Mouse Ribonucleotide Reductase Control

INFLUENCE OF SUBSTRATE BINDING UPON INTERACTIONS WITH ALLOSTERIC EFFECTORS

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Using ribonucleotide reductase encoded by vaccinia virus as a model for the mammalian enzyme, our laboratory developed an assay that allows simultaneous monitoring of the reduction of ADP, CDP, GDP, and UDP. That study found ADP reduction to be specifically inhibited by ADP itself. To learn whether this effect is significant for cellular regulation, we have analyzed recombinant mouse ribonucleotide reductase. We report that allosteric control properties originally described in single-substrate assays operate also under our four-substrate assay conditions. Three distinctions from the vaccinia enzyme were seen: 1) higher sensitivity to allosteric modifiers; 2) higher activity with UDP as substrate; and 3) significant inhibition by ADP of GDP reduction as well as that of ADP itself. Studies of the effects of ADP and other substrates upon binding of effectors indicate that binding of ribonucleoside diphosphates at the catalytic site influences dNTP binding at the specificity site. We also examined the activities of hybrid ribonucleotide reductases, composed of a mouse subunit combined with a vaccinia subunit. As previously reported, a vaccinia R1/mouse R2 hybrid has low but significant activity. Surprisingly, a mouse R1/vaccinia R2 hybrid was more active than either mouse R1/R2 or vaccinia R1/R2, possibly explaining why mutations affecting vaccinia ribonucleotide reductase have only small effects upon viral DNA replication.

Ribonucleotide reductase (RNR), which catalyzes the first reaction committed to DNA synthesis, is distinctive in many ways. First, the interplay between two different allosteric sites evidently acts to ensure that the four deoxyribonucleotide products of the enzyme are synthesized at rates proportional to the base composition of the genome of the organism (1). Because deoxyribonucleotides have no known functions in eukaryotic cells except as DNA precursors, it makes good metabolic sense for rates of synthesis and utilization to be balanced.

Second and less thoroughly investigated, the four ribonucleotide substrates are reduced at one active site. To what extent does competition among the four ribonucleotide substrates for binding to this site contribute toward the ability of the enzyme to act in concert with the need of the cell for dNTPs? It was partly for this reason that our laboratory developed an assay protocol that allows all four RNR reactions to be monitored simultaneously, in one reaction mixture (2, 3). Our results, obtained with ribonucleotide reductases encoded by bacteriophage T4 (2) and vaccinia virus (3) support the conclusion that ribonucleotide substrate concentrations are equal in importance to concentrations of nucleoside triphosphate effectors as determinants of the ability of the enzyme to produce its four products at rates proportional to the base composition of the genome.

We have used the vaccinia virus enzyme as a model for mammalian ribonucleoside diphosphate reductases, because of its close structural relationship to the mammalian enzymes and because the viral enzyme subunits have been available in our laboratory for some time as purified recombinant proteins (4). In applying the four-substrate assay to vaccinia virus RNR, we observed one striking phenomenon: a fairly strong and quite specific inhibition of the reduction of ADP by ADP itself (3). We speculated that this effect might represent a metabolic control system that links the energy status of a cell with systems for DNA replication. Before carrying out experiments to test this hypothesis, it was essential to learn whether this effect is peculiar to the viral model system we were using or whether a mammalian ribonucleotide reductase behaves similarly. Within the past few years, cDNAs for the large (R1) and small (R2) subunits of the mouse RNR have become available (5, 6). Therefore, using recombinant mouse ribonucleotide reductase, we have now been able to ask whether ADP behaves similarly as an apparent regulator of enzyme activity. It was also of interest to confirm that the effects of allosteric modifiers, seen in vitro in analysis of single-substrate reactions, are similar when the enzyme is acting upon all four substrates simultaneously. Finally, we wished to extend our earlier observation (3) that interspecific hybrid RNRs, formed by mixing mouse and vaccinia virus subunits, are enzymatically active.

Like all known mammalian RNRs, the enzymes encoded by phage T4 and vaccinia virus are type I ribonucleotide reductases (1), which act upon ribonucleoside diphosphate (rNDP) substrates. Each enzyme is a heterotetramer containing the homodimeric R1 and R2 proteins. The R2 protein contains a catalytically essential tyrosine free radical formed and stabilized with the help of a nearby iron center consisting of two ferric ions bridged by an oxygen atom. The R1 protein contains the catalytic site and two allosteric sites: the activity site, which binds either ATP or dATP and regulates overall catalytic activity, and the specificity site, which binds either ATP, dATP, dGTP, or dTTP and regulates specificity; for example, dTTP, when bound in the specificity site, activates the reduction of GDP and inhibits the reduction of both CDP and UDP.

EXPERIMENTAL PROCEDURES

Expression of Mouse cDNAs for R1 and R2 and Purification of the Recombinant Proteins—Mouse R1 and R2 cDNAs were expressed in

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Escherichia coli BL21(DE3), from plasmids developed in Lars Thelander’s laboratory (Umeå, Sweden) (5, 6) and kindly provided by Dr. Thelander. Induction and purification of mouse R1 protein were carried out as described (6), except that bacteria after harvesting were lysed in a French pressure cell at 15,000 p.s.i. Also, elution of the protein from a dATP-Sepharose affinity column was carried out with column buffer containing 75 mM ATP, a condition that we had found earlier to be effective in purifying vaccinia virus R1 (4). Mouse R2 protein was induced and purified as described by Mann et al. (5) and as modified in our laboratory (3). Recombinant vaccinia virus R1 and R2 proteins were expressed and purified as described previously by this laboratory (4, 7).

The pH value used for the assay reactions, 8.2, was based upon our earlier determination of the pH optima for RNRs of vaccinia virus and cultured BSC40 monkey kidney cells (8). When we ran the four-substrate assay with the mouse enzyme at pH 7.6 under our quasi-physiological conditions, we found the activity to be decreased by about 24% compared with the activity determined at pH 8.2 and the overall product profile to be substantially the same (data not shown).

**Nucleotide Binding Experiments—**Nucleotide binding to mouse R1 protein was measured according to the ultrafiltration assay devised by Ormø and Sjöberg (9). For each assay a 3H-labeled dNTP, with or without added unlabeled rNDPs, as indicated, was mixed with R1 in a solution containing 50 mM Tris-HCl, pH 7.6, 10 mM MgCl2, and 2 mM dithiothreitol in a total volume of 150 μl. After incubation on ice for 15 min, a 30-μl aliquot was taken for scintillation counting to quantitate total nucleotide concentration. The remainder (120 μl) was applied to a Nanosep centrifugal separator (Gelman; cut-off value, 30 kDa), and centrifugation at 6000 rpm was carried out for 2 min. Aliquots of 30 μl were then withdrawn from the filtrate for scintillation counting to quantitate unbound nucleotide. Concentrations of bound nucleotides were determined by subtracting unbound nucleotide from total nucleotide. Control reactions run in the absence of added protein showed that no significant amounts of nucleotide were bound nonspecifically to the filters; in these control reactions the counts in “unbound” nucleotide were virtually identical to counts in “total” nucleotide.

**RESULTS**

*Regulatory Effects of Individual Nucleoside Triphosphate Effectors—*With the exception of our preliminary studies on T4 and vaccinia virus ribonucleotide reductases, most of what we know about allosteric control of this crucially important enzyme has been learned from assay protocols in which the enzyme is exposed to only one substrate at a time. Within the cell, the enzyme is exposed to all four rNDP substrates. How might competition among these substrates influence the activities of individual allosteric effectors?

Fig. 1 explores the effects of individual allosteric modifiers in the four-substrate assay, as well as illustrating the kind of data generated by this assay. As noted earlier (2, 3), we remove unreacted rNDP substrates and ATP from the reaction mixture by boronate column chromatography, and then the deoxyribonucleoside diphosphate products of the reactions are resolved by ion exchange high pressure liquid chromatography. These profiles also include the individual dNTP effectors added, but not ATP, which is removed by the boronate column. In this experiment the four rNDP substrates were present initially at equal concentrations of 0.15 mM each. ATP was present at 2.5 mM, its approximate intracellular concentration, whereas each individual dNTP was present at 40 μM. At this level, dATP was expected to bind primarily to the specificity site and thus have no significant effect on the binding of any added nucleoside triphosphate (data not shown). The data in Fig. 1 show that individual dNTPs have effects similar but not identical to those described for calf thymus (10) and mouse (11) RNRs in single-substrate assays. dCTP strongly activated reduction of GDP and ADP, whereas inhibiting CDP reduction. dGTP greatly stimulated ADP and GDP reduction, while inhibiting

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**TABLE I**

Effects of allosteric modifiers on activities of mouse ribonucleotide reductase

| dNTP effector | Rate of product formation (nmol dNDP/min/mg R1 protein) | dCDP | dUDP | dADP | dGDP |
|---------------|--------------------------------------------------------|------|------|------|------|
| None          | 108.0                                                  | 11.1 | 2.4  | <0.1 |      |
| dCTP          | 108.2                                                  | 11.3 | 2.4  | <0.1 |      |
| dTTP          | 31.8                                                   | 10.9 | 13.3 | 78.4 |      |
| dATP          | 7.6                                                    | <0.1 | 5.1  | <0.1 |      |
| dGTP          | 11.1                                                   | <0.1 | 92.7 | 5.3  |      |

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**FIG. 1.** Regulation of mouse ribonucleotide reductase activities in the four-substrate assay by individual allosteric effectors. All reaction mixtures contained the four rNDP substrates at 0.15 mM each and ATP at 2.5 mM. Each of the four activities was negligible when ATP was omitted from the reaction mixture.

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Four-substrate Assay for Ribonucleotide Reductase—Enzyme assays were carried out essentially as described previously (2, 3), with modifications as indicated in individual tables and figure legends. Reaction mixtures contained 50 mM HEPES, pH 8.2, 5 mM MgCl2, 50 mM dithiothreitol, 20 mM Fe(NH4)2(SO4)2, 1 mM mouse R1 protein, 4 μM R2 protein, and ribonucleoside diphosphate substrates and nucleoside triphosphate effectors at the indicated concentrations and in a total volume of 100 μl. Incubation was carried out at 37 °C for 5 min. Reactions were terminated by addition of 5 μl of 50% (v/v) perchloric acid, and this was followed by chromatographic analysis of the reaction mixture (2, 3).
the reduction of both pyrimidine nucleotides. dTTP activated GDP reduction much more strongly than ADP reduction, whereas dGTP had the converse effect. The activation of ADP reduction by dTTP and the activation of GDP reduction by dGTP are effects not seen in single-substrate analysis of calf thymus RNR (10) or recombinant mouse RNR (11).

dATP slightly activated ADP reduction and strongly inhibited CDP and UDP reduction, whereas GDP reduction was undetectable. This suggests that dATP added at 40 μM does bind significantly to the activity site. In additional experiments (not shown), dATP was seen to inhibit all four activities at higher concentrations, as has been seen repeatedly in single-substrate assays. Numerical values are presented in Table I. These data show the mouse enzyme to be significantly more sensitive to individual allosteric effectors than vaccinia virus RNR, assayed under similar conditions (compare with Fig. 2 in Ref. 3). For example, the GDP reductase activity of the viral enzyme was approximately doubled by dTTP and halved by dATP. By contrast, the mouse GDP reductase activity was absolutely dependent upon added dTTP and was completely inhibited by added dATP at the concentration used. Similarly, the inhibitory effect of dGTP upon CDP and UDP reduction appeared to be stronger for the mouse than for the viral enzyme.

Enzyme Behavior in the Presence of Four rNDP Substrates at Estimated in Vivo Concentrations—We presume that ribonucleotide reductase in vivo acts upon the four rNDP substrates at relative rates corresponding to the nucleotide composition of the genome. Mouse DNA contains 58% A + T and 42% G + C (12). Table II presents enzyme product profiles for reactions run under various conditions. For these experiments, quasi-physiological or in vivo concentrations of nucleoside triphosphates were estimated from pool size determinations in our laboratory for a variety of cultured mammalian cell lines (3), and quasi-physiological concentrations of rNDP substrates were estimated from nucleotide pool data compiled by Traut (13) for cultured mammalian cell lines. Traut estimated the following values for intracellular rNDP concentrations from averaged data from many studies of nonhuman cell lines: ADP, 849 μM; CDP, 71 μM; GDP, 159 μM; and UDP, 155 μM. We rounded off these values as follows for our quasi-physiological conditions: ADP, 850 μM; CDP, 70 μM; GDP, 160 μM; and UDP, 160 μM. Of course, these estimates could be quite inaccurate because of variation among different cell lines, compartmentation, variation of activity coefficients within the intracellular milieu, and so forth. Nevertheless, our data clearly establish the essentiality of allosteric modifiers for directing the enzyme to produce four deoxyribonucleoside diphosphates at rates corresponding to their rates of utilization.

The importance of substrate concentrations as a determinant of physiological specificity is evident but less pronounced than what was seen earlier for the vaccinia virus enzyme (3). At equimolar rNDPs (150 μM each), ADP reduction was significantly higher and GDP reduction was significantly lower than expected for biologically balanced deoxyribonucleotide production. At quasi-physiological concentrations, ADP reduction was close to the expected value, and GDP reduction was slightly closer but still below the expected value.

This presents a contrast from comparable results with the vaccinia virus RNR, in which we found that GDP reduction is higher than expected under quasi-physiological conditions. The more striking contrast, however, is for UDP reduction, where we found the vaccinia enzyme to have little activity under all conditions tested. Table II, however, shows the relative rate of UDP reduction by the mouse enzyme to correspond fairly closely to the representation of dTMP in the mouse genome. Others have reported (14) that most of the dTMP in mammalian genomes arises from dCMP, via the dCMP deaminase reaction followed by thymidylate synthase and that intracellular reduction of UDP to dUDP is negligible under most conditions. The data of Table II show CDP reduction at quasi-physiological rNDP concentrations to be considerably higher than the proportion of dCMP in the mouse genome. Therefore, even though UDP reduction is significant, our data are consistent with the concept that a significant fraction of the dTMP residues in mouse DNA arises via deamination of deoxycytidine nucleotides.

Effects of a Range of Substrate Concentrations—Although our estimates of effective intracellular rNDP concentrations may be inaccurate for the above-mentioned reasons, we felt

![Fig. 2](https://via.placeholder.com/150)

**Fig. 2. Effects upon mouse rNDP reductase specificity of proportional variations in rNDP substrate concentration.** The bi-proportional substrate mixture contained ADP, CDP, GDP, and UDP at 120, 1.0, 2.0, and 2.0 mM, respectively. Allosteric effectors were present at their estimated in vivo concentrations (2.5 mM ATP, 60 μM dATP, 15 μM dGTP, and 50 μM dTTP).

| Assay conditions | Percentage of total product formed |
|------------------|-----------------------------------|
|                  | dCDP | dUDP | dADP | dGDP |
| Equimolar substrates, no dNTPs | 78   | 18   | 4    | <1   |
| Equimolar substrates, in vivo dNTPs | 28   | 26   | 33   | 14   |
| In vivo substrates, no dNTPs | 78   | 19   | 3    | <1   |
| In vivo substrates, in vivo dNTPs | 34   | 25   | 27   | 15   |
| Percentage of total nucleotides in mouse genome | 21   | 29   | 29   | 21   |

* Present in mouse DNA as dTMP.

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Table II: Effects of allosteric modifiers and substrate concentrations upon mouse ribonucleotide reductase product profiles

All reaction mixtures contained ATP at 2.5 mM. Equimolar substrates refers to 0.15 mM each of ADP, CDP, GDP, and UDP. In vivo substrates refers to 850 μM ADP, 70 μM CDP, 160 μM GDP, and 160 μM UDP. ATP was present in each reaction mixture at 2.5 mM. In vivo dNTPs refers to 60 μM dATP, 15 μM dGTP, and 50 μM dTTP.
that the rNDP concentrations relative to each other should more closely reflect the in vivo situation. Therefore, we carried out a substrate concentration-velocity experiment, using a bioproportional mixture of ribonucleoside diphosphates as the variable substrate rather than a single substrate. According to the mammalian rNDP pool data compiled by Traut (13), the ratio of ADP to CDP to GDP to UDP is about 12:1:2:2. Fig. 2 shows results of an experiment in which ADP concentration was varied from 300 to 1800 μM and the other three rNDPs were varied proportionately. None of the concentrations tested yielded a closer fit between the product profile in the four-substrate assay and the nucleotide composition of mouse DNA. This result suggests either that our assumptions about rNDP concentration ratios in mouse cells are inaccurate, that control of rNDP reductase specificity in vivo involves additional factors, or both.

The most interesting feature of Fig. 2 is the marked inhibition of purine nucleotide reduction seen at the highest substrate concentrations. In this respect the mouse RNR resembles vaccinia virus RNR (3), and this result suggests that the apparent control of RNR activity by ribonucleoside diphosphates might be physiologically significant. To further investigate the basis for this effect, we varied the concentration of each rNDP individually, in the presence of each of the other three rNDPs at its respective estimated in vivo concentration. As shown in Fig. 3, ADP inhibited its own reduction at high concentrations, although the effect was not as pronounced as seen in the experiments with bioproportional substrate mixtures. The effect of ADP is evidently more complex than simple competition with other rNDPs for catalytic sites on the enzyme, because CDP reduction was activated at high ADP concentrations; if only competition at the catalytic site were involved, CDP reduction would decline at higher ADP concentrations, and ADP reduction would increase. By contrast, the effects of the other rNDPs did seem to involve mostly competition for binding at the catalytic site. In Fig. 4, for example, note that increasing GDP concentration in the presence of fixed amounts of the other three rNDPs increased the rate of GDP reduction but decreased the activity of the enzyme toward the other three substrates. A nearly identical result was seen with CDP, namely, increased CDP reduction at high levels coupled with decreased activities toward the other three substrates.

Analysis of Nucleotide Binding to Mouse R1 Protein — The enzyme activity data presented above indicate that the effect of ADP on the RNR product profile is more complex than simple competition between ADP and other rNDP substrates at the catalytic site. Does ADP bind to a previously unknown site on R1, or does it bind at high concentrations of other rNDPs to R1 in the absence of added rNDP substrates. Data from these experiments are shown in Fig. 5. Both dGTP and dTTP displayed hyperbolic binding behavior as expected for single-site binding. Curve fitting for the dGTP data (Fig. 5A) yielded a $K_d$ of $4.4 \pm 0.38 \mu M$ and for the dTTP data (Fig. 5B) a $K_d$ of
1.8 ± 0.30 μM. Because dATP binds at two sites with differing affinities, we carried out dATP binding experiments at two different R1 protein concentrations and displayed the data on a Scatchard plot (Fig. 5C). Linear regression analysis yielded for the high affinity (specificity) site a $K_d$ of 12.6 ± 2.3 μM and for the low affinity (activity) site a $K_d$ of 54.3 ± 4.0 μM.

Our first experiment tested the idea that ADP at high concentrations can bind, as an ATP analog, either to the specificity site on R1, to the activity site, or to both. Accordingly, we asked whether ADP at high concentrations could inhibit the binding of dATP to R1. As shown in Fig. 6, however, ADP at concentrations as high as 1800 μM had no discernible effect upon the binding of dATP, present at 1.5 μM (open circles). Because ADP in this experiment is expected to bind to the catalytic site as well as to possible other sites, we modified the protocol by asking whether ADP could inhibit dATP binding under conditions where little if any ADP was bound at the catalytic site. This was accomplished by running the binding assays in the presence of CDP at a constant concentration of 320 μM, 10-fold higher than the reported $K_m$ for CDP with a mammalian RNR (11), so that ADP would be largely displaced from catalytic sites. Under these conditions also (Fig. 6, closed circles) we saw no effect of ADP upon amount of dATP bound. However, at all concentrations of ADP, the presence of CDP had a slight but consistent depressive effect upon the amount of dATP bound.

The latter finding suggested that CDP itself might have a direct effect upon the binding of nucleotides at one or both allosteric sites. We tested this idea by observing the effect of CDP upon the binding of its prime allosteric inhibitor, dGTP. As shown in Fig. 7A (open circles), CDP at high concentrations inhibited dGTP binding by nearly 10-fold. This finding suggests that allosteric and catalytic sites are in two-way communication. Most literature on allosteric enzymes focuses upon the concept that effector binding at an allosteric site influences substrate binding at the catalytic site. The data of Fig. 7A indicate that the converse relationship also holds. The metabolic rationale for this behavior of the enzyme is that CDP can promote its own reduction by driving release from the enzyme of its prime inhibitor. A similar relationship is shown in Fig. 7B (open circles), where we show that GDP stimulates the binding of its allosteric activator, dTTP. In this case the data suggest that GDP promotes its own reduction by causing a shift in RNR specificity toward GDP, through binding of an effector that is both inhibitory to CDP and UDP reduction and stimulatory toward its own reduction.

Because the primary goal of these experiments was to understand the effect of ADP upon ADP and GDP reduction, the nucleotide binding experiments depicted in Fig. 7 were carried out in the presence of ADP at a constant concentration. That concentration was set at 500 μM, a value permitting ADP to compete effectively with CDP and GDP, respectively, for binding at the catalytic site. Again, as in the experiment of Fig. 6, ADP displayed a small but consistent effect. In Fig. 7A, note that ADP depressed the inhibition of dGTP binding by CDP, and in Fig. 7B, the effect of ADP was to depress the stimulation of dTTP binding by GDP.

These experiments suggest a model for understanding the effects of ADP upon activities of mouse RNR. As suggested by the data of Fig. 6, ADP may be relatively inert in the sense that its binding to the catalytic site does not directly influence affinities for effectors at the specificity site. However, it might influence these affinities indirectly, by competing with the other RNR substrates for binding to the catalytic site and thereby depressing the effects of those other rNDPs on effector binding. This idea was tested in the experiments of Fig. 8, where ADP concentration was varied in the presence of other rNDP substrates at constant concentrations. As seen in Fig. 6, ADP by itself exerts little or no direct influence on effector binding. Note from the squares in both panels A and B of Fig. 8...
that ADP had little or no effect, in the absence of other rNDPs, on the binding of either dGTP or dTTP. Note also confirmation of the results shown in Fig. 7; in the absence of added ADP, CDP strongly inhibited dGTP binding (compare circles with squares, Fig. 8A), and GDP activated dTTP binding (compare circles with squares, Fig. 8B). As ADP concentration was increased, dGTP binding was increased in the presence of CDP (circles, Fig. 8A), and dTTP binding in the presence of GDP was decreased (circles, Fig. 8B). In Fig. 8A an even greater stimulation of dGTP binding by ADP was seen when the experiment was carried out in the presence of a mixture of CDP, UDP, and GDP, each at its estimated physiological concentration (triangles). Taken together, these data support the hypothesis upon which the experiment was based, namely, that the effect of ADP binding to allosteric effectors is indirect. From the experiment of Fig. 8A, we suggest that ADP stimulates dGTP binding by displacing CDP from the catalytic site, thereby limiting the inhibition of dGTP binding by CDP. Similarly, in Fig. 8B, we suggest that ADP binding displaces GDP, thereby limiting the ability of GDP to stimulate dTTP binding.

Activities of Mouse/Vaccinia Virus Chimeric rNDP Reductases—Although this study has documented some significant differences between the ribonucleotide reductases of mouse cells and vaccinia virus, the two enzymes are closely related, with amino acid sequence identities near 80% (15). Partly to examine whether the close structural relationship connotes a close functional relationship, in our earlier study (3), we showed that a hybrid enzyme containing vaccinia virus R1 protein and mouse R2 protein was enzymatically active, although the specific activity was lower than our values for the native viral enzyme (4) and those determined by Davis et al. (6) for the mouse enzyme. Because for the present study we had prepared homogeneous recombinant mouse R1 protein, we repeated the experiment, using both mouse/vaccinia hybrid enzymes. For this experiment we assayed equimolar rNDP substrates (150 μM each) in the presence of ATP as the sole allosteric effector, conditions that maximize the reduction of CDP. As shown in Fig. 9, the vaccinia R1/mouse R2 hybrid is active, as reported.
previously, with a specific activity significantly lower than that of either native holoenzyme. Unexpectedly, the mouse R1/vaccinia R2 hybrid was more active than either native enzyme, with a specific activity about twice that of the vaccinia virus holoenzyme.

**DISCUSSION**

Simultaneous analysis of the four activities of ribonucleotide reductase has revealed novel aspects of the regulation of this pivotal enzyme, as shown both by our earlier studies of type I RNRs of T4 phage and vaccinia virus (2, 3) and by more recent studies by Andersson et al. (16) of the type III anaerobic RNR of T4 phage. Recombinant mouse ribonucleotide reductase shows allosteric behavior in the four-substrate assay similar to that earlier described for other mammalian RNRs (10, 11), such as strong dependence upon dGTP for ADP reduction, upon dTTP for GDP reduction, and upon ATP for all activities of the enzyme. General inhibition by dATP and inhibition of CDP and UDP reduction by dGTP are among the feedback inhibition phenomena described previously. Our studies reveal two regulatory effects, less strong but still significant, which have not been reported before: activation of ADP reduction by dTTP and of GDP reduction by dGTP. Whether these effects play a significant role in regulating dNTP pool sizes in vivo cannot yet be determined from our data.

The relatively high activity of the mouse enzyme toward UDP was unexpected in light of the negligible activity of the vaccinia virus enzyme toward this substrate, and in vivo data suggesting that the pathway dCMP → dUMP → dTMP → dTTP is more significant in vivo for dTTP synthesis than is the pathway involving RNR, viz. UDP → dUDP → dUMP → dTMP.

Regulation of mouse RNR activity by ADP seems to be less specific than was reported previously (3) for the vaccinia virus enzyme, in the sense that the bioproportional mixture of rNDPs exerts a stronger regulatory influence under our conditions than does ADP alone. For example, in the experiment of Fig. 2, the percentage of dADP + dGDP in the product mix dropped from 64 to 28% in going from the lowest to the highest rNDP concentration tested. By contrast, in the experiment of Fig. 3, the corresponding change, in going from 800 to 1800 μM ADP, was from 48% of the total to 34%.

Whatever the specific regulatory mechanisms involved, however, it is apparent that ADP interacts with the enzyme differently from the other rNDPs, because its effects upon the product profile in the four-substrate assay cannot be explained simply in terms of competition among the four substrates for the catalytic site. The nucleotide binding experiments depicted in Figs. 6–8 suggest a model for understanding this interaction. According to this model, ADP binds exclusively at the catalytic site. Note from Fig. 6 that ADP at a 1200-fold molar excess over dATP had no detectable effect upon dATP binding, suggesting that ADP does not interact with the two allosteric sites. We cannot rule out the possibility of some binding of ADP at the activity site, because this experiment was carried out at 1.5 μM dATP, a concentration so low that binding should have occurred primarily at the high affinity specificity site. Nor can
we yet rule out the possibility that ADP binds to a previously undescribed site on the R1 protein. More direct approaches are needed, and these are in progress.

If we assume that the effects of ADP result from its interaction with the catalytic site, we can understand its effects upon the specificity of mouse ribonucleotide reductase, namely, activation of CDP reduction and inhibition of ADP and GDP reduction. ADP evidently has little direct effect on the binding of either dATP (Fig. 6), dGTP (Fig. 8A, squares), or dTTP (Fig. 8B, squares), whereas CDP and GDP both have strong effects upon binding of allosteric effectors (Fig. 7). However, ADP can modulate the effects of CDP and GDP by competing with them for binding to the catalytic site. The intracellular pool size of ADP, which is much higher than those of the other three rNDPs (13), suggests that this competitive binding is metabolically significant. A consequence of the displacement of CDP and GDP from the catalytic site is to depress the binding of dTTP (promoted by GDP) and increase binding of dGTP (inhibited by CDP) at the specificity site; these events in turn should inhibit reduction of ADP and GDP and increase reduction of CDP. Our observation (Fig. 8A, triangles) that ADP shows a 3-fold stimulation of dGTP binding in the presence of quasi-physiological concentrations of CDP, GDP, and UDP is consistent with this interpretation.

The experiment of Fig. 5 yielded $K_a$ values for the binding of dATP, dGTP, and dTTP to mouse R1 protein. The values reported here (12.6 and 54.3 μM for dATP and 4.4 μM for dGTP) are considerably higher than corresponding values recently reported (11) for mouse R1 (0.07 and 1.5 μM for dATP and 0.2 μM for dGTP). Although we do not know the basis for the discrepancy, we note that the values we have determined are closer to what one might expect a priori if these interactions are to participate in metabolic regulation, because they lie closer to actual intracellular concentrations of these effectors. The average intracellular concentrations estimated by Traut (13) are 24 μM for dATP and 5.2 μM for dGTP; these values lie close to those determined in our own laboratory with several different cultured mammalian cell lines (17).

Finally, the high activity of the chimeric RNR containing mouse R1 and vaccinia virus R2 was unexpected; it would be of interest to determine whether this higher activity results from particularly efficient iron binding in vitro by the hybrid enzyme. However, our observation may explain why early studies on targeted deletion of the vaccinia virus R1 gene led to mutant viruses with no significant phenotype (18, 19). Perhaps a chimeric enzyme is formed in cells infected by this mutant. In vaccinia virus-infected monkey kidney cells, the R2 protein accumulates in significant excess over R1 (20). The viral R2 present may suffice to drive the formation of a significant amount of an enzymatically hybrid enzyme. Because levels of cellular R1 vary with growth phase of a cell culture (21), a prediction from our observations would be that the ability of a cell line to support productive vaccinia virus infection would be a function of the level of its own R1 protein.

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