Oligomerization Status Directs Overall Activity Regulation of the Escherichia coli Class Ia Ribonucleotide Reductase

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Ribonucleotide reductase (RNR) is a key enzyme for the synthesis of the four DNA building blocks. Class Ia RNRs contain two subunits, denoted R1 (α) and R2 (β). These enzymes are regulated via two nucleotide-binding allosteric sites on the R1 subunit, termed the specificity and overall activity sites. The specificity site binds ATP, dATP, dTTP, or dGTP and determines the substrate to be reduced, whereas the overall activity site binds dATP (inhibitor) or ATP. By using gas-phase electrophoretic mobility macromolecule analysis and enzyme assays, we found that the Escherichia coli class Ia RNR formed an inhibited αβ4 complex in the presence of dATP and an active αβ2 complex in the presence of ATP (main substrate: CDP), dTTP (substrate: GDP) or dGTP (substrate: ADP). The R1-R2 interaction was 30–50 times stronger in the αβ2 complex than in the αβ2 complex, which was in equilibrium with free α2 and β2 subunits. Studies of a known E. coli R1 mutant (H59A) showed that deficient dATP inhibition correlated with reduced ability to form αβ2 complexes. ATP could also induce the formation of a generally inhibited αβαβ complex in the E. coli RNR but only when used in combination with high concentrations of the specificity site effectors, dTTP/dGTP. Both allosteric sites are therefore important for αβ2 formation and overall activity regulation. The E. coli RNR differs from the mammalian enzyme, which is stimulated by ATP also in combination with dGTP/dTTP and forms active and inactive αβ2 complexes.

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2 The abbreviations used are: RNR, ribonucleotide reductase; GEMMA, gas-phase electrophoretic mobility macromolecule analysis/analyser; NDP, ribonucleoside-5′-diphosphate; DTT, dithiothreitol; SPR, surface plasmon resonance; HPLC, high performance liquid chromatography.

Class Ia RNRs are found almost in all eukaryotic organisms and some bacteria, viruses, and bacteriophages (see the Ribonucleotide Reductase Database (RNRdb)). The first RNR studied was from Escherichia coli, which is often referred to as a prototype for the class Ia enzymes. However, complementary studies in the mouse enzyme were also important to fully understand its sophisticated allosteric regulation. This regulation is needed to ensure that the intracellular levels of the four DNA building blocks are balanced (specificity site regulation) and not generally too high (overall activity regulation). The specificity site can bind ATP, dATP, dTTP, or dGTP and selects the substrate to be reduced. Class I RNRs use ribonucleoside-5′-diphosphates (NDPs) as substrates. Binding of ATP or low concentrations of dATP (Kd = 0.43 μM at 25 °C for the E. coli RNR (5)) to the specificity site promotes reduction of CDP and UDP. In a similar manner, GDP reduction is stimulated by dTTP and ADP reduction by dGTP.

The overall activity site is an ATP cone domain that binds ATP or dATP (6). The ATP cone domain is located in the N-terminal region of the R1 protein and is, according to the holoenzyme model, located close to the R1-R2 interaction surface (7, 8). This allosteric site determines the overall activity of the enzyme. ATP stimulates enzyme activity, whereas high concentrations of dATP (Kd = 5.7 μM (5)) turn it off. Although several models have been proposed (9–11), it is not known how the two almost identical nucleotides (ATP or dATP) can have opposite effects on enzyme activity. The allosteric regulation of the E. coli enzyme differs from the general model in that dTTP, when assayed with one substrate at a time, stimulates the reduction of all four substrates (12–16). However, the physiological effect of dTTP is ambiguous because the combination of dTTP and ATP leads to general inhibition of enzyme activity (ATP is always present in the cell).
The enzymatically active form of class Ia RNRs has previously been described to be a tetramer with $\alpha_2\beta_2$ structure (1). However, studies of the mouse enzyme suggest that the R1 subunit is a hexamer at physiological R1 protein and ATP/dATP concentrations. The R1 hexamer can interact with 1–3 R2 dimers to form an $\alpha_3\beta_2$ complex at physiological nucleotide and R1-R2 protein concentrations (17) and an $\alpha_3\beta_2$ complex in the presence of gemcitabine diphosphate (18), a substrate analog that stabilizes R1-R2 interaction. It is unclear which role these large complexes have in allosteric regulation; ATP and dATP have opposite effects on enzyme activity, but they still seem to stimulate the formation of similar complexes. A role of large complexes in allosteric regulation has also been demonstrated in the bacterial class Ia RNRs from *Pseudomonas aeruginosa* and *E. coli*. The *P. aeruginosa* enzyme possesses two adjacent ATP cones (19). Deletion of the most N-terminal ATP cone abolishes both the dATP-induced inhibition and the formation of large oligomeric complexes (19). Early ultracentrifugation studies on the *E. coli* class Ia RNR showed that dATP induces large complexes that had a 1:1 ratio of $\alpha$- and $\beta$-proteins and was suggested to be a dimer of the classical $\alpha_2\beta_2$ complex (14).

In contrast to the conventional techniques used for the characterization of the *E. coli* RNR, a previous study of mammalian allosteric RNR complexes (17) introduced a high resolution technique termed gas-phase electrophoretic mobility macromolecule analysis (GEMMA) (20, 21). In GEMMA, protein complexes are brought into gas phase to measure their diameter. Because there is a good correlation between diameter and mass in the gas phase, it is usually possible to measure the molecular mass of protein complexes within an error of 5.6% (21). In comparison with traditional methods such as ultracentrifugation or size exclusion chromatography, weaker RNR complexes can be studied with good separation of the different protein complexes in a wide molecular mass range (between 10 and >1000 kDa). Furthermore, the short run time (<5 min/run) enables extensive testing of various conditions.

To clarify how the *E. coli* class Ia RNR is regulated and what role large complexes have in this regulation, we have studied enzyme was for the first time studied with all four substrates simultaneously. The conclusion from this study was that the *E. coli* enzyme is in equilibrium between an active $\alpha_2\beta_2$ complex formed in the presence of ATP, dTTP, or dGTP with similar substrate specificity as other class Ia RNRs and an inactive $\alpha_3\beta_2$ complex formed in the presence of dATP or effector combinations of ATP + dTTP/dGTP. The *E. coli* RNR is therefore different from the mouse enzyme, which forms an active $\alpha_2\beta_2$ octamer in the presence of ATP (or effector combinations of ATP + dTTP/dGTP) and an inactive $\alpha_2\beta_2$ octamer in the presence of dATP (17). Contrary to current models for the mouse enzyme (22), the specificity site in the *E. coli* enzyme seems to have a central role in the overall activity regulation because the inactive complex is formed regardless whether ATP or dATP occupies the overall activity site as long as a deoxyribonucleotide occupies the specificity site (an inactive complex is not formed when ATP binds both allosteric sites). However, dATP is still the main regulator of overall activity at physiologically relevant concentrations of nucleotides, which is in common with the mouse enzyme.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of the R1 and R2 Proteins**—Purified procedures were used for the expression and purification of the *E. coli* R1 protein (23), the *E. coli* H59A mutant R1 protein (11), the *E. coli* R2 protein (24), the mouse R1 protein (25), and the mouse R2 protein (26). The *E. coli* R2Δ30C protein with a functional Tyr-122 residue was purified according to the protocol described for the Y122FΔ30C protein (27). All proteins in this study (except the R2Δ30C protein) were verified to be enzymatically active.

**RNR Activity Assay**—The *E. coli* RNR was assayed at 25°C in the presence of allosteric effector(s), substrate(s), 20–30 mM magnesium acetate, and 33 mM Hepes-KOH at pH 7.6. As reductant, either the thioredoxin system (0.4 mM NADPH, 13 μM *E. coli* thioredoxin, 0.5 μM *E. coli* thioredoxin reductase, and 30 μM EDTA) or 75 mM dithiothreitol (DTT) was used. DTT gave a lower specific activity of the enzyme than when the thioredoxin system was used but had no obvious effect on the allosteric properties of the enzyme. The specific activity of each protein (R1 or R2) was measured in nmol × min⁻¹ ng⁻¹ (units/mg) with 0.5 mM CDP as substrate and 1.5 mM ATP as effector in the presence of an excess of the other protein. Two assay procedures were used: the radioactivity assay and the HPLC assay.

In the radioactivity assay (50-μl reaction volume), tritium or $^{14}$C-labeled substrate was used. The reaction product was separated from the substrate using Dowex-50 (28) or boronate chromatography (29). The mouse RNR was assayed at 37°C with 10 mM DTT as reductant (28), but otherwise, the conditions were similar to the *E. coli* assay conditions described above to make the experiments comparable.

The HPLC assay (50 μl) was performed with all four substrates (non-radioactive CDP, UDP, GDP, and ADP) present at 0.7 mM each. The procedure was essentially as described previously (30) with the differences mentioned below. The assay buffer consisted of 30 mM magnesium acetate, 30 μM EDTA, 33 mM Hepes-KOH, pH 7.6, 20 mM NaF (to inhibit phosphatases), and 75 mM DTT. The products were separated from the substrates by boronate chromatography (29), and the amount of produced dCDP, dUDP, dGDP, and dADP was quantified by HPLC chromatography using a 100 × 4.6 mm, 3-μm 1500-Å PolyWAX A column (PolyLC, Columbia, MD), and a gradient between solution A (water) and solution B (0.4 mM potassium phosphate, pH 8.5). The gradient started with 20% solution B and increased gradually to 52% solution B (5 min), 45% solution B (30 min), 88% solution B (40 min), continued at 88% solution B (until 62 min), and decreased to 20% solution B (the complete run was finished in 72 min). The HPLC assays were generally performed with low concentrations (0.1 mM) of dNTP effectors to minimize the interference from dNDP impurities (the dNTP effectors dTTP, dGTP, and dATP generally contained ~0.5% dNDPs; dTDP was not well separated from dUDP in the HPLC...
chromatography)). A control sample where the R1 protein was omitted was used as background for these experiments. The reaction products were quantified by measuring the peak heights of the dNDPs, subtracting the background coming from effector dNTP impurities (control sample), and comparing the peak heights with a dNTP standard. The peak heights of the dNDPs in the control samples were severalfold lower than in the real samples for the experiments where an effector dNTP concentration of 0.1 mM was used.

Cloning of NusA-tagged E. coli R2—The E. coli R2 cloning sequence was inserted into the NusA-containing pETM-60 expression vector (European Molecular Biology Laboratory). To make the NusA-R2 fusion construct, the E. coli R2 gene in the pTB2 plasmid (27) was PCR-amplified. The forward and reverse primers used in the amplification procedure contained NcoI or BamHI restriction sites (underlined): forward, 5'-GAC ATG CCA TGG CAT ATA CCA CCT TTG C-3' (NcoI); and reverse, 5'-GGG GGA TTC TCA GAG CTG GAA GTT ACT C-3' (BamHI). The PCR product and pETM-60 expression vector were digested with both restriction enzymes, ligated, and transformed into E. coli, and purified. The resulting pETM60-R2 plasmid was verified by sequencing from both directions.

NusA-R2 Expression and Purification—The pETM60-R2 vector was introduced into E. coli Rosetta BL21(DE3) cells (Novagen) and expressed. Typically, 3 liters of cultures were cultivated by shaking at 150–200 mM potassium phosphate, pH 7.0 (15 ml of concentrated culture in 1 liter of running buffer). The cell pellets were disintegrated by freezing and thawing. Glycerol (20% (v/v)) was added to the thawed cells prior to ultracentrifugation (30,000 g for 30 min), resuspended in 50 mM Tris-HCl, pH 7.6, and centrifuged again. The resulting pellet was dissolved in 5 ml ammonium sulfate (0.36 g/ml, corresponds to 60% saturation) and centrifuged (30,000 g). The supernatant was injection of 15 ml of carrier buffer containing 20 mM ammonium acetate, pH 7.5, and 2 mM DTT was added to the R1 protein solutions to increase protein stability. Prior to GEMMA analysis, the protein samples were diluted to a concentration of 0.025–0.1 mg/ml in a buffer containing 20 mM ammonium acetate, pH 7.5, 0.005% (v/v) Tween 20, nucleotides, and magnesium acetate. All GEMMA experiments were performed at a MgCl2/nucleotide ratio of 1:1. At higher concentrations of magnesium, the efficiency of nucleotide-induced 510-kDa complex formation generally decreased but could be compensated for by adding more nucleotides. The GEMMA system contained the following components: 3480 electrospray aerosol generator, 3080 electrostatic classifier, 3085 differential mobility analyzer, and 3025A ultrafine condensation particle counter (TSI Corp., Shoreview, MN). The parameters used in the GEMMA system were similar to those previously described (17). A capillary pressure drop of 1.4–2 p.s.i. was used to minimize a broad nucleotide peak in the beginning of the graphs. When several experiments were plotted in the same graph (see Figs. 1 and 2), their baselines were plotted at intervals of 4–17.5 units. The samples were generally scanned several times (1–4 times) to increase the signal-to-noise ratio.

SPR Biosensor Analysis of E. coli R1-R2 Complex Formation in the Presence of ATP and dTTP—A detailed procedure will be published elsewhere4 but is briefly described here. SPR protein binding studies were performed using a Biacore 3000 biosensor instrument (GE healthcare). A sterile-filtered and degassed mixture of 10 mM HEPES, pH 7.4, 0.15 mM NaCl, 20 mM MgCl2, 2 mM DTT, and 0.005% (v/v) surfactant P20 was used as running buffer. Effector nucleotides dTTP (2 mM) and/or ATP (100 μM) (GE Healthcare) were included in the running buffer when required. The E. coli R2 protein was biotinylated with EZ-Link Sulfo-NHS-LC-LC-Biotin from Pierce Biotechnology Inc. to a ratio of approximately one biotin per R2 dimer. The biotinylated R2 proteins (ligand) were subsequently immobilized to streptavidin-coated sensor chips (GE Healthcare). A biotin-deactivated flow cell was used as reference cell in the experiments. Concentration series of six different R1 protein concentrations (analyte) between 0.01 and 5 μM were injected at a flow rate of 30–90 μl/min. Bound R1 proteins were removed from the immobilized R2 proteins by injection of 15 μl of 0.5 M KCl between the R1 injections. Data were analyzed using steady-state affinity calculations within the BIAevaluation 4.1 software (GE Healthcare).

RESULTS

Allosteric Regulation of the E. coli Class Ia RNR Is Not Affected by the GEMMA Buffer—GEMMA is restricted to volatile buffers/salts such as ammonium acetate. Before making a GEMMA analysis of the E. coli RNR, it was confirmed that the allosteric properties of the enzyme were not affected by the GEMMA buffer (20 mM ammonium acetate, pH 7.5). Similar specific activities, dATP inhibition constants, and specificities of the reaction were obtained in the absence or presence of ammonium acetate (data not shown).

E. coli RNR Forms a 510-kDa Complex in the Presence of dATP or ATP + dTTP—Gel filtration experiments show that the E. coli R1 protein is in a monomer-dimer equilibrium and is predominantly monomeric at 0.025 mg/ml (31). However, dimer formation can be induced by elevating the protein con-

4 M. Crona, E. Furrer, E. Torrents, and B.-M. Sjöberg, manuscript in preparation.
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GEMMA analysis of R1-R2 complex formation in the presence of 50 μM dATP showed a 510-kDa peak of unknown subunit composition (Fig. 1C, top trace). The formation of this complex was reversible (the complex dissociated upon removal of dATP (data not shown)) and dependent on R1-R2 interaction because it was not formed when the R2Δ30C protein was used instead of the wild-type R2 protein (Fig. 1C, bottom trace). The 510-kDa complex could also be formed in the presence of ATP in combination with dTTP (Fig. 1D, top trace) but not with dTTP used alone (Fig. 1D, bottom trace). The overall activity site is therefore involved in 510-kDa complex formation; this site binds dATP or ATP but neither dTTP nor dGTP. No 510-kDa complex was observed with ATP as sole effector (Fig. 1E, top trace). The formation of R1 dimers in Fig. 1E (bottom trace) is indicative that the concentration of ATP (100 μM) in the experiments was high enough to bind to both allosteric sites (ATP binds to both allosteric sites with equal affinity in the E. coli class Ia RNR (5)). A conclusion from the GEMMA experiments was that the formation of the 510-kDa complex requires occupancy of both allosteric sites and that the specificity site effector must be a deoxyribonucleotide because the complex was not formed with ATP alone. This conclusion is in agreement with previous ultracentrifugation studies where it was found that dATP (and combinations of ATP + dTTP) but not ATP alone could induce a larger protein complex than α2β2 (14).

The effect of substrates on E. coli RNR complex formation was also investigated. The substrates did not induce complex formation by themselves, but CDP had a stimulatory effect on dATP-induced 510-kDa complex formation. This effect was particularly obvious for the R1 mutant N238A (31), which is altered in the R1-R1 dimer interaction surface (see the supplemental data). ADP had a dual role in the E. coli RNR because this substrate could mimic ATP as an allosteric effector (see the supplemental data).

Concentration Units and Molecular Mass Determinations by GEMMA—The data in Fig. 1, A–E, were plotted as raw counts (i.e., the amount of particles measured by the machine in each size channel), but the GEMMA software (Aerosol Instrument Manager, TSI Inc., St. Paul, MN) could also plot the data in mass concentration units (see example in Fig. 1F), albeit at lower resolution (each data point in Fig. 1F is calculated from several data points in the original raw count data in Fig. 1C, top trace). Therefore, we only used the mass concentration unit when it was necessary to calculate the concentration of each complex.

The software could calculate molecular masses from the type of low resolution graphs shown in Fig. 1F (but not from the original data shown in Fig. 1C, top trace). The calculation gives approximate masses; even if the size of the largest complex is located between 510 and 568 kDa, it was still measured as 510 kDa in Fig. 1F. The measured size of the complex was also dependent on the concentration of salts, such as nucleotides and magnesium, as a crust of salt formed around the protein concentration or by the addition of allosteric effectors (31). GEMMA analysis of the R1 subunit at 0.05 mg/ml showed that it is primarily a monomer (theoretical molecular mass of 86 kDa) in the absence (Fig. 1A, top trace) and presence of 50 μM dATP (bottom trace). The molecular masses and subunit compositions of the complexes are shown on the top of each peak. B, analyses of 0.025 mg/ml R2 (top trace) and R2Δ30C proteins (bottom trace). C, analysis of a mixture of 0.05 mg/ml R1 and 0.025 mg/ml R2 proteins in the presence of 50 μM dATP (top trace). The bottom trace shows a negative control experiment where R2Δ30C was used instead of the R2 protein. D, analysis of a mixture of R1 (0.05 mg/ml) and R2 proteins (0.025 mg/ml) in the presence of a combination of 50 μM ATP and 50 μM dTTP (top trace) or 50 μM dTTP used alone (bottom trace). E, analysis of R1 (0.05 mg/ml) and R2 proteins (0.025 mg/ml) in the presence of 100 μM ATP (top trace). The R2 protein is omitted in the bottom trace. F, reanalysis of panel C (top trace), which is now plotted in concentration units instead of raw counts.

GEMMA analysis of R1-R2 interaction studies. The measured size of the complex was also indicative that the concentration of ATP (100 μM) in the experiments was high enough to bind to both allosteric sites (ATP binds to both allosteric sites with equal affinity in the E. coli class Ia RNR (5)). A conclusion from the GEMMA experiments was that the formation of the 510-kDa complex requires occupancy of both allosteric sites and that the specificity site effector must be a deoxyribonucleotide because the complex was not formed with ATP alone. This conclusion is in agreement with previous ultracentrifugation studies where it was found that dATP (and combinations of ATP + dTTP) but not ATP alone could induce a larger protein complex than α2β2 (14).

The effect of effectors on E. coli RNR complex formation was also investigated. The effectors did not induce complex formation by themselves, but CDP had a stimulatory effect on dATP-induced 510-kDa complex formation. This effect was particularly obvious for the R1 mutant N238A (31), which is altered in the R1-R1 dimer interaction surface (see the supplemental data). ADP had a dual role in the E. coli RNR because this substrate could mimic ATP as an allosteric effector (see the supplemental data).
particles makes them appear larger in GEMMA (34). If magnesium-nucleotide levels at 50 μM or below were used, this effect was usually not large enough to shift the measured molecular mass in the low resolution graphs. In Fig. 1E (top trace), when 100 μM ATP was used, the size of the R1 dimer shifted to 193 kDa, and a salt peak was seen in the beginning of the graph.

The dATP-induced R1-R2 Complex Is Determined to Have an αβ₄ Composition—The similar molecular masses of the E. coli R1 monomer (86 kDa) and R2 dimer (2 × 43 kDa) made it difficult to analyze the subunit composition of the 510-kDa complex by GEMMA. To overcome this problem, the size of the R2 subunit was increased by making a fusion construct between E. coli NusA (a protein unrelated to nucleotide metabolism) and the R2 protein. The addition of the N-terminal fusion protein increased the molecular mass of the R2 protein to 2 × 100 kDa. The NusA-R2 fusion protein was enzymatically active with a specific activity of 57 units/nmol R2 polypeptide (570 kDa). The NusA-R2 fusion protein was enzymatically active with a specific activity of 57 units/nmol for the wild-type R2 protein and 750 kDa using the NusA-R2 protein.

Because there was a tobacco etch virus protease site located between the NusA protein and the R2 protein, the NusA part could be cleaved off by proteolysis. After tobacco etch virus protease cleavage, the 705-kDa peak shifted back to 510 kDa in GEMMA analyses, and a new peak corresponding to free NusA (a protein unrelated to nucleotide metabolism) appeared (data not shown). The specific activity of the R2 protein was not affected by tobacco etch virus protease cleavage (data not shown).

Correlation between Octamer Formation and dATP Inhibition in the H59A R1 Mutant—The H59A mutation in the E. coli R1 protein (analogous to D57N in mouse (2, 35)) is located in the vicinity of the overall activity site (11). The mutant (which has normal affinity to dATP) requires higher dATP concentrations than the wild-type protein to be inhibited and was suggested to have a reduced ability to communicate dATP inhibition from the overall activity site (11). GEMMA analysis showed that the H59A mutant was able to form αβ₄ octamers in the presence of R2 protein and 50 μM dATP (Fig. 3A). It was possible to quantify the amount of each protein complex in Fig. 3A by summing up the data points and subtracting the background (the area is shown on top of each peak). Although this procedure is only semiquantitative (the sensitivity depends on which protein is used), it gives a general idea about the amount of octamers in the sample. In Fig. 3B, the ability to form octamers is plotted as a function of the dATP concentration for the wild-type R1 and H59A proteins in the presence of R2 protein and CDP (substrate). The dATP concentration needed to induce 50% octamers (and to cause dATP inhibition (11)) was higher in the mutant than in the wild-type protein; the mutant required 5.4 ± 1.3 μM dATP for 50% octamer formation, whereas octamer formation was nearly saturated at 2.5 μM dATP for the wild-type protein. When the protein concentration was decreased 4-fold as compared with Fig. 3B, 1.2 ± 0.08 μM dATP was needed for 50% octamer formation in the wild-type protein (5–10 μM was now needed for saturation), whereas the mutant required 16 ± 3.8 μM dATP for 50% octamer formation (Fig. 3C). The dATP concentrations needed for octamer formation were thus higher at low protein concentrations (Fig. 3C) than at high protein concentrations (Fig. 3B).

The difference between the wild-type and mutant R1 proteins was also tested in activity assays with dATP as allosteric effector (Fig. 3D). In these assays, dATP has two effects; at low concentrations, it binds only to the specificity site, which leads to activation of enzyme activity, and at higher concentrations, the effector binds to both allosteric sites, which leads to inhibition. The activity assays were performed with the same protein concentrations as in GEMMA (0.025 and 0.1 mg/ml R1 protein and equimolar amount of R2 protein). The H59A mutant required higher dATP concentrations to be inhibited than the
wild-type protein, and both proteins needed higher dATP concentrations to be inhibited with 0.025 mg/ml than with 0.1 mg/ml R1 protein. In these experiments, we looked at general trends rather than absolute correlations because the buffer conditions and substrate/magnesium concentrations were not the same in GEMMA and activity assays (see “Experimental Procedures”). The conclusion from enzyme assays and GEMMA analyses of the wild-type and mutant proteins was that inefficiency of dATP-induced octamer formation was always accompanied by inefficient dATP inhibition.

The E. coli RNR Could Be Inhibited by Combinations of ATP and dNTPs—The E. coli (wild-type and H59A mutant) and mouse RNRs were compared in enzyme assays where GDP was used as a substrate, and a combination of 2 mM dTTP and variable concentrations of ATP were used as allosteric effectors. The binding of ATP is mainly to the overall activity site in these experiments, and binding of ATP to the specificity site can be assumed to be negligible because dTTP has much higher affinity than ATP to this site (the $K_d$ for ATP is about 40 times higher than dTTP for the E. coli enzyme at 25 °C (5)). The E. coli and mouse RNRs had different overall activity regulation (Fig. 4A). Both wild-type and H59A mutant E. coli enzymes were inhibited with ATP + 2 mM dTTP because the activity was less than with dTTP alone (beginning of curve). The inhibition seemed to be general because CDP reduction was also decreased with the effector combination as compared with either effector alone (data not shown). In contrast, the mouse enzyme activity increased when ATP was added to the dTTP-stimulated enzyme. The stimulatory effect of ATP on dTTP-induced GDP reduction has also been observed for mammalian RNRSi before (17, 22).

In Fig. 4B, the ATP + dTTP inhibition data from wild-type and H59A E. coli RNR (Fig. 4A) were replotted together with corresponding dATP inhibition data (Fig. 3D). Surprisingly, the ATP + dTTP inhibition was as efficient as the dATP-induced inhibition, indicating that the affinity for ATP in the presence of 2 mM dTTP and R2 is similar to the affinity for dATP to the overall activity site. Another interesting observation that is clearly seen in Fig. 4B was that the ATP + dTTP inhibition is equally efficient in the H59A protein as in the wild-type protein (in contrast to the dATP inhibition, which is inefficient in the H59A protein).

E. coli RNR activity seemed to be generally inhibited in situations when ATP was combined with dNTPs because the reduction of GDP/CDP was also inhibited by ATP + dGTP (the GDP reduction is shown in Fig. 4C) but neither with dGTP (beginning of curve) nor with ATP used alone (ATP stimulated CDP reduction (data not shown)). ADP was not tested as substrate in these assays because it may act as both allosteric effector and substrate (see supplemental data). These findings are important pieces of information for the understanding of the mechanism behind the overall activity regulation because they show that both allosteric sites are involved in inhibition. Another aspect is whether the inhibition induced by ATP + dNTP has a physiological function. The intracellular concentrations of dTTP and ATP in logarithmically growing E. coli have been estimated to be 0.08–0.3 and 3–4 mM, respectively (36, 37). An additional set of experiments was performed with effector combinations of ATP (variable concentrations) + 0.2 mM dTTP (Fig. 4C). The inhibition was much less efficient when ATP was combined with 0.2 mM dTTP than with 2 mM dTTP, possibly because the equilibrium between ATP and dTTP in the specificity site disturbs the formation of the inhibited complex (see “Discussion”). No significant inhibition was seen with effector combinations of 0.2 mM dTTP and ATP concentrations above 1 mM (Fig. 4C). Thus, the inhibition of the E. coli enzyme activity by the combination of ATP + dTTP might be of limited physiological relevance. In contrast, dATP was a strong inhibitor at physiologically relevant concentrations (the intracellular concentration of dATP in logarithmically growing E. coli has been estimated to be ~0.2 mM (36, 37)) because 50% inhibition occurred already at ~0.25 mM dATP when assayed in the presence of 3 mM ATP as effector and 0.7 mM CDP as substrate (Fig. 4D). The H59A mutant required twice as high dATP concentrations to be inhibited as the wild-type enzyme in this assay. The difference between the two proteins is therefore less pronounced in Fig. 4D than the ~5-fold difference when dATP was used as the only allosteric effector (Fig. 3D). A possibility is that dATP (in analogy with dTTP/dGTP) bound to the specificity site enables ATP bound to the overall activity site to promote allosteric inhibition and that the H59A mutant has deficient dATP inhibition but normal ATP + dATP inhibition (Fig. 4B suggests that the ATP + dATP inhibition is not affected in the H59A mutant). Consequently, there was a more pronounced difference between the...
wild-type and H59A proteins at high dATP concentrations in Fig. 4D than at lower dATP concentrations when the overall activity site is mostly occupied with ATP (given that the affinity for ATP and dATP to the overall activity site is equal in the presence of a dNTP bound to the specificity site (Fig. 4B)).

**DISCUSSION**

GEMMA and SPR biosensor analyses of the *E. coli* class Ia RNR show that all nucleotide effectors are able to induce the formation of an active αβ4 complex by stimulating the α3 formation and R1-R2 interaction (Fig. 6A). Although the enzyme can reduce each of the four substrates when activated with dTTP, we now show that the dTTP-activated enzyme prefers GDP as substrate when all substrates are present simultaneously. Although the allosteric regulation of RNRs contain many universal features (1), some differences between RNRs can be found as well; the *E. coli* class Ia RNR formed an inhibited αβ4 octamer in the presence of dATP or combinations of ATP + dNTP (dTTP, dGTP or possibly also dATP) (Fig. 6A), whereas the mouse enzyme was stimulated in the presence of ATP + dTTP. Already during the pioneer work on the *E. coli* RNR in the late 1960s, it was found that RNR forms an active αβ2 complex in the presence of ATP, dTTP, and dGTP and an inhibited larger complex with 1:1 R1-R2 stoichiometry in the presence of dATP or effector combinations of ATP with dTTP (14). In biochemistry textbooks, the inhibitory role of ATP through the overall activity site in the *E. coli* enzyme is generally not mentioned. However, a schematic model of the *E. coli* structure/allosteric regulation is often used to illustrate the allosteric regulation of class Ia RNRs (40), although it is the regulation of the mouse enzyme (22) that is actually described.
An advantage of our studies as compared with the ones performed four decades ago is that GEMMA is sensitive enough to quantify the different oligomeric structures and to show that the E. coli enzyme can form the \( \alpha_4\beta_4 \) complex also in the R1-R2 concentration range that is present in enzyme assays (<0.1 mg/ml) and E. coli cells (0.18 – 0.36 mg/ml R1 protein and 0.07 – 0.14 mg/ml R2 protein, from immunological estimates of aluminum-extracted E. coli cell pellets (41)).

GEMMA analysis of dTTP/dGTP-induced E. coli R1-R2 protein complexes showed that only minor quantities of the \( \alpha_3\beta_4 \) octamer. Studies of the E. coli H59A R1 mutant give further support to the connection between allosteric inhibition, tight R1-R2 interaction, and formation of large oligomeric complexes. This mutant, which was previously known to show deficient dATP inhibition and weaker R1-R2 interaction as compared with the wild-type enzyme (11), is here shown to possess inefficient \( \alpha_4\beta_4 \) complex formation. Intriguingly, the H59A mutant protein also highlighted differences between the dATP inhibition and the ATP + dTTP inhibition. Although higher dATP concentrations are needed to cause inhibition in the mutant protein as compared with the wild-type protein, there is no obvious difference between the two proteins in the inhibition mediated by ATP + dTTP. The His-59 residue seems therefore to have different roles in the inhibition mediated by dATP and ATP + dTTP.

In addition to the correlation between allosteric inhibition and formation of tight large complexes, both earlier studies and this study have identified influences from the specificity site and binding of the R2 protein on the overall activity site and allosteric inhibition. The specificity site effector dTTP can increase the inhibitory effect of dATP (14) by mediating stronger interaction of dATP to the overall activity site (5, 15). In addition, the R2 subunit has been observed to increase the affinity for dATP to the overall activity site but not to the specificity site (15). Our enzyme assay results suggest that the affinity to ATP (in analogy with dATP) increases in the presence of R2 protein and dTTP because ATP + dTTP inhibition occurs at a low of a concentration of ATP as the concentration of dATP needed to cause dATP inhibition (Fig. 4B). In comparison, the affinity for ATP is ~10 times lower than for dATP to the overall activity site when measured in the absence of dTTP and R2 protein (5), and dTTP alone does not have a major effect on ATP affinity to this site. The favorable effect of the R2 subunit on ATP/dATP affinity could possibly be explained by the fact that the \( \alpha_2\beta_4 \) complex has a higher affinity for ATP than the \( \alpha_2 \) complex.

The pronounced inhibition of enzyme activity in the presence of ATP plus high concentrations of dTTP is an important result for our delineation of the mechanisms of allosteric regulation because it tells us that both allosteric sites are involved in inhibition and \( \alpha_4\beta_4 \) complex formation but may be of less importance in a physiological situation. As shown in Fig. 4C, the inhibition is less efficient when a physiological dTTP concentration was used, although the dTTP concentration was high enough to be the major nucleotide in the specificity site. As illustrated in Fig. 6B, optimal enzyme activity would be achieved when the \([\text{ATP}] / [\text{dTTP}] \) ratio is high enough to allow ATP to compete at the specificity site and disturb the formation of an inhibited complex but low enough to prevent ATP from being the major nucleotide at this site (ATP causes a switch in substrate specificity to CDP/UDP reduction). If formation of the ATP + dTTP inhibited \( \alpha_4\beta_4 \) complex is much slower than the rapid equilibrium of nucleotides in the allosteric sites, a physiological ratio of \([\text{ATP}] / [\text{dTTP}] \) may efficiently disturb the formation of the inactive form.

The overall activity regulation of the E. coli RNR has both similarities and differences as compared with the overall activity regulation of the mouse enzyme. One common feature is that inhibitory concentrations of dATP induce large oligomeric complexes with tight R1-R2 interactions. Furthermore, the dATP inhibition-deficient E. coli H59A protein and the corresponding mouse D57N protein both show weaker R1-R2 interactions as compared with the wild-type proteins (10, 11). Another similarity is that dTTP has been shown to increase the affinity for dATP in both the E. coli and the mouse wild-type enzymes (5, 10, 15). The differences are mainly that different large complexes are formed (\( \alpha_4\beta_4 \) in E. coli and \( \alpha_5\beta_4 \) in mouse) and the response to ATP. ATP alone, which does not induce large complex formation in E. coli, induces an \( \alpha_5\beta_2 \) complex in the mouse enzyme with a fairly tight R1-R2 binding (half the affinity as compared with the dATP-induced \( \alpha_5\beta_2 \) complex (42)). Furthermore, the E. coli enzyme is inhibited by the combination of ATP + dTTP, whereas the mouse enzyme activity was stimulated 2–3-fold by ATP via the overall activity site in the presence of dTTP as specificity site effector and GDP as substrate. It is tempting to speculate that overall activity regulation has evolved in two different steps. The evolution of dATP inhibition seems to be an early event because it is common to nearly all class Ia RNRs (1). The stimulatory/inhibitory effects of ATP via the overall activity site on dTTP (or dGTP)-induced enzyme activities might represent more recent evolutionary events because they differ among different class Ia RNRs. The different allosteric regulation of the E. coli and mouse RNRs is reflected by the different complexes formed; the E. coli RNR is in equilibrium between an active \( \alpha_2\beta_2 \) tetramer and an inactive complex.

5 The values are based on the assumptions that the cytosol contributes with 50–100% of the volume in pelleted bacteria and that the density of the cell pellets is ~1 g/ml (see “E. coli Statistics” at the CyberCell Database (CCDB) site).
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$\alpha_4\beta_4$ octamer, whereas the mouse enzyme equilibrates between active and inactive $\alpha_6\beta_2$ complexes. Despite these differences, the physiological outcome effect by ATP or dATP in the *E. coli* enzyme is fairly similar to the mouse enzyme; ATP can be regarded an activator also in the *E. coli* enzyme (high concentrations of ATP prevent $\alpha_4\beta_4$ formation via the specificity site), and dATP is the most efficient general inhibitor of the deoxyribonucleotides. The differences between the allosteric regulations in the two species could reflect the different cellular environments; cells in multicellular organisms are exposed to constant environments, whereas unicellular organisms are exposed to fluctuating environments. The allosteric properties of RNR need to be adjusted to the levels of other nucleotide metabolizing enzyme activities and nucleotide levels as exemplified by the unicellular eukaryote *Trypanosoma brucei*. This species has very low intracellular CTP and CDP pools (29, 43), and consequently, the RNR seems to have lost the dATP inhibition (29, 44) as a way to boost dCTP biosynthesis (dATP stimulates CDP reduction).

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