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Role of Arginine 293 and Glutamine 288 in Communication between Catalytic and Allosteric Sites in Yeast Ribonucleotide Reductase

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Abstract

Ribonucleotide reductases (RRs) catalyze the rate-limiting step of *de novo* deoxynucleotide (dNTP) synthesis. Eukaryotic RRs consist of two proteins, RR1 (α) that contains the catalytic site and RR2 (β) that houses a diferrityrosyl radical essential for ribonucleoside diphosphate reduction. Biochemical analysis has been combined with isothermal titration calorimetry (ITC), X-ray crystallography and yeast genetics to elucidate the roles of two loop 2 mutations R293A and Q288A in *Saccharomyces cerevisiae* RR1 (ScRR1). These mutations, R293A and Q288A, cause lethality and severe S phase defects, respectively, in cells that use ScRR1 as the sole source of RR1 activity. Compared to the wild-type enzyme activity, R293A and Q288A mutants show 4% and 15%, respectively, for ADP reduction, whereas they are 20% and 23%, respectively, for CDP reduction. ITC data showed that R293A ScRR1 is unable to bind ADP and binds CDP with 2-fold lower affinity compared to wild-type ScRR1. With the Q288A ScRR1 mutant, there is a 6-fold loss of affinity for ADP binding and a 2-fold loss of affinity for CDP compared to the wild type. X-ray structures of R293A ScRR1 complexed with dGTP and AMPPNP–CDP [AMPPNP, adenosine 5-(β,γ -imido)triphosphate tetralithium salt] reveal that ADP is not bound at the catalytic site, and CDP binds farther from the catalytic site compared to wild type. Our *in vivo* functional analyses demonstrated that R293A cannot support mitotic growth, whereas Q288A can, albeit with a severe S phase defect. Taken together, our structure, activity, ITC and *in vivo* data reveal that the arginine 293 and glutamine 288 residues of ScRR1 are crucial in facilitating ADP and CDP substrate selection.

Keywords

ribonucleotide reductase; cancer; dNTP; ITC; X-ray crystallography

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The atomic coordinates and structure factors for wild type–dGTP–ADP, R293A–dGTP, Q288A–dGTP–ADP, wild type–AMPPNP–CDP, R293A–AMPPNP–CDP and Q288A–AMPPNP–CDP ScRR1 (codes 3S87, 3S8A, 3TBA, 3S8B 3S8C and 3TB9, respectively) have been deposited in the Protein Data Bank.

Introduction

Ribonucleotide reductases (RRs) use radical-based chemistry to catalyze the reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates and thus provide essential precursors for DNA synthesis.^{1,2} Hence, RR links the RNA and DNA worlds.³ All RRs are divided into three classes on the basis of their metallo-cofactors that are involved in radical generation.^{4–6} Eukaryotic RRs belong to class Ia that use a tyrosyl-based free radical.⁷ The holoenzyme of class Ia RR consists of two proteins RR1 (α) and RR2 (β). The budding yeast *Saccharomyces cerevisiae* has two RR2 proteins that form a heterodimer ScRR2-RR4 ($\beta\beta'$). RR2 (β) houses the diferric-tyrosyl radical cofactor ($\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}\text{-Y}\cdot$). RR4 (β') lacks key residues required for iron binding and is unable to assemble its own metallo-cofactor⁸ but essential for Fe binding and cofactor assembly in RR2 (β).⁹ The R1 is the business end of RR containing the catalytic and two allosteric sites.

Tight control of dNTP pools is vital for homeostasis; increased or decreased dNTP pools result in mutator phenotypes.^{10,11} RR plays a central role in maintaining both the levels and relative ratios of dNTPs; hence, it is highly regulated transcriptionally¹² and allosterically^{13–15} and, in *S. cerevisiae*, by subunit compartmentalization¹⁶ and by its protein inhibitor Sml1.^{17–20} The allosteric regulation involves three sites on RR1: the catalytic site (C-site), the specificity site (S-site) and the activity site (A-site). RR uses allosteric communication to determine C-site substrate preference based on the nucleotide effector bound at the S-site.^{13,21–23} ATP/dATP binding in the S-site promotes C-site selection for CDP or UDP, TTP binding selects for GDP and dGTP binding selects for ADP.¹ CDP reduction has been observed without a corresponding effector in *Escherichia coli* and mouse RRs, albeit at a reduced rate.^{24,25} Structural studies of *S. cerevisiae* RR1 (ScRR1) provide direct evidence that binding of a specific effector nucleoside triphosphate at the S-site accompanies characteristic conformational changes that favor binding of its cognate substrate at the C-site.^{13,23}

The RR1 loop 2 (residues 286–295, yeast numbering), spanning the S- and C-sites, is known to be required for substrate selection.²² The mechanism of selection mediated by loop 2 has been the subject of two crystallographic studies conducted on *Thermotoga maritima* class II RR¹³ and *S. cerevisiae* class I RR.²³ Both *T. maritima* class II RR and *S. cerevisiae* class I RR share a common allosteric mechanism involving key residues in loop 2. The apo form of *S. cerevisiae* RR holds loop 2 in a partially closed conformation, with no or very little room for substrate binding. Upon nucleoside triphosphate binding to the S-site, loop 2 shifts toward the effector site, concomitantly creating room for substrate binding. Once the substrate is bound, loop 2 yet again changes conformation and moves away from the effector toward the substrate. Two residues in loop 2, R293 and Q288, have been proposed to be critical for substrate recognition.²³ A recent study by Kumar *et al.* has shown the impact of a number of single point mutations in ScRR1 loop 2 on cellular dNTP pools and cell growth phenotype in the presence of ScRR3, an alternative RR1 that is highly induced under genotoxic stress.²⁶ Also, substitutions at multiple positions in loop 2 give rise to mutants bearing imbalanced dNTP pools; only R293A and Q288A exhibit synthetic lethality with deletion of ScRR3 based on meiotic progeny analysis,²⁷ pointing to significant roles played by Q288 and R293 in ScRR1.

To gain a detailed mechanistic understanding of the roles of Q288 and R293 in ScRR1 function, we have carried out X-ray crystallographic studies, isothermal titration calorimetry (ITC) assays of substrate/effector binding and *in vitro* and *in vivo* RR functional analyses on the R293A and Q288A ScRR1 mutants relative to the wild-type ScRR1. We demonstrate that the R293A and Q288A mutants both lead to significant loss of reductase activity toward substrates ADP and CDP. The decrease in reductase activities of the two mutants is well

correlated with a decrease in substrate binding affinity. X-ray structures of R293A complexed with dGTP and AMPPNP-CDP [AMPPNP, adenosine 5-(β,γ -imido)triphosphate tetralithium salt] reveal that ADP is not bound at the catalytic site, and CDP binds farther from the catalytic site compared to CDP binding in wild type. Q288A ScRR1-dGTP-ADP structures show that ADP binds at the catalytic site with a loss of a key interaction when compared to the wild type. In the Q288A ScRR1-AMPPNP-CDP structure, CDP binds similarly to wild type. However, loop 2 becomes disordered. Furthermore, we show that, in cells lacking ScRR3, ScRR1 R293A results in lethality, whereas Q288A can support mitotic growth, albeit with severe S phase defects. Our study highlights the important roles played by R293 and Q288 residues in maintaining the structural integrity of loop 2 for correct substrate recognition.

Results

Activity of wild-type, R293A and Q288A ScRR1

We have investigated the effects of the mutations R293A and Q288A on ScRR1 enzymatic activity by measuring the rate of ribonucleoside diphosphate reduction using an *in vitro* RR activity assay in the presence of excess RR2.^{28,29} We tested the structure-function relationship of one pyrimidine and one purine substrate by conducting RR activity assays using [^3H]CDP and [^{14}C]ADP as substrates. The specific activity for CDP reduction for the wild-type, R293A and Q288A ScRR1 when ScRR1 was limiting was 190 ± 10 , 50 ± 4 and 60 ± 7 nmol/min/mg, respectively. The specific activity for ADP reduction was 258 ± 12 , 10 ± 2 and 40 ± 3 nmol/min/mg for the wild-type, R293A and Q288A ScRR1, respectively. Under ScRR2 limiting conditions, the specific activity for CDP reduction for wild-type, R293A and Q288A ScRR1 was 354 ± 10 , 65 ± 5 and 70 ± 5 nmol/min/mg, respectively. The specific activities obtained for the wild-type ScRR1 are in agreement with previously reported values.³⁰ Hence, both R293A and Q288A mutations cause significant decreases in ADP and CDP reduction activities.

ITC studies of effector (dGTP) binding to wild-type, R293A and Q288A ScRR1

We have investigated dGTP binding to the wild-type ScRR1 by ITC. When dGTP was injected into the buffer alone, a relatively small exothermic heat change was observed (data not shown). A similar titration experiment with wild-type ScRR1 resulted in large exothermic heat changes exhibiting characteristic binding isotherms (Fig. 1a). The heat changes at various molar ratios of dGTP added to the wild-type ScRR1 could be best fitted to a one-binding-site model that gave a K_d of $0.27 \mu\text{M}$. The isotherm profile of dGTP binding to wild-type ScRR1 shows a high negative enthalpy change and a small negative entropic change, suggesting that the binding is enthalpically favorable while entropically unfavorable.

The ITC profiles of dGTP binding to R293A and Q288A ScRR1 are not significantly different from wild-type ScRR1 (Fig. 1b and g). The K_d calculated from the association constant derived from the binding isotherm of dGTP to R293A and Q288A ScRR1 is $0.57 \mu\text{M}$ and $0.61 \mu\text{M}$, respectively. There is a 2-fold difference in the K_d of dGTP binding to wild-type ScRR1 compared to either mutant ScRR1. The free-energy change ΔG , enthalpic change ΔH and $-T\Delta S$ are provided in Table 1. Taken together, the ITC data indicate that the R293A and Q288A substitutions at loop 2 have only mild effect on effector dGTP binding to ScRR1.

ITC studies of substrate (ADP) binding to the wild-type, R293A and Q288A ScRR1

We performed further ITC analyses to determine the effects of R293A and Q288A mutations on substrate (ADP) binding. Prior to ADP binding to wild-type ScRR1, the

protein was incubated with its effector dGTP (500 μ M) to ensure effector-based substrate selection,^{1,13,23} without effector binding, substrates are catalyzed only at 10% efficiency.³¹ The heat of dilution of the blank buffer gave a very small constant exothermic heat change. Titration experiment using the wild-type ScRR1 resulted in large exothermic heat change indicative of substrate ADP binding to the protein (Fig. 1c). The heat changes at various molar ratios of ADP to the wild-type ScRR1–dGTP could be best fitted to a single-site binding model that gave a K_d of 11 μ M. The ITC profile of the substrate ADP binding to wild-type ScRR1–dGTP shows a large negative enthalpic change, suggesting that the binding reaction is predominantly enthalpically driven. Moreover, a small positive entropic change was observed indicative of hydrophobic interactions during ADP binding.

In comparison to the wild-type ScRR1, ADP binding to R293A ScRR1 is very weak or undetectable as evident from the binding isotherm profile (Fig. 1d). Titration of substrate ADP to R293A ScRR1–dGTP led to no significant exothermic or endothermic heat changes (Fig. 1d). Instead, a very small exothermic heat change is observed at the various molar ratios of ADP. This small exothermic heat change upon ADP binding is comparable to the heat of dilution of ADP or very minimal binding. In contrast to R293A ScRR1, substrate ADP titration to Q288A ScRR1 does lead to significant exothermic heat change and that could be best fitted to a single-site binding model that gave a K_d of 65 μ M, 6-fold lesser than that of the wild-type protein (Fig. 1h). Taken together, the ITC profiles indicate that the R293A mutation severely impaired ADP binding, whereas Q288A significantly weakens the ADP binding at the C-site.

ITC studies of substrate CDP binding to wild-type, R293A and Q288A ScRR1

We also measured the binding of substrate CDP in the presence of the ATP analogue AMPPNP as the effector. Titration of CDP to the wild-type ScRR1–AMPPNP leads to a significant exothermic heat change reflecting binding. The integrated isotherm profile is best fitted to a one-site binding model that gave a K_d of 25 μ M (Fig. 1e). The CDP binding is predominantly enthalpy driven with a small positive entropic value, suggesting that hydrophobic interactions are also involved. Similarly, we have investigated CDP binding to the R293A and Q288A ScRR1–AMPPNP complex. Heat changes at various molar ratio of CDP could be best fitted to a one-site binding model that gave a K_d of 45 and 50 μ M for R293A and Q288A ScRR1, respectively (Fig. 1f and i). Similarly, in the case of the wild-type ScRR1, enthalpy is the major driving force for the CDP binding to R293A and Q288A ScRR1–AMPPNP with a small entropic contribution (Fig. 1e, f i and Table 1). The % error in data fitting for CDP isotherm profile is more than 10% but less than 20%, which is within acceptable limit in ITC analyses. The binding profile was repeated several times with similar results. Our data indicate that substrate CDP binding affinity is decreased by 2-fold in the R293A and Q288A mutants.

X-ray structures of wild-type, R293A and Q288A ScRR1

We used X-ray crystallography to gain further insight into the mechanism underlying the enzyme inactivation and difference in the binding of substrates upon R293A and Q288A mutations in ScRR1. Prior to the data collection, wild-type, R293A and Q288A ScRR1 crystals were soaked with 1 mM dGTP and 0.5 mM ADP or 1 mM AMPPNP and 0.5 mM CDP for 1–2 h. In spite of the fact that cocrystallization is a preferred method over soaking, we were unable to obtain co-crystals of the cognate effector–substrate complexes. Although the wild-type ScRR1 crystals were soaked with nucleotides at lower concentrations to those previously reported,²³ the structures appear to be identical. The three-dimensional structure of the wild-type ScRR1–dGTP–ADP, R293A ScRR1–dGTP, Q288A ScRR1–dGTP–ADP, wild-type ScRR1–AMPPNP–CDP, R293A ScRR1–AMPPNP–CDP and Q288A ScRR1–AMPPNP–CDP complexes were determined to 2.2 Å, 2.9 Å, 2.8 Å, 2.8 Å, 2.8 Å and 2.5 Å

resolutions, respectively. The structures were refined to an acceptable range of R and R_{free} values with good geometrical parameters (Table 2).

In the crystal structure of wild-type ScRR1–dGTP–ADP complex loop 2, dGTP at the S-site and ADP at the C-site are clearly visible in the $|F_o| - |F_c|$ omit map (Fig. 2a). Moreover, there is clear electron density for residue R293, which is important for substrate binding. In the case of R293A ScRR1, the electron density clearly shows that R293 has been mutated to alanine and dGTP is bound at the S-site (Fig. 2b). Interestingly, no detectable electron density is observed for the substrate ADP and side chain of residue Q288 on loop 2 in the R293A ScRR1 (Fig. 2b). The overall RMS difference for C α atoms between the wild type–dGTP–ADP structure and the R293A–dGTP structure is 0.37 Å. Although we do not observe the ADP binding in our crystal structure, we cannot rule out the possibility of the existence of a minor population of R293A ScRR1 where ADP is bound (since we observe 4% of enzyme activity compared to wild-type ScRR1). This is because a minor population is unlikely to be observed in a time- and space-average crystal structure. In the case of Q288A ScRR1–dGTP–ADP structure, electron density shows that Q288 has been mutated to alanine and both effector dGTP and substrate ADP binds to S- and C-sites, respectively (Fig. 2c).

In the wild-type and R293A ScRR1–AMPPNP–CDP structures, both AMPPNP and CDP are visible in the $|F_o| - |F_c|$ omit maps (Fig. 2d and e). Loop 2 appears to be mostly ordered; however, residues 290–295 of loop 2 undergo a conformational change accompanied by a significant movement toward the C-site. Thus, CDP binds farther away from the catalytic C218 due to steric restraint (Figs. 2e and 3a and b). In Q288A ScRR1–AMPPNP–CDP, AMPPNP and CDP are visible in the $|F_o| - |F_c|$ omit map (Fig. 2f). However, loop 2 is disordered.

Effect of R293A and Q288A mutations on ScRR1 function *in vivo*

To investigate the functional consequence of the R293A and Q288A mutations *in vivo*, we performed plasmid shuffle experiment in a $\Delta rnr1\Delta rnr3$ double mutant that was kept alive by a wild-type ScRR1 on a *URA3CEN* plasmid (one to two copies per cell).³² *TRP1CEN* plasmids expressing *RNR1* promoter-driven, N-terminally 3×Myc-tagged wild-type (^{Myc}WT) or mutant ScRR1 (^{Myc}R293A and ^{Myc}Q288A) were introduced into $\Delta rnr1\Delta rnr3$, and the transformants were grown on plates containing 5-fluoroorotic acid (5-FOA). *URA3* encodes orotidine-5'-monophosphate decarboxylase that converts 5-FOA to 5-fluorouracil, which is toxic to the cell. Therefore, growth on 5-FOA plates indicates loss of the *URA3CENRNR1* plasmid and that the ^{Myc}RR1 encoded by the *TRP1CEN* plasmid can provide the essential *RNR1* activity. The R293A ScRR1 failed to grow on 5-FOA plate, while Q288A ScRR1 gave rise to colonies that exhibited slower growth relative to the WT control (Fig. 4a). Failure of R293A to support mitotic growth is not due to change in protein expression, as the ^{Myc}WT, ^{Myc}R293A and ^{Myc}Q288A proteins were detected at comparable levels *in vivo* (Fig. 4b).

For determination of whether the slow growth phenotype of the Q288A mutant was due to a defective S phase, the WT- and Q288A-ScRR1-expressing cells were released from G1 arrest and monitored throughout the first cell cycle by flow cytometry. WT cells enter the first S phase ~50 min after being released from G1 (Fig. 4c). By contrast, the Q288A mutant exhibited a predominantly S phase profile even when grown asynchronously and a severe defect in S phase progression after G1 release (Fig. 4c).

Discussion

In this study, we investigated the *in vitro* and *in vivo* effect of mutations at two critical residues of ScRR1 loop 2, R293 and Q288. The R293A mutation severely compromised

ADP binding at C-site (Fig. 1d and Table 1). The crystal structure of the R293A ScRR1–dGTP complex shows that ADP is undetectable in the $|F_o| - |F_c|$ electron density map (Fig. 2b). This is not entirely surprising as ADP makes the greatest number of interactions with residue R293 when bound to wild-type ScRR1.²³ The loss of activity in Q288A ScRR1 is likely caused by the 6-fold decrease in ADP binding derived from the ITC data (Table 1). The loss of affinity for ADP binding can be rationalized by examining the crystal structure of the Q288A ScRR1–dGTP–ADP complex (Figs. 2c and 5). The Q288A mutation directly results in the loss of two hydrogen bonds formed by the glutamine side chain with ADP and with R293. The later interaction is important for positioning the guanidinium side chain of R293, which in the wild-type ScRR1 forms a hydrogen bond and a stacking interaction with ADP. The loss of the hydrogen bond and the stacking interaction contributes to the weaker binding of ADP to Q288A that may result in the loss of activity.

The major impact of the R293A mutation on CDP binding results in it binding farther from the catalytic site, which may partly explain the loss of activity (20% enzyme activity compared to wild type). Comparison of the wild-type ScRR1–AMPPNP–CDP structure with the R293A–AMPPNP–CDP (Fig. 3a and b) shows that the mutation results in a conformational change in loop 2 accompanied by a shift toward the C-site. The new position of loop 2 will cause a steric clash between P294 and CDP if it binds to its original position as observed in the wild-type ScRR1.²³ Thus, CDP binding in the R293A ScRR1 mutant is stabilized by new hydrogen bonds. N3 of the base interacts with the O atom of K292, O2 of the base interacts with the N of G246 and 2' oxygen of the ribose interacts with the O atom of S217 and the N atom of G247 (Fig. 3a and b). Our previous structure of ScRR1–AMPPNP–CDP indicates that R293 forms an inter-loop 2 hydrogen bond with N291 in the wild-type ScRR1–AMPPNP–CDP complex.²³ R293A mutation disrupts the abovementioned key hydrogen bond, which is required for stabilizing the loop 2 conformation (Fig. 3c). The loss of this hydrogen bond leads to a conformational rearrangement and movement of loop 2 toward loop 1 (residues 265–270). The new conformation of loop 2 is stabilized by several hydrogen bonds made by the main chain and side chain of Q288 to the loop 1 residues N270, T268 and T265 (Fig. 3c). Compared to the wild-type ScRR1, ribose–C-site interactions with catalytic residues are not present in R293A ScRR1 (Fig. 3b). As a consequence of this, all the catalytic residues are too far away from the substrate for catalysis to occur. It should be noted that we did observe a small amount of activity, and it is quite possible that there is a mixed population of CDP binding mode among the R293A mutant protein where the wild-type ScRR1-like binding would be the minor population responsible for the small amount of activity, while the major population is what we have observed in the crystal structure of the Q288A–AMPPNP–CDP mutant. Unfortunately, at the resolution of our structure, we are unable to resolve the different species.

The loss of CDP reductase activity for Q288A ScRR1 likely results from the 2-fold decrease in CDP binding affinity as evident from the ITC data. The Q288A mutation results in loss of van der Waals interactions, which may account for the weaker binding. Furthermore, in the Q288A–AMPPNP–CDP structure, loop 2 is disordered. This disordering may result from the loss of two hydrogen bonds by the glutamine side chain to the main-chain carbonyl and amide atoms of G246. The disordered loop 2 may lead to the disruption of the allosteric communication between the specificity and catalytic sites (Fig. 2f).

Our *in vivo* functional analyses in the $\Delta rnr1\Delta rnr3$ double mutant cells indicate that R293A ScRR1 cannot provide enough RR activity to support mitotic growth, whereas Q288A can. A previous study by Kumar *et al.* showed that R293A and Q288A were both synthetically lethal with $\Delta rnr3$.²⁷ The difference in the Q288A mutant phenotype may result from the different strategies in generating the double mutant. While no viable spores of the

$\Delta rnr3rnr1(Q288A)$ genotype arose from the cross between $\Delta rnr3$ and $rnr1(Q288A)$ in the previous study,²⁷ we were able to obtain viable $rnr1(Q288A)$ mutant cells using plasmid shuffle in a $\Delta rnr1\Delta rnr3$ strain. Thus, the compromised RR activity of the Q288A mutant may be sufficient for mitotic growth but not for spore germination. Although Q288A mutant is viable, it exhibits severe S phase defect, indicating that the residual RR activity greatly impedes DNA replication. Thus, our biochemical data demonstrate that R293A and Q288A mutations in ScRR1 almost abolish ADP reduction and markedly reduce CDP reduction. However, the Chabes group has previously shown that expressing the R293A and Q288A ScRR1 in yeast cells skewed the dNTP pool toward higher dATP/dCTP and dGTP/dCTP ratios.²⁷ While the decreased dCTP pool in the Q288A mutant is consistent with the observed low CDP reduction activity, the direction of the dATP/dCTP skewing in the R293A mutant is opposite to its *in vitro* activities. A likely explanation for the discrepancy between cellular dNTP pool distributions and *in vitro* RR activities is the uncharacterized contribution from ScRR3 in the study by Chabes group. Although deletion of ScRR3 alone has an apparent affect on cellular growth or dNTP pools, the level and activity of ScRR3 may make significant contribution to RR1 activity in ScRR1 loop 2 mutants,^{14,26} considering the severely compromised RR activity in the R293A and Q288A ScRR1. Moreover, the relative contribution of ScRR1 (α_2) and ScRR3 (α'_2) homodimers and heterodimer ($\alpha\alpha'$) toward the activity and specificity of RR1 also needs further characterization.

One of the crucial roles of RR is to maintain a balanced dNTP pool. Previous studies from other group and our current study show that RR achieves this by maintaining strict cognate effector–substrate recognition via exquisite conformational changes undergone by loop 2.^{13,23,27} Our study further underscores the importance of loop 2 in substrate selection and highlights the crucial roles of two residues, R293 and Q288, in maintaining proper loop 2 conformations for substrate recognition.

Materials and Methods

Expression and purification of ScRR1

The cloning and expression of wild-type ScRR1 have been previously reported in the references.^{23,33} Expression plasmids for the R293A and Q288A ScRR1 mutants were constructed by introducing the mutations into the wild-type ScRR1 expression plasmid using the Quick Change (TM) site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers used to mutate R293A and Q288A in ScRR1 are listed below.

R293A forward primer 5'-GGTGGTAATAAAGCCCCTGGTGCG-3'

R293A reverse primer 5'-CGCACCAGGGGCTTTATTACCACC-3'

Q288A forward primer 5'-CGTTATGTTGACGCTGGTGGTAATAAA-3'

Q288A reverse primer 5'-TTTATTACCACCAGCGTCAACATAACG-3'.

Activity assays

The activities of wild-type, Q288A and R293A ScRR1 were determined using *in vitro* [³H]CDP and [¹⁴C]ADP reduction assays as previously described.^{28,30} Briefly, the buffer solution (50 mM Hepes, 5% glycerol and 0.1 M KCl at pH 7.6) and the ScRR2-RR4 protein solution were brought inside a glove box under deoxygenated conditions. A solution of FeNH₄SO₄ containing 5 equivalents of Fe(II) per ScRR2-RR4 heterodimer was made based on the Ferozine assay.³⁴ This solution was added to the protein solution and incubated at 4 °C in the glove box. The protein solutions were removed from the glove box, and the oxygen saturated buffer was added. Excess iron was removed by S200 10/300 size-exclusion

chromatography. For determination of the specific activity of wild-type, Q288A and R293A ScRR1, the reaction mixture contained 2.1 μM ScRR2-RR4 and 0.3 μM wild-type or Q288A or R293A ScRR1 in an activity assay buffer of 50 mM Hepes (pH 7.6), 15 mM MgCl_2 , 1 mM ethylenediaminetetraacetic acid, 100 mM KCl, 5 mM DTT, 3 mM ATP and 1 mM [^3H] CDP (~ 3000 cpm/nmol). When assaying ADP reductase activity, the assay buffer contained 3 mM ATP, 100 μM dGTP and 1 mM [^{14}C]ADP (~ 3000 cpm/nmol). The reaction mixture was pre-incubated for 3 min at 30 $^\circ\text{C}$, and 30- μl aliquots were sampled at fixed time intervals after reaction initiation. The above reactions were quenched by placement in a boiling water bath, allowance for cooling and treatment with alkaline phosphatase. Product [^3H] dCDP or [^{14}C]dADP that formed during the reaction was separated from substrate [^3H]CDP or [^{14}C]ADP using boronate affinity chromatography.³⁵ The amount of [^3H] dCDP or [^{14}C]dADP formed was quantified by liquid scintillation counting using a Beckman LS6500 liquid scintillation counter.

Isothermal titration calorimetry

Effector (dGTP) and substrate (ADP or CDP) binding was performed by ITC using a VP-ITC₂₀₀ instrument (Microcal 432 Inc., Northampton, MA). For the wild-type ScRR1 dGTP titration experiment, 1.5 μl aliquots (except the first injection used, 0.5 μl) of 0.5 mM dGTP in binding buffer [50 mM Hepes buffer (pH 7.0), containing 5 mM MgCl_2 , 5 mM DTT and 5% v/v glycerol] were injected into the cell containing 20 μM wild-type ScRR1 protein in the same buffer at 25 $^\circ\text{C}$. A similar titration experiment was performed with the R293A and Q288A ScRR1 mutants. Blank titrations for wild-type, R293A and Q288A ScRR1 were conducted in the binding buffer at 25 $^\circ\text{C}$. To investigate ADP binding to wild-type, we saturated R293A and Q288A ScRR1 proteins with nucleotide dGTP in binding buffer prior to the assay. CDP binding to wild-type, R293A and Q288A ScRR1 was assayed in a similar manner, except the proteins were saturated with adenosine 5'-(β,γ -imido)triphosphate nucleotide (AMPPNP) instead. The integrated heat of injection, after correcting for the appropriate blank buffer, was used to fit the one-site binding model using Microcal Origin 7.0 (Microcal Inc.). The observed binding constants were used to calculate the Gibbs free-energy relationship (ΔG) using $\Delta G = -RT \ln K$ (obs), and ΔS and $-\Delta H$ were calculated from ΔG using the Gibbs free-energy equation $\Delta G = \Delta H - T\Delta S$.

Crystallization, soaking and X-ray data collection

The crystals were grown as described in the reference.³⁶ The soaking conditions were partly guided by our ITC data. Instead of using extremely saturating nucleotide concentrations as previously used,²³ we used 0.5 mM ADP in our soaking experiments. Crystals were incubated for 1 h in reservoir solution containing 20–25% polyethylene glycol 3350, 0.2 M NaCl and 100 mM Hepes (pH 7.5) with 1 mM effector and 0.5 mM substrate. Effector dGTP was assayed with ADP as substrate, and effector AMPPNP (ATP analogue) was used instead of ATP in order to compare with our previously reported structure assayed with CDP.²³ Moreover, AMPPNP is less susceptible to hydrolysis than ATP.³⁷ Subsequently, crystals were soaked in 20–25% polyethylene glycol 3350, 0.2 M NaCl and 100 mM Hepes (pH 7.5) supplemented with 20% glycerol and cryo-cooled. All the data for each complex were collected from a single flash-cooled crystal at cryogenic temperatures at the beam-lines NECAT 24IDC-24IDE and GMCA ID-23B at the Advanced Photon Source using a Quantum-315 CCD and a MAR-300 detector, respectively. All the crystals belong to the orthorhombic space group $P2_12_12$, with unit cell parameters as given in Table 2. The data were integrated and scaled using HKL2000.³⁸

Structure determination

The complex crystals are all isomorphous to the native $P2_12_12$ form (Protein Data Bank codes: 2CVX and 2CVU), and the structures were directly determined by the difference

Fourier technique. The graphics software Coot³⁹ was used for model building interspersed with refinement using REFMAC5.⁴⁰ To avoid model bias, we have excluded 10% of reflections as that of previously reported in wild-type structure.²³ The final models were evaluated with PROCHECK.⁴¹ Bound substrates and effectors were located using $|F_o| - |F_c|$ electron density maps. The electron density for ligands was confirmed by calculating omit maps using the program CNS1.2.⁴² Figures were prepared in PyMOL.⁴³

Yeast strains, immunoblotting and cell cycle analysis

The yeast strain used in this study were MHY798 of genotype *MATa, rnr1::HIS3, rnr3::kan, ade2, can1, his3, leu2, trp1, ura3, pMH352 (URA3CENRNR1)*. Plasmid pMH975 encoding N-terminally 3×Myc-tagged ScRR1 was derived from pRS314 (*TRP1CEN*). R293A and Q288A mutations were introduced into pMH975 by site-directed mutagenesis and confirmed by sequencing of the entire coding region. Yeast cells were grown in rich YPD medium (1% yeast extract, 2% peptone and 2% glucose) or defined medium (6.7 g/l yeast nitrogen base without amino acids from Difco and 2% glucose) with appropriately supplemented amino acids.

For immunoblotting to detect the ScRR1 protein, trichloroacetic acid was employed to extract protein from 1×10^7 to 1×10^8 mid-log-phase cells for each loading as described previously.⁴⁴ Proteins were resolved by 8% SDS-PAGE, transferred to nitrocellulose membranes and probed with primary and secondary antibodies. Blots were developed by an enhanced chemiluminescence substrate (Perkin-Elmer). Cell cycle synchrony and flow cytometry analysis were performed as described previously.⁴⁵

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Abbreviations used

ScRR1	<i>Saccharomyces cerevisiae</i>
RR1	RR, ribonucleotide reductase
ITC	isothermal titration calorimetry
5-FOA	5-fluoroorotic acid

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Fig. 1.

ITC profiles of effector (dGTP) and substrate (ADP or CDP) binding to wild-type, R293A and Q288A ScRR1. Effector and substrate binding was derived from the nonlinear least-squares fit of the isotherms. The isotherm profile of dGTP, ADP and CDP to the wild-type, R293A and Q288A ScRR1 could be best fitted to the one-site binding model. (a) Binding isotherm of dGTP to the wild-type ScRR1. (b) dGTP binding to the R293A ScRR1. (c and d) Binding isotherms of substrate ADP to dGTP-saturated wild-type and R293A ScRR1. (e and f) ITC profiles of substrate CDP binding to wild-type ScRR1 and R293A ScRR1. (g) Binding isotherm of dGTP to the Q288A ScRR1. (h) Binding isotherm of ADP to the Q288A ScRR1. (i) Binding isotherm of CDP to the Q288A ScRR1.

Fig. 2.

Stereo view of effectors and substrates (dGTP–ADP complex or AMPPNP–CDP) bound to wild-type, R293A and Q288A ScRR1. (a) $|F_o| - |F_c|$ electron density for effector dGTP, loop 2 and substrate ADP of wild-type ScRR1 (blue density) contoured at 3σ . (b) $|F_o| - |F_c|$ electron density for effector dGTP and loop 2 of R293A ScRR1 (blue density) contoured at 3σ . (c) $|F_o| - |F_c|$ electron density for effector dGTP and loop 2 of Q288A ScRR1 (blue density) contoured at 3σ . (d) $|F_o| - |F_c|$ electron density for effector AMPPNP, loop 2 and substrate CDP of wild-type ScRR1. (e) $|F_o| - |F_c|$ electron density for effector AMPPNP, loop 2 and substrate CDP of R293A ScRR1 (blue density) contoured at 3σ . (f) $|F_o| - |F_c|$ electron density for effector AMPPNP, disordered loop 2 and substrate CDP of Q288A ScRR1 (blue density) contoured at 3σ .

Fig. 3. Comparison of AMPPNP- and CDP-bound wild-type and R293A ScRR1 structures. (a) Stereo view of the substrate CDP binding and its proximity to loop 2. (b) Comparison of catalytic site of wild-type and R293A ScRR1 with CDP bound. All catalytically important residues and their interactions with the wild-type ScRR1 CDP substrate are shown as broken lines. (c) Effect of the mutation on the loop 2 conformation. In all of the panels, wild-type ScRR1 is colored magenta and R293A ScRR1 is colored green and the interactions are shown as broken lines.

Fig. 4.

Growth defects of *rnr1(R293A)* and *rnr1(Q288A)* mutants. (a) R293A ScRR1 causes lethality. *TRP1CEN* plasmids harboring N-terminally 3×Myc-tagged wild-type *RNR1* (WT), *rnr1(Q288A)* and *rnr1(R293A)* were introduced into a $\Delta rnr1\Delta rnr3$ double deletion strain that was kept alive by a copy of wild-type untagged *RNR1* on a *URA3CEN* plasmid. Growth on 5-FOA-containing plates indicates loss of the *URA3CENRNR1* plasmid and that the 3×Myc-tagged Rnr1 expressed from the *TRP1CEN* plasmid can provide the essential *RNR1* activity. (b) *In vivo* expression of Q288A and R293A ScRR1 proteins. Left panel: $\Delta rnr1\Delta rnr3$ mutant cells harboring both the *URA3CENRNR1* plasmid and the *TRP1CEN* plasmid encoding 3×Myc-tagged wild-type (^{Myc}WT), R293A or Q288A ScRR1 (^{Myc}R293A

and ^{Myc}Q288A) were grown to log phase and harvested for protein extraction and Western blotting with anti-Rnr1 and anti-Myc antibodies. Right panel: Western blotting detection of Rnr1 proteins using the polyclonal anti-Rnr1 antibodies in whole-cell extracts. Lanes 1 and 2 were from cells that grew on 5-FOA-containing plate. Only the 3×Myc-tagged Rnr1 (^{Myc}WT and ^{Myc}Q288A) were detected by anti-Rnr1. Lane 3 is from cells without selection on the 5-FOA plate; both untagged wild-type (the lower band, WT) and 3×Myc-tagged (the upper band, ^{Myc}R293A) Rnr1 proteins were detected. (c) Comparison of cell cycle progression of the wild-type and the *rnr1*(Q288A) mutant cells by flow cytometry. $\Delta rnr1\Delta rnr3$ double deletion strains harboring *RNR1*(WT) or *rnr1*(Q288A) were synchronized in G1 phase of the cell cycle by α -factor-mediated arrest and then released into cell cycle by washing off α factor. Cells were harvested at 10-min intervals at room temperature (23 °C) and processed for flow cytometry analysis. The shaded profiles are cells from asynchronously growing, log-phase cultures.

Fig. 5.
Comparison of ADPbound wild-type and Q288A ScRR1 structures. Wild-type ScRR1 is colored green, and Q288A ScRR1 is colored magenta.

Table 1

Thermodynamic parameters derived from the binding of dGTP, ADP and CDP with wild-type, R293A and Q288A ScRR1

Protein	Number of binding sites	Ligand	K_d (dissociation constant, μM)	ΔG (kcal mol ⁻¹)	ΔH (kcal mol ⁻¹)	$-T\Delta S$ (kcal mol ⁻¹)
Wild-type ScRR1	0.97 ± 0.0103	dGTP	0.27	-8.9	-10.5	1.6
R293A ScRR1	1.08 ± 0.013	dGTP	0.57	-8.5	-10.6	2.1
Q288A ScRR1	1.07 ± 0.007	dGTP	0.52	-8.5	-10.1	1.6
Wild-type ScRR1	1.2 ± 0.12	ADP	11	-6.7	-6.6	-0.1
R293A ScRR1	Nd ^a	ADP	Nd ^a	Nd ^a	Nd ^a	Nd ^a
Q288A ScRR1	0.96 ± 0.1	ADP	68	-5.6	-4.3	-1.3
Wild-type ScRR1	1.07 ± 0.018	CDP	25	-6.2	-4.5	-1.7
R293A ScRR1	1.08 ± 0.03	CDP	45	-5.4	-2.8	-2.6
Q288A ScRR1	1.2 ± 0.13	CDP	44	-5.9	-3.3	-2.6

^aNote that substrate ADP titration to R293A ScRR1 does not involve a significant heat change (weak signal), and hence, thermodynamic parameters were not obtained.

Table 2
Data collection and refinement statistics for X-ray crystal structures of wild-type, R293A and Q288A ScRR1 complexes

	Name of data set					
	Wild-type ScRR1- dGTP-ADP	R293A ScRR1-dGTP	Q288A ScRR1- dGTP-ADP	Wild-type ScRR1- AMPNP-CDP	R293A ScRR1- AMPNP-CDP	Q288A ScRR1- AMPNP-CDP
Space group	$P2_12_12$	$P2_12_12$	$P2_12_12$	$P2_12_12$	$P2_12_12$	$P2_12_12$
Cell dimensions a , b , c (Å)	65.5, 107.6, 117.2	64.8, 108.4, 117.7	67.7, 107.7, 118.0	64.9, 107.8, 117.5	64.1, 107.7, 116.8	64.3, 107.9, 117.0
Wavelength (Å)	1.03	1.03	0.98	0.98	0.98	0.98
Resolution (Å)	20.04–2.25	20.4–2.90	41.13–2.80	49.03–2.80	40.29–2.77	43.50–2.53
Monomer per asymmetric unit	1	1	1	1	1	1
Unique reflections	39,265	19,963	20,226	21,212	20,834	28,484
R_{sym}^a , $I/\sigma(I)$	7.2 (54.4) ^b	13 (54.2)	9.0 (52.2)	13.2 (52.4)	14.4 (58.7)	10.5 (51.9)
I/σ	15 (2.1)	10.1 (2.2)	16.0 (2.4)	12.5 (2.8)	15.2 (2.2)	25.0 (5.0)
Completeness (%)	99.8 (95.4)	96.5 (98.0)	96.0 (94)	98.8 (97.0)	98.3 (99.6)	99.9 (100)
Redundancy	5.0 (3.6)	4.1 (4.3)	5.7 (5.6)	3.4 (3.4)	7.2 (7.4)	7.9 (8.0)
<i>Refinement</i>						
Resolution (Å)	20.00–2.25	20.00–2.90	41.13–2.80	49.03–2.80	40.29–2.77	43.50–2.53
Number of reflections	35,900	16,450	16,821	18,651	18,704	25,067
$R_{\text{work}}/R_{\text{free}}^c$	20.4/26.0	22.7/29.9	19.5/26.3	20.8/25.0	19.6/25.50	19.6/23.7
Number of atoms	5540	4997	5353	5328	5350	5278
Protein	5244	4980	5234	5229	5255	5107
Ligand/ion	58	31	58	57	57	57
Water	238	14	61	42	38	114
Average B -factor						
Protein	47.6	65.6	55	47.4	57	40.1
Ligand/ion	38	69.5	67.7	57	57	51.3
Water	45.8	47.2	47	41.6	49.5	39.8
RMSDs from ideal						
Bond length (Å)	0.014	0.014	0.012	0.010	0.017	0.012
Bond angle (°)	1.6	1.6	1.6	1.3	1.7	1.5
Ramachandran plot, residues in (%)						

	Name of data set					
	Wild-type ScRR1–dGTP–ADP	R293A ScRR1–dGTP	Q288A ScRR1–dGTP–ADP	Wild-type ScRR1–AMPPNP–CDP	R293A ScRR1–AMPPNP–CDP	Q288A ScRR1–AMPPNP–CDP
Core regions	87.9	85.1	86.5	88.0	89.4	90.4
Allowed regions	11.0	13.1	12.6	10.7	9.2	8.2
Generously allowed regions	1.0	1.9	0.9	1.4	1.4	1.4
Disallowed regions	0.0	0.0	0.0	0.0	0.0	0.0

^a $R_{\text{Sym}} = \sum_{hk} E_i^2 I_k(hkl) - \langle I(hkl) \rangle \sum_{hk} E_i I_k(hkl)$, where $I_k(hkl)$ is the k th observation of reflection hkl and $\langle I(hkl) \rangle$ is the weighted average intensity for all observations i of reflection hkl .

^b Values in parenthesis are used for the highest-resolution shell.

^c R_{work} and $R_{\text{free}} = \sum ||F_o| - |F_c|| / \sum ||F_o|$, where F_o and F_c are the observed and calculated structure factor amplitudes, respectively. For the calculation of R_{free} , 10% of the reflection data were selected and omitted from refinement.