Enhancement by Effectors and Substrate Nucleotides of R1-R2 Interactions in *Escherichia coli* Class Ia Ribonucleotide Reductase*

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Ribonucleotide reductases are a family of essential enzymes that catalyze the reduction of ribonucleotides to their corresponding deoxyribonucleotides and provide cells with precursors for DNA synthesis. The different classes of ribonucleotide reductase are distinguished based on quaternary structures and enzyme activation mechanisms, but the components harboring the active site region in each class are evolutionarily related. With a few exceptions, ribonucleotide reductases are allosterically regulated by nucleoside triphosphates (ATP and dNTPs). We have used the surface plasmon resonance technique to study how allosteric effects govern the strength of quaternary interactions in the class Ia ribonucleotide reductase from *Escherichia coli*, which like all class I enzymes has a tetrameric \(\alpha_4\beta_2\) structure. The \(\alpha_4\) component called R1 harbors the active site and two types of binding sites for allosteric effector nucleotides, whereas the \(\beta_2\) component called R2 harbors the enzyme active site region and the allosteric binding sites. All class I RNRs consist of two non-identical homodimeric components denoted R1 (or \(\alpha_4\)) and R2 (or \(\beta_2\)). The active site region and the allosteric binding sites are located in the R1 component, whereas the radical required for catalysis is harbored by the R2 component. The three-dimensional structures are known for both components of *E. coli* class Ia RNR, protein R1 of 171 kDa and protein R2 of 87 kDa (3–5). A quaternary structure of the holoenzyme is not known but involves the interaction of the C-terminal part of the R2 component with two helices (\(\alpha_1\) and \(\alpha_2\)) in R1 (3, 5–9). Based on this knowledge on the known location of the interaction site between the R1 protein and a peptide mimicking the R2 C terminus and on symmetrical complementarities in the R1 and R2 structures, a quaternary structure for the holoenzyme was suggested (cf. Fig. 1) (5). The proposed quaternary structure offers a plausible mechanism for how the active site in R1 and the radical site in R2 may interact via long range radical transfer during catalysis (3, 10, 11).

Whereas the active site in R1 is responsible for the actual reduction of the ribonucleoside diphosphate substrates, the allosteric regulatory sites are responsible for determining the substrate specificity and for switching on or off the enzyme activity. The allosteric regulation is controlled by two types of nucleotide binding sites denoted the “specificity” site and the “overall activity” site. Each R1 homodimer harbors two identical specificity sites at the homodimer interface and two identical overall activity sites at the N-terminal domains (12). The specificity sites can bind dTTP, dGTP, dATP, and ATP, whereas the overall activity sites only bind ATP and dATP. ATP is a positive effector when bound to the specificity site as well as to the overall activity site, whereas dATP has a positive regulatory role only when bound to the specificity site. When dATP is bound to the overall activity site (which has a 10 times lower affinity for dATP compared with the specificity site), it acts as a general negative effector and shuts down catalysis.

The enzyme ribonucleotide reductase (RNR) catalyzes the reduction of ribonucleotides to the corresponding deoxyribonucleotides, the building blocks needed for DNA synthesis. The reaction is based on radical chemistry. A sophisticated allosteric regulation manifested by the binding of deoxyribonucleoside triphosphates and ATP allows the enzyme to coordinate a balanced production of precursors for DNA replication and repair.

RNRs can be grouped into three different classes (1–3). The focus of this study is the class Ia RNR of *Escherichia coli* (the subgrouping of class I into Ia and Ib is based interalia on the presence or the absence of one of the two allosteric sites). All class I RNRs consist of two non-identical homodimeric components denoted R1 (or \(\alpha_4\)) and R2 (or \(\beta_2\)). The active site region and the allosteric binding sites are located in the R1 component, whereas the radical required for catalysis is harbored by the R2 component. The three-dimensional structures are known for both components of *E. coli* class Ia RNR, protein R1 of 171 kDa and protein R2 of 87 kDa (3–5). A quaternary structure of the holoenzyme is not known but involves the interaction of the C-terminal part of the R2 component with two helices (\(\alpha_1\) and \(\alpha_13\)) in R1 (3, 5–9). Based on this knowledge on the known location of the interaction site between the R1 protein and a peptide mimicking the R2 C terminus and on symmetrical complementarities in the R1 and R2 structures, a quaternary structure for the holoenzyme was suggested (cf. Fig. 1) (5). The proposed quaternary structure offers a plausible mechanism for how the active site in R1 and the radical site in R2 may interact via long range radical transfer during catalysis (3, 10, 11).

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1 The abbreviations used are: RNR, enzyme ribonucleotide reductase; CM5, carboxymethylated dextran matrix; DTT, dithiothreitol; P20, non-ionic detergent (Amersham Biosciences); PMSF, phenylmethylsulfonyl fluoride; RU, resonance units.
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(13). When bound at the specificity site, ATP and dATP are preferentially promoting the reduction of CDP and UDP, dTTP promotes the reduction of all four substrates, and dGTP promotes the reduction of GDP and dGDP (14). In vivo, the enzyme may have both types of allosteric sites occupied by nucleotides with the effect that the physiological allosteric regulation of substrate specificity in the presence of ATP (at the overall activity site) is slightly different. ATP and dATP now promote the reduction of CDP and UDP, dTTP promotes the reduction of GDP (and ADP), and dGTP promotes the reduction of GDP (and GDP) (15). The allosteric regulation of most RNRs, including all of the eukaryotic RNRs, follows this general scheme (16).

Several ultracentrifugation studies have demonstrated qualitatively that the allosteric effectors strengthen the E. coli R1-R2 holoenzyme complex (6, 13, 17). The allosteric effects on E. coli R1-R2 holoenzyme complex (6, 13, 17). The allosteric effectors strengthen the E. coli R1-R2 holoenzyme complex (6, 13, 17). The allosteric effects on E. coli R1-R2 holoenzyme complex (6, 13, 17). The allosteric effects on E. coli R1-R2 holoenzyme complex (6, 13, 17).

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Expression and Purification of the Hexahistidine-tagged Proteins—The E. coli BL21(DE3) cells containing a mutagenized pTB1 or pTB2 plasmid were grown in five flasks each with 1 liter of LB medium (total 5 liters of medium) with 50 μg/ml carbenicillin. The cultures were shaken vigorously (250 rpm) at 37 °C. When the cultures had grown in the exponential phase for at least 10 generations and reached an absorbance of 0.8, the expression of the cloned gene was induced by the addition of isopropyl-β-d-thiogalactopyranoside to a final concentration of 1 mM. After ~3 h of induction when the cultures reached the stationary phase at an absorbance of 2.1–2.4, the cultures were harvested by centrifugation. Cell pellets were frozen on dry ice and stored at −80 °C.

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facilitate coupling to the membranes. The interaction area between proteins R1 and R2 has not been identified experimentally but is strongly suggested based on symmetries in the separately solved crystal structures for the two components and on a number of biochemical data involving site-directed mutagenesis. Therefore, we sought to introduce the smallest possible tag to avoid disturbing the natural protein-protein interactions within the complex and to use parts of the polypeptide chains not likely to be involved in the holoenzyme interactions. We decided to introduce hexahistidine tags at the N termini of proteins R1 and R2, and in addition, we introduced a hexahistidine sequence into the middle of the R2 polypeptide at a position where the largely α-helical structure of R2 is interrupted by two consecutive β-strands (Fig. 1). This part of the structure forms a tip of the heart-shaped R2 structure and is considered to be opposite to the part of R2 that interacts with protein R1 in the enzymatically active holoenzyme complex (Fig. 1). The N-terminal hexahistidine addition to protein R1 and the internal hexahistidine addition to protein R2 resulted in proteins that behaved similarly to their respective wild type counterparts with regards to solubility and protein stability. However, the N-terminal hexahistidine addition to protein R2 gave a highly insoluble protein that eluded several refolding attempts and therefore could not be used for further studies. The N-terminal part of each R2 polypeptide chain interacts with the other polypeptide chain in the R2 homodimer, which may explain why this construct suffered from solubility and/or folding problems.

The internal R2-His6 protein had a $K_D$ for interaction with R1 and a holoenzyme $k_{cat}$ that were similar to the $K_D$ and $k_{cat}$ of wild type R2 (Table I). The iron content of R2-His6 was lower than the theoretically expected value of 4, but this was also the case for the wild type R2 protein (Table I) and has been consistently found in many earlier studies (37). The catalytic activity of R2-His6 and wild type R2 is comparable to the values published earlier (37) as are their dissociation constants toward protein R1 as well (8). Also, the catalytic efficiency of the N-terminal R1-His6 protein and its dissociation constants toward protein R2 were similar to those of wild type R1 (data not shown).

**R1-R2 Interaction Studies by Surface Plasmon Resonance**—We performed amine-based chemical coupling of each hexahistidine-tagged protein to CM5 membranes. Studies of R1-R2 protein interactions have to be performed in buffers where oxidation of protein R1 can be avoided and magnesium ions can be included. Therefore, it is not possible to use nitrotriacetic acid membranes for coupling. Also, using membranes with hexahistidine-specific antibodies would require that all of the experiments be run anaerobically, which would impose additional complications.

In initial studies, it turned out that R1-His6 coupled to the CM5 membrane did not bind any R2 protein and could not be used for protein interaction studies. One possible explanation is that the N-terminal domain of R1, which harbors the allosteric activity site, is involved in the formation of the R1-R2 holoenzyme complex (cf. Fig. 1). Other explanations may be that the R1 interaction surface was not exposed on the membrane or that R1 was inactivated by the coupling conditions. The internal R2-His6 protein was resistant to the coupling conditions (data not shown) and was consequently used for all of the subsequent studies on protein interactions between the ribonucleotide reductase components proteins R1 and R2. Initial Biacore binding experiments showed that there was a high background of unspecific binding of protein R1 to the CM5 membrane (activated and deactivated), even in the absence of coupled R2-His6. Similar problems have been observed previ-

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**Subunit Interaction in E. coli Ribonucleotide Reductase**
The conditions required by the Biacore assay will adequately reflect the behavior of the wild type ribonucleotide reductase enzyme under these conditions.

**Positive Allosteric Effectors Induce a Specific R1-R2 Interaction That Is Strengthened by Substrate Nucleotides**—The R2-His6 protein coupled to the CMS membrane interacts weakly with the R1 protein in the absence of the allosteric effector nucleotide. The association and dissociation phases are very fast, and saturation could not be obtained (Fig. 2C). In contrast, the presence of the positive allosteric effector dTTP induces a specific interaction between R1 and R2-His6 bound on the CMS membrane (Fig. 2A). The association and dissociation phases are still fast, but plots of bound R1 versus injected R1 concentration show saturation kinetics (Fig. 2C) with a $K_D$ of 6.5 μM in the presence of 100 μM dTTP (Table III). The $K_D$ was constant over a range of 0.3–2 ng of R2-His6 coupled/mm² of the membrane and was essentially the same in the presence of 1 mM dTTP. The maximal amount of R1 interacting with bound R2-His6 on the membrane is constant over the entire range of coupled R2-His6 (Fig. 2D), indicating that only approximately one-third to one-half of the bound R2-His6 is available for R1 interaction (Table III). Inclusion of other positive allosteric effectors instead of dTTP gave similar results as shown in Table III for dGTP and ATP. The $K_D$ values were similar to those in the presence of dTTP, and the amount of bound R1 protein was typically 0.4–0.6 mol/mol R2-His6 bound to the membrane. The inclusion of thioredoxin together with the R1 analyte gave similar $K_D$ values and relative amounts of R1 bound per R2-His6 on the membrane as in experiments without thioredoxin. This result agrees well with the lack of thioredoxin effects seen in solution studies with the Biacore buffer (cf. Table II). We also revisited dCTP as a potential allosteric effector (40) and found that it behaves similarly to dTTP and promotes reduction of all four substrates (data not shown). However, an ~15-fold higher concentration of dCTP compared with dTTP is required. In the surface plasmon resonance experiments, 1 mM dCTP was required to promote a positive effect on R1-R2 interaction (Table III).

Interestingly, in the presence of both a positive allosteric effector (dTTP) and a substrate nucleotide, i.e. GDP (Fig. 2, B and C) or CDP, the binding of protein R1 to the bound R2-His6

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**TABLE I**

**Characteristics of R2-His6 compared with wild type R2**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Iron content</th>
<th>$K_D$ (μM)</th>
<th>$k_{cat}$ (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R2 wild type</td>
<td>2.9 ± 0.2</td>
<td>0.23 ± 0.04</td>
<td>600 ± 35</td>
</tr>
<tr>
<td>R2-His6</td>
<td>2.7 ± 0.2</td>
<td>0.21 ± 0.01</td>
<td>549 ± 47</td>
</tr>
</tbody>
</table>

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**FIG. 1. Three-dimensional structure of modeled R1-R2 complex.** The two polypeptides of R1 are shown in two dark gray shades, and the two polypeptides of R2 are shown in two light gray shades. The location of the different His₆ tags is indicated on the left R1 and R2 polypeptides, and the substrate and allosteric effector binding sites are on the right R1 polypeptide.
on the membrane was more pronounced (Table IV). This was manifested both as a stronger $K_D$ and as a higher amount of R1 protein bound to R2 on the membrane. As shown in Table IV, the presence of GDP plus dTTP induced a 3.3 times stronger R1-R2 complex and the presence of GDP plus dTTP induced a 2.2 times stronger R1-R2 complex than did dTTP alone. Control experiments with a non-substrate nucleotide (GMP) in the presence of dTTP instead caused a weaker R1-R2 interaction (beyond the detection limits of the assay) compared with the interaction in the presence of dTTP.

**Nucleotide Binding Affinities to Protein R1**—For comparative reasons, we also performed nucleotide binding assays to protein R1 as binary complexes with dTTP or GDP and as ternary complexes with both dTTP and GDP using the ultrafiltration assay (35). In binary complexes, the $K_D$ for dTTP was $\sim$50 times stronger than the $K_D$ for GDP (Fig. 3). At 25 °C, the ternary complex had a $\sim$4-fold stronger $K_D$ for dTTP compared with the $K_D$ for dTTP in the binary complex and the ternary complex also had a 4-fold stronger $K_D$ for GDP as compared with GDP binding in the binary complex (Fig. 3). These results imply an energetically equivalent stabilization at both binding sites and a random order binding of nucleotides to R1. Interestingly, the magnitude of these allosteric effects of nucleotides on R1 is comparable to the effects that these nucleotides have manifested both as a stronger $K_D$ and as a higher amount of R1 protein bound to R2 on the membrane (Table III). Because drastic differences in the allosteric behavior of R1 at high and low temperatures have been observed previously (41), these nucleotide binding experiments

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**Table II**

<table>
<thead>
<tr>
<th>Type of buffer</th>
<th>$K_D$ (μM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type R2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R2-(His)$_6$</td>
<td>$0.23 \pm 0.04$</td>
<td>$792 \pm 31$</td>
</tr>
<tr>
<td>R2-(His)$_6$</td>
<td>$0.94 \pm 0.23$</td>
<td>$304 \pm 32$</td>
</tr>
<tr>
<td>SPR buffer (+ thioredoxin)</td>
<td>$1.73 \pm 0.68$</td>
<td>$272 \pm 47$</td>
</tr>
<tr>
<td>SPR buffer (+ thioredoxin)</td>
<td>$1.69 \pm 0.36$</td>
<td>$253 \pm 25$</td>
</tr>
</tbody>
</table>

**Table III**

<table>
<thead>
<tr>
<th>Effector nucleotide</th>
<th>Concentration (mM)</th>
<th>$K_D$ (μM)</th>
<th>Bound R1 (mol/mol R2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dTTP</td>
<td>0.1</td>
<td>$6.5 \pm 0.2$</td>
<td>$0.35 \pm 0.01$</td>
</tr>
<tr>
<td>dGTP</td>
<td>1</td>
<td>$8.2 \pm 0.9$</td>
<td>$0.52 \pm 0.03$</td>
</tr>
<tr>
<td>dCTP</td>
<td>0.1</td>
<td>$8.6 \pm 0.6$</td>
<td>$0.49 \pm 0.01$</td>
</tr>
<tr>
<td>ATP</td>
<td>1</td>
<td>$8.8 \pm 0.4$</td>
<td>$0.60 \pm 0.02$</td>
</tr>
<tr>
<td>CDP</td>
<td>0.1</td>
<td>ND$^a$</td>
<td>$9.7 \pm 0.9$</td>
</tr>
<tr>
<td>ATP</td>
<td>0.1</td>
<td>ND$^a$</td>
<td>$6.1 \pm 0.2$</td>
</tr>
</tbody>
</table>

$^a$ ND, not detected.

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**Table IV**

<table>
<thead>
<tr>
<th>Additions</th>
<th>$K_D$ (μM)</th>
<th>Bound R1 (mol/mol R2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$6.5 \pm 0.2$</td>
<td>$0.35 \pm 0.01$</td>
</tr>
<tr>
<td>GDP (1 mM)</td>
<td>$2.0 \pm 0.1$</td>
<td>$0.61 \pm 0.06$</td>
</tr>
<tr>
<td>CDP (1 mM)</td>
<td>$2.9 \pm 0.1$</td>
<td>$0.44 \pm 0.01$</td>
</tr>
</tbody>
</table>

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**Figure 2**

Surface plasmon resonance binding curves of protein R1 (analyte) interacting with protein R2-R2-(His)$_6$ (ligand) bound to CM5 membranes. Binding curves in the presence of 100 μM dTTP (A, 801 RU of R2-R2-(His)$_6$) as allosteric effector and 1 mM GDP (B, 801 RU of R2-R2-(His)$_6$) as substrate are shown. The different response curves represent injections of 0, 0.05, 0.15, 0.3, 0.6, 1, 1.5, 2, and 4 μM R1 in running buffer plus 2 mM DTT and dTTP or dTTP/GDP as indicated above. C, a comparison of the response in the absence of effector nucleotide ( bulldog, 840 RU of R2-R2-(His)$_6$) and in the presence of dTTP ( △ ) or dTTP/GDP ( ● ) is as noted above. D, the amount of R1 bound to different amounts of R2-(His)$_6$ bound to CM5 membrane in the presence of 100 μM dTTP.
at 25 °C also were performed at 4 °C. Both effector binding and substrate binding in the binary complexes were approximately four times stronger at 4 °C as compared with 25 °C. Nucleotide binding in the ternary complex at 4 °C was on the other hand the same as in the binary complexes at 4 °C. Our results suggest that the allosteric effects only occur at physiological temperatures, as was suggested previously from a transition at 12 °C in the Arrhenius plot of E. coli ribonucleotide reductase activity (41).

Concentrations of dATP Promoting Negative Allosteric Effects Result in Very Tight R1-R2 Interaction—The effector nucleotides ATP and dATP can bind to both types of allosteric sites in E. coli protein R1. Most intriguing is the effect of dATP, which is a positive allosteric effector, when bound to the specificity site where dTTP, dGTP, and ATP also bind but impose negative allosteric effects on enzyme activity when bound to the overall activity site (where only ATP can also bind). The $K_D$ values for dATP bound to the specificity and the overall activity sites of protein R1 are 0.9 and 6.3 μM, respectively (23), and the apparent $K_D$ values for positive and negative allosteric effects on enzyme activity are 0.2 and 1.7 μM, respectively (23). Therefore, the R1-R2 interaction on the Biacore membrane was studied at a dATP concentration between 1 μM and 1 mM. Fig. 4A shows that a 1 μM dATP concentration promotes an R1-R2 interaction reminiscent of what was seen with dTTP (cf. Fig. 2A), dGTP, and ATP. In contrast, at dATP concentrations of ≥10 μM, the R1-R2 interaction becomes gradually much stronger, most prominently seen as drastically slower dissociation phases (Figs. 4, B–D). In addition, the relative amount of R1 bound to R2-His$_6$ on the membrane increases gradually from ~0.24 mol of R1 bound/mol R2 at 1 μM dATP to 0.57 mol/mol at 1 mM dATP (Table V). The dissociation constant is almost 100 times stronger at 1 mM dATP as compared with that at 1 μM dATP. Comparative R1-R2 binding experiments using the enzyme activity assay (7) gave similar results with $K_D$ values decreasing from 0.27 (at 1 μM dATP) to 0.015 μM (at 100 μM dATP, data not shown). This trend holds in solution also in the Biacore buffer where the $K_D$ values drop from 4.3 μM (at 1 μM dATP) to 0.001 μM (at 100 μM dATP, Table V).

**DISCUSSION**

Protein-protein interaction studies in class Ia ribonucleotide reductase from E. coli were initiated in the late 1960s (13), and the separate three-dimensional structures of the holoenzyme component proteins, R1 and R2, were solved to a high resolution in the 1990s (4, 5); however, the structure of the holoenzyme complex and the forces that influence the complex are still not known. In this study, we have adopted the surface plasmon resonance technique to study protein-protein interactions within E. coli class Ia ribonucleotide reductase. This is the first systematic study of how allosteric effector nucleotides, substrate nucleotides, and combinations thereof influence the R1-R2 holoenzyme complex.

To bind protein R2 to CM5 sensor chips, we introduced a hexahistidine tag in the middle of the R2 protein such that the His-tagged protein would be oriented specifically on the membrane. The His-tagged R2 protein in solution had the same solubility, stability, catalytic activity, and interaction with protein R1 as wild type R2. Bound to the CM5 membrane, the His-tagged R2 protein bound R1 with a constant $K_D$ within a span of 0.3–2 nM of R2 bound/mm$^2$ of membrane surface. The R1-R2-His$_6$ interaction on the membrane appears highly specific, because only known allosteric effector nucleotides but not non-interacting nucleotides can promote interaction of the two protein components. However, unspecific binding of protein R1 to the membrane initially disturbed the studies but could be overcome by the inclusion of increased concentration of detergent in the buffer system. This increased the numerical value of the $K_D$ compared with the $K_D$ obtained in solution experiments but still allowed relative comparisons of $K_D$ values for allosteric combinations not studied earlier with other techniques.

An important result of the surface plasmon resonance binding studies was that the combinations of substrate and effector nucleotides enhanced the R1-R2 interaction 2–3-fold compared with the $K_D$ in the presence of only effector nucleotide. This finding corresponds to a free energy change$^3$ of ~0.7 kcal/mol for dTTP/GDP compared with dTTP. Interestingly, we observed mutual allosteric effects of effector and substrate binding to protein R1 alone of the same magnitude (free energy change$^3$ of ~0.8 to ~0.9 kcal/mol) at 25 °C (the same temperature as for the surface plasmon resonance studies) but not at 4 °C. This finding strongly supports that the allosteric effects of nucleotide binding to R1 are the major determinants for the nucleotide-mediated changes in R1-R2 dissociation constant.

The remarkable dual effects of dATP as a positive effector at low concentrations (when bound to the allosteric specificity site) and negative effector at high concentrations (when bound to the overall activity site) have brought about several different molecular explanations. It has been suggested that the negative effects of dATP on catalysis results from the formation of multimeric R1 complexes at high dATP concentrations (13, 17). Another explanation based on studies of mouse ribonucleotide reductase is that high concentrations of dATP promote the formation of an R1-R2 complex that disturbs the catalytically essential radical transfer between R2 and R1 (25). A third explanation also relating to the mouse enzyme is that a high concentration of dATP (and ATP) induces an octameric complex (two R1 dimers and two R2 dimers) that isomerizes to an inactive form and that only ATP by binding to a third type of allosteric site can induce an enzymatically active dodecameric (three R1 dimers and three R2 dimers) form (27). We could observe a drastically (almost 100 times) decreased dissociation constant for E. coli R1-R2 in the presence of increasing concentrations of dATP. This increase of R1-R2 affinity coincides with the inhibition of enzyme activity (Ref. 23 and this study). Even though our results show increasing amounts of R1 bound to R2 when the interaction becomes tighter, similarly high stoichiometries were also obtained in the presence of positive effectors (Table III) and in the active complex (Table IV). Therefore, it is unlikely that the inhibitory effect of dATP is caused by the formation of multimeric R1 forms as proposed earlier (13, 17, 42). We propose that the negative allosteric effect of dATP occurs in a 1:1 complex of E. coli R1 and R2 and that the inhibition of enzyme activity is caused by the formation of the tight complex.

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$^3$Calculated according to: $\Delta G = RT \ln(K_{binary}/K_{ternary})$, where $R$ is the gas constant and $T$ is the temperature in Kelvin.
in running buffer plus 2 mM DTT and but the modeled holoenzyme complex of R1 and R2 has a cavity for thioredoxin to reduce the C-terminal disulfide of R1 (11), has been speculated that the R1-R2 complex has to come apart disulfide is reduced very fast by the C-terminal cysteine pair. It another study (44) establishes that the oxidized active site "

**Fig. 4.** Surface plasmon resonance binding curves of R1-R2 interaction in the presence of increasing dATP concentrations. The concentrations of dATP in the buffer and the load of R2-His6 on the CM5 membranes were 1 μM and 1146 RU (A), 10 μM and 1148 RU (B), 100 μM and 1382 RU (C), and 1000 μM and 1171 RU (D). The different response curves represent injections of 0, 0.025, 0.05, 0.15, 0.3, 0.6, 1, 1.5, 2, and 4 μM R1 in running buffer plus 2 mM DTT and dATP as indicated above.

**Table V**

<table>
<thead>
<tr>
<th>Concentration of dATP (μM)</th>
<th>SPR results (Bound R1)</th>
<th>Activity measurements (kcat)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_D (μM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mol/mol R2</td>
<td></td>
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<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>1.8 ± 0.5</td>
<td>4.3 ± 1.8</td>
</tr>
<tr>
<td>10</td>
<td>0.16 ± 0.02</td>
<td>0.80 ± 0.44</td>
</tr>
<tr>
<td>100</td>
<td>0.11 ± 0.06</td>
<td>0.001 ± 0.002</td>
</tr>
<tr>
<td>1000</td>
<td>0.023 ± 0.004</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>0.57 ± 0.01</td>
<td>26.5 ± 5.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.8 ± 0.03</td>
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</tbody>
</table>

*ND, not determined because of a lack of enzyme activity.

Most intriguingly, we discovered that thioredoxin enhances the R1-R2 interaction by a factor of 4 (corresponding to a free energy change of ~0.8 kcal/mol). No complex between thioredoxin or glutaredoxin (another physiological RNR reductant) and components of the ribonucleotide reductase system has ever been trapped. The active site cleft of R1 is not wide enough to allow thioredoxin to enter to reduce the Cys225/Cys462 disulfide formed during catalysis (5). How- ever, a working model based on studies with mutant R1 proteins lacking one or both of the C-terminal cysteines Cys754 and Cys759 (full-length R1 is 761 residues) predicts that thioredoxin and glutaredoxin works by reducing the C-terminal disulfide of R1 (32, 39). The C-terminal part of R1 (~20 residues) is too flexible to be visible in the electron density map of R1 (5). A plausible explanation is that the flexibility is functionally important and that the reduced C-terminal cysteine pair reaches into the active site of R1 to shuttle reducing equivalents to the active site. The structure of a mixed disulfide between a 25-mer peptide corresponding to the R1 C-terminal residues 737–761 and E. coli glutaredoxin provides insight into one of the potential intermediates in this sequence of redox reactions (43), and another study (44) establishes that the oxidized active site disulfide is reduced very fast by the C-terminal cysteine pair. It has been speculated that the R1-R2 complex has to come apart for thioredoxin to reduce the C-terminal disulfide of R1 (11), but the modeled holoenzyme complex of R1 and R2 has a cavity that may allow the C terminus of R1 to interact with the active site in the holoenzyme complex (3). One possible explanation to our discovery that thioredoxin strengthens the R1-R2 interaction is that oxidized R1 has a weaker interaction with R2 than reduced R1. Even mild oxidation of R1 (short exposure to air in the absence of chemical reductant) causes it to dissociate to monomers (17). If thioredoxin ensures that R1 is C-terminally reduced at the end of each turnover, the reduction of the active site cystine would be fast (44). The chemical reductant DTT gives a 2–5 times lower kcat value (32, 39) and presumably reacts directly with the active site cystine of R1. Another explanation is that the flexible C-terminal domain of R1 weakens the R1-R2 interaction and that its competing interaction with thioredoxin helps stabilize the R1-R2 complex. The last visible side chain in the R1 structure (Asp745) is close to the N-terminal domain, which harbors the allosteric activity site that is implicated in the R1-R2 interaction (cf. Fig. 1). Earlier perplexing observations that the C754S/C759S double mutant (39) and the C754A single mutant (32) of R1 had much higher kcat values with DTT as reductant than wild type R1 or single mutants C754S and C759A/S may also be explained by the inhibitory effects of only those flexible C-terminal domains (wild type, C754S, and C759A/S) that can interact with the active site disulfide in R1. If proper functioning of the reduced C-terminal domain of R1 required that the R1-R2 complex is weakened or comes apart, the tight R1-R2 complex promoted by dATP bound to the allosteric activity site would undoubtedly be a deadlock to catalytic turnover.

4 A. Holmgren, personal communication.
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Enhancement by Effectors and Substrate Nucleotides of R1-R2 Interactions in Escherichia coli Class Ia Ribonucleotide Reductase
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