Redox Studies of Subunit Interactivity in Aerobic Ribonucleotide Reductase from *Escherichia coli*®

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Ribonucleotide reductase is a heterodimeric (α2β2) allosteric enzyme that catalyzes the conversion of ribonucleotides to deoxyribonucleotides, an essential step in DNA biosynthesis and repair. In the enzymatically active form aerobic *Escherichia coli* ribonucleotide reductase is a complex of homodimeric R1 and R2 proteins. We use electrochemical studies of the dinuclear center to clarify the interplay of subunit interaction, the binding of allosteric effectors and substrate selectivity. Our studies show for the first time that electrochemical reduction of active R2 generates a distinct Met form of the diiron cluster, with a midpoint potential (−163 ± 3 mV) different from that of R2Met produced by hydroxyurea (−115 ± 2 mV). The redox potentials of both Met forms experience negative shifts when measured in the presence of R1, becoming −233 ± 6 and −226 ± 3 mV, respectively, demonstrating that R1-triggered conformational changes favor one configuration of the diiron cluster. We show that the association of a substrate analog and specificity effector (dGDP/dTTP or GDP/dTTP) with R1 regulates the redox properties of the diiron centers in R2. Their midpoint potential in the complex shifts to −192 ± 2 mV for dGDP/dTTP and to −203 ± 3 mV for GDP/dTTP. In contrast, reduction potential measurements show that the diiron cluster is not affected by ATP (0.35–1.45 mM) and dATP (0.3–0.6 mM) binding to R1. Binding of these effectors to the R1-R2 complex does not perturb the normal docking modes between R1 and R2 as similar redox shifts are observed for ATP or dATP associated with the R1-R2 complex.

Ribonucleotide reductases (RNRs) catalyze the reduction of the four canonical ribonucleotides to the corresponding deoxyribonucleotides in all organisms, thus providing the precursors for DNA synthesis. Allosteric regulation ensures a balanced dNTP pool needed for DNA replication and repair. At present, three major classes of RNRs have been identified that differ in their protein structure and in the cofactors essential for catalysis (1). Despite these differences, all RNRs catalyze the reduction of ribonucleotides by similar radical-based mechanisms, including formation of a transient thyl radical at the active site that in turn produces a transient 3'-substrate radical (2). In class I enzymes, it is proposed that the thyl radical within the R1 subunit is formed during each catalytic cycle via long range electron/proton transfer from a stable tyrosyl radical located in the R2 subunit (1, 2).

The enzymatic activity of aerobic *Escherichia coli* RNR, which belongs to the class I RNR like the mammalian enzyme, depends on the formation of the complex between the two different proteins, R1 (α2) and R2 (β2), and is regulated by the binding of dNTPs and ATP, which act as allosteric effectors. The R1 homodimer carries two types of regulatory sites in addition to the catalytic binding site. Overall activity is stimulated by ATP and inhibited by dATP through the binding of these effectors to the activity site (3). The regulation of substrate specificity involves a second type of positive regulatory site, called the specificity site, which binds dTTP, dGTP, dATP, and ATP. Binding of effectors to this site modulates either substrate selectivity or rate of reduction. With ATP or dATP bound, the enzyme catalyzes the reduction of pyrimidine nucleotides (CDP and UDP). With dTTP bound to the specificity site GDP is the preferred substrate, whereas binding of dGTP favors ADP reduction (4).

Recently, the crystal structures of R1 complexed with an ATP analog, the specificity effector dTTP, and the substrate GDP in combination with dTTP have identified the two allosteric sites and the catalytic site (5). Crystallographic data suggest that during catalysis R1 undergoes significant conformational changes that dictate the redox cycling of the catalytic redox-active cysteines and the adjustment of the catalytic site for the selected substrate (5, 6). In addition, substrate binding is shown to occur only to the reduced form of R1.

The *E. coli* R2 protein harbors in each of its two polypeptide chains an essential free radical at Tyr-122 and a non-heme diiron center. The function of the metal site appears to be the generation and stabilization of the radical (7–10). Protein R2 having the tyrosyl radical reduced to normal tyrosine is called Met-R2 (R2Met) and is enzymatically inactive. Reduction of the cluster and subsequent oxidation by molecular oxygen are required for the generation of the radical from Met-R2. In *E. coli*, a flavin reductase supplies the electrons needed for the reduction of the Met cluster (11). Many different R2 structures are available, including different states of the wild type protein (i.e. apo-, Met-, red-R2) and various mutant forms, but there is no crystal structure of R2 containing the radical (6, 12–14).
Formation of the R1-R2 complex allows the controlled delivery of the radical function from Tyr-122 in R2 to Cys-439 in R1 via the proposed hydrogen-bonded path (12, 14). The process of radical transfer to the active site occurs only when substrate and effectors are present, to prevent uncontrolled redox processes and loss of the radical. The R1-R2 complex has not yet been structurally characterized. Kinetic studies of truncated R2 and R2 C-terminal peptides have determined that R1 and R2 interact primarily via the C-terminal 30 amino acids in R2 (15). A model of the complex was developed on the basis of docking studies, and the crystal structure of R1 co-crystallized with synthetic 20-residue peptide corresponding to the C terminus of R2 (16). According to this model the C terminus of R2 binds into a cleft in R1, thus completing the specific electron transfer path between the two subunits required for radical shuttling. Unfortunately, no significant spectroscopic changes are observed upon complex formation between R1 and R2 (17, 18). Moreover, in wild type RNR reactions the tyrosyl radical is always present, and no change in the radical spectroscopic properties is detected with naturally occurring substrates. Nevertheless, the redox potential of the diiron site shifts to a considerably more negative value upon complex formation between R2 and R1, and the hydrogen bonding pathway proposed to operate during catalysis may involve at least one of the ferric ions (12, 16, 19, 20). This shift in the redox potential is the only evidence to date of a conformational change at the metal/radical site induced by R1 binding.

In this work we test the hypothesis that the activity of the R1-R2 complex can be modulated by conformational changes at the dinuclear center induced by binding of substrate and allosteric regulators to R1. By studying the conformational transitions of the diiron centers in different regulatory states of the enzyme, we can gain insight into the interactions that promote long range electron transfer in RNR. The redox properties of the active radical-containing R2 and its complex with R1 are determined and then compared with those obtained in the presence of ATP, dATP, dTTP, and GMP/dTTP. These observations are discussed in terms of the factors known to regulate the reaction of the protein and working electrode. Solutions were made anaerobic through several cycles of argon and vacuum over a total period of 2 h. These experimental conditions were the same as those applied in our previous redox measurements of R2red and its complex with R1 (20). The protein concentrations used in all present studies were about 18–22 μM radical containing R2 and 24–28 μM R1, a sufficient excess of R1 was added to ensure complete complexation based on studies showing that R1 and R2 components form a 1:1 complex of active enzyme with dissociation constant of 0.09–0.18 μM (15). Concentrations of 2–4 μM were used for indicator dyes, selected to cover the experimental potential range of protein titration, with a starting volume of 5 ml. The reaction mixture for typical R1-R2 experiments contained a final concentration of 0.35 mM DTT. At pH 7.4 DTT does not affect the properties of the diiron centers (24).

The effect of substrate analogs and allosteric effector binding on the redox potential of the active R1-R2 complex was determined in the presence of saturating amounts of the physiological ligands (25). R1 in solution was present as an effector or effector/substrate analog-saturated complex prior to the addition of R2. The standard incubation mixture was used with the activity effector ATP added at variable concentrations (0.35, 0.6, 0.7, and 1.45 mM) or with the negative effector DATP at concentrations 0.3, 0.4, and 0.6 mM. Reductive titrations of R1-R2 with substrate analogs dGDP (2.6–3.0 mM) or GMP (3.0–3.4 mM) were performed in the presence of 45 μM dTTP (a specificity effector). Mg2+ ions are required for complex formation between R1 and R2 and for enzyme activity. In our experiments Mg2+ concentration used was 0.35 or 0.7 mM in experiments with ATP and dATP and 0.35 mM in experiments with substrate analog/specificity effector.

The equilibrium potential was determined to be stabilized when the measured potential changed by less than ±1 mV per 30 min. All potential values reported herein are referred to the standard hydrogen electrode at 25 °C. 4 mV were added to the final values for temperature correction (20). The Nernst equation (Equation 1) was applied to calculate the midpoint potential (E0) of the diiron cluster in R2 from the plot of E versus log([Enzox]/[Enzred]) (Eq. 1)

$$E = E_0 + (0.059/n) \log \left( \frac{[Enz_{ox}]}{[Enz_{red}]} \right)$$

(Eq. 1)

UV-visible spectroscopy was made on a PerkinElmer Life Sciences Lambda 12 Spectrophotometer with a thermostated cell compartment and PerkinElmer Life Sciences Computerized Spectroscopy Software (PECSS), version 4.31, was used for spectral acquisition and manipulation.

The bioanalytical system BAS-100 electrochemical analyzer was used for potentiometric titrations. All collected UV-visible spectra were corrected for turbidity and dye contributions. All the potential data reported here are believed to be accurate, because the Nernst plots obtained are close to the ideal, and this is the most rigorous criterion for achievement of equilibrium. The observed shifts in the potential are thought to be representative of the expected complexes as almost no turbidity was observed during redox experiments.

Miscellaneous—Structural diagrams were constructed using the program WebLab from crystallographic coordinate files deposited in the Protein Data Bank. The files 1R1B (differf R2), 1MXR (differf R2), 1XIK (differf R2), and 1AVS (Tyr-122 → Phe R2) were used.

RESULTS

R2 can exist in one of three stable redox states, the fully oxidized active R2ox form [Fe(III)Fe(III) ... TyrO], the one-electron reduced R2red form [Fe(III)Fe(III) ... TyrOH], and the fully reduced R2redred form [Fe(II)Fe(II)]. The midpoint potential of the inactive Met form of R2 produced by reduction of R2ox with the radical scavenger H2O2 was measured in our previous work for both free R2red and its complex with R1 (20). In this...
work we extend those studies to the enzymatically active radical containing R2ox and to the Met form obtained from the electrochemical reduction of R2ox. We designate as R2Met,e, the form of R2Met produced by reduction of R2ox with H2 and as R2Met,c the form produced during the reductive titration of R2ox.

The electrochemical reductive titration of R2ox occurs as two distinct processes. The first one corresponds to the one-electron reduction of the tyrosyl radical, at a potential of 1.0 ± 0.1 V (20) to generate R2Met,e (Fig. 1). R2Met,e is subsequently reduced to R2Met,c at a potential E_m = −163 ± 3 mV (Table I) in a single two-electron process, as indicated by a slope of 35 mV in the corresponding Nernst plot (Fig. 1, inset). This value is about 50 mV more negative than the one previously recorded for the R2Met,c/R2red couple (−115 mV) (20). Exposure of the protein thus reduced to air leads to oxidation by O2 and regeneration of R2ox. A second titration carried out on the same sample generates an identical titration curve with the same potential (E_m = −166 ± 3 mV). Under the equilibrium conditions utilized for potentiometric experiments, the heterogeneity of metal sites previously seen in kinetic experiments is not observed (26), as both diiron clusters of R2 are reduced at the same potential.

We investigated the effect of R1 association on the redox properties of R2ox. Similarly to the free R2ox, the R1-R2 complex is reduced in two stages. The first stage corresponds to the one-electron reduction of the tyrosyl radical to generate R2Met,e. The potential of this reduction step is approximately −1.0 V, as in the case of free R2ox. It is important to note that this value is an estimate, with limited accuracy (26), and changes of the order of 10s of mV induced by R1 binding would go undetected. The second stage corresponds to the two-electron reduction of R1-R2Met,e to R1-R2red. The midpoint potential for this step was determined to be −233 ± 6 mV (Table I), corresponding to a shift of 60 mV relative to the uncomplexed protein. By comparison, reductive titration of the R1-R2Met,c complex, involving the chemically generated Met form, provides a reduction potential of −226 ± 3 mV, virtually identical to the value measured for R1-R2Met,e.

The redox properties of the diiron centers in the R1-R2ox complex were investigated in the presence of the allosteric effectors ATP (general activator) and dATP (general inhibitor). As for the R1-R2 complex in the absence of effectors, reduction of the radical is observed first, at ~1.0 V, to generate the Met protein, followed by bielectronic reduction to the diferric form. The midpoint potential values for the Met form in the R1-R2 complex associated with ATP (at concentrations 0.35, 0.6, 0.7, and 1.45 mM) or dATP (at concentrations 0.3, 0.4, and 0.6 mM) were determined to be −215 ± 5 and −216 ± 3 mV, respectively (Table I). A slope of 30–34 mV was obtained in the Nernst plots, which compares reasonably with the theoretical slope of 27 mV for a two-electron transfer process. Overall, the potentiometric titration of both R1-R2/ATP and R1-R2/dATP gives redox potentials that are nearly identical to the ones exhibited by the R1-R2ox complex in the absence of effectors. Exposure of the reduced complex to air at the end of the titration resulted in generation of the characteristic spectrum of R2ox, showing 60% recovered radical for the dATP complex and 40–50% for the ATP complex. The midpoint potential of the diiron cluster in the R1-R2Met,e complex examined in the presence of dGDP/dTTP or GMP/dTTP was determined to be −192 ± 2 and −203 ± 3 mV, respectively (Table I), appreciably lower than the value obtained in the absence of nucleotides (Fig. 2, dGDP/dTTP-complex is shown). As in the other measurements reported in this work, the potential traces show a Nernstian behavior, corresponding to a bielectronic reduction (28–31 mV slope for the Nernst plot; Fig. 2, inset). Addition of air to the reduced complex at the end of the titration regenerated R2ox with 60% recovery.

As a reference, Table II (Supplemental Material) lists the different redox forms of R2 discussed in this work and their preparation.

**DISCUSSION**

**Effect of Tyrosine Reduction Mode on the Redox Properties of the Diiron Centers in R2Met**—In our previous work we studied R2Met obtained from the reduction of R2ox with H2 (now called R2Met). In contrast, in the current set of experiments we generate R2Met by electrochemical reduction of R2ox (now called R2Met,c). A difference in redox potentials of about 50 mV is reported, indicating that the Met form obtained via electrochemical reduction of R2ox is different from the form obtained via chemical reduction. To our knowledge this is the first time

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**TABLE I**

Midpoint potential for the reduction of diferric R2Met to diferric R2red

| Protein form | E_m (mV) |
|--------------|----------|
| R2Met,c      | −163 ± 3 |
| R2Met,e      | −115 ± 3 |
| R2-Y122F     | −178 ± 2 |
| R1-R2Met,c   | −226 ± 3 |
| R1-R2Met,e   | −233 ± 6 |
| ATP/R1-R2Met,c | −215 ± 5 |
| dATP/R1-R2Met,c | −216 ± 3 |
| dGDP/dTTP/R1-R2Met,c | −192 ± 2 |
| GMP/dTTP/R1-R2Met,c | −203 ± 3 |

*Values taken from previous work (20).*

*Values obtained from both previous (pH 7.6) and current work (pH 7.4).*
That the properties of the dinuclear center in \textit{R2\textsubscript{Met}} are explicitly related to the mode of tyrosine reduction. Nonetheless, analysis of past published work is consistent with this observation. Studies of \textit{R2\textsubscript{ox}} inhibition by several reducing agents have shown that different classes of reactants followed different kinetics, suggesting that different reduction mechanisms were at play (24–26). It is believed that the use of organic radicals (for example derived from methyl viologen, riboflavin, and phenosafranin) reproduces the conditions for reduction in \textit{vivo} via long range outer-sphere electron transfer (30). This is also the condition realized in the electrochemical cell, where methyl viologen/flavins are employed to favor electron transfer from the electrode surface to the protein. In contrast, the involvement of a different mechanism of radical reduction has been proposed for \textit{HU} on the basis of differences observed in reduction kinetics (12). A different reduction mechanism can account for the formation of \textit{R2\textsubscript{Met}} forms with different metal clusters. \textit{R2\textsubscript{Met,e}} and \textit{R2\textsubscript{Met,c}} are likely to be distinguished by changes in water coordination, hydrogen bonding, and/or reorientation of the amino acid side chains coordinated to iron.

Comparison of the published structures of \textit{R2\textsubscript{Met}} reveals differences among different structures in first and outer sphere geometry of the dinuclear site (Fig. 3, a and b). The most obvious variations are observed in \textit{H}-bonding between the radical bearing Tyr-122 and the iron ligand Asp-84 and in the coordination geometry of Asp-84 around the iron center (12, 14, 31). In the two structures Asp-84 adopts alternatively a monodentate (Fig. 3b) (31) or bidentate (Fig. 3a) coordination mode (12). Different protocols were used to generate the different Met forms reported above, neither of which included \textit{HU} or electrochemical reduction. Nonetheless, the variations indicate that this region of the dinuclear cluster is particularly sensitive to the way an electron is delivered to the radical.

In this respect, it is interesting to compare the redox potential of \textit{R2\textsubscript{Met,e}} and \textit{R2\textsubscript{Met,c}} with the previously determined values for the \textit{R2\textsubscript{Met}} mutant \textit{TYR-122 \textrightharpoondown PHE} (20). \textit{TYR-122 \textrightharpoondown PHE} is unable to generate a stable radical and can exist in two oxidation states only, corresponding to the fully oxidized (“\textit{Met}”) \textit{Fe(III)/Fe(III)} and fully reduced \textit{Fe(II)/Fe(II)} forms. The midpoint potential of the \textit{Met/red} couple is \(-175 \pm 2 \text{ mV}\) in the mutant protein (Table I), showing the distinct effect of Tyr and Phe on the environment of the diiron cluster. Presumably, the difference is mainly due to the absence of the hydrogen-bonding hydroxyl function connecting Tyr-122 with the bidentate iron ligand Asp-84 (Fig. 3) and to the different coordination of Asp-84 itself (32). The change in redox potential ascribed to these differences is comparable with the change observed between \textit{R2\textsubscript{Met,e}} and \textit{R2\textsubscript{Met,c}}. Indeed a change of 50 mV falls within the energy range of weak hydrogen bonds (2–12 kcal/mol) (20).

A hydrogen bond between Tyr-122 and Asp-84 is observed in the reduced form of the protein (13) (Fig. 3c). If establishing this interaction is necessary during reduction to stabilize the reduced form of the protein, the absence of this bond in \textit{R2\textsubscript{Met,e}} makes the formation of the reduced structure more unfavorable. This can rationalize the negative shift of redox potential in going from the couple \textit{R2\textsubscript{Met}/R2\textsubscript{red}} to the couple \textit{R2\textsubscript{Met,e}/R2\textsubscript{red}}.

Recent high field EPR spectra show that the \textit{H}-bonding proton is inserted between Tyr-122 and Asp-84 during reduction of the radical to give the Met form (31). As a consequence the proton is probably significant from the mechanistic point of view and is a putative component of the electron/proton transfer pathway proposed to allow radical transfer from Tyr-122 to the active site of R1. Our redox measurements suggest that the coordination sphere of the \textit{R2\textsubscript{Met}} cluster can access different conformations, possibly determined by differences in the same H-bond between Tyr-122 and Asp-84, and suggest that the mechanism of radical reduction has a role in generating this difference.

The sensitivity of the redox potential of the dinuclear cluster to relatively small perturbations of its coordination sphere is mechanistically significant, as it provides a mode for controlling electron transfer processes involving the iron atoms. It has been proposed that transfer of the radical function to the active site during turnover proceeds via such pathway. Therefore, we suggest that indirect interaction with other components of the RNR system can tune the reactivity of the protein complex by inducing conformational changes in the cluster itself.

\textbf{Effect of R1 Binding on the Redox Properties of the Diiron Cluster}—Our earlier redox studies of \textit{R2\textsubscript{Met}} and the \textit{TYR-122 \textrightharpoondown PHE} mutant, both complexed with \textit{R1}, showed that the redox potential of the diiron cluster for the \textit{R2\textsubscript{Met}}/\textit{R2\textsubscript{red}} couple shifted in the negative direction by over 100 mV in the complex, becoming \(-226 \pm 3 \text{ mV}\) in \textit{R2\textsubscript{Met}}/\textit{R1} and \(-281 \pm 3 \text{ mV}\) in \textit{TYR-122 \textrightharpoondown PHE}/\textit{R1} (20). In this work we report the redox potential of the couple \textit{R2\textsubscript{Met,e}}/\textit{R2\textsubscript{red}} as \(-223 \pm 6 \text{ mV}\), indistinguishable from the value reported for \textit{R2\textsubscript{Met}}/\textit{R2\textsubscript{red}} (Table I). The identity in redox potentials suggests that the dinuclear clusters in both \textit{R2\textsubscript{Met}}/\textit{R1} and \textit{R2\textsubscript{Met,e}}/\textit{R1} complexes have a very similar structure, contrary to that observed for the isolated \textit{R2\textsubscript{Met}} forms. From the direction of the redox shift we can conclude that \textit{R1} binds the \textit{R2\textsubscript{Met}} forms significantly more tightly than the reduced form, thereby hindering formation of the reduced ferric cluster under turnover conditions in \textit{vivo}.

In the hypothesis that the structural differences between \textit{R2\textsubscript{Met,e}} and \textit{R2\textsubscript{Met,c}} arise mostly from differences in cluster

\footnote{Pär Nordlund, personal communication.}
structure, binding of R1 appears capable of stabilizing only one conformation of the metal site, irrespective of how the Met form has been generated. Binding of R1 provides a mechanism to tune the structure of the dinuclear site toward one form, which is presumably more active in favoring transfer of the radical function from the tyrosine 122 to the catalytic site.

Although the unbound Tyr-122 → Phe R2 is found to have a reduction potential comparable with R2_Met,e, binding of R1 to the mutant causes a much larger shift in the redox potential (−103 mV as opposed to −60 mV determined for R1-R2_Met,e) (Table II). This indicates that R1 appears unable to tune the structure of the diiron centers in the mutant protein to one similar to wild type R2 (Fig. 3), in agreement with conclusions from EPR studies of the mixed valent S = 9/2 form of the mutant (33). The thermodynamic results for R1-R2_Met and R1-Tyr-122 → Phe suggest that the hydrogen bond between Tyr-122 and Asp-84 is an important factor in controlling both the diiron cluster potential as well as the normal binding modes between R1 and R2.

Influence of Activity Effectors on the Diiron Cluster in the R1-R2 Complex—In the model of the R1-R2 structure, the activity effector site on R1 is positioned far from the substrate-binding site but close to the C terminus of R2 and may influence the relative orientation of the two subunits. Competitive binding of ATP/dATP to this site controls overall enzyme activity. To assess whether this allosteric effect is mediated by perturbation of the electron transfer reaction, we measured the effect of ATP/dATP binding on the redox potential of the dinuclear site.

In contrast to their effect on the enzymatic activity (stimulation by ATP and inhibition by dATP), neither effector induces changes in the redox properties in the R1-R2 complex (Table I). According to redox data, the local structural rearrangements around the activity binding sites that may have an influence on the rates of NDP reduction are not communicated to the diiron centers.

Most interesting, enzyme activity analysis of aerobic E. coli RNR has shown that the reaction proceeds also in the absence of an effector (4). Moreover, it has been shown by centrifugal sucrose density gradients and glycerol gradients that the binding affinity between R1 and R2 does not change when effectors are present (34–36). These observations confirm that no major effector-induced reorganization is required for catalysis. The fact that other RNR proteins of the same class, such as mammalian and mouse enzymes, display an absolute requirement for effectors prevents a generalization of this conclusion (1, 6).

The redox data concerning ATP binding to the R1-R2 complex presented herein are consistent with the recently published crystal structure of the AMPPNP complex with R1, showing that the binding of this effector analog does not lead to any significant conformational changes in the resting state of the R1 protein (5).

Substrate Analog Specificity Effector Binding Modulates the Midpoint Potential of the Diiron Centers—Enzymatic selectivity for a given nucleotide substrate is affected by binding of a specificity allosteric effector. As for the activity effector case, we assessed the possibility that allosteric control by the specificity effector/substrate couple is mediated by changes in the diiron center by measuring the redox potential of the couple R2_Met,eR1/R2_redR1. The binding affinity between the two subunits will change if the effector molecules perturb the R1-R2 binding equilibrium between the reduced and oxidized enzyme. Therefore, this experimental result may give insight into the mechanism of electron transfer regulation of RNR. The two natural substrate analogs dGDP and GMP both in combination with dTTP have been used for this purpose.

The x-ray crystal structure of the R1 homodimer identified two specificity effector sites located at the polypeptide interface and two catalytic sites located deep inside the protein (5). The
distance between the dTTP-binding site and the closest substrate, GDP, is about 15 Å, requiring that the effect of the allosteric effector must be mediated by long range protein interactions. Combined biochemical studies and crystallographic analyses have demonstrated that structural changes taking place upon substrate/specificity effector association do affect R1 structure and thus its specific interactions with the R2 component, leading to rearrangements in hydrogen bonding network along the radical transfer pathway (5, 37). In agreement with these observations, our titrations confirm changes in specific R1-R2 interactions upon substrate/effector binding (Table I).

The positive shift of 31 mV in the redox potential of the diron cluster with respect to the R1-R2 complex suggests that dGDP/dTTP binding to R1 causes the metal sites in R2 to undergo conformational rearrangements controlled by the new protein environment. Association of dGDP/dTTP appears to regulate the interaction of the two subunits. Most interesting, substrate analog/effector binding is linked to the increased ease of reduction of the cluster, suggesting that effectors favor the reductive process probably by affecting the electron transfer pathway. The binding of the mononucleotide substrate analog GMP in combination with dTTP to the active R1-R2 complex also induced a positive shift of 20 mV in the midpoint potential of the diiron cluster, indicative of perturbation of the metal sites in R2 (Table I). The smaller redox change in the latter case can be explained with structural differences in the R1-diiron cluster with respect to the R1-R2 complex suggests that substrate analog/effector association to R1 results in conformational perturbation of the metal sites in R2 subunit. The redox data have shown for the first time that electron transfer in E. coli RNR is regulated by complex formation between R1 and R2, and the binding of substrate analogs and specificity effectors.

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