model. Model-dependence global analysis was performed using

SEDANAL (27) to define the association constants and sedimentation coefficients of the protein-RNA complexes. For data acquired with the fluorescence detector, the amplitude of the stochastic noise depends strongly on the signal intensity. Therefore, the curve fits were weighted by the standard deviation at each point. This also served to convert the RMSD reported by SEDANAL from arbitrary fluorescence intensity units to χ , where a value of 1.0 corresponds to a perfect fit. Joint confidence intervals were obtained using the F-statistic to define a statistically significant increase in the variance.

Results

The enhanced sensitivity of fluorescence detection in sedimentation velocity measurements allows the dsRNA concentrations to be greatly reduced, from $\sim 1 \mu\text{M}$ using absorbance detection at 260 nm to 20-30 nM for fluorescence analysis of 5'-fluorescein labeled RNAs. Figure 1 shows an analysis of PKR binding to dsRNA using a long sequence (40 bp) at low salt (75 mM NaCl), conditions that previously were found to promote aggregation. The signal/noise of the raw sedimentation velocity traces for the RNA alone is excellent (Figure 1A). The data fit well to a model of a single species with a corrected sedimentation coefficient of $s_{20,w} = 3.69 \text{ S}$ and a molecular weight of 30,979 g/mol consistent with a predicted mass of 26,128 g/mol based on the RNA sequence (Table S1, supporting information). Thus, this dsRNA preparation is homogenous and suitable for analysis of PKR binding.

The binding of PKR to the 40 bp dsRNA was characterized by a sedimentation velocity titration using fluorescence detection. Although the general approach is similar to the method we have previously described by analysis of PKR-RNA interactions using absorbance detection (24), the RNA concentration here is much lower and we are able to access a much broader range of PKR concentrations, ranging from nM to μM . Another difference is that the PKR contributes, albeit weakly, to the absorbance signal measured at 260 nm whereas only the fluorescein-labeled RNA contributes to the fluorescence signal.

Initially, the velocity data are analyzed using a model-independent approach to help define the correct binding mode. Figure 1B shows a titration of the 40 bp dsRNA over a broad range of PKR concentration from 50 nM to $7 \mu\text{M}$, represented as an overlay of normalized $g(s^*)$ sedimentation coefficient distributions. The formation of PKR-dsRNA complexes causes the distributions to shift far to the right, to a saturating value of $\sim 9.46 \text{ S}$ at $2 \mu\text{M}$ PKR. Hydrodynamic calculations indicate that the magnitude of this shift is greater than expected for binding of a single PKR but is consistent with binding of three PKR monomers (Table S1). The $g(s^*)$ distribution at $7 \mu\text{M}$ exhibits a shoulder at higher S , indicating formation of higher aggregates. However, the data obtained over the range 50 nM - $2 \mu\text{M}$ can be globally fit to a sequential binding model to resolve the three equilibrium constants (Figure 1C). The fit is of good quality, with small systematic deviations in the residuals and $\text{RMSD} = 1.70$. As we have previously observed for PKR binding to dsRNA lattices (15, 17, 19), the first PKR binds most strongly, with $K_{d1} \sim 30 \text{ nM}$ and the second and third ligand bind progressively more weakly, with $K_{d2} \sim 50 \text{ nM}$ and $K_{d3} \sim 250 \text{ nM}$ (Table 1). Note that these are stepwise, macroscopic dissociation constants and are not corrected for statistical effects. Fitted values for the sedimentation coefficients of the three complexes RNA-PKR, RNA-PKR₂ and RNA-PKR₃ (Table S1) correspond to frictional ratios of 1.42, 1.46, and 1.40, respectively, which lie in the range typically observed for RNA-PKR complexes (24).

These results obtained with the 40 bp dsRNA in AU75 demonstrate that it is feasible to analyze PKR binding to relatively long dsRNAs, even at low ionic strength, by reducing the dsRNA concentration into the nM range using the high sensitivity afforded by fluorescence detected analytical ultracentrifugation. We have employed this approach to rigorously define

the PKR binding stoichiometries and affinities for a truncation series of dsRNAs ranging from 20 to 40 bp in 5 bp increments. Binding parameters were obtained at both high (200 mM) and low (75 mM) concentrations of NaCl. As described above for the 40 bp duplex, the binding stoichiometries were based on the limiting sedimentation coefficients estimated from the shift in $g(s^*)$ distributions with increasing PKR concentration. The models were verified and binding constants were resolved by global analysis of the data using SEDANAL. For the measurements performed in 75 mM NaCl, where the binding affinity is high, it was not possible to reliably fit for the sedimentation coefficients of the intermediate dsRNA-PKR complexes due to low population of these species. The sedimentation coefficients for these species were fixed at the values obtained in 200 mM NaCl (corrected for buffer density and viscosity). The results of the fits are summarized in Table 1 and the corresponding hydrodynamic parameters are presented in Table S1.

There are several trends that emerge from the analysis of PKR binding to the RNA truncation series at high- and low-salt conditions. For several of the dsRNAs (20, 25 and 40bp) the apparent binding stoichiometries increase at lower salt. This effect is likely associated with the enhancement in binding affinity that occurs at lower salt (16). The K_d s decrease by as much as 10-fold upon reducing [NaCl] from 200 to 75 mM. Thus, the second (20 and 25 bp) or third (40 bp) PKR is not detectable at high salt because K_d becomes too high. In most cases, the dissociation constants sequentially increase such that $K_{d1} < K_{d2} < K_{d3}$. This successive decrease in binding affinities is not due to a negative cooperativity but represents a statistical effect. The macroscopic, stepwise binding constants measured in sedimentation velocity experiments correspond to the product of an intrinsic binding constant k and a statistical factor corresponding to the number of microscopic configurations of the ligand on the dsRNA (19, 28). As the RNA is sequentially saturated with ligand the statistical factors decrease, resulting in successively weaker binding. As expected for a nonspecific protein-nucleic acid interaction, the limiting binding stoichiometries measured in AU75 increase with the length of the dsRNA. However, this trend is surprisingly weak: upon doubling the lattice length from 20 to 40 bp the number of PKRs that bind does not double and only increases from 2 to 3.

It has previously been reported that activation of PKR requires a minimum of 30 bp of dsRNA (13) and we have correlated PKR activation with the stoichiometry of binding measured in AU200 buffer for the dsRNA truncation series (15). Our PKR binding data indicate a strong dependence of binding stoichiometry on salt concentration. Thus, we have re-examined the dependence of PKR activation of dsRNA length in a lower salt, AU75 buffer (Figure 2). The activation profiles are very similar to those we previously observed at higher salt. A minimum of 30 bp are required to detect activation with a maximum near 30 nM and the maximal extent of activation increases with the length of the dsRNA. Thus, although the 20 and 25 bp dsRNAs can each bind two PKRs in AU75 buffer, they are incapable of stimulating autophosphorylation under the same conditions.

Discussion

The enhanced sensitivity afforded using fluorescein-tagged dsRNAs with fluorescence-detected analytical ultracentrifugation has allowed us greatly reduce RNA concentrations from micromolar to nanomolar. In contrast to previous studies of PKR-dsRNA interactions by analytical ultracentrifugation (15), at lower RNA concentrations the analysis of binding stoichiometries and affinities is not complicated by aggregation formation and we were able to directly characterize the interaction of PKR with duplex RNAs ranging from 20-40 bp at multiple salt concentrations. The binding stoichiometries obtained here at 200 mM NaCl agree with those obtained earlier except for the 40 bp dsRNA, where we previously detected three PKR ligands by fluorescence anisotropy and sedimentation equilibrium (15) but only

two in the present sedimentation velocity measurements. Note that sedimentation velocity has greater resolving power than the earlier techniques and small amounts of aggregate may have contributed to a higher apparent stoichiometry. The dissociation constants for the 20 and 30 bp dsRNAs in 200 mM NaCl differ slightly from those previously obtained using absorbance detection. The origin of the discrepancies is not clear but the broader PKR concentration range used for the present experiments make them more reliable.

The dependence of the PKR binding stoichiometries and affinities on salt concentration and RNA length provides insight into the mechanism of assembly of PKR on duplex RNAs and formation of active complexes. There is a large electrostatic component to the PKR-dsRNA interaction (16) that results in increased binding affinity as the NaCl concentration is decreased from 200 to 75 mM. The concomitant increase in binding stoichiometries for several of the RNAs is likely associated with the enhancement in binding affinity. Limited solubility of PKR-RNA complexes precludes measurements at NaCl concentrations below 75 mM and we make the assumption that the stoichiometries measured under these conditions correspond to the maximum. We have previously characterized the binding of the PKR dsRBD, containing both dsRBM1 and dsRBM2, and dsRBM1 alone to the same dsRNA truncation series in AU75 buffer (17, 19). The stoichiometries for the full-length enzyme are consistently lower for either domain construct across the range of RNA lengths. Presumably, the presence of the kinase domain and linker in PKR result in steric hindrance which increases the effective binding site size relative to the domain constructs.

The dependence of the limiting PKR binding stoichiometries on dsRNA length does not conform to standard models (29-31) for nonspecific binding of proteins to finite nucleic acid lattices. The stoichiometries are expected to regularly increase with RNA length, but for PKR they remain fixed at 2 PKR/RNA from 20-35 bp and only increase to 3 for the 40 bp sequence. In contrast, the stoichiometries for dsRBM1 binding to RNA increase strongly with length and conforms to a modified overlapping ligand binding model (19) where the motif binds to multiple faces of the dsRNA duplex and overlaps along the helical axis with a site size of 12 bp and a minimum overlap of 4 bp (17). The dsRBD also follows this model for RNAs up to a length of 30 bp but deviates strongly for longer RNAs, and the stoichiometries actually decrease between 35 and 40 bp. These data indicate a change in binding mode to a larger size with increasing RNA length. This model is supported by NMR mapping experiments showing that only the N-terminus of dsRBM2 interacts with a 20 bp dsRNA but extensive interactions are observed upon binding to the 40 bp sequence (17). In addition, affinity cleavage experiments demonstrate that simultaneous binding of both dsRBMs of PKR is correlated with PKR activation (32). We suggest that an analogous change of binding mode occurs as a function of RNA length for the full-length enzyme, resulting in an increase in the site size and a constant binding stoichiometry from 20-35 bp.

Although dimerization clearly plays a critical role in the PKR mechanism (8), the ability of a single dsRNA to bind 2 PKR monomers is not sufficient to induce autophosphorylation. In AU75 buffer the 20 and 25 bp RNAs each bind 2 PKRs yet are incapable of inducing measurable autophosphorylation (Figure 2). Previously, we suggested that the inability of these shorter RNAs to activate in AU200 is associated with low binding affinities, resulting in low population of the active $(\text{PKR})_2\text{:RNA}$ species (15). However, the 20 and 25 bp RNAs also do not activate in AU75, yet the binding affinities measured in this buffer are as strong as those measured for a 30 bp RNA in AU200 where this RNA is capable of activating PKR autophosphorylation. Thus, additional factors must be limiting the ability of short RNAs to induce the functional, active dimeric form of PKR. We propose that activation of PKR by longer RNAs requires a binding mode that inaccessible upon binding to shorter RNAs (Figure 3). Our RNA binding data indicate that this active binding state is characterized by a larger size. Possibly, this mode is required to allow for direct interaction of the kinase

domains to mediate functional dimerization. Experiments are now underway to directly test this hypothesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

dsRBD	dsRNA binding domain
dsRBM	dsRNA binding motif
eIF2α	eukaryotic initiation factor 2 α
PKR	RNA activated protein kinase
RMSD	root mean square deviation

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Figure 1.

Sedimentation velocity analysis of PKR binding to a 40 bp dsRNA in AU75. (A) Raw sedimentation velocity profiles for 40 bp dsRNA. For clarity, only every 13th scan is shown. (B) Normalized $g(s^*)$ distributions of 0.03 μM 40 bp dsRNA (black), dsRNA + 0.05 μM PKR (blue), dsRNA + 0.3 μM PKR (red), dsRNA + 0.8 μM PKR (cyan), dsRNA + 2 μM PKR (green), dsRNA + 7 μM PKR (brown). (C) Global analysis of difference curves. The data were subtracted in pairs to remove systematic noise and the four data sets at the PKR concentrations were fit to a 2:1 binding model using SEDANAL (27). 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 7th difference curve of the 165 scans that were fit is shown.

Figure 2.

Activation of PKR by varying length duplex RNAs in 75 mM NaCl. Experiments were performed as previously described (10) in AU75 buffer at 32°C. Each reaction contains 200 nM PKR, 0.4 mM ATP, 4 μCi [γ - ^{32}P]-ATP and the indicated amount of RNA. The reaction was allowed to proceed for 20 min at 32°C before quenching with SDS loading buffer and resolved on SDS-PAGE. 20bp dsRNA (black), 25bp dsRNA (blue), 30bp dsRNA (red), 35bp dsRNA (green), 40bp dsRNA (brown).

Figure 3. Model for PKR binding to short and long dsRNAs. (A) Domain organization of PKR. (B) Two PKRs are able to bind to the 20 bp dsRNA at low salt via dsRBM1. PKR binds to dsRNAs of ≥ 30 bp with a larger site size via interaction of both dsRBM1 and dsRBM2. This binding model facilitates dimerization of the kinase domains, resulting in PKR activation.

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Table 1
PKR-dsRNA dissociation constants measured in AU75 and AU200 buffers at 20°C

dsRNA (bp)	[NaCl] mM	Model ^a	K _{d1} (nM)	K _{d2} (nM)	K _{d3} (nM)	RMSE ^b
20	75	2	45.2 (35.2, 59.5)	54.1 (40.9, 69.9)	-	1.54
20	200	1	303 (263, 349)	-	-	1.50
25	75	2	7.8 (6.8, 9.0)	473 (391, 572)	-	1.83
25	200	1	438 (380, 497)	-	-	1.27
30	75	2	100.1 (83.1, 122.5)	22.6 (18.6, 26.8)	-	1.54
30	200	2	52.0 (46.0, 58.3)	6270 (3920, 11800)	-	1.31
35	75	2	7.6 (6.7, 8.6)	69.1 (50.8, 95.2)	-	1.67
35	200	2	61.1 (47.9, 72.8)	4910 (2950, 8260)	-	1.38
40	75	3	32.1 (27.3, 38.0)	52.4 (40.6, 67.8)	245 (212, 280)	1.70
40	200	2	38.1 (29.5, 47.3)	813 (645, 1070)	-	1.52

Parameters were obtained by global nonlinear least square analysis of sedimentation velocity experiments. The values in parentheses represent the 95% joint confidence intervals obtained using the F-statistic.

^aThe model is specified by the stoichiometry of PKR binding.

^bThe root mean square deviation of the fit. Because the fits were weighted by the standard deviation of the data at each point, a perfect fit corresponds to an RMSE of unity.