Glutamate 52-β at the α/β subunit interface of *Escherichia coli* class Ia ribonucleotide reductase is essential for conformational gating of radical transfer

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Ribonucleotide reductases (RNRs) catalyze the conversion of nucleoside diphosphate substrates (S) to deoxynucleotides with allosteric effectors (e) controlling their relative ratios and amounts, crucial for fidelity of DNA replication and repair. *Escherichia coli* class Ia RNR is composed of α and β subunits that form a transient, active αβ2 complex. The E. coli RNR is rate-limited by S/e-dependent conformational change(s) that trigger the radical initiation step through a pathway of 35 Å across the subunit (α/β) interface. The weak subunit affinity and complex nucleotide-dependent quaternary structures have precluded a molecular understanding of the kinetic gating mechanism(s) of the RNR machinery. Using a docking model of αβ2 created from X-ray structures of α and β and conserved residues from a new subclassification of the *E. coli* la RNR (Iag), we identified and investigated four residues at the α/β interface (Glu350 and Glu52 in β2 and Arg129 and Arg639 in α2) of potential interest in kinetic gating. Mutation of each residue resulted in loss of activity and with the exception of E52Q-β2, weakened subunit affinity. An RNR mutant with 2,3,5-trifluorotyrosine radical (F3Y122) replacing the stable Tyr122 in WT-β2, a mutation that partly overcomes conformational gating, was placed in the E52Q background. Incubation of this double mutant with Hisα-α2/S/e resulted in an RNR capable of catalyzing pathway-radical formation (Tyr356-β2), 0.5 eq of dCDP/F3Y122, and formation of an αβ2 complex that is stable in pulldown assays over 2 h. Negative stain EM images with S/e (GDP/TTP) revealed the uniformity of the αβ2 complex formed.

Ribonucleotide reductases (RNRs) are macromolecular machines that convert nucleoside diphosphates (NDP) to deoxynucleoside diphosphates (dNDP) supplying de novo the pools of monomeric building blocks required for DNA biosynthesis, and controlling in a sophisticated fashion the relative ratios of these pools and their amounts, essential for fidelity of DNA replication and repair (1–3). The class Ia RNRs are found in both humans and *Escherichia coli*, with the latter serving as the prototype that has been studied for decades. Despite this, the molecular structure of the machine and its gymnastics on binding nucleotides at three distinct sites still remains a mystery. These proteins are composed of two subunits, α and β, which in the case of the *E. coli* RNR form an active αβ2 complex (3, 4). The NDP substrates, dNTP, and ATP allosteric effectors bind in three sites within α: the catalytic site (C-site), the specificity site (S-site), which controls which NDP is reduced, and the activity site (A-site), which controls the rate of turnover (5–7). The β subunit contains the diferric-tyrosyl radical (Y122∗) cofactor essential for nucleotide reduction (8, 9). The initiation of nucleotide reduction requires oxidation of Cys439-α by Tyr122∗-β over a distance of 35 Å, utilizing a specific pathway shown in Fig. 1 (3, 4, 10).

Although studies from the Eklund lab (4, 11) have provided us with atomic resolution structures of α and β, the structure of the active complex has remained a challenge as the subunit interactions are weak (0.2 to 0.4 μM) even in the presence of NDPS and dNTPs and β can act catalytically (12–15). In addition, the C-terminal 30 to 35 residues of all βs are always disordered and this is the region that Sjöberg and co-workers (13) showed was largely responsible for α/β affinity. Within this disordered tail of β reside the conserved residues: Tyr356 and Cys439. Tyr356 is an essential component of the 35-Å pathway involved in the oxidation of Cys439 where NDP reduction occurs (3, 13, 16, 17). Glu350, which has recently shown, plays an essential role in initiation of the conformational gating of this long-range oxidation when NDPS and dNTPs bind to α (18).

5 The abbreviations used are: RNR, ribonucleotide reductases; NDP, nucleoside diphosphates; dNTP, deoxynucleoside diphosphates; RT, radical transfer; UAA, unnatural amino acid; SEC, size exclusion chromatography; N3CDP, 3′-azido-2′-deoxycytidine diphosphate; F3Y122, 2,3,5-trifluorotyrosine radical; Ni-NTA, nickel-nitrilotriacetic acid; TR, thioredoxin; TRR, thioredoxin reductase; NO2Y122∗, 3-nitrotyrosine radical.

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Briefly, initiation of the radical transfer (RT) process is thought to involve proton transfer from the water on FeI in the diferriec-Y cofactor to Tyr122, and electron transfer from Tyr356 forming the Tyr122 phenol (Fig. 1) (18–21). Recent RT studies and new Ia RNR subclassifications (22) have helped us to identify conserved residues that could play an important role at the subunit interface of the RNR in conformational gating. The results of these studies are reported herein.

Currently our thinking about the RNR structure is governed by a docking model of αββ generated by Eklund and co-workers (3, 4) using the crystal structures of α2 and β2 and their shape complementarity. Their model is supported by four distance measurements (3) made using pulsed electron electron double resonance (PELDOR) spectroscopy and recent biophysical studies including small angle X-ray scattering and single particle electron microscopy (EM) (23–25). For the most part these methods have taken advantage of RNRs with site-specific incorporations of unnatural amino acids (UAAs) (3).

Using E350X-α2β2 (X = Ala, Asp, or Gln) mutants in WT and mutant backgrounds in which Tyr122 was replaced with tyrosine analogs that are hotter oxidants (3-nitrotyrosine, NO2Y122 or 2,3,5-trifluorotyrosine, F3Y122) (20, 31), we found an inability of E350X-β2 to initiate RT even in the case of the Glu to Asp substitution (18). This result suggested that charged residues might play an important role in gating RT at the interface where the Glu350 residue resides.

This paper focuses on our efforts to identify additional interface residues using mutagenesis and our ability to site-specifically incorporate UAAs into each subunit. Recently using sequence information, the class Ia RNRs (rnrdb.pfitmap.org) now designated NrdAg and NrdBg were subcharacterized (22). This information and our current structural understanding of αβ2 resulted in the identification and examination of mutations in four conserved residues: Glu52 and Glu350 in β2 and Arg329 and Arg639 in α2 in E. coli RNR. The inactivity of the E. coli mutants established that these residues are essential and the binding studies of α/β interactions established that with the exception of E52Q, the binding affinities decreased 5–20-fold relative to WT-α/β. The tight affinity and inactivity of the E52Q mutant led to further investigation of its properties in the F3Y122 background. Unlike WT-β2, F3Y122-β2 results in partial uncoupling of the conformational gating that rate limits NDP reduction (20, 32) by rapidly producing the Tyr356- (now detectable). It is likely being reduced to the F3Y122-O− (phenolate) instead of the phenol (18). Despite the inactivity of E52Q-β2, the double mutant, E52Q/F3Y122-β2, when incubated with α2/S/e (CDP/ATP or GDP/TPP) resulted in formation of 0.5 eq of the Tyr356 intermediate and in the case of CDP, 0.5 eq of dCDP per F3Y122. Pull-down experiments of the α/β mixture after 5 min and 2 h using a His6-α, gave a high recovery of a 0.6–0.8/1.0 ratio of subunits in the β/α complex. Negative
served residue in the disordered C-terminal tail of ND1 analog binds in the active site and is enzymatically converted to a nitrogen-centered nucleotide radical (N'), that becomes covalently bound to a cysteine in the active site. The inactivation is stoichiometric with the WT-β2, with complete loss of activity resulting from 1 Tyr122/β2 being converted to 0.5 eq of N', leaving 0.5 eq of the Tyr122 remaining (34–36). This unusual stoichiometry is associated with the half-sites reactivity of all class I RNRs. The N' has been extensively characterized by isotopic labeling and EPR methods. With mutant β2s the rate of formation of N' is often slow and the radical is quenched slowly with time; the kinetics often preclude N' detection, thus analysis of total radical loss as a function of time is monitored (13). The results of experiments in which E52X-β2/α2/N3CDP/TTP (X = Ala, Asp, and Gln) were incubated and analyzed by EPR over 120 min are summarized in Fig. 3A (33). No N’ is observed and the total Tyr122 varies no more than 10% over the 2-h time period. With WT-β2, 0.5 eq of N’ is formed within 30 s. Thus, no activity of E52X mutants is apparent by this method either.

A third method to assess RNR activity is to place E52X into a different background: specifically one in which the Tyr122 is replaced with F3Y122. The F3Y122-β2 mutant when incubated with α2/CDP/ATP has been studied in detail and shown to generate dCDP and the pathway Tyr356→Tyr122 within 10 sec in the first turnover and then reoxidize the putative F3Y122-O→F3Y122 in the rate-limiting step in the steady-state (20). This mutant is a hotter oxidant than Tyr122 and disrupts conformational gating of the RT process (20, 32, 37). The E52Q mutant in this background has 0.1% WT activity (Table 1), likely associated with endogenous levels of co-purifying WT-β2. Thus, all assays pointed to inactivity of E52Q-β2.

![Image 313x526 to 563x733](https://example.com/image.png)

**Figure 2.** Kd between α2 and β2 in the presence of CDP/ATP determined by the competitive inhibition spectrophotometric assay (12). The data were fit (solid line) to Equation 1. All data are representative of two independent experiments and are expressed as mean ± S.D. Subscript a, b, and t are the bound, free, and total protein concentrations, respectively. A, Kd for α2/E52X-β2 (X = Ala, Asp, or Gln). E52A (blue), E52D (orange), E52Q (red) and B, Kd for mutant α2/β2: R329A (blue), R329K (red), R329Q (black), and R639Q (green). C, binding for α2/E52Q/F3Y122-β2 shows a stoichiometric titration under standard assay conditions (blue) and an expanded version of α2/E52Q-β2 shown in A (red). D, analysis of activity with increasing concentrations of E52Q/F3Y122 (0.7 F3Y122; see text).

**Table 1**

| Specific activity and Kd for E52X (X = Ala, Asp, or Gln)–β2 | Specific activity | Wild-type activity | Kd
|----------------|-----------------|------------------|-----|
| β2             | Y122/β2         | nmol/min/mg/μg  | %   | μM |
| WT             | 1.2             | 7000             | 100.0 | 0.18a |
| E52A           | 1.0             | 13.4             | 0.2  | 0.96 |
| E52D           | 1.2             | 10.2             | 0.2  | 2.33 |
| E52Q           | 1.1             | 8.6             | 0.1  | 0.12 |
| F3Y122         | 0.7             | 686             | 9.8  | <4 nm |a
| E52Q/F3Y122    | 0.9             | 5.8             | 0.1  | <4 nm |a

a Specific activity was determined by the radioactive assay (49) and Kd was determined by the competitive binding method (12). All data are representative of at least two independent experiments.

b Previously reported (12).

c Upper limit for Kd of E52Q/F3Y122.

stain electron microscopy (EM) analysis and size exclusion chromatography (SEC) studies revealed that the predominant species is α2β2. The implications of these results on conformational gating and potential structural insight of the active complex are discussed.

**Results**

**Identification of conserved α/β interface residues, their mutation and assay for activity, and subunit binding affinity**

Our recent studies investigating the role of Glu350, a conserved residue in the disordered C-terminal tail of β2, suggested that this residue was essential for the conformational gating of the RT initiation process (13, 18). We therefore looked at other conserved charged residues using the α2β2 docking model, to identify those that might reside at the α/β interface. Alignment of 80 sequences in the NrdAg/NrdBg subclass revealed that Glu350 and Glu122 in β2 and Arg329 in α were conserved in 80 of 80 sequences, whereas Arg329 in α was conserved in 79 of 80. These residues and additional ones, Arg323 (not conserved) and Arg735 (76/80) in α, became candidates for investigation by mutagenesis.

In the case of the glutamates, each residue was changed to Ala, Gln, and Asp, whereas in the case of the arginines, each was changed to Ala, Gln, and Lys. The proteins were expressed and purified to homogeneity based on SDS-PAGE analysis using the WT protocols (supplemental Fig. S1). In the case of the β2 mutants, the diferric-Y122 mutant was self-assembled to give a cofactor with a Y122· content similar to WT-β2 (Table 1). All mutants were assayed for activity and a Kd for each α/β interaction was determined (Table 1). The E52X-β2 (X = Ala, Asp, or Gln) mutants have activity ~0.15% of WT-β2, within the levels typically observed for endogenously co-purifying WT-β2. The Kd measurements revealed that the Ala and Asp mutants are 5- and 10-fold higher than WT, whereas Gln is similar to WT (Fig. 2A). These studies suggest that Glu122 plays an important role in catalysis.

**N3CDP as a probe of E52X-β2 (X = Ala, Asp, or Gln)**

Because RNR is essential, the issue of endogenous WT-RNR co-purifying with the mutants always hinders determination of a lower level of enzymatic activity. An alternative way to assess activity has been to use the mechanism-based inhibitor 2'-azido-2'-deoxycytidine diphosphate (N3CDP) (13, 33). This NDP analog binds in the active site and is enzymatically converted to a nitrogen-centered nucleotide radical (N'), that becomes covalently bound to a cysteine in the active site. The inactivation is stoichiometric with the WT-β2, with complete loss of activity resulting from 1 Tyr122/β2 being converted to 0.5 eq of N', leaving 0.5 eq of the Tyr122 remaining (34–36). This unusual stoichiometry is associated with the half-sites reactivity of all class I RNRs. The N' has been extensively characterized by isotopic labeling and EPR methods. With mutant β2s the rate of formation of N' is often slow and the radical is quenched slowly with time; the kinetics often preclude N' detection, thus analysis of total radical loss as a function of time is monitored (13). The results of experiments in which E52X-β2/α2/N3CDP/TTP (X = Ala, Asp, and Gln) were incubated and analyzed by EPR over 120 min are summarized in Fig. 3A (33). No N’ is observed and the total Tyr122 varies no more than 10% over the 2-h time period. With WT-β2, 0.5 eq of N’ is formed within 30 s. Thus, no activity of E52X mutants is apparent by this method either.

A third method to assess RNR activity is to place E52X into a different background: specifically one in which the Tyr122 is replaced with F3Y122. The F3Y122-β2 mutant when incubated with α2/CDP/ATP has been studied in detail and shown to generate dCDP and the pathway Tyr356→Tyr122 within 10 sec in the first turnover and then reoxidize the putative F3Y122-O→F3Y122 in the rate-limiting step in the steady-state (20). This mutant is a hotter oxidant than Tyr122 and disrupts conformational gating of the RT process (20, 32, 37). The E52Q mutant in this background has 0.1% WT activity (Table 1), likely associated with endogenous levels of co-purifying WT-β2. Thus all assays pointed to inactivity of E52Q-β2.

![Image](https://example.com/image.png)
Importance of glutamate 52 in β of class Ia RNR

Figure 3. Time-dependent inactivation of RNR mutants in the presence of N₃CDP at 25 °C. A, time-dependent radical formation of E52X-β2 (X = Ala, or Asp, or Glu), WT-α2, and TTP in the absence (orange, red, or green) and presence (blue, purple, or brown) of N₃CDP. B, time-dependent radical loss of R329X-α2 (X = Ala, Lys, or Glu), WT-β2, and TTP in the absence (orange, red, or green) and presence (blue, purple, or brown) of N₃CDP. Each point represents the average of two independent trials.

Efforts to determine the $K_d$ for subunit interactions with this double mutant gave data distinct from the single E52Q mutant (Fig. 2C) and the other mutants (Figs. 2A). The sharp break suggests a “stoichiometric” titration. Reanalysis of these data in which activity is monitored with increasing concentrations of E52Q/F₃Y₁₂₂-β2 reveal that for 0.1 μM αβ2 complex, 0.28 μM of the double mutant was required for complete inactivation (Fig. 2D). Given that the mutant protein used in this experiment has 0.7 F₃Y₁₂₂/β2 with the radical equally distributed between the two β monomers and assuming that the diferric-cluster without radical binds much more weakly, then one would predict the requirement for 0.29 μM mutant, very similar to the experimental observation.

CDP/ATP, GDP/TTP, and N₃CDP/TTP to probe E52Q/F₃Y₁₂₂-β2 activity by EPR methods

Although no activity of E52Q-β2 or E52Q/F₃Y₁₂₂-β2 was observed under steady-state conditions, additional experiments were performed on E52Q/F₃Y₁₂₂-β2 to determine whether chemistry could be observed in the first turnover. As noted above, addition of CDP/ATP/α2 to the single mutant, F₃Y₁₂₂-β2, results in formation of Tyr₃⁵⁶e and a burst of dCDP (0.5 eq/F₃Y₁₂₂). The double mutant, E52Q/F₃Y₁₂₂-β2 was incubated with CDP/ATP and analyzed by EPR spectroscopy for production of the Tyr₃⁵⁶e. The results are shown in Fig. 4A and are summarized in Table 2. The data reveal that only 4% of the total radical is lost within 1 min and that it increases to 30% by 5 min. Also within the 1-min time frame, 0.50 eq of Tyr₃⁵⁶e is formed. The rate of loss of the total radical is substantially reduced when CDP is omitted. When ATP is omitted, however, the total radical is reduced to 50% by 5 min and the amount of Tyr₃⁵⁶e is increased to 40% of the total radical by 1 min and remains unchanged at 5 min. Thus CDP is the predominant driver of Tyr₃⁵⁶e formation and the effector (ATP) appears to stabilize the F₃Y₁₂₂ radical in β2 when no substrate is present for reduction.

An identical set of experiments carried out with purine substrates and effectors have the same phenotypes. The results are summarized in Table 2. With GDP/TTP, by 5 min 30% of the total radical is lost, whereas 0.5 eq of Tyr₃⁵⁶e is formed within 2 min. The effector TTP stabilizes total radical and limits Tyr₃⁵⁶e formation, whereas GDP is the predominant driver of Tyr₃⁵⁶e. What is most amazing about these results is that under steady-state conditions where neither E52Q nor E52Q/F₃Y₁₂₂-β2 make dCDP, E52Q/F₃Y₁₂₂-β2 can initiate RT subsequent to S/e binding.
As noted above, a second way to look for activity, uses N₄CDP or N₃CDP/TPP. The results of this set of experiments are shown in Fig. 4C and summarized in Table 2. In contrast to the results with the single mutant (E52Q), N' is formed and accounts for 49% (N₄CDP/TPP) versus 43% (N₃CDP) of the total radical at 10 min (compare Fig. 4, C with D, an authentic standard for N'). Thus these data also support the activity of the double mutant, E52Q/F₃Y₁₂₂,-β2, at least on the first turnover.

**E52Q/F₃Y₁₂₂,-β2 with pre-reduced α2, CDP, and ATP can produce dCDP**

The above observation that the double mutant, E52Q/F₃Y₁₂₂,-β2, is capable of RT to the α2 catalytic site suggests that this protein may be able to make dCDP, even though no (or very low) activity is observed in the steady-state. To test for dCDP formation, an assay was carried out with a 1:1 ratio of subunits at 20 μM in the presence of CDP alone (blue), CDP/ATP (red), and CDP/ATP with reductant TR/TRR/NADPH (green) and the reaction was monitored as a function of time (Fig. 5). The amount of the Tyr₃⁵⁶₉ (0.5 eq) observed (Table 2) is likely formed during reverse RT and suggested that 0.5 eq of dCDP would be generated. The results shown in Fig. 5 suggest that this is the case. There is a burst of dCDP formation and it is independent of the presence of reductant. The size of the burst in all three experiments is similar to the amount of Tyr₃⁵⁶₉ formed, consistent with half-sites reactivity and one turnover. In all experiments, the burst phase is followed by a slow phase that occurs from 0.2 to 0.6% (1.6, 3.4, and 4.4 nmol/min/mg in Fig. 5, blue, red, and green, respectively) of that observed with the single mutant, F₃Y₁₂₂,-β2 (686 nmol/min/mg). The rate is fastest with TR/TRR/NADPH/CDP/ATP > CDP/ATP > CDP. A number of explanations are possible for the slow phase observed in all experiments. In the absence of reductant (red and blue, Fig. 5) the slow phase could be associated with endogenous β2 acting catalytically, with very slow completion of the catalytic cycle in which Tyr₃⁵⁶₉ must reoxidize the F₃Y-O⁻ or with slow release of cytosine catalyzed by the oxidized form of RNR. This issue remains unresolved. However, the interesting result is that E52Q/F₃Y₁₂₂,-β2 is able to carry out one turnover! Thus, although the steady-state assays do not reveal significant activity (0.1% WT, Table 1), the double mutant is capable of the radical-based reactions that result in dCDP formation.

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![Graph showing dCDP formation by WT-α2 and E52Q/F₃Y₁₂₂,-β2 (0.91 Y₁₂₂/β2) in the presence of CDP (blue), CDP/ATP (red), or CDP/ATP and reductant TR/TRR/NADPH (green).](image)

As noted above, a second way to look for activity, uses N₄CDP or N₃CDP/TPP. The results of this set of experiments are shown in Fig. 4C and summarized in Table 2. In contrast to the results with the single mutant (E52Q), N’ is formed and accounts for 49% (N₄CDP/TPP) versus 43% (N₃CDP) of the total radical at 10 min (compare Fig. 4, C with D, an authentic standard for N’). Thus these data also support the activity of the double mutant, E52Q/F₃Y₁₂₂,-β2, at least on the first turnover.

**Interaction of Hisᵦ-α2 and E52Q/F₃Y₁₂₂,-β2 using pulldown assays and SDS-PAGE analysis**

Our previous studies showed that incubation of Hisᵦ⁻NH₂Y₇₃₀-α2 with β2, CDP, and ATP resulted in formation of NH₂₃₂₉⁻ concomitant with Tyr₁₂₂⁻ loss. Rapid purification of Hisᵦ⁻NH₂Y₇₃₀-α2 from this mixture using a Ni-NTA affinity resin by centrifugation followed by SDS-PAGE analysis showed that α and β co-purified (24). Given these results and the apparent stoichiometric titration of E52Q/F₃Y₁₂₂,-β2 with α2 in our binding assay (Fig. 2, C and D), similar pulldown experiments with Hisᵦ⁻α2/E52Q/F₃Y₁₂₂,-β2/ATP/CDP using a Ni-NTA resin were undertaken. Purification was carried out by centrifugation (Fig. 6A) or column gravity workup (Fig. 6B) with quantitation by SDS-PAGE and densitometry using α and β standards (Fig. 6A, left). Centrifugation analysis monitoring supernatants from time 0 to 120 min incubation prior to workup revealed that when no CDP was present (time 0), no E52Q/F₃Y₁₂₂,-β2 was pulled down, but within 1 min of its addition, the pulldown was maximized and remained unchanged (Fig. 6A, right). The majority of the pulldown experiments were carried out using a column gravity workup (Fig. 6B), as it typically gave higher recoveries of Hisᵦ⁻α2 (>80%). A variety of experiments were carried out in which the S (CDP or GDP), e (ATP or TTP), or S/e pairs and the incubation times, 5 or 30 min, were varied. In addition, controls with β2, E52Q/Y₁₂₂,-β2, and F₃Y₁₂₂,-β2, or E52Q/Y₁₂₂,-β2 without S/e were also examined. The results summarized in Table 3 reveal that with S alone or S/e that a β2/α2 ratio of 0.5–0.8 was observed, where with e alone, the ratio was lower at 5 min, but increased by 30 min (experiments 8 and 12). The data together suggest that the appropriate S/e pair form

![Image showing pulldown assays of different β2s by Hisᵦ⁻WT-α2 analyzed by 10% SDS-PAGE.](image)
In these experiments, the peaks corresponding to reaction of \(\text{NH}_2\text{Y}_730^-\) were examined by negative stain EM. Our previous studies on the functional uncoupler that generates the Tyr356 pathway radical in pulldowns. These studies suggest the F3Y122 combination with the E52Q mutation are important for successful \(\alpha2\beta2\) complex formation.

**Characterization of the reaction mixture by SEC and negative stain EM**

Two additional types of experiments were carried out to support an \(\alpha2\beta2\) complex structure and the tightness of the complex. In one set of experiments the reaction of E52Q/F3Y\(\text{Y}_{122}^-\)-\(\beta2\) was incubated with 0.5 eq of a2 (1:2, \(\alpha2\)-\(\beta2\) subunit ratio), GDP, and TTP and loaded on a Superdex 200 SEC column and then eluted with assay buffer containing 50 \(\mu\text{M}\) GDP and 10 \(\mu\text{M}\) TTP. The results shown in Fig. 7A reveal a peak eluting at 12.1 ml and a broad peak at 13.7 ml. Comparison with molecular weight standards in Fig. 7B suggests that the former is \(\alpha2\beta2\) and the latter is \(\beta2\) and the ratio is 1:1 based on a comparison of the relative peak areas as expected from experimental design (Fig. 7, red). When the FPLC experiment was carried out in the absence of nucleotides in the elution buffer, peaks were observed at very similar elution volumes (Fig. 7, black), but the ratio of the peak intensities suggest only \(\sim 40\%\) \(\alpha2\beta2\) complexation. In a control with F3Y\(\text{Y}_{122}^-\)-\(\beta2\)-a2/GDP/TTP, no \(\alpha2\beta2\) complex was observed (Fig. 7A, blue). Control experiments with E52Q/Y\(\text{Y}_{122}^-\)-\(\beta2\) in place of E52Q/F3Y\(\text{Y}_{122}^-\)-\(\beta2\) showed \(\alpha2\beta2\) complex formation with GDP/TTP in the elution buffer, whereas no \(\alpha2\beta2\) was observed without GDP/TTP (not shown). In these experiments, the peaks corresponding to \(\alpha2\beta2\) at 12 ml eluted 25 min after reaction initiation with GDP and TTP. Thus although the pulldown experiments allow isolation of \(\alpha2\beta2\) with very high recovery and no GDP/TTP in the elution buffer, the SEC data tell us that on the 30-min time scale of the SEC analysis, the two subunits come apart in the absence of nucleotides during chromatography.

In a second set of experiments, \(\alpha2\beta2\) complex formation was examined by negative stain EM. Our previous studies on the reaction of \(\text{NH}_2\text{Y}_{730}^-\)-a2, \(\beta2\), CDP, and ATP reported our first efforts to look for the “active” \(\alpha2\beta2\) complex by this method (24). The resulting low resolution (\(\sim 32\) \(\text{Å}\)) model revealed a subunit arrangement that was consistent with the \(\alpha2\beta2\) docking model (Fig. 1). Interestingly, when WT-a2 and WT-\(\beta2\) were mixed and observed on an EM grid with negative stain, almost all observed particles were of free a2 and almost no \(\alpha2\beta2\) complex was observed. Free \(\beta2\) is too small (87 kDa) to be visualized. \(\text{NH}_2\text{Y}_{730}^-\)-a2 with WT-\(\beta2\) gave rise to \(\sim 70\%\) \(\alpha2\beta2\) particles (24).

Here, negative stain EM experiments with WT-a2 and E52Q/F3Y\(\text{Y}_{122}^-\)-\(\beta2\) with GDP/TTP were carried out under similar conditions to the SEC (Fig. 7) and pulldown (Table 3) experiments. What is immediately striking is the large number of \(\alpha2\beta2\) complexes that are present (Fig. 8), estimated to be 90%. The ratio of 1:2 for \(\alpha2\beta2\) was chosen to maximize the chemistry (Tyr\(\text{Y}^{356}\) formation) as typically there are \(\sim 0.8\) E52Q/F3Y\(\text{Y}_{122}^-\)-\(\beta2\). Taken together, the pulldown studies, EM, and SEC analysis reveals \(\alpha2\beta2\) complexes that are supported by biochemical analysis that shows active RT and dCDP formation. The SEC data reveal that further work, such as our stopped flow fluorescence studies on \(\text{NH}_2\text{Y}_{730}^-\)-a2, will be informative in determining a quantitative assessment of the subunit affinity in the complex observed.
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Table 4
Specific activities for mutant-α2s with 5-fold WT-β2 or 10-fold F₁Y₁22-β2 determined by the radioactive assay (49) and Kₐ for mutant-α2/WT-β2 interaction determined by the competitive inhibition assay (12)

All data are representative of at least two independent experiments.

| α2   | WT-β2 Specific activity | F₁Y₁22-β2 Specific activity | β2 | Kₐ |
|------|-------------------------|----------------------------|----|----|
|      | nmol/min/mg             | Wild-type activity (%)      | nmol/min/mg | Wild-type activity (%) | μM   |
| WT   | 2428                    | 100.0                      | 805 | 100.0 | 0.18² |
| R329A | 0°                      | 0.00                      | 5.2 | 0.6 | 4.65 |
| R329K | 2.8                     | 0.12                      | 22.3 | 2.8 | 2.56 |
| R329Q | 0°                      | 0.00                      | 8.0 | 1.0 | 2.79 |
| R639Q | 837                     | 34.47                     | ND  | ND  | ND   |
| R735Q | 1492                    | 61.45                     | ND  | ND  | ND   |

¹ Previously reported by Climent et al. (12).
² The counts were the same as the background control.
³ ND, not determined.

Activity and subunit binding affinity of additional α and β mutants (Table 4)

In addition to Glu52, our search for charged interface residues suggested that Arg329 and Arg639 in α were also of interest. Arg329 is located in loop 3 of α and is adjacent to a second Arg at 323 that is not conserved (see supplemental Fig. S2). Mutants of Arg329 (Ala, Lys, and Gln) and a control of Arg323 to Lys are shown in Table 4. R329A and R329Q have no detectable activity, whereas R329K has 0.12% activity of WT-α2. Binding studies (12) revealed that all three mutants exhibit 10–20-fold weaker binding than WT, similar to the phenotypes of the E52X mutants with the exception of Gln (Fig. 2, A and B). Given the weak Kₐ values, the mutants were assayed at higher protein concentration and still found to have no activity (Table 4). In the case of R329K, an additional experiment was carried out with N₆CDP to look for N₆ formation and total radical loss. These results (Fig. 3B) also indicate that this mutant is inactive and hence important. The control, a lysine mutant of Arg329 has 34% the activity of the WT-α2 and was thus not considered further.

The R329X-α mutants were also studied with F₁Y₁22-β2. Activity of 1 to 3% WT was observed with the Lys mutant having the highest level (Table 4). These studies also suggest that Arg329 plays an important role in the RNR catalysis. Finally, studies with an R639Q-α mutant revealed that it is inactive, whereas mutations of the non-conserved Arg350, also proposed to be at the interface, results in active enzyme.

Discussion

In the past decade using technology to site-specifically incorporate UAA coupled to time-resolved kinetic measurements to study the consequences of the incorporation, much has been learned about the long distance RT process required to initiate nucleotide reduction in RNR (3, 26, 38). The UAA mutants have been one of the crucial perturbants to allow uncoupling of the rate-limiting conformational gating that masks the RT and the nucleotide reduction chemistry in the WT enzyme (20).

Binding of the appropriate S and e pairs to α2 followed by binding β2 has long been known to trigger the essential conformational change(s) that occurs over the 37 Å (C-site) or 39 Å (S-site) in α2 to the RT initiation site in β2 (Fe²⁺ to S of Cys439 or 2-O of TTP, respectively) (19, 32). The binding of CDP/ATP or CDP (or GDP/TTP or GDP) changes the loop 2 structure in α2 and also induces a closure of the barrel structure around the catalytic site on α2 (39). These changes must be transmitted across the α/β subunit interface, likely through a conserved network of residues to position the water bound to the Fe¹ in the dipheric cluster, so that the proton can be efficiently delivered to Tyr122 concomitant with its reduction (Fig. 1).

Our recent studies on the conserved Glu350 in β2, suggested that it likely plays a very important role in conformational gating (3, 18). We thus decided to investigate the possible role of other conserved interface residues including Glu52-β2, Arg329-α2, and Arg639-α2.

The results with the E52Q in the WT and F₁Y₁22-β2 backgrounds are most striking. In the WT background it is unable to make dNDPs and is inactive in the formation of the N₃ from N₄CDP. E52Q-β2, in contrast to the Glu and Asp mutants, binds similarly to α2 as WT-β2 (Table 1, Fig. 2A) and for E52Q/F₁Y₁22-β2 the binding to α2 is stoichiometric (Fig. 2, C and D). Additionally, although the E52Q/F₁Y₁22-β2 is inactive in the steady-state assay, it is able to make N₃ from N₄CDP, Tyr356⁺ in the presence of CDP/ATP, and catalyze 1/2 turnover (one CDP/two F₁Y₁22), consistent with the half-site reactivity of RNR (20, 23). It is likely that the reoxidation of F₁Y₁22⁻O⁻ to the F₁Y₁22⁺ by Tyr356⁺ is too slow to compete with loss of the total radical (Table 2) (18, 20, 37), potentially explaining the lack of activity under steady-state conditions. This reoxidation is also slow for F₁Y₁22⁻O⁻ in the WT background, but the E52Q mutation appears to result in an even slower process.

To investigate α/β binding, we used several pulldown approaches. The experiment with His₄-α2, S/e (where S is CDP or GDP and e is ATP or TTP), and E52Q/F₁Y₁22-β2 allowed isolation of a complex by Ni-NTA affinity chromatography with a β2/α2 subunit ratio of ~0.6. In contrast, the ratio of 0.01 was observed with the WT control after a 5-min incubation (Table 3).

Interestingly, the double mutant complex has a longer lifetime than the pathway (Tyr356⁺) radical in the pulldown assays. The total amount of radical (F₁Y₁22⁻ and Tyr356⁺) decreases 30 to 50% over 5 min (Table 2), yet the complex can be isolated over 2 h (Fig. 6A, right, and time course data not shown with the other experiments in Table 3). Thus, the conformation of the αβ2 complex that allowed its isolation appears to have a “kinetic” memory, that it, remains in an altered conformation
after much of the pathway radical has decayed. This observation of a kinetic memory is strikingly similar to our recent studies with α on the human RNR. This subunit forms a hexameric structure, α6, in the presence of dATP or the phosphorylated drugs clofarabine di- or triphosphate (CIFDP or CIFTP) (40–42). When dATP dissociates from α6, the hexamer returns to a monomeric state. However, when CIFDP or CIFTP dissociate, the hexameric structure remains. The molecular basis for the continued tight binding of αβ2 in the case of the E. coli RNR double mutant and α6 in the hRNR remain unknown. However, it is intriguing in the case of the E. coli RNR that a conservative chemical substitution Gln for Glu in the F3Y122→Trp48 that in turn connects to Asp237, which connects to Tyr122 of loop 3 of the essential enzyme.

From the many β2 structures available, we know that Glu52 located on the surface of β is conformationally flexible with “out,” “in,” and “intermediate” conformations (supplemental Fig. S2, B and C). Its “in” conformation connects through waters to a conserved residue, Arg236, within β. Arg236 has connectivity to Trp48 that in turn connects to Asp327, which connects to His118, a ligand to Fe3 of the cofactor (supplemental Fig. S2C). It is the water on Fe1 that is proposed to deliver the proton to Tyr122 upon Tyr122→Trp48 reduction (supplemental Fig. S2C) (11, 19, 43). Also shown in supplemental Fig. S2 is the location of the “out” conformation of Glu52 relative to the conserved Arg319 in loop 3 of α in the αβ2 docking model. Supporting the importance of Arg319, mutants (Gln, Lys, and Ala) show weak binding to β2, with $K_d$ values elevated 10-fold relative to WT, similar to the results with Glu52 and Glu52 mutants. The inactivity of Glu52 and Arg319 mutants might result from their altered conformations in this region of α2. The studies with E52Q/F3Y122→β2 and the requirement for S/e suggest its importance in conformational triggering of RT across α/β. The unexpected observation of the high percentage of the α2β2 complex formed in the double mutant may provide the opportunity to gain insight into the structure of this complex based on our negative stain EM images (Fig. 8).

Finally, the least well studied mutant, Arg639-α has very low activity and has weakened binding to β. Recent structures from the Drennan lab (39) show that in the presence of the correct S/e pairs, loop 2 (yellow, supplemental Fig. S3A) becomes ordered, the barrel clamps around the catalytic site, and the β-hairpin (supplemental Fig. S3B; blue to orange) moves to potentially protect the active site. Arg639, which is adjacent to this hairpin may play a role in stabilizing the differential hairpin conformations. Interestingly, this β-hairpin is conserved in the class II RNRs and is observed to move when the adenosylcobalamin cofactor, the radical initiator, binds to initiate nucleotide reduction via formation of a thiol radical (44).

Conclusions

The reversible long distance RT between α and β continues to be a fascinating feature of the class I RNRs. RT is gated subsequent to binding the appropriate S and e pairs on the α subunit, requiring communication across the subunits over a distance of 35 to 40 Å. The transient nature of the α and β interactions in the E. coli RNR, the flexibility of its α and β tails both essential in catalysis, the complexity and number of nucleotide-binding sites, have all made an understanding of the molecular mechanism of conformational gating and a structure of an active RNR elusive. Here we have identified conserved residues likely to control conformational gating at the α/β interface. The most intriguing results are that the double mutant of E52Q/F3Y122→β2 when incubated with α2, S, and e, potentially forms the “tightest” complex thus far reported based on pulldown assays, SEC, and negative stain EM studies. The conservative mutation of Glu52 to Asp, on the other hand, weakens subunit affinity compared with WT. Clearly the design of the subunit interface is intricate, providing the exquisite control that is needed for the RT chemistry mediated by S/e in this essential enzyme.

Experimental procedures

Materials

All primers and plasmids utilized in this study are shown in supplemental Table S1. All primers were provided by Integrated DNA Technologies. Site-directed mutagenesis was performed using the Stratagene QuickChange kit and all constructs were confirmed by sequencing at QuintaraBio (Boston). WT-β2 (7000 nmol/min/mg) and E52X-β2 (X = Ala, Asp, or Gln) were isolated as previously reported with typical yields of 20–30 mg/g of cell paste (45). WT-α2 (2428 nmol/min/mg) and mutant-α2s were purified following the published protocol with typical yields of 20–30 mg/g of cell paste (29). Thioredoxin (TR, 40 units/mg) and thioredoxin reductase (TRR, 1400 units/mg) were purified following the standard protocols (46, 47).

F3Y was enzymatically synthesized from the corresponding phenol using tyrosine phenol lyase TPL (27). The pBAD-nrdB-TAG122 and pEVOL-F3Y/RYS-E3 plasmids were generated and isolated as described (20). Apo-F3Y122→β2 and apo-E52Q/F3Y122→β2 were expressed, purified, and reconstituted as previously reported (20). Typical yields were 8–10 mg/g of cell paste.

[5′-3H]CDP was purchased from ViTrax (Placentia, CA). Roche Applied Science provided the calf alkaline phosphatase (20 units/μl). Sigma provided Hesper, MgSO4, EDTA, LB, 2× YT microbial medium, ampicillin, chloramphenicol (Cm), hydroxyurea, ATP, CDP, TTP, GDP, deoxyctydine (dC), and NADPH. Isopropyl β-D-thiogalactopyranoside and dithiothreitol (DTT) were obtained from Promega. N3CTP (2′-Azido-2′-deoxyctydine 5′-triphosphate) was purchased from Trilink Biotechnologies and converted to the diphosphate (N3CDP) as previously described (48). Assay buffer consisted of 50 mM Hesper (pH 7.6), 15 mM MgSO4, 1 mM EDTA. The temperature was controlled using a Lauda circulating water bath for all experiments: at 25 °C for E. coli RNR. All E. coli α2 and β2 concentrations are reported per dimer.

RNR activity assays

The activity of E. coli WT-α2 or WT-β2 (0.15 μM) was determined in the presence of 5-fold excess of the second subunit, β2 or α2 (0.75 μM), E52X-β2 (X = Ala, Asp, or Gln, 0.5, 1.0, and 2 μM), F3Y122→β2 (0.5 μM), E52Q/F3Y122→β2 (2 μM), or mutant-α2s (2 μM) were also assayed in a 5-fold excess of WT-α2 or WT-β2. A typical assay mixture of 140 μl contained RNR, TR (30 μM), TRR (0.5 μM), and ATP (3 μM) in assay buffer and the
reaction mixture was initiation with [5-^3H]CDP (1 mm, 3769 cpm/nmol). The method of Steeper and Stuart (49) was used for analysis.

**Time-dependent inactivation of RNR mutants in the presence of N_2CDP**

A 250-μl reaction mixture contained: protein (30 μM WT-α2 with 30 μM E52X-β2 (X = Ala, Asp, or Gln) or 30 μM R329X-α2 (X = Ala, Lys, or Gln) with 30 μM WT-β2), 0.2 mM TTP, 50 mM Hepes (pH 7.6), 1 mM EDTA, 15 mM MgSO_4_ and was incubated at 25 °C for 1 min. The time 0 sample was frozen in liquid nitrogen and the EPR spectrum was recorded. The sample was then thawed and the reaction started by addition of 0.25 mM N_3CDP. The sample was then monitored by chemical quench twice that is always present in the reaction mixture.

**Importance of glutamate 52 in β of class Ia RNR**

In a final volume of 250 μl the reaction mixture contained WT-α2 (15 to 50 μM), with 1 eq of β2, substrate (CDP (1 mm) or GDP (1 mm) or N_2CDP (0.25 mm)), ± effector (ATP (3 mM) or TTP (0.2 mM)) in assay buffer. Samples were incubated for a specified time in a circulating water bath at 25 °C and quenched for EPR analysis in liquid nitrogen. EPR spectra were recorded at 77 K in the Department of Chemistry Instrumentation Facility on a Bruker ESP-300 X-band spectrometer equipped with a quartz finger Dewar filled with liquid nitrogen. Typical EPR parameters were as follows: microwave frequency = 9.45 GHz, power = 32 μW, modulation amplitude = 1.5 G, modulation frequency = 100 kHz, time constant = 40.9 ms, scan time = 41.9 s. Analysis of the resulting spectra was carried out using WinEPR (Bruker) and an in-house written program in Matlab R2016a (50). EPR spin quantitation was carried out using Cu^{II} as standard.

**Pulldown assays**

A final volume of 100 μl contained untagged-β2s (10 μM), His_{6}-WT-α2 (10 μM), ATP (3 mM), or TTP (0.2 mM) in assay buffer at 25 °C. CDP (1 mM) or GDP (1 mM) or alternatively mutant β2 was added to initiate the reaction. The reaction mixture was incubated for 1 to 120 min at 25 °C and then combined with a nickel-nitrilotriacetic acid resin (~60 or 300 μl from Qiagen) suspended in the EDTA-free assay buffer and rotated by hand at room temperature for 1 min. The sample was then centrifuged (30 s, 3,000 × g, 4 °C) and the supernatant was removed. Alternatively, the NTA resin (300 μl) was placed in a small column and eluted by gravity. In the former case, the resin “pellet” was rapidly resuspended in 600 μl of wash buffer (EDTA-free assay buffer with 300 mM NaCl and 15 mM imidazole (pH 7.6)) and centrifuged (30 s, 3,000 × g, 4 °C). This wash step was repeated a second time. Resin-bound protein was then eluted by resuspending it in elution buffer (100 μl, EDTA-free assay buffer with 250 mM imidazole (pH 7.6)), followed by centrifugation (30 s, 3,000 × g, 4 °C). The procedure (flow through, washes (W1 and W2), and elution (E)) took 5 min. The recovery of α is typically 40 to 50%.

In the latter case, gravity elution, the procedure (loading, washes, and elution) is the same except that the procedure takes 2 to 3 min and the recovery of α is typically ~90%. The contents of each fraction were assessed by SDS-PAGE (10%) and compared with the fractions obtained in a control experiment with standards made from stock solutions: 1 μM His_{6}-WT-α2 and 1 μM WT-β2.

**Negative stain EM on α2 with E52Q/F_{3}Y_{122}^{-}β2**

A reaction mixture was prepared with 5 μM α2, 10 μM E52Q/F_{3}Y_{122}^{-}β2, 1 mM GDP, and 0.2 mM TTP in assay buffer (50 mM Hepes, pH 7.6, 15 mM MgSO_4_ and 1 mM EDTA) where β2 was added last to initiate the reaction. The mixture was incubated 3 min at 25 °C and then diluted 130-fold in assay buffer containing 1 mM GDP and 0.2 mM TTP giving final protein concentrations of 40 nM α2 and 80 nM E52Q/F_{3}Y_{122}^{-}β2. The solution was applied to a 300-mesh continuous carbon grid (EMS) and stained three times with a 1% uranyl acetate solution. The total
time between reaction initiation and application onto the grid was ~15 min.

Data collection
All images were collected at the W. M. Keck Institute for Cellular Visualization at Brandeis University. The grids were imaged at 200 kV on a Tecnai F20 electron microscope (FEI) equipped with an UltraScan 4000 CCD camera (Gatan) using SerialEM operated in manual low-dose mode at a magnification of 62,000 with a pixel size of 1.79 Å at the specimen level.

Size exclusion chromatography
A reaction of 300 µl contained 50 mM Hepes (pH 7.6), 15 mM MgSO₄, 2 µM α₂, 4 µM E52Q/F₃Y₁₂₂Δβ2, 1 mM GDP, and 0.2 mM TTP. The reaction mixture was loaded into a 200-µl loop and injected onto a Superdex 200 10/300 GL preequilibrated in 50 mM Hepes (pH 7.6), 15 mM MgSO₄, 50 mM GDP, 10 mM TTP, and 150 mM NaCl. The flow rate was 0.5 ml/min. Ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), and ovalbumin (44 kDa) were used as the protein standards to generate a standard curve for molecular weight estimation.

Author contributions—Q. L., M. J. P., and J. S. designed the study and wrote the paper. Q. L. made the mutants and performed activity assays, EPR experiments, and pulldown assays. M. J. P. identified the interface residues and proposed the structural models for their role. A. T. T. analyzed EPR data and performed pulldown assays and SEC experiments. K. R. played an important intellectual role in experimental design. A. K. performed assays related to Arg339 and Arg335 in α G. H. K., A. T. T., and C. L. D. carried out the negative stain EM experiments and analysis and provided structural insight into the function of the mutants. All authors approved the final version of the manuscript.

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**Importance of glutamate 52 in β of class Ia RNR**