Cross-talk between the Allosteric Effector-binding Sites in Mouse Ribonucleotide Reductase*

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We compared the allosteric regulation and effector binding properties of wild type R1 protein and R1 protein with a mutation in the “activity site” (D57N) of mouse ribonucleotide reductase. Wild type R1 had two effector-binding sites per polypeptide chain: one site (activity site) for dATP and ATP, with dATP-inhibiting and ATP-stimulating catalytic activity; and a second site (specificity site) for dATP, ATP, dTTP, and dGTP, directing substrate specificity. Binding of dATP to the specificity site had a 20-fold higher affinity than to the activity site. In all these respects, mouse R1 resembles Escherichia coli R1. Results with D57N were complicated by the instability of the protein, but two major changes were apparent. First, enzyme activity was stimulated by both dATP and ATP, suggesting that D57N no longer distinguished between the two nucleotides. Second, the two binding sites for dATP both had the same low affinity for the nucleotide, similar to that of the activity site of wild type R1. Thus the mutation in the activity site had decreased the affinity for dATP at the specificity site, demonstrating the interaction between the two sites.

All ribonucleotide reduction is allosterically controlled to ensure an appropriate supply of each of the four dNTPs required for DNA replication and repair (1–3). Three different classes of ribonucleotide reductases exist in nature. For all of them the substrate specificity of a single protein is regulated by binding of nucleoside triphosphate effectors to specific sites on the protein to provide the required mixture of dNTPs. In recent years, the allosteric regulation of the different classes was studied in detail in various microorganisms (4–7). X-ray studies of the R1 protein (NrdA) of the class Ia reductase from Escherichia coli (8, 9) and of the large protein (NrdD) of the class III reductase from phage T4 (10) provided detailed structural information concerning effector binding.

All three classes are present in bacteria; in some cases one finds all three in the same organism (6). In contrast, only class Ia is found in higher eukaryotes (1–3, 11). To this class belongs also one of the three reductases of E. coli that has become the prototype of class Ia reductases (12). This reductase consists of two tightly bound homodimeric proteins: the larger R1 protein (coded by the nrdA gene) and the smaller R2 protein (coded by the nrdB gene). R1 is the business end of the enzyme, containing both catalytic and allosteric sites. R2 harbors a tyrosyl radical, located at a specific position of the polypeptide chain, and an oxygen-linked di-iron center. The radical is generated through the interaction of the iron center with oxygen. During catalysis the radical function moves from R2 to a cysteine residue in the catalytic site of R1 (2, 13, 14) and there provides the activation of the ribonucleotide that is required for the reduction of the ribose moiety (3, 15).

Each polypeptide of E. coli R1 contains two allosteric sites: one specificity site capable of binding ATP, dATP, dGTP, or dTTP and one activity site accepting only ATP or dATP (16). Effector binding to the specificity site modulates the catalytic activity so that binding of ATP or dATP prepares the protein for the reduction of UDP or CDP, binding of dTTP prepares the protein for the reduction of GDP, and binding of dGTP prepares the protein for the reduction of ADP. In the E. coli enzyme, this regulation of substrate specificity is only found when ATP binds to the activity site. In the x-ray structure, the specificity site is located at the interface between the two polypeptides of the R1 homodimer (9). The activity site regulates the overall activity of the enzyme so that binding of ATP stimulates enzyme activity, whereas binding of dATP inhibits it. These effects are independent of the occupation at the specificity site. In the x-ray structure, the activity site is located at the N terminus of the polypeptide chain (9). This part of the R1 protein is in direct contact with the R2 protein in the model-built structure of the E. coli R1-R2 enzyme complex (8, 9). Fig. 1 shows a schematic representation of the allosteric sites of the E. coli R1 protein, their occupancy by various effectors, and how they influence the catalytic activity of the ribonucleotide reductase.

The regulation of mammalian ribonucleotide reductases was found to follow the pattern set by the E. coli enzyme. As long ago as 1979, experiments with a highly purified R1 preparation from calf thymus demonstrated the same effector specificity for substrate reduction, as well as stimulation by ATP and inhibition by dATP (17). However, in contrast to the bacterial enzyme, the mammalian enzyme showed almost no activity in the absence of effectors. Furthermore, binding of dTTP or dGTP to the specificity site did not require that ATP be bound to the activity site to give full specificity. Binding experiments suggested the presence of two distinct binding sites. However, the binding stoichiometry was different: the calf thymus R1 contained only one specificity site and one activity site per homodimer, whereas E. coli R1 contains two of each. Strong evidence for the independent functions of two separate allosteric sites was obtained with purified reductases from mouse T-lymphoma cell lines resistant to growth inhibition by deoxyguanosine (18, 19). One cell line, dGuo-L, provided an R1 protein insensitive to allosteric regulation by dGTP, suggesting a mutation in the specificity site (18). R1 from the other cell line, dGuo-200–1, was insensitive to inhibition by dATP, suggesting a mutation in the activity site (19). This R1 contained

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Allosteric Regulation of Mouse Ribonucleotide Reductase

EXPERIMENTAL PROCEDURES

Preparation of Mouse R1 and R2 Proteins—Recombinant mouse R1 and R2 proteins were overexpressed in BL21(DE3)pLysS bacteria using the pET 3a expression vector (22, 23). Purification of the R1 and R2 proteins was made as described earlier. The final preparation of the wild type R1 protein was homogeneous as determined by SDS-gel electrophoresis (Fig. 2, lane 3).

Overlap extension polymerase chain reaction (24) with mutagenized oligonucleotides was used to introduce the D57N mutation into the wild type R1 protein pETR1 vector. The resulting pETD57NR1 construct was verified by DNA sequencing.

The purification of wild type R1 protein uses affinity chromatography on dATP-Sepharose as a major purification step (22). This purification procedure could not be applied directly to the mutant D57N R1 protein because of its lower affinity for dATP (see “Binding of Effectors to Mouse D57N R1”). Instead we developed the following procedure. Before loading on the dATP-Sepharose column, the dissolved ammonium sulfate pellet was equilibrated with 50 mM Tris-Cl, pH 7.6, 0.1 M KCl. Elution of the D57N R1 protein from the column was made with 10 mM ATP instead of 50 mM. Most of the experiments were made with this ATP eluate, and the degree of homogeneity is shown in Fig. 2, lane 1. To obtain a higher purity of the D57N R1 protein for use in some of the binding studies, the ATP eluate was further purified by chromatography on a HiLoad 16/60 Superdex 200 preparative grade column (Amersham Pharmacia Biotech) in 50 mM Hepes-KOH, pH 7.6, 0.15 M KCl, 10 mM MgCl₂, 2 mM dithiothreitol, 20 μM dTTP (Fig. 2, lane 2). After purification, D57N was unstable. Frozen solutions had a tendency to precipitate after thawing and were centrifuged at high speed before use for binding experiments.

R1/R2 Protein Interactions—The interaction of wild type or mutated R1 proteins with the R2 protein was studied at 22 °C by a biosensor technique, as described earlier (21).

Other Methods—The reductions of 0.5 mM CDP, GDP, or ADP were determined with dithiothreitol as hydrogen donor, as described previously (25, 26). Incubations were at 37 °C for 50 min with 1–5 μg of wild type or mutated R1 and 4 μg of R2. Binding of the allosteric effectors was measured at 4 °C by a rapid filtration method (27). Protein was determined colorimetrically (28). The method was standardized by amino acid analysis of wild type R1 protein.

RESULTS

Allosteric Regulation of the Catalytic Activity of Wild Type Mouse R1—In accordance with earlier results (17) concerning the calf thymus reductase, we found that the mouse enzyme reduced CDP, ADP, and GDP very poorly in the absence of allosteric effectors. Reduction of CDP required millimolar concentrations of ATP. Replacement of ATP by increasing concentrations of dATP resulted first in minor stimulation but reverted to inhibition at concentrations above 20 μM dATP (Fig. 3A). In addition, the ATP-stimulated enzyme was strongly inhibited by dATP (Fig. 3A). These data are in full agreement with the model developed from earlier results with E. coli and calf thymus enzymes. ATP binding induces CDP reduction when bound to both the specificity and activity sites. Addition of dATP then displaces ATP from the activity site and inhibits the enzyme. The reductions of GDP and ADP also followed predictions from the model. GDP reduction was dependent on binding of dTTP to the specificity site (data not shown). It was stimulated more than 6-fold by ATP binding to the activity site but was inhibited completely when dATP was bound instead (Fig. 3B). The small inhibition by dGTP (Fig. 3B) results from competition with dTTP at the specificity site. ADP reduction presents a similar story (Fig. 3C). This reaction primarily requires binding of dGTP to the specificity site. When bound to

FIG. 1. Scheme for the allosteric properties of E. coli R1 showing the two classes of sites (substrate specificity and overall activity), the binding of various effectors to these sites, and the effects of binding on the catalytic activity of the R1-R2 complex.

a single point mutation in the N-terminal region, transforming aspartic acid to asparagine (D57N) (20). In E. coli R1, dATP is bound in this region (9).

 Biosensor studies (21) indicated that allosteric effector binding strongly influenced mouse R1/R2 protein interaction. Binding to the specificity sites promoted formation of R1 dimers, which is believed to be a prerequisite for binding to the R2 dimer. The low affinity between the R1 monomers in the absence of effectors may explain why the mammalian enzyme requires effectors for activity. Additional binding of ATP/dATP to the activity sites further increased mouse R1/R2 interaction.

Using current technology and the availability of large amounts of recombinant proteins, we wished to resolve the paradoxical findings in the literature about the numbers of allosteric sites on the mammalian enzyme. To this purpose both the wild type form and the D57N mutant of mouse R1 were overproduced in E. coli, purified to homogeneity, and investigated with respect to their allosteric properties. For the wild type R1 protein, it was of particular interest to investigate the reported difference from the E. coli class Ia prototype in the effector binding stoichiometry. For the D57N mutant, one important task was to determine whether the protein no longer was able to bind dATP, or whether the lack of inhibition was caused by a different mechanism. We hoped that the results would help us to understand why the mere addition of a 2'-OH to dATP transforms an inhibitor to an activator for the wild type enzyme.

FIG. 2. SDS gel electrophoresis of the ATP eluate of the D57N mouse R1 protein (lane 1, 3.2 μg), the D57N R1 protein after chromatography on Superdex 200 (lane 2, 5 μg), and wild type mouse R1 protein (lane 3, 2.8 μg). The gel contained 6% acrylamide.
the activity site, ATP stimulated catalytic activity, whereas when dATP bound to the activity site, catalytic activity was inhibited. The small inhibition by dTTP arose from competition with dGTP at the specificity site (Fig. 3C).

Fig. 3. Allosteric regulation of the substrate specificity of wild type mouse ribonucleotide reductase. The postulated binding of the various effectors to the two classes of sites shown in Fig. 1 is given at the extremes of the curves. A, CDP reduction. The effects of dATP alone (■) or in the presence of 2 mM ATP (▲) are shown. 100% represents the formation of 2.0 nmol of dCDP during a 30-min incubation with only ATP as effector. B, GDP reduction. All reaction mixtures contained 2 mM dTTP. The effects of increasing amounts of dATP (■), ATP (▲), or dGTP (▼) are shown. 100% is the activity in the presence of only dTTP (0.25 nmol of dGDP formed during 30 min). The insets to A and B show the results of experiments at lower concentrations of dATP. C, ADP reduction. All reaction mixtures contained 2 mM dGTP. The effects of dATP (■), ATP (▲), or dGTP (▼) are shown. 100% is the activity in the presence of only dGTP (0.35 nmol of dADP formed during 30 min).

Fig. 4. Allosteric regulation of the substrate specificity of mouse D57N R1 mutant ribonucleotide reductase. The designations are as in Fig. 3. 100% is 16 nmol of dCDP (A) in the presence of only ATP as effector, 1.1 nmol of dGDP (B) in the presence of only dTTP, and 3.0 nmol of dADP (C) in the presence of only dGTP.

Allosteric Regulation of the Catalytic Activity of the Mouse D57N R1 Mutant—Fig. 4 shows results from experiments with the mutant enzyme similar to those depicted in Fig. 3 for the wild type enzyme. The major difference from the wild type R1 concerns the function of dATP, which stimulated CDP reduction over the whole concentration range and did not inhibit the ATP-stimulated reaction (Fig. 4A). In addition, in the case of GDP (Fig. 4B) and ADP reduction (Fig. 4C), dATP stimulated the reaction in the presence of the prime effectors dTTP (for GDP reduction; Fig. 4B) and dGTP (for ADP reduction; Fig. 4C). Note also that GDP reduction was inhibited by dGTP and that ADP reduction was inhibited by dTTP, as was the case for the wild type R1. All results suggest that the single amino acid mutation resulted in an R1 protein that binds both ATP and dATP to the activity site but no longer distinguishes between...
them when it comes to their effect on catalysis.

**Binding of Effectors to Wild Type Mouse R1**—The availability of relatively large amounts of homogeneous mouse R1 prepared in *E. coli* made possible a more detailed study of effector binding than was possible 20 years ago with the scarce calf thymus R1 protein. In particular, we could now address the puzzling earlier discrepancy in binding stoichiometry between the mammalian and *E. coli* enzymes. Fig. 5A shows Scatchard plots for dATP and dGTP binding to the R1 dimer. A linear curve (as for dGTP) suggests homogeneous binding to identical sites; non-linearity (as for dATP) suggests separate sites, each binding the ligand with a different affinity. Extrapolation to the abscissa gives the number of sites per dimer, and the slope of the curve gives the dissociation constant *K*<sub>D</sub>. According to these criteria, dGTP was bound by two identical sites with a *K*<sub>D</sub> of 0.2 μM. dATP was bound to four sites, even though the non-linearity of the curve made extrapolation difficult. Assuming the curve to be the resultant of two separate classes of sites, as indicated in Fig. 5A, we can calculate approximate binding constants of 0.07 μM and 1.5 μM for the two dATP-binding sites.

To obtain an understanding of the structural basis for the modulation of catalytic activity by the interplay between different effectors, we determined dATP binding curves in the presence of an excess of other effectors. Fig. 5B shows competition with dGTP and dTTP. In both cases we tested two concentrations of competing effector to ascertain saturating conditions. dATP binding was now linear and extrapolated to close to two sites with binding constants of 0.3 μM (competing nucleotide, dGTP) and 0.2 μM (competing nucleotide, dTTP). A 20-fold increase in concentration of the competitor did not change this. With ATP, the results depended on the concentration of the competitor (Fig. 5C). At 0.33 mM ATP, dATP binding was linear and extrapolated to two sites with a *K*<sub>D</sub> of 1 μM. When the ATP concentration was increased 4-fold, ATP blocked dATP binding almost completely, and no meaningful curve could be drawn from the data (Fig. 5C). These experiments show that all four dATP-binding sites also bind ATP but that only two sites bind dGTP and dTTP.

**Binding of Effectors to Mouse D57N R1**—As mentioned under “Experimental Procedures,” we tested two different preparations of the protein. First, we show the results with the ATP eluate (Fig. 2, lane 1), because this preparation was available in relatively large amounts. In contrast to the wild type R1, the mutated R1 bound dATP with a single *K*<sub>D</sub> of 0.5 μM (Fig. 6A). The linear curve extrapolates to a value of 1.2 sites per dimer. In the presence of an excess of competing dGTP or dTTP (Fig. 6B), the affinity for dATP was essentially unchanged (*K*<sub>D</sub> = 0.2–0.3 μM) but now extrapolated to 0.6 sites. Thus both competing nucleotides blocked half of the dATP-binding sites, suggesting that the blocked sites also can bind dTTP and dGTP. In agreement with this, the binding curve for dGTP in Fig. 6A extrapolates to 0.4 sites, with a *K*<sub>D</sub> of 0.2 μM. We return to the significance of these data under “Discussion.”

In a different set of experiments, we tested the inhibition of dATP binding by an excess of competing effectors where dATP was present at close to saturating concentrations (Table I). dATP was bound at 1.5 sites in the absence of competitor in this experiment. This value was halved in the presence of either dGTP or dTTP, and a combination of the two resulted in no further decrease. ATP by itself instead decreased dATP binding to less than one-fifth, and, in combination with either dGTP or dTTP, abolished it completely. Together, the data in Table I suggest that, similar to wild type R1, half of the dATP-binding sites also can bind dGTP and dTTP, that dGTP and dTTP bind to the same site, and that ATP binds to both dATP-binding sites. However, ATP binding is weak and at the concentration

![Fig. 5. Scatchard plots of effector binding to wild type R1 protein. A, binding of dATP (• and ○) and dGTP (▲ and ■). The separate symbols represent in each case the results from two different experiments. Approximate extrapolations to the abscissa are given for the two postulated dATP-binding sites. B, binding of dATP in the presence of an excess of dGTP (▲, 13 μM; ●, 330 μM) or dTTP (■, 23 μM; ○, 670 μM). Each nucleotide competes for two dATP-binding sites, and increasing the concentration 20-fold does not affect the result. C, binding of dATP in the presence of ATP (▲, 0.33 mM; ■, 1.33 mM). At the lower concentration ATP competes for two dATP-binding sites, but increasing the concentration blocks all sites. v, the average number of ligand molecules bound per R1 homodimer.](image-url)
now the linear curve extrapolated to a value of 2.4 sites per dimer (Fig. 6A). In the presence of an excess of competing dTTP, the curve for dATP binding extrapolated to around 1.5 sites per dimer (data not shown).

**Influence of Allosteric Effectors on the Binding of Wild Type or D57N R1 Protein to R2 Protein**—Protein R2 was immobilized to a sensor chip, and solutions containing different concentrations of R1 protein in the absence or presence of allosteric effectors were injected while R1 binding was recorded. The equilibrium response data from increasing concentrations of wild type or mutated R1 protein were analyzed in Scatchard plots (Fig. 7). With wild type R1 protein, we found much steeper slopes with ATP and dATP than with dTTP or dGTP, indicating a lower dissociation constant for R1/R2 binding (Fig. 7, upper panel). This steep plot was also present for the D57N R1 protein in the presence of ATP, whereas the dATP plot was indistinguishable from the plots obtained with dTTP or dGTP (Fig. 7, lower panel). We interpret the result to mean that in the mutant R1 protein, dATP binding to the activity site is much less efficient in inducing the high affinity conformation of R1 than in the wild type protein, whereas the effects of ATP binding are the same.

**DISCUSSION**

The experiments with the wild type enzyme provided clear-cut results. The effector binding stoichiometries (four dATP or ATP and two dTTP or dGTP per R1 dimer) are now the same as those found earlier for *E. coli* R1, and the lower values reported earlier for calf thymus R1 in all probability were caused by impurities in the preparation. Mouse R1 has two classes of sites, one site binding all the tested effectors and the second site binding only dATP and ATP. All these data are identical to earlier results with *E. coli* R1, where the first site is considered to regulate substrate specificity and the second site is considered to regulate enzyme activity. The present data depicted in Figs. 3 and 5 fully support a similar pattern of regulation for the mouse reductase and confirm the earlier work with the calf thymus reductase that led to the conclusion that the allosteric regulation of the mammalian ribonucleotide reductase follows the same rules as the regulation of the *E. coli* enzyme.

In addition, the results concerning the catalytic activity of the D57N mutant are quite straightforward. There are several lines of evidence demonstrating that the aspartate 57 residue is located in the allosteric activity site. First, the allosteric activity site is absent from all microbial ribonucleotide reductases lacking a sequence homologous to the 100 N-terminal amino acids of *E. coli* R1. Second, in a complex between AMPPNP\(^1\) and *E. coli* R1, histidine 59 (which corresponds to aspartate 57 of mammalian R1) binds to the ribose moiety of the analog. Third, dGuo-200–1 mutant cells containing the D57N mutation are not inhibited in their growth by deoxyadenosine, and CDP reduction by the reductase isolated from these cells is not inhibited by dATP. The present data (Fig. 4) provide conclusive evidence on this point. They show that not only CDP reduction but also GDP and ADP reductions catalyzed by the mutated enzyme were strongly stimulated by dATP. During reduction of GDP and ADP, the specificity site is blocked by dTTP and dGTP, indicating a lower dissociation constant for R1/R2 binding (Fig. 7, upper panel).

\(^1\) The abbreviations used are: AMPPNP, adenosine 5′-(β, γ-imino)triphosphate.
dGTP, respectively, and the stimulation by dATP has to involve the activity site. With CDP, the distinction between the two sites was more difficult, as dATP in these experiments binds to both sites. In conclusion, the D57N mutation affects the activity site, resulting in stimulation of the enzyme by both ATP and dATP, i.e. the enzyme no longer distinguishes between hydroxyl and hydroxyl at C-2′ of ATP. This agrees with the presumed interaction between aspartate 57 and the ribose moiety of the ligand, as suggested from the AMPPNP- E. coli structure.

The interpretation of the effector binding data with the D57N mutant is less clear. The low binding stoichiometries might be caused by a functional loss of sites, with only two dATP-binding sites in the mutated R1 dimer, instead of four. If this were the case, the protein would have lost one specificity site and one activity site, because the catalytic experiments demonstrate the function of both classes. More likely, impurities in the preparations decreased the stoichiometry. Because of the lability of D57N, some molecules may also have lost the ability to bind effectors. We favor this explanation, because the binding stoichiometry increased for the D57N R1 protein with increasing purity of the preparation.

The dATP binding curve for D57N R1 (Fig. 6A) differs from that of wild type R1 (Fig. 5A) in two aspects; it shows no curvature, and the $K_D$ value calculated from the straight line is almost an order of magnitude larger than the first $K_D$ value for wild type R1. We have presented above conclusive evidence for the presence of both a specificity site and an activity site on the mutated R1. Our finding of a single slope has two alternative explanations. Both sites have the same or closely similar $K_D$ values of 0.5 μM or the curve measures the $K_D$ for the specificity site, and the affinity at the activity site is too low to be measured. Both alternatives lead to the unexpected conclusion that the $K_D$ for the specificity site is 0.5 μM and not 0.07 μM as in wild type R1, demonstrating that a mutation in the specificity site decreased the affinity for dATP at the specificity site almost 10-fold. Note that this effect is not seen with dGTP.

The competition experiments with other effectors suggest strongly that the first alternative is correct (Fig. 6D). An excess of either dGTP or dTTP halved the number of sites available for dATP binding but only minimally affected the $K_D$ in the Scatchard plot. As both dGTP and dTTP bind to the specificity site, the site remaining for dATP binding is the activity site with a $K_D$ of 0.2–0.3 μM. In addition, the data in Table I show that dTTP and dGTP, either alone or in combination, blocked half of the dATP-binding sites. In contrast, competition experiments with ATP (Table I) show that ATP bound to all four sites.

Combining the results from the allosteric effects on enzyme activity and the binding data, we conclude that in the D57N mutant the activity site has the ability to bind both ATP and dATP but that the protein no longer can distinguish between them. A similar situation has been described for the R1 protein of *Trypanosoma brucei* (7) and some class II ribonucleotide reductases (6). The mutation also decreases the affinity of the specificity site for dATP almost 10-fold, so that it can no longer be distinguished from the activity site in the Scatchard plot. This explains why the protein no longer could be easily purified by dATP-Sepharose chromatography. At first sight it may seem surprising that a mutation in one allosteric site primarily affects binding at the second site. The interdependence of the two sites, however, became evident already from the finding that the $K_D$ for dATP at the activity site of *E. coli* R1 depended on the type of effector bound to the specificity site (16). Our concepts concerning the allosteric regulation of ribonucleotide reductases illustrated by Fig. 1 depend on a high degree of flexibility of the enzyme structure, which permits the conformational transitions required for the postulated interactions between the two allosteric sites and the catalytic site. The present result further adds to this picture by suggesting similar interactions also between the two allosteric sites.

Our new data combining enzyme assays, nucleotide binding experiments, and studies by a biosensor technique of R1/R2 interaction clearly define the function of the various allosteric effectors. Still they do not tell us why the removal of the 2′-OH from ATP transforms an activator to an inhibitor of the wild type enzyme. The mouse enzyme does not absolutely require ATP to be bound to the activity site for activity, because GDP or ADP reduction stimulated by dTTP or dGTP, respectively, is active also in an enzyme with an empty activity site. Therefore, the conformation of the mouse R1 protein with effectors bound to the specificity sites allows the formation of an active enzyme complex together with the R2 protein, even in the absence of ATP bound to the activity sites. However, binding of ATP greatly stimulates activity, and this may be a result of the tighter R1/R2 complex induced by ATP (21). Because both ATP and dATP, on binding to the activity sites, induce the same high affinity complex between the R1 and R2 proteins in the wild type mouse ribonucleotide reductase, the mechanism for dATP inhibition seems not to involve different enzyme complexes. Instead, dATP binding may interfere with the catalytically essential radical transfer pathway between the active site of R1 and the tyrosyl radical of R2 (9, 21). Hopefully, the crystal structure of an R1/R2 complex will reveal the mechanism of dATP inhibition.

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