Enzymatically Active Mammalian Ribonucleotide Reductase Exists Primarily as an \( \alpha_6\beta_2 \) Octamer

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Ribonucleotide reductase synthesizes deoxyribonucleotides, which are essential building blocks for DNA synthesis. The mammalian ribonucleotide reductase is described as an \( \alpha_6\beta_2 \) complex consisting of R1 (\( \alpha \)) and R2 (\( \beta \)) proteins. ATP stimulates and dATP inhibits enzyme activity by binding to an allosteric site called the activity site on the R1 protein. Despite the opposite effects by ATP and dATP on enzyme activity, both nucleotides induce formation of R1 oligomers. By using a new technique termed Gas-phase Electrophoretic-Mobility Macromolecule Analysis (GEMMA), we have found that the ATP/dATP-induced R1 oligomers have a defined size (hexamers) and can interact with the R2 dimer to form an enzymatically active protein complex (\( \alpha_6\beta_2 \)). The newly discovered \( \alpha_6\beta_2 \) complex can either be in an active or an inhibited state depending on whether ATP or dATP is bound. Our results suggest that this protein complex is the major form of ribonucleotide reductase at physiological levels of R1-R2 protein and nucleotides.

Ribonucleotide reductase is a key enzyme to synthesize a balanced supply of the four dNTPs used as building blocks for DNA synthesis (1). The mammalian ribonucleotide reductase reduces ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates, which are further metabolized in the cell to become dCTP, dTTP, dATP, and dGTP. This enzyme consists of two different proteins called R1 (\( \alpha \)) and R2 (\( \beta \)) that are both required for enzymatic activity. The R2 protein is a dimer (2 \( \times \) 45 kDa), and each polypeptide contains a tyrosyl radical that is generated and stabilized by an iron center. Electrons are shuttled between the tyrosyl radical and the active site in the R1 protein where the actual catalysis occurs. The R1 protein contains a substrate-binding site and two allosteric effector-binding sites termed the specificity and activity sites, respectively. ATP and dATP bind to both allosteric sites, whereas dGTP and dTTP bind to only the specificity site. In the absence of nucleotide effectors, the mammalian R1 protein is a 90-kDa monomer. Allosteric effectors that only bind to the specificity site (dTTP or dGTP) induce the formation of an enzymatically active \( \alpha_6\beta_2 \) complex by stimulating R1 dimer formation and R1-R2 interaction (2).

The specificity site determines which substrate is to be reduced. When ATP (or dATP used at low concentration) is bound to this site, the enzyme reduces CDP and UDP. In a similar manner, dTTP stimulates GDP reduction and dGTP stimulates ADP reduction. By having this regulation, the enzyme ensures that there will be a balanced supply of all four dNTPs in the cell. The mechanism behind the specificity site function is known from experiments where the R1 dimer has been crystallized together with various allosteric effectors and substrates. When a specificity site effector is bound to one of the two R1 polypeptides, a conformational change is induced in a connecting loop that influences binding of the correct substrate to the second R1 polypeptide (3). Therefore, R1 dimer formation is a prerequisite for the specificity site regulation to function properly.

The activity site regulates the overall activity of the enzyme, which is important for controlling the optimal cellular levels of dNTPs as too high levels (or an imbalance between the four dNTPs) lead to an increased mutation rate (4). The overall activity is controlled by ATP and dATP binding to the activity site (1). The binding of ATP to this site induces an increased enzyme activity (hyperactivity) compared with when only the specificity site is occupied (5). When total cellular dNTPs are high, dATP out-competes ATP for binding to the activity site and the enzyme is shut off. The effect of dATP is concentration dependent (6). At low concentrations (\( \sim 2 \mu M \)), it acts as a pure specificity site regulator and directs CDP and UDP reduction. At higher concentrations, it binds to both allosteric sites and then the enzyme activity is turned off.

The mechanism behind the activity site regulation is not well understood, but it has been suggested that R1 oligomer formation might play a role in the function of the activity site (7–8). It has been observed in glycerol gradients and dynamic light scattering experiments that the R1 protein forms oligomers in the presence of the activity site effectors ATP or dATP (7–9). However, it was not clear whether these oligomers can bind the R2 subunit. A problem when studying ribonucleotide reductase with traditional methods such as glycerol gradients and gel filtration is that the R1-R2 complexes are unstable and fall apart during analysis. Another uncertainty was that many of these experiments were performed at a 100-fold higher R1 protein concentration than in the cell where the concentration is estimated to be 0.05 mg/ml (10). With Surface Plasmon Resonance (Biacore), it was possible to measure R1 protein binding to an immobilized R2 subunit. The conclusion from this study was that all nucleotide effectors strongly induce the binding of R1 dimers to the R2 subunit (2). It is a paradox that dATP/ATP, which stimulates the formation of R1 oligomers rather than dimers in glycerol gradients and dynamic light scattering...
Mammalian Ribonucleotide Reductase Is an $\alpha_6\beta_2$ Octamer

experiments, is the strongest inducer of $\alpha_2\beta_2$ complexes in Bia-
core. Therefore, it has been questioned whether the R2 protein
is immobilized in such a way that higher complexes cannot bind
or that the R1 oligomers are destabilized by the chip surface.
Obviously, new techniques are needed to clarify how ATP and
dATP influence the mammalian ribonucleotide reductase qua-
ternary structure.

Gas-phase electrophoretic-mobility macromolecule analysis
(GEMMA)2 is a relatively new method to study protein com-
plexes in solution: a diluted protein sample (usually 10 ng/µl) is
transmitted into the gas phase by a charged reduced electrospray
process (11–12). The generated particles, each containing
one protein molecule with a +1 charge, are separated according
to size in a differential mobility analyzer and subsequently
quantified by a particle counter. In contrast to mass spectrom-
etry, this method is run at atmospheric pressure and measures
the diameter of the particle rather than the mass. However,
because particle diameter and mass are correlated to each
other, the mass can usually be determined with an error of ±
5.6% (12). Electrospray ionization mass spectrometry can be
used to obtain exact masses of protein complexes, but the sen-
sitivity is strongly biased toward small protein complexes (13).
Taken together, we think the two methods complement each
other, with GEMMA providing quantitative information about
the protein complexes while mass spectrometry determines the
exact size of these complexes.

In this study, we analyzed how various nucleotide effectors
affect the quaternary structure of mouse ribonucleotide reduc-
tase by gel filtration, GEMMA, and mass spectrometry. In
agreement with previous studies, we found that nucleotide
effectors that bind only to the specificity site induce the forma-
tion of R1 dimers. The R1 dimers can interact with the R2
dimer, forming an enzymatically active $\alpha_2\beta_2$ complex. How-
ever, in the presence of ATP or dATP both allosteric sites
become occupied and then R1 hexamers are formed. These
hexamers can interact with the R2 dimer, forming $\alpha_6\beta_2$ com-
plexes. The $\alpha_6\beta_2$ complex could either be in a hyperactive form
in the presence of ATP or in an inactive form in the presence of
dATP.

EXPERIMENTAL PROCEDURES

Protein Purification—The R1 protein was expressed in a
ClpB-negative Escherichia coli pLysS (DE3) strain (see
“Acknowledgments”). The R1 protein was induced with 0.05
mM isopropyl-1-thio-$\beta$-d-galactopyranoside overnight at 15 °C
and purified by ammonium sulfate precipitation and dATP-
Sepharose chromatography as described previously (14). The
mouse R2 protein (as well as mutants of the R2 protein)
was expressed and purified by ammonium sulfate precipitation
and DEAE chromatography as described previously (15). The R1
and R2 proteins were quantified by UV light absorption using
extinction coefficients of $E^{1%}_{1\ cm} (280 nm) = 12.0 \ (9)$ and
$E^{1%}_{1\ cm} (280–310 \text{ nm}) = 13.8$, respectively (15). The specific
activities of the R1 and R2 proteins varied between different
preparations but were relatively similar (±50%) to those previ-
ously reported (14–15).

Gel Filtration Analysis of the R1 Protein—The R1 protein was
analyzed on a Superdex 200 10/300 GL column (Amersham
Biosciences). The mobile phase contained 150 mM KCl, 6 mM
MgCl$_2$, 50 mM Tris-HCl, pH 7.6, and various concentrations
of different nucleotides. The R1 protein was premixed with
the mobile phase to a concentration of 0.02–0.25 mg/ml (100-µl
sample) before analysis. The column was connected to a UV
detector (UV 2075 Plus; Lasco), and detection was performed at
290 nm to minimize the background absorbance from the
nucleotides in the mobile phase without losing too much sen-
sitivity for protein detection. The molecular masses of the R1
complexes were obtained by comparing the retention time to a
molecular mass standard composed of thyroglobulin (669 kDa;
17.14 min), ferritin (440 kDa; 20.08 min), IgG (159 kDa; 23.39
min), transferrin (80 kDa; 26.76 min), ovalbumin (43 kDa; 29.63
min), and myoglobin (16.9 kDa; 33.87 min).

Ribonucleotide Reductase Assays—Enzyme assays were per-
formed using R1 protein (varied between 0 and 2 µg) and R2
protein (4 µg) in 50 µl of a buffer containing 3 mM ATP, 6 mM
MgCl$_2$, 0.5 mM [5-3H]CDP (Amersham Biosciences), 10 mM
dithiothreitol, 10 mM FeCl$_3$, 100 mM KCl, 8 mM Hepes-KOH,
and 50 mM Tris-HCl, pH 7.6 (Tris/KCl buffer). The reaction
mixture (50 µl) was incubated at 37 °C for 30 min, and the
amount of [5-3H]dCDP formed was measured (16). In some
cases, we wanted to use similar buffer conditions for the
enzyme assay as we use in GEMMA (GEMMA is not compatibil-
ity with non-volatile salts). In these experiments KCl was
excluded and 20 mM ammonium acetate, pH 7.6, and 0.005%
TWEEN 20 were added. The reduction of [8-14C]GDP (Moravek
Biochemicals) was performed with 2.5 µg of R1 and 4 µg of R2
proteins under similar conditions as with CDP. However, 2 mM
dTTP was then used in combination with variable amounts of
ATP as effectors and the [14C]dGDP formed quantified after
separation by boronate chromatography (17). An alternative
preincubation-based procedure was also sometimes used (8):
5.5 µg of R1 and 4.48 µg of R2 protein were mixed with a buffer
consisting of 2 mM dTTP, 10 mM MgCl$_2$, 25 mM dithiothreitol,
50 mM FeCl$_3$, 10 mM KCl, 7 mM NaF, and variable amounts
of ATP. After 5 min of preincubation at 25 °C, the assay was initi-
ated by adding 100 µM [14C]GDP and incubated at 37 °C for
5 min.

GEMMA—The non-volatile salts present in the R1 protein
solution were removed by gel filtration using a buffer contain-
ing ammonium acetate, pH 7.6, used at 20 mM for the R2
protein and 100 mM for the R1 protein. The protein samples were
diluted to a concentration of 0.01–0.1 mg/ml in a buffer con-
taining 20 mM ammonium acetate, pH 7.6, 0.005% Tween 20,
and various amounts of nucleotides up to a maximum concen-
tration of 150 µM. Magnesium was generally used at the same
centration as the nucleotides. The GEMMA system con-
tained the following components: 3480 electrospray aerosol
generator, 3080 electrostatic classifier, 3085 differential mobi-

2The abbreviations used are: GEMMA, gas-phase electrophoretic-mobility macromolecule analysis/analyser; h-site, hexamerization site.
the signal became stable. After obtaining a stable signal, the flow rate could be reduced by decreasing the capillary pressure drop from 3.7 to ~1.5–2 psi. The spray cone was difficult to observe at this low pressure, and the stability of the spray was then monitored by the current reading. The proteins were scanned at the size range of 2.55–25.5 nm for 105 s/scan, and a particle density of 0.58 g/cm³ was used for the molecular mass calculations. The particle density was obtained from the average of 20 different standard proteins and was valid in the size range of 15–900 kDa.

Mass Spectrometry—The nanospray mass spectrometry was run on a QTOF Ultima API machine (Micromass Ltd., Manchester, UK) using gold-coated borosilicate needles loaded with ~5 μl of sample. The following parameters were used: 2-kV needle voltage, 25 °C source temperature, 100–200-V cone voltage, 100–200-V RF lens 1 settings, 5–10-eV collision energy, and 2200-V microchannel plate voltage. The machine was calibrated using a 2 mg/ml CsI solution in 50% isopropanol. The masses of protein complexes were measured from smoothed (2 × 200 channels by the Savitzky-Golay smoothing method) and centroided (100 channels, 80%) data by Masslynx software (Waters Corp.).

RESULTS

Gel Filtration Experiments Show That the R1 Protein Forms Hexamers in the Presence of ATP/dATP—Analysis of protein complex formation by gel filtration was performed in the absence or presence of various allosteric effectors and an R1 protein concentration of 0.2–0.25 mg/ml (Fig. 1A). The concentration of effectors was high enough to saturate the specificity site (dTTP) or both allosteric sites (dATP/ATP). It was also needed to have the effectors present in the running buffer to avoid dissociation of the protein complexes (data not shown). In agreement with previous results (7–9), we found that dTTP induced the formation of R1 dimers and dATP or ATP induced the formation of a large R1 complex. By comparing the size of this complex to a standard of known globular proteins, the size was determined to be ~544 kDa, which fits well with a hexamer. ATP and dATP had similar effects on oligomerization. Protein complex formation was efficient throughout a wide protein concentration range. Decreasing the protein concentration to 0.0125 mg/ml also demonstrated that dTTP induces dimer formation and ATP/dATP induces hexamer formation (Fig. 1B). R1 complex formation seemed independent of magnesium ions because R1 hexamers (0.05 mg/ml) were efficiently formed in the presence of 3 mM ATP and the absence of magnesium (Fig. 1B, bottom graph).

Enzyme Activity and Allosteric Regulation Are Not Altered by the GEMMA Buffer—We wanted to confirm that GEMMA conditions do not affect enzyme activity. For GEMMA analysis we developed a buffer consisting of 20 mM ammonium acetate, pH 7.6, and 0.005% Tween 20 to prevent non-specific R1 aggregation. We measured the ribonucleotide reductase activity (CDP reduction) in the presence of the GEMMA buffer and normalized the results to the activity when using the standard Tris/KCl-containing buffer. The average of two measurements of the specific activity of the R1 protein was 100 ± 4% in the GEMMA buffer (the R2 protein was in excess). The addition of the inhibitory nucleotide effectors 50 μM dATP or 100 μM dGTP to the GEMMA buffer resulted in enzyme activities of 10 ± 2% and 12 ± 2%, respectively. Therefore, neither the specific activity of the R1 protein nor the allosteric regulation of the enzyme seemed to be altered when the GEMMA buffer was used. We also studied the effect of Mg²⁺ on the enzyme activity (GEMMA is limited to Mg²⁺ concentrations of 100 μM or below), and we found that the Mg²⁺ concentration needs to be at least equal to the total nucleotide concentration for maximal activity (data not shown). There was no advantage to increasing the amount of Mg²⁺ to higher than the total nucleotide concentration.

GEMMA Experiments Show That the R1 Protein Is a Monomer and the R2 Protein Is a Dimer—The buffer used in GEMMA is different compared with the gel filtration buffer, and first we wanted to confirm that we get the same protein complexes in GEMMA as in gel filtration. The R1 and R2 subunits were analyzed separately in the absence of allosteric effectors (Fig. 2, A and B). The R1 protein shown in Fig. 2A gave a size of 91 ± 5 kDa, suggesting that it is a monomer (theoretical
Mammalian Ribonucleotide Reductase Is an $\alpha_6\beta_2$ Octamer

![Graphs showing size distributions of R1 complexes](image)

FIGURE 3. GEMMA analysis of the mouse R1 protein (0.1 mg/ml) in the presence of various nucleotide effectors. The nucleotide effectors were 50 $\mu$M Mg-dATP (A), 50 $\mu$M Mg-dATP (reduced flow rate) (B), no effector (C), 50 $\mu$M Mg-dGTP (D), combination of 50 $\mu$M Mg-dGTP and 1 $\mu$M Mg-dATP (E), and combination of 50 $\mu$M Mg-dGTP and 100 $\mu$M Mg-ATP (F). Experiments B–F were performed at reduced flow rate (1.6 psi).

size 90 kDa). Three variants of the R2 protein were used in this study: the wild-type protein, a C-terminal-truncated protein, and an N-terminal-truncated protein. The three variants were analyzed separately by GEMMA, showing that they all formed dimers. The first two variants gave a size of 91 ± 5 kDa (theoretical size 2 × 45 kDa), whereas the N-terminal-truncated variant shown in Fig. 2B gave a size of 81 ± 5 kDa (theoretical size 2 × 38 kDa).

GEMMA Experiments Show That ATP/dATP Always Induces R1 Hexamer Formation, Regardless of Which Effector Is Bound to the Specificity Site—We needed to optimize the GEMMA experiments because the method is not compatible with high concentrations of nucleotides (or other non-volatile salts). In Fig. 3A, the R1 protein was measured in the presence of 50 $\mu$M Mg-dATP (50 $\mu$M magnesium acetate and 50 $\mu$M dATP). The graph shows four peaks resulting from salt, R1 monomers, R1 dimers, and R1 oligomers. The estimated molecular mass of the oligomers was 510 ± 29 kDa, which fits well with the size of R1 hexamers. The salt peak comes from Mg-dATP clusters. A problem encountered during analysis was that the Mg-dATP clusters non-specifically bound to the R1 monomer, which had the appearance of being 101 kDa instead of 91 kDa. The effect of salts on the measured size of proteins is a known problem in GEMMA, especially for the measurement of small proteins (18). The same mixture was re-analyzed at a reduced liquid flow rate (Fig. 3B), reducing the size of the primary droplets generated during the electrospray process. The reduced flow rate resulted in the R1 monomer being correctly measured. A drawback of the reduced flow rate is an ~2-fold drop in sensitivity, but this was only a minor problem that could be compensated for by doubling the number of scans. The relative peak heights (and areas) of the monomer and dimer were fairly stable between similar samples, whereas the absolute values in particles/cm³ varied depending on factors such as the flow rate used. All subsequent measurements of the R1 and R2 proteins were performed at reduced flow rate.

There is good agreement between the results from GEMMA (Fig. 3) and the gel filtration experiments (Fig. 1). In the absence of allosteric effectors, the R1 protein was mainly a monomer (Fig. 3C), and after addition of a specificity site effector (50 $\mu$M Mg-dGTP) the protein formed dimers (Fig. 3D). The addition of effectors that bound to both allosteric sites (e.g. dATP) induced R1 hexamer formation (Fig. 3B). The concentration of dGTP or dATP in these experiments (50 $\mu$M) was enough to saturate the specificity site (dGTP) or both allosteric sites (dATP) as judged from filter binding studies (19) and enzyme activity measurements (5, 8).

Reducing the flow rate improved the molecular mass measurements in most cases, except for the R1 dimer (theoretical size 180 kDa), which was measured as 173 kDa in the absence of salt (Fig. 3C) and 193 kDa in the presence of nucleotides (Fig. 3, A and B, D–F).

We also analyzed R1 complex formation in the presence of various combinations of allosteric effectors. To investigate which R1 complexes were formed when the activity site was occupied with dATP and the specificity site occupied by another effector (e.g. dGTP), GEMMA analysis of the R1 protein was carried out in the presence of 50 $\mu$M dGTP and 1 $\mu$M dATP (Fig. 3E). The dATP concentration was low enough to allow dGTP to fully occupy the specificity site (19). Also, in this case R1 hexamers were formed. Finally, it was investigated which complexes are formed in the presence of 100 $\mu$M ATP. In this case some hexamers were also formed, but the oligomerization process was not so efficient at this low concentration of ATP (data not shown). The efficiency of hexamer formation was increased when 100 $\mu$M ATP was used in combination with a specificity site effector (50 $\mu$M dGTP) (Fig. 3F). Similar beneficial effects on effector affinities have been previously observed and were explained by cross-talk between the allosteric sites (19). We conclude that when ATP or dATP binds to the activity site, hexamer formation is induced. The good agreement between GEMMA data and gel filtration experiments indicates that the GEMMA buffer is appropriate for the measurement of R1 complexes.

Mass Spectrometry Analysis Confirms That the Oligomers Induced by dATP (or ATP) Are Hexamers—To confirm that the dATP-induced complex is a hexamer, the R1 protein was analyzed by electrospray ionization mass spectrometry (a more accurate method than GEMMA or gel filtration). The mass spectrum of the R1 protein in the presence of 20 $\mu$M Mg-dATP and 20 mM ammonium acetate, pH 7.5, gave a series of peaks centered on 5000 m/z units (coming from the R1 monomer; Fig. 4A). A similar series of peaks was also formed in the absence of dATP, and the size was then determined to be 90.2 kDa (data not shown). A second series of peaks centered on 12,000 m/z units was only formed in the presence of dATP, but the resolution was too low to assign the mass of the corresponding protein complex. To increase the resolution, the magnesium was excluded and the ammonium acetate buffer was replaced with 50 mM ammonium formate, pH 7.5 (Fig. 4B). The mass of the
corresponding protein complex was calculated to be $549.75 \pm 0.07$ kDa, which fits well with a hexamer (theoretical size 547.7 kDa, including 2 dATP/polypeptide). The deviation between theoretical and experimental values can be explained by the extra mass coming from nonspecific binding of dATP and other salts.

**GEMMA Analysis Shows That the R1 Hexamers Are Able to Interact with the R2 Protein, Forming an $\alpha_6\beta_2$ Complex**—To investigate whether the various R1 complexes were able to bind the R2 dimer, R1 and R2 protein complexes in the presence of dTTP were measured by GEMMA (Fig. 5A). Compared with the results when using each protein alone, a new peak with a mass of ~266 kDa was observed, corresponding to an $\alpha_6\beta_2$ structure (theoretical size 270 kDa). The peaks at 91 and 193 kDa could not be assigned. To assign the unknown complexes in Fig. 5A, a truncated variant of the R2 protein was used. This protein lacks 60 N-terminal amino acids encoding a recognition site for ubiquitin-mediated proteolysis in mitosis (20), but it has a similar specific activity as the wild-type protein in enzyme assays (data not shown). The first complex (now shifted to 81 kDa) was mainly from R2 dimers (2 $\times$ 38 kDa), and the second complex (shifted to 173 kDa) was from $\alpha_6\beta_2$ complexes (Fig. 5B). To prove that the observed interactions were real, a second truncated variant of the R2 protein was used. This protein lacks 7 amino acids in the C terminus that had previously been shown to be crucial for R1-R2 interaction (21). With this variant of the R2 protein there was no R1-R2 interaction and only the $\beta_2$ and $\alpha_2$ peaks were observed (Fig. 5C). The effect of dATP on R1-R2 interaction using the same set of R2 protein variants was studied (Fig. 5, D–F). The R1 hexamer shifted in size to 633 $\pm$ 35 kDa when the R2 protein or the N-terminal-deleted variant of the R2 protein was added (Fig. 5, D and E) but not with the R2 protein, which was unable to interact with the R1 protein (Fig. 5F). The size of this complex corresponded to an $\alpha_6\beta_2$ structure (theoretical size 637/623 kDa (including 2 dATP/polypeptide), depending on whether wild-type- or N-terminal-truncated R2 protein is used). Surprisingly, only one R2 dimer was bound to the R1 hexamer. The mass of this complex was not increased when the concentration of the R2 protein was doubled (data not shown). It appears that binding of one R2 dimer prevents the binding of a second R2 dimer.

**Correlation between $\alpha_6\beta_2$ Complex Formation and Stimulation of Enzyme Activity by ATP**—Partially based on enzyme activity assays, the mammalian ribonucleotide reductase was recently suggested to contain three allosteric sites (7–8); at low concentrations ATP acts as a pure specificity site regulator but inhibits the reaction when used at high enough concentrations to bind to the activity site ($\sim$1 mM). When ATP is used above 1 mM, it also binds to the hexamerization site (h-site) and the enzyme activity increases again. A dip in the enzyme activity at 1 mM ATP was thus taken as evidence for the existence of an additional regulatory site (the h-site). The dip in the enzyme activity was claimed to be even more pronounced if the enzyme were preincubated with allosteric effectors before the substrate was added (8). To investigate how many allosteric sites ribonucleotide reductase contains, the reduction of GDP in the presence of a constant amount of dTTP (2 mM) and an increasing concentration of ATP was measured (Fig. 6A). Three different assay conditions were used, the standard protocol (KCl buffer, no preincubation), GEMMA conditions (KCl was replaced by 20 mM ammonium acetate), and the published preincubation-based procedure (8). The concentration of dTTP (2 mM) was sufficient to exclude ATP binding to the specificity site (19), simplifying interpretation of the results. As seen in the figure, the enzyme is active with only dTTP as effector and this activity increases gradually with the ATP concentration. Already at a concentration of 100 $\mu$M ATP, we had almost 2-fold stimulation of enzyme activity. Similar results were obtained if we
**Mammalian Ribonucleotide Reductase Is an $\alpha_6\beta_2$ Octamer**

**FIGURE 6. Correlation between ribonucleotide reductase activity and GEMMA.** A, enzyme activity assays at 37 °C in the presence of 0.5 mM GDP (substrate), 2 mM dTTP (effector), and increasing concentrations of ATP. Three different buffer conditions were used, standard conditions (●), and a preincubation-based assay procedure (●). B, enzyme activity using GDP as substrate and dTTP as effector (similar conditions were used as in panel A, inset). C, GEMMA analysis under enzyme assay conditions with GDP as substrate and dTTP as effector (similar conditions were used as in panel A, inset, but FeCl₃ was excluded). D, a similar setup as in panel C but with the addition of 100 μM Mg-ATP in the buffer. Also in this case two separate experiments were done using R1 alone and in combination with the R2 protein. The spacing between the two experiments is 2 × 10⁵ units. All GEMMA experiments were performed at reduced flow rate (1.2 psi).

Reduced the nucleotide and magnesium concentrations to fit the requirements for GEMMA analysis (Fig. 6A, inset). Also in this case we had an almost 2-fold stimulation at 100 μM ATP. We did not observe any dip in the enzyme activity at any of the three assay conditions (Fig. 6A). The effect of ATP on enzyme activity can thus be explained by activity site binding, and there is no support for the existence of an additional regulatory site.

The stimulation of enzyme activity is nearly complete at 0.5 mM ATP (Fig. 6A). Higher concentrations of ATP were needed to saturate the enzyme activity in the absence of dTTP using CDP as substrate (Fig. 6B). Maximal stimulation was then obtained at ~1 mM ATP. It is possible that the affinity of ATP to the activity site is increased in the presence of a specificity site effector (e.g., dTTP). Similar effects have been observed previously and were explained by cross-talk between the allosteric sites (19).

Finally, parallel GEMMA experiments and enzyme assays were carried out using ammonium acetate buffer with GDP as substrate and dTTP as effector. A nearly 2-fold stimulation of enzyme activity was observed when 100 μM ATP was added to the reaction mixture (Fig. 6A, inset). The GEMMA analysis showed that in the presence of dTTP, the R1 protein exclusively formed dimers that interacted with the R2 protein in an $\alpha_6\beta_2$ complex (Fig. 6C), whereas in the presence of ATP and dTTP there were approximately equal amounts of R1 dimers and hexamers, which interacted with the R2 protein to form $\alpha_6\beta_2$ and $\alpha_6\beta_2$ complexes (Fig. 6D). We conclude that there is a correlation between the $\alpha_6\beta_2$ complex formation and stimulation of enzyme activity.

**DISCUSSION**

GEMMA is a quick method allowing high-throughput analysis. Therefore, we have been able to study the influence of many factors on R1-R2 complex formation. R1 complex formation mainly comes from the allosteric effectors, although the strength of the interactions could be modulated by substrates, reducing agents, the pH value, and salt concentrations. Effectors that only bind to the specificity site always induced the formation of R1 dimers, whereas effectors that bind to both allosteric sites always induced R1 hexamers. Because there is no effector known that only binds to the activity site, it is not known if it is enough to bind to this site for the formation of hexamers or if both allosteric sites need to be occupied. The GEMMA results showed for the first time that all forms of the R1 protein bind the R2 protein. The concentration of R1 dimers and hexamers, which were approximately equal amounts in the presence of ATP and dTTP there were approximately equal amounts of R1 dimers and hexamers, which interacted with the R2 protein to form $\alpha_6\beta_2$ and $\alpha_6\beta_2$ complexes (Fig. 6D). We conclude that there is a correlation between the $\alpha_6\beta_2$ complex formation and stimulation of enzyme activity.

Based on dynamic light scattering experiments and enzyme activity assays, Cooperman’s laboratory recently proposed a new model for the allosteric regulation of ribonucleotide reductase (7–8). This model involves three allosteric sites: the specificity site, the activity site, and a third hypothetical site termed the hexamerization site (h-site). Accordingly, dATP or ATP binds to the activity site, inducing the formation of inactive R1 tetramers. However, when high concentrations of ATP are used the h-site is also occupied, inducing the formation of R1 hexamers that can form a hyperactive complex together with the R2 protein. Our data do not support the proposed model because we could not find support for the h-site in enzymatic assays and because we did not observe any dATP/ATP-induced tetramers. ATP or dATP always induced the formation of R1 hexamers as measured by three independent methods. However, under
Mammalian Ribonucleotide Reductase Is an $\alpha_6\beta_2$ Octamer

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certain conditions we observed that ATP also induced nonspecific aggregation/precipitation leading to clogging of GEMMA capillaries and altered gel filtration peaks (reduced peak intensities/changed peak positions (data not shown)). The aggregation/precipitation problems could be limited by either using a high concentration of ATP (10 mM) or by leaving out magnesium from the buffer. It cannot be excluded that nonspecific aggregation/precipitation might have influenced the average protein size in dynamic light scattering experiments and caused enzyme inhibition at certain concentrations of ATP in previous analyses.

The mammalian ribonucleotide reductase belongs to the class Ia family of ribonucleotide reductases that contains enzymes from eukaryotes, some bacteria, and some viruses (1). We do not know whether the newly discovered $\alpha_6\beta_2$ complex is unique to mammals or whether other class Ia enzymes are able to form this complex as well. Previous results from the *E. coli* class Ia ribonucleotide reductase are in conflict with each other. It was reported that the *E. coli* class Ia enzyme is able to form a large protein complex in the presence of dATP (22–23), but a more recent study failed to detect this complex (24). To clarify this issue, we have started to study the *E. coli* enzyme as well as other representatives of the class Ia family with the GEMMA technique.

The GEMMA, mass spectrometry, and gel filtration results are all in perfect agreement with previous results that the specificity site effectors stimulate R1 dimer formation (7–9) and that these dimers can interact with the R2 protein, forming an $\alpha_6\beta_2$ structure (2). However, the ATP concentration in the cell has been estimated to be $3 \pm 1.7$ mM (25), and at this concentration the activity sites will also be at least partially occupied. In our enzyme activity assays, ATP had a saturating effect in this concentration range, and in our gel filtration experiments the hexamers dominated when using 3 mM ATP. Therefore, the $\alpha_6\beta_2$ complex is likely the major form of ribonucleotide reductase in vivo. We have also shown that there is a correlation between hexamer formation and ATP activation. Perhaps the hexamer has a higher activity than the dimer, but the binding of dATP can block this activity by inducing a small change in the protein structure. The next logical step in our project will be to try to localize the interaction surfaces in the hexamer and see whether we can make a mutant R1 protein that is unable to form hexamers. The study of this mutant will hopefully give further insight into the activity site regulation of ribonucleotide reductases.