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STRUCTURAL BASIS FOR ALLOSTERIC REGULATION OF HUMAN RIBONUCLEOTIDE REDUCTASE BY NUCLEOTIDE-INDUCED OLIGOMERIZATION

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Abstract

Ribonucleotide reductase (RR) is an $\alpha_n\beta_n$ (RR1•RR2) complex that maintains balanced dNTP pools by reducing ribonucleoside diphosphates to deoxyribonucleoside diphosphates. RR1 is the catalytic subunit and RR2 houses the free radical required for catalysis. RR is allosterically regulated by its activator ATP and its inhibitor dATP, which regulate RR activity by inducing oligomerization of RR1. Here, we report the first X-ray structures of human RR1 bound to TTP-only, dATP-only, TTP•GDP, TTP•ATP, and TTP•dATP. These structures provide insights into ATP/dATP regulation of RR. At physiological dATP concentrations, RR1 forms inactive hexamers. We determined the first X-ray structure of the RR1•dATP hexamer and used single-particle electron microscopy to visualize the $\alpha_6\bullet\beta\beta'$ 1•dATP holo complex. Site-directed

¹ $\beta\beta'$ denotes the yeast RR2•RR4 hetero-dimer. While RR2 is a functional β subunit, RR4 (β') lacks key residues required for producing the free radical.

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ACCESSION CODES

The hRRM1•TTP structure has been assigned the PDB ID code 3HNC.

The hRRM1•dATP structure has been assigned the PDB ID code 2WGH.

The hRRM1•TTP•GDP structure has been assigned the PDB ID code 3HND.

The hRRM1•TTP•ATP structure has been assigned the PDB ID code 3HNE.

The hRRM1•TTP•dATP structure has been assigned the PDB ID code 3HNF

The ScRR1•dATP hexamer structure has been assigned the PDB ID code 3PAW

The 3D map of yeast RR holocomplex has been assigned the EMDB code EMD-1807

mutagenesis and functional assays confirm that hexamerization is a prerequisite for inhibition by dATP. Our data provide an elegant mechanism for regulating RR activity by dATP-induced oligomerization.

INTRODUCTION

Ribonucleotide reductase (RR) plays a crucial role in *de novo* DNA synthesis by reducing ribonucleoside diphosphates to 2'-deoxy ribonucleoside diphosphates¹ and maintains balanced pools of deoxynucleoside triphosphates (dNTPs) in the cell². RRs are divided into three classes, I to III, based on the method of free-radical generation^{3–8}. All eukaryotic organisms encode a class I RR, consisting of an $\alpha_n\beta_n$ multi-subunit protein complex, in which the minimally active form is $\alpha_2\beta_2$ ^{9,10}. The α or RR1 subunit contains the catalytic (C-site) and two allosteric sites¹¹, while the β or RR2 subunit houses a stable tyrosyl free radical that is transferred some 35 Å to the catalytic site to initiate radical-based chemistry on the substrate^{12,13}. RR is regulated transcriptionally¹⁴, allosterically¹ and, in the yeast *S. cerevisiae*, RR is further regulated by subunit localization¹⁵ and by its protein inhibitor Sml1¹⁶. In mammalian cells, RR activity is also controlled by the RR2 levels^{17,18}. Consistent with the varying RR2 levels, dNTP pools also vary with the phases of the cell cycle, reaching the highest concentration during S-phase^{17,19}.

RR is regulated by an intricate allosteric mechanism^{11,20}. The two allosteric sites of RR are the specificity site (S-site), which determines substrate preference, and the activity site (A-site), which stimulates or inhibits RR activity depending on whether ATP or dATP is bound^{11,21}. While it is known that dATP, which is less abundant in the cell than ATP, has a 100-fold higher affinity for RR^{22,23–24,25}, the mechanism for this is unknown. Most of our knowledge on RR has been derived from studies on the *E. coli* and other prokaryotic enzymes^{1,8,10,11,26–29}, which exist as $\alpha_2\beta_2$ hetero-tetramers³⁰. This organization failed, however, to mechanistically explain how dATP inactivates and ATP activates the enzyme. Recent biochemical studies suggest that both dATP and ATP regulate mouse RR (mRR) by altering its oligomeric state in an ATP/dATP concentration-dependent manner^{23,31}. The Cooperman group proposed that dATP induces an inactive mRR1 tetramer, while ATP induces active mRR1 dimers and hexamers²³. Interestingly, in a previous study Thelander and co-workers observed dATP and ATP induced tetramers for calf thymus RR1³². In contrast, the Hofer group proposed that both dATP and ATP induce mRR1 hexamers³¹. In fact, the cancer drug gemcitabine has been shown to inactivate a higher order oligomer of human RR³³. Additionally, recent reports suggest that the *E. coli* RR1 instead of forming hexamers forms tetramers³⁴. However, the structural basis for dNTP regulation by RR oligomerization is not known.

In the present study, we report the first X-ray crystal structures of human RR1 (hRRM1) in complex with TTP, dATP, TTP•GDP, TTP•ATP, and TTP•dATP. The TTP, dATP and TTP•GDP structures reveal the structural basis for substrate selection, while the TTP•ATP and the TTP•dATP structures explain the different affinities of the A-site for ATP and dATP. We also show that RR activity is lowest at high dATP concentrations when the hexamer population is high. In addition, we report the X-ray structure of *S. cerevisiae* RR1 (Yeast RR1) α_6 and a structure of the $\alpha_6\beta_6$ holo complex based on a single-particle electron microscopy (EM) 3D reconstruction. These structures provide a first glimpse of the structural basis underlying dATP-induced hexamerization, and, together with structure-function and mutagenesis data, provide an elegant model for regulation of RR by dATP-induced oligomerization.

RESULTS AND DISCUSSION

CRYSTAL STRUCTURE OF hRRM1

We report the first X-ray crystal structures of hRRM1 co-crystallized with (1) TTP bound at the S-site, (2) dATP bound at the S-site, (3) TTP bound at the S-site and GDP at the C-site, (4) TTP bound at the S-site and ATP at the A-site, and (5) TTP bound at the S-site and dATP at the A-site at resolutions of 2.4 Å, 2.3 Å, 3.2 Å, 3.1 Å, and 3.1 Å, respectively (Fig. 1a and Table 1). Electron densities for the first RRM1 structures with ATP and dATP bound at the A-site are shown in Figs. 1b and 1c.

To assess the similarity of hRRM1 with its class I and II homologues, the structures of RRM1 from *S. cerevisiae* (ScRRM1), *Escherichia coli* (EcRRM1), *Thermotoga maritima* (TmNrd1), and *Salmonella typhimurium* (StRRM1) were aligned with all four structures of the hRRM1 complexes in a pair-wise fashion (Supplementary Table 1). hRRM1 shares the highest structural homology with ScRRM1 with an RMSD of 0.8 Å, while EcRRM1, TmNrd1, and StRRM1 have RMSDs of 1.4 Å, 1.3 Å, and 1.3 Å, respectively. A full comparison and description of the C-site and the S-site are described in Supplementary Discussion.

ALLOSTERIC REGULATION AT THE ACTIVITY SITE

dATP functions as an allosteric inhibitor by having higher affinity for the A-site than ATP^{23,25}. Hence, dATP is able to outcompete ATP, which is more abundant in the cell than dATP. Compared to ATP, dATP thus binds the A-site with an approximately 13-times and 40 to 100-times better K_d for EcRRM1 and mRRM1, respectively^{23,25}. The structural basis for why dATP functions as an inhibitor and ATP as an activator has remained unknown. We have now determined structures of hRRM1 with ATP or dATP bound at the A-site (Figs. 1b, c and 2a, b). The A-site is formed by helices H1–H4, with H1 spanning residues 15–26, H2 spanning 36–46, H3 spanning 53–70, and H4 spanning 74–90 (Fig. 2c), that form a four-helix bundle. This ATP-binding cone is covered at one end by the previously named “ β -cap”, a β -hairpin formed by the first fourteen N-terminal amino acid residues and residues 48–51¹⁰.

While binding of both ATP and dATP requires helix H1 and the β -cap to shift from their positions in the native (TTP-only) form (Fig. 2c), resulting in some induced fit, the two binding modes show striking differences. dATP binds more deeply inside the four-helix bundle and its ribose adopts a half-chair conformation with the 2'-carbon out of plane. In contrast, ATP binds less deeply inside the four-helix bundle and its ribose adopts a 2'-endo conformation (Fig. 2c, d and Supplementary Fig. 1a). The distance between the positions of the 3'-OH of ATP and dATP is 2.4 Å (Fig. 2d). Using the program DPX³⁵ to calculate the atomic depth, the distance from the closest solvent accessible atom, dATP has a depth of 1.53 Å while ATP has a shallower depth of 1.33 Å. Furthermore, surface accessibility calculations with AREAIMOL³⁶ show that upon binding to the A-site ATP buries 297 Å², while dATP buries 310 Å². Since the binding energy of protein-ligand interactions can be expressed as a change in solvent-accessible surface area between the bound and unbound states³⁷, the deeper binding of dATP and the larger buried surface area compared to ATP, must contribute to its higher affinity. The different ribose conformations seen in the bound ATP and dATP molecules may further contribute to the difference in their affinities for the A-site.

It is the chemical difference at the 2' position on the ribose moiety between ATP and dATP that causes the difference in their binding at the A-site. The 2'-OH of ATP sterically precludes the ribose from binding more deeply inside the four-helix bundle, whereas dATP, lacking the 2'-OH, can penetrate the pocket more deeply. We propose that Ile 18 acts as a steric gate as it would clash with the 2'-OH of ATP binding at the dATP position (Fig. 2d

and Supplementary Fig. 1a). The 2'-carbon of the ribose in dATP makes several interactions with hydrophobic residues (Fig. 2b and Supplementary Table 2). Interestingly, while the 3'-OH of dATP makes no hydrogen bonds with hRRM1, the 3'-OH of ATP makes a hydrogen bond to the side-chain of Asp 57. The mutation of Asp 57 to Asn abolishes the ability of hRRM1 to discriminate between ATP and dATP at the A-site³⁸. In our ATP-bound structure, Asp 57 forms a salt-bridge with Arg 21, a residue that interacts with both the phosphate and ribose moieties (Fig. 2a). The mutation of Asp 57 to Asn is likely to abolish the salt-bridge and change the electrostatic environment of the A-site. The resulting loss of allosteric regulation by dATP is discussed below under dATP-induced oligomerization.

The phosphate groups of dATP and ATP bound at the A-site adopt different conformations (Fig. 2c, d). The phosphate moiety of bound ATP is held in place by a ring of positively charged residues (Lys 5, Arg 6, Lys 17, Arg 21, and Lys 88) (Fig. 2a). We also observe two magnesium ions interacting with the negatively charged phosphate groups in the ATP-bound structure, which appear to be absent in the dATP-bound form (Fig. 2a, b). In contrast to ATP bound at the A-site, the phosphate groups of bound dATP are extended rather than folded back to place the γ -phosphate close to the adenine ring, and Lys 17 no longer interacts with the γ -phosphate as in the ATP-bound structure (Fig. 2a, d). However, the difference in phosphate conformations between ATP and dATP bound at the A-site may be partly due to the absence of magnesium binding in the dATP structure.

The adenine ring in dATP binds more deeply in the nucleotide binding pocket than that in ATP. As a consequence, unlike ATP, dATP is not within hydrogen-bonding distance of the side-chain atoms of the surface residues Lys 5 and Glu 11. Conversely, the amide nitrogen of Met 14 hydrogen bonds with the adenine base of dATP, an interaction not observed in the ATP-bound structure (Fig. 2a, b). Both bases form hydrophobic interactions with residues Val 3, Ile 18, and Leu 56, which pack against both faces of the adenine ring (for clarity Leu 56 is not shown in Fig. 2a, b).

The only other RR1 structure with an effector bound at the A-site currently available is that of EcRR1 containing the bound ATP analogue AMPPNP³⁹ (Supplementary Fig. 1b). The adenine base of AMPPNP occupies a different position in EcRR1 from that of ATP in hRRM1 (Supplementary Fig. 1b). The differences in binding can be attributed to (1) different conformations of the β -cap, (2) displacement of the β -cap in EcRR1 towards solvent by at least 6 Å, and (3) the N-terminal portion of helix H1 in the EcRR1 structure protruding towards the ribose, unlike its position in the hRRM1 structure.

dATP-INDUCED OLIGOMERIZATION

We examined the dATP concentration-dependent oligomerization of wild-type and mutant hRRM1 using size-exclusion chromatography (SEC) and multi-angle light scattering (MALS) (Fig. 3 and Supplementary Fig. 2). Our measurements show that in the absence of any effector molecules, hRRM1 is a monomer (Fig. 3a, **blue line**). Interestingly, at a dATP concentration of 5 μ M, we observe a reduction in monomers with concomitant emergence of dimers and hexamers (Fig. 3a, **red line**). At a dATP concentration of 20 μ M, within the reported concentrations for S-phase^{17,19}, the hexamer dominates and less dimers are present (Fig. 3a, **green line**). These results have been independently corroborated by MALS (Supplementary Fig. 2a). At 20 μ M dATP, MALS shows peaks at 521 kDa and 185 kDa corresponding to an hRRM1 hexamer and dimer, respectively. Similar observations have been reported for the mouse RR1³¹. Interestingly, when the first 74 residues of hRRM1 belonging to the ATP-binding cone are deleted (t-hRRM1), dATP no longer induces the formation of hexamers. Instead, in the presence of hRRM2, dATP induces the formation of an $\alpha_2\beta_2$ holohuman RR complex. This result further illustrates the importance of the A-site for oligomerization (Fig. 3c).

The activity of the dATP-induced oligomers was assessed by *in vitro* functional assays. In the first assay the rate of dCDP formation was measured using [3H]-CDP as the substrate and ATP as the effector³³. In the second assay [14C]-ADP was used as the substrate and dGTP and ATP as the effectors. The specific activities of hRR and Yeast RR are given in Table 2. At increasing concentrations of dATP, when hRRM1 exists mainly as a hexamer, the specific activity of hRRM1 diminishes (Supplementary Fig. 2b). Similar results were observed for Yeast RR (Supplementary Fig. 2c).

These findings deviate from previous studies by Kashlan and co-workers that reported that the dATP tetramer is the least active RR form and that the hexamer was only observed at non-physiologically high dATP concentrations²³. This discrepancy may not be due to the results obtained by dynamic light scattering but rather to their interpretation. Since dynamic light scattering experiments performed on mixtures of oligomeric species lack the capability to resolve differences in radius that are less than a factor of 4–5 in magnitude, the reported value may well have been the average mass of all the species in solution. It is thus possible that the measured value of ~380 kDa in the presence of 10 – 100 μ M dATP reported by Kashlan and co-workers did not represent an RR tetramer but rather a mixture of dimers and hexamers.

X-RAY CRYSTAL STRUCTURE OF THE SCRR1-dATP HEXAMER

To obtain insight into the structural basis of RR1 oligomerization, we worked on crystallizing hRRM1 α_6 . Although dATP-induced hexamers of hRRM1 crystallized in a hexagonal space group, the crystals only diffracted to a resolution of 10 Å. We were able, however, to obtain crystals of dATP-induced hexamers of ScRR1 in the P6₃ space group that diffracted to 6 Å (Table 1). We expect that the ScRR1 hexamer is similar to the hRRM1 hexamer as they both form mainly inactive hexamers at 20 μ M dATP (Fig. 3a and Supplementary Fig. 2d), and the structures of the dimers are similar (Supplementary Table 1). We were able to determine a low-resolution structure of the ScRR1•dATP hexamer using molecular replacement. Since no structure exists of a ScRR1 with dATP bound at the A-site, the hRRM1•TTP•dATP structure (Fig. 1a) was used as the search model for molecular replacement. Rigid body refinement at 6.6 Å gave an R-free of 44.3% (Table 1), which, at this resolution, is indicative of a correct solution. Although low-resolution structures do not provide details at the atomic level, they are useful for considerations of oligomer organization.

The packing of the ScRR1 subunits in the crystal is consistent with two different hexamer models, which we will call models A and B (Fig. 4a, b). Multiple crystal packing arrangements are not uncommon, so it is necessary to identify the physiologically relevant one. In both models, the ScRR1 α_6 is a trimer of dimers, in which the three dimers are related to each other by a three-fold axis. However, models A and B differ in the diameter of the central pore of the hexamer and in how dATP would mediate hexamerization. In model A, only three of the six dATP-bound four-helix ATP-binding cones participate in forming the hexamer interface, leaving the other three free to interact with the small subunit (Fig. 4a, c). Hence, only three dATP molecules are at the hexamer interfaces. Unlike in model A, in model B the interfaces between the dimers that stabilize the hexamer are exclusively formed by the six dATP-bound four-helix ATP-binding cones (Fig. 4b). Each of the three interfaces is formed by two dATP-bound four-helix cones from adjacent RR1 dimers that contact each other in an antiparallel conformation and are related by 2-fold symmetry.

VALIDATION OF THE dATP-INDUCED HEXAMER BY SITE-DIRECTED MUTAGENESIS

The hexamer interfaces for both models were inspected for interactions that could be targeted by mutagenesis to disrupt hexamerization. Several site-directed mutations were

designed based on the interface in model B, but with the exception of D16R and H2E (Supplementary Fig. 3a), mutations in the N-terminal ATP-binding cone resulted in insoluble protein. Packing interactions in model A are poor when compared to model B, and only a single site-directed mutation, D182R, was chosen to test this model.

Using SEC, each mutant was tested for its ability to form hexamers at the approximate S-phase concentration of 20 μ M dATP. As previously observed, most of the wild-type protein formed hexamers at this dATP concentration (Fig. 3d, **green trace**). The H2E mutation resulted in a shift of the equilibrium from mainly hexamer with little dimer to more dimer and less hexamer, while the D16R mutation disrupted hexamer formation completely (Fig. 3d, **orange and purple traces**). The molecular weights of the oligomers formed by the D16R mutant protein were independently derived from MALS to be 190 kDa and 88 kDa, corresponding to a dimer and monomer, respectively (Supplementary Fig. 2e). To further confirm that Asp 16 was involved in hexamerization, the D16R mutation was also examined in ScRR1. As expected, ScRR1 D16R mutant protein also failed to form dATP-induced hexamers (data not shown), demonstrating that the same mechanism must underlie dATP-induced hexamerization of ScRR1 and hRRM1. Interestingly, the aforementioned D57N mutant that is not inhibited by dATP also forms dimers but not hexamers at physiological concentrations (Supplementary Fig. 3b)⁴⁰. The D182R mutation at the hexamer interface according to model A in ScRR1 did not disrupt dATP-induced hexamerization (data not shown).

Since the D16R mutation abolished the ability of hRRM1 to form dATP-induced hexamers, we hypothesized that D16R like D57N, would also prevent allosteric inhibition of hRRM1 by dATP at physiological concentrations. To test this hypothesis, purified wild-type, D16R, and H2E proteins were subjected to *in vitro* activity assays using [3H]-CDP and [14C]-ADP as substrates. The D16R and H2E mutant proteins retained 55% and 56% of the wild-type activity for CDP reduction and 67% and 56% of the wild-type activity for ADP reduction, respectively (Fig. 3e). The D16R mutant has similar activities in the presence or absence of 20 μ M dATP (Fig. 3f). Furthermore, circular dichroism spectroscopy showed that all mutants were properly folded (Supplementary Fig. 3c), and isothermal titration calorimetry showed that the D16R protein retained its ability to bind dATP (data not shown). Since the D16R and D57N mutants are not inhibited and do not form hexamers at physiologically relevant dATP concentrations, these results confirm that allosteric inhibition of RR by dATP under physiologic conditions requires hRRM1 to be in a hexameric form.

Both SEC/MALS and *in vitro* activity assays provide experimental evidence that the dATP-induced hexamer in solution takes the form of model B (Fig. 4b). Only site-directed mutations designed based on model B, but not model A, interfered with hexamer formation. To test whether mutants interfering with dATP-induced hexamerization also disrupt the interface needed for ATP-induced oligomerization, we conducted SEC analysis of the mutants in the presence of ATP. The D16R mutant, which was specifically designed to disrupt the dATP hexamer, retained its ability to form hexamers in the presence of ATP (Supplementary Fig. 3d). The H2E and D57N mutants were also able to form ATP hexamers (data not shown). Furthermore, we found that wild-type and mutant hRRM1 are similar in their apparent dissociation constant, $K_{d(\text{app})}$, for ATP binding (Supplementary Fig. 3d).

MODELING THE HOLO COMPLEX

To further examine how hexamer models A and B fit with published data, we performed modeling studies using the previously determined crystal structure of the StRR1•StRR2 $\alpha_2\beta_2$ holo complex³⁰ (see Supplementary Methods). In the case of model A, the ScRR2 subunits would bind to the outside of ScRR1•dATP α_6 , allowing up to six ScRR2 subunits to bind to ScRR1•dATP α_6 and, hence, permitting the formation of $\alpha_6\beta_2$, $\alpha_6\beta_4$, and $\alpha_6\beta_6$

complexes (Figs. 4c). In the case of model B, the ScRR2 subunits bind in the central cavity of ScRR1•dATP α_6 , which is only large enough to accommodate two small Yeast RR subunits. Therefore, the dATP-induced holo complex could only be $\alpha_6\beta_2$ (Fig. 4d).

SINGLE-PARTICLE EM RECONSTRUCTION OF THE YEAST RR•dATP HOLO COMPLEX

The ScRR•dATP holo complex was isolated using SEC. The molecular weight derived from SEC was consistent with an $\alpha_6\beta\beta'$ complex (Supplementary Fig. 4). EM images of the purified Yeast RR•dATP holo complex in negative stain revealed a homogeneous particle population (Fig. 5a), and class averages (Fig. 5a, small panels) showed particles with well-defined features consistent with different views of the holo complex (see Supplemental Methods). A 3D reconstruction of the holo complex in cryo-negative stain was then calculated using 50°/0° tilt pair images and the random conical tilt approach⁴¹ (see Supplemental Methods for details). The density map at 28 Å resolution (Fig. 5b) could be used to fit in the X-ray structures of the ScRR1•dATP hexamer and the ScRR2•ScRR4 hetero-dimer (Fig. 5c). The resulting model of the holo complex is consistent with an $\alpha_6\beta\beta'$ complex and, as the ScRR2•ScRR4 hetero-dimer is clearly located inside the ring formed by ScRR1•dATP α_6 , supports model B for the hexameric arrangement of ScRR1.

DISCUSSION

Based on structure-function data, we present a model that accounts for the down-regulation of RR activity by dATP-induced oligomerization (Fig. 5d). Our SEC/MALS data show that there is a dynamic equilibrium between the α , α_2 , and α_6 forms of hRRM1 and that the hexamer population increases with increasing dATP concentrations (Fig. 3a, Supplementary Fig. 2a and Ref. 31). In the absence of nucleotide effectors, RR1 exists as an inactive monomer. Binding of the effectors ATP, dATP, TTP or dGTP to the S-site causes RR1 to form dimers, which can then form $\alpha_2\beta_2$ hetero-tetramers. Partial occupation of the A-site by dATP may cause two α_2 subunits to associate into a transient tetramer intermediate that is not observable in our SEC/MALS experiments, presumably because once the S-site is occupied by nucleoside triphosphate effectors and the α_2 subunit is formed, higher-order oligomerization will occur by the association of sets of RR1 dimers rather than monomers with dimers. Hence, transient tetramers may be formed by the association of two RR1 dimers. The tetramers may be very unstable, and either fall apart again or immediately pick up an additional dimer to form a hexamer, making them difficult to observe. However, we cannot rule out the possibility that three dimers may associate to form a hexamer without the requirement of a tetramer. RR activity will also continue to diminish with the increase in dATP-induced hexamers in response to increased dATP levels. Consistent with previously published data³¹ and our EM structure, α_6 can associate with β to form an $\alpha_6\beta_2$ holo complex in the presence of dATP. Finally, when dATP levels become depleted during DNA replication or repair, dATP will dissociate from the A-site and active ATP-bound RR oligomers will be formed to replenish the dNTP supply.

Several groups have published data on the quaternary structure of mammalian RRs. Hofer's laboratory observed an $\alpha_6\beta_2$ complex for both ATP and dATP³¹, while the Cooperman and Stubbe laboratories observed ATP-induced $\alpha_6\beta_6$ holo complexes^{23,33}. The structure of the Yeast RR•dATP holo complex shows that only a dimer of the small subunit can be accommodated inside the hexamer pore, forming an $\alpha_6\beta_2$ complex (Fig. 5c). Since both ATP and dATP can form hexamers, how can dATP be an allosteric inhibitor while ATP is an allosteric activator? Our SEC data on the mutants suggest that dATP and ATP form different types of hRRM1 hexamers. If the ATP and dATP hexamers have different packing arrangements as our data suggest, this may offer clues to why ATP and dATP exert opposite allosteric effects on RR. It is conceivable that the conformational changes accompanying dATP hexamerization may lead to the disruption of free-radical transfer to the active site.

Indeed, while a higher resolution structure will be needed to be certain, our low-resolution model of the Yeast RR holo complex indicates that the small subunit (ScRR2●ScRR4) may bind further away from ScRR1 (Supplementary Fig. 5) compared to the StRR holo complex, which is considered to be an intermediate of the active form. The packing of the RR●ATP holo complex, on the other hand, may only lead to conformational changes that promote the activation of RR.

METHODS

Wild-type hRRM1 was expressed in *E. coli* BL21-CodonPlus-RIL (Stratagene). Cells were grown in TB, and the proteins were purified using peptide affinity chromatography. Crystallization experiments were carried out at room temperature by the hanging drop vapor diffusion method, and X-ray diffraction data were collected at the Advanced Photon Source. The different oligomers of hRRM1 and ScRR1 were characterized using SEC and MALS. ScRR1•dATP holo complex was purified by SEC and analyzed by EM. RR activities were determined using *in vitro* ¹⁴C-ADP and ³H-CDP reduction assays and the amount of [3H-CDP] and [14C]-ADP formed was quantified by liquid scintillation counting. Detailed experimental procedures are described in the supplement.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Structure of hRRM1: **(a)** Ribbon diagram of the hRRM1 dimer. Chains A and B are colored yellow and cyan, respectively. The four-helix ATP-binding cones of both subunits are shown in red. TTP (green), GDP (orange), and ATP/dATP (magenta) bound at the S-, C- and A-sites, respectively, are represented as transparent surfaces. **(b)** The four-helix cone with ATP-bound. $2F_o-F_c$ electron density for ATP (carbon, oxygen and nitrogen atoms are colored yellow, red and blue, respectively) contoured at 1σ is shown in green wire mesh. **(c)** The four-helix cone with dATP-bound. $2F_o-F_c$ electron density for dATP (carbon, oxygen and nitrogen atoms are colored black, red and blue, respectively) contoured at 1σ is shown in blue wire mesh.

Figure 2.

ATP and dATP binding at the A-site of hRRM1. **(a)** ATP binding. The protein is represented as ribbons. Carbon, oxygen and nitrogen atoms are colored in cyan, red, and blue, respectively. Interacting residues are drawn as sticks, and ATP is represented as sticks with yellow carbon and phosphorous atoms. Hydrogen bonds and electrostatic interactions are shown as black dashed lines. Magnesium atoms are represented as lime-green spheres. **(b)** dATP binding. The protein is represented as ribbons with carbons colored in magenta, and interacting residues drawn as sticks. dATP is represented as sticks with its carbon and phosphorous atoms colored in green. Hydrogen bonds and electrostatic interactions are shown as dashed black lines. **(c)** The ATP-binding cones of hRRM1•TTP•ATP (blue) and hRRM1•TTP•dATP (magenta) were aligned to that of hRRM1•TTP (orange). The proteins are represented as ribbons, and the ATP nucleotide is colored in yellow and the entire dATP nucleotide is colored in green. **(d)** Comparison of ATP and dATP binding by superposing **(a)** and **(b)**. The same coloring scheme as in **(a)** and **(b)** is used.

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Figure 3. SEC analysis of hRRM1 oligomers and enzyme activities of wild-type and mutant hRRM1. (a) The protein concentration of hRRM1 was kept at 1.25 μM in these experiments. hRRM1 forms monomers in the absence of dATP (blue trace) and a mixed population of monomers, dimers, and hexamers at a dATP concentration of 5 μM (red trace). At 20 μM dATP, the hexamers are the dominant species, with a small amount of dimer (green trace). (b) Standard curve used for the determination of molecular weights of RR. (c) SEC analysis of RR holo complex with t-hRRM1 and hRRM2. The t-hRRM1 in the presence of 20 μM dATP formed a dimer that eluted at a molecular weight of 186 kDa. When the two species were mixed, they eluted at a molecular weight of 278 kDa, corresponding to an $\alpha_2\beta_2$ holo complex. (d)

Wild-type (green trace), D16R (purple trace), and H2E (orange trace) hRRM1 proteins at a concentration of 10 μ M were tested for their ability to form hexamers in the presence of 20 μ M dATP. (e) The specific activity of wild-type and mutant hRRM1 was determined using [3H]-CDP and [14C]-ADP reduction assays. (f) The specific activity of D16R in the presence of 20 μ M dATP. [3H]-CDP reduction was carried out in the presence of 3mM ATP and with and without 20 μ M dATP. When [14C]-ADP was used as the substrate, D16R activity was measured in the absence of ATP and with and without 20 μ M dATP. Error bars illustrate s.d.

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Figure 4.

Hexameric packing of RR1 based on the low-resolution X-ray crystal structure of the ScRR1 hexamer. **(a and b)** Ribbon diagrams of the two possible hexamer packing arrangements.

ScRR1 monomers are colored in forest green and limon or blue and cyan. All the four-helix ATP-binding cones are colored in red. **(c)** Model of the RR holo complex based on the ScRR1 hexamer shown in **(a)** and the positions of the ScRR2 subunits modeled based on the StRR1•StRR2 holo complex. ScRR1 monomers are colored in forest green and limon with the ATP-binding cones in red. ScRR2 subunits are colored in purple and violet. **(d)** Model of the RR holo hexamer complex based on the ScRR1 hexamer shown in **(b)** and the positions of the ScRR2 subunits modeled based on the StRR1•StRR2 holo-complex. ScRR1

monomers are colored in blue and cyan with the ATP-binding cones in red. ScRR2 subunits are colored purple and violet.

Figure 5.

Electron microscopy of the $\alpha_6\bullet\beta\beta'$ •dATP holo complex. (a) Raw image of the holo complex in negative stain. The scale bar is 50 nm. The panels to the right show representative class averages. The side length of the individual panels is 35 nm. (b) Different views of the 28 Å density map calculated using the random conical tilt approach with 50°/0° image pairs of cryo-negatively stained holo complex. The scale bar is 5 nm. (c) Model of the $\alpha_6\bullet\beta\beta'$ •dATP holo complex. The grey contour shows the crystal structure of the RR1•dATP hexamer resolution-filtered to 28 Å. The golden contour shows the difference density obtained by subtracting the density of the RR1•dATP hexamer from the EM density map of the holo complex. The green ribbon diagram represents the RR1•dATP. (d) Schematic diagram of the assembly pathway. It shows the dimerization of α and α_2 subunits into an $\alpha_2\beta_2$ complex, followed by tetramerization to an α_4 complex, and finally hexamerization to an α_6 complex. The final complex is labeled $\alpha_6\beta_2$ and DEALWIS.

hexamer, and the red ribbon diagram represents the yeast $\beta\beta'$ heterodimer (PDB ID 1JKO; Ref,⁴²) docked into the difference peak. **(d)** Model for dATP-dependent oligomerization of eukaryotic RRs. Binding of effectors to the S-site causes dimerization, and binding of dATP to the A-site causes the formation of hexamers via a hypothesized short-lived tetramer intermediate or the immediate association of three dimers to form a hexamer (signified by ?). Effectors bound at the S-site are shown as magenta spheres, and dATPs bound at the A-site are shown as marine spheres.

Table 1

Data collection and refinement statistics (molecular replacement)

	hRRM1 +TTP	hRRM1 +dATP	hRRM1 +TTP + dATP	hRRM1 +TTP +ATP	hRRM1 +TTP +GDP	ScRR1 +dATP
Data collection						
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P6 ₃
Cell dimensions						
<i>a</i> , <i>b</i> , <i>c</i> (Å)	69.0,141.1,219.3	67.0,129.3,76.7	68.9,114.4,220.0	69.2,114.5,222.5	73.2,115.8, 221.3	166.5,166.5,381.7
<i>α</i> , <i>β</i> , <i>γ</i> (°)	90,90,90	90,95.5,90	90,90,90	90,90,90	90,90,90	90,90,120
Resolution (Å)	50.0–2.4	48.8–2.30	50.0–3.15	50.0–3.1	50.0–3.2	50.0–6.6
<i>R</i> _{sym} or <i>R</i> _{merge}	8.3(51.4)	10.0(45.0)	14.9(48.9)	11.5(45.3)	10.0(49.9)	12.2(47.9)
<i>I</i> / <i>σ</i>	13.1(2.3)	10.7(3.0)	10(2.5)	13(2.5)	12.8(3.1)	14.3(1.6)
Completeness (%)	97.9(97.7)	98.4(97.7)	90.1(92.6)	89.4(93)	98.9(99.2)	88.3(52.4)
Redundancy	3.9(3.0)	3.8(3.8)	5.5(4.8)	4.6(4.8)	4.9(4.8)	5.3(1.3)
Refinement						
Resolution (Å)	50.0–2.4	48.85–2.30	50.0–3.15	50.0–3.10	50.0–3.2	50.0–6.6
No. reflections	62082	51047	26016	27243	29455	9377
<i>R</i> _{work} / <i>R</i> _{free}	18.6/23.7	19.2/25.8	19.2/26.4	19.3/27.7	18.3/24.8	39.1/44.2
No. atoms	11770	10661	11545	11384	11510	23162
Protein	11327	10362	11391	11215	11337	23162
Ligand/ion	90	68	110	89	136	0
Water	353	231	44	80	37	0
<i>B</i> -factors						
Protein	45.0	26.1				
Ligand/ion	52.6	22.6				
Water	43.2	22.0				
R.m.s. deviations						
Bond lengths (Å)	.009	.017	.011	.015	.014	
Bond angles (°)	1.231	1.613	1.428	1.672	1.550	

* Single crystal was used for each data set.

* Values in parentheses are for highest-resolution shell.

Table 2

Specific Activities of Yeast RR and human RR

Species	[dATP] μM	Specific Activity ($\text{nmol min}^{-1}\text{mg}^{-1}$)	
Human		CDP Reductase [#]	ADP Reductase ^{\$}
hRRM2		1101.7	648.6
Wild type hRRM1	0	440.1	244.1
	20	104.5	40.3
<i>S.cerevisiae</i>			
ScR2R4		616.2	412.2
ScRR1	0	190.7	228.45
	20	46.7	46.7

^{\$} 3 mM ATP[#] 3 mM ATP 100 μM dGTP