Yeast DNA Damage-inducible Rnr3 Has a Very Low Catalytic Activity Strongly Stimulated after the Formation of a Cross-talking Rnr1/Rnr3 Complex*

The ribonucleotide reductase system in Saccharomyces cerevisiae includes four genes (RNR1 and RNR3 encoding the large subunit and RNR2 and RNR4 encoding the small subunit). RNR3 expression, nearly undetectable during normal growth, is strongly induced by DNA damage. Yet an rnr3 null mutant has no obvious phenotype even under DNA damaging conditions, and the contribution of RNR3 to ribonucleotide reduction is not clear. To investigate the role of RNR3 we expressed and characterized the Rnr3 protein. The in vitro activity of Rnr3 was less than 1% of the Rnr1 activity. However, a strong synergism between Rnr3 and Rnr1 was observed, most clearly demonstrated in experiments with the catalytically inactive Rnr1-C428A mutant, which increased the endogenous activity of Rnr3 by at least 10-fold. In vivo, the levels of Rnr3 after DNA damage never reached more than one-tenth of the Rnr1 levels. We propose that heterodimerization of Rnr3 with Rnr1 facilitates the recruitment of Rnr3 to the ribonucleotide reductase holoenzyme, which may be important when Rnr1 is limiting for dNTP production. In complex with inactive Rnr1-C428A, the activity of Rnr3 is controlled by effector binding to Rnr1-C428A. This result indicates cross-talk between the Rnr1 and Rnr3 polypeptides of the large subunit.

DNA damage in eukaryotic cells leads to arrest of the cell cycle and activation of the genes involved in DNA repair (1). The DNA damage checkpoint pathway responsible for activation of DNA damage-inducible genes in yeast Saccharomyces cerevisiae has been the focus of intensive research during the past decade (2). The emerging conservation between the DNA damage checkpoint pathway in yeast and humans promotes a better understanding of this vital process (3, 4). One of the frequently used tools in the dissection of the DNA damage checkpoint pathway in yeast is the gene RNR3 (5). This gene is induced 5–10-fold (6–8), and in some reports more than 100-fold (5), in response to DNA damage. In addition, it is not essential, and the rnr3 null mutation has no phenotype under all studied conditions (5). These properties of the RNR3 gene have been exploited in the identification of a number of important genes involved in the DNA damage checkpoint function such as CRT1, TUP1, SSN6, and DUN1 (9–12). On the basis of homology with the RNR1 gene encoding the large subunit of yeast ribonucleotide reductase (RNR),1 it has been proposed that RNR3 encodes a second large subunit of the yeast RNR (5). In support of this hypothesis, it was demonstrated that overexpression of RNR3 could rescue rnr1 null mutants (5).

Ribonucleotide reductase is the rate-limiting enzyme in DNA precursor biosynthesis present in all living cells (13). All RNRs use free radical chemistry to reduce ribonucleotides to deoxyribonucleotides. Based on the nature of the cofactor providing the free radical for the reaction, RNRs were divided into three classes (14). Nearly all eukaryotes have an RNR belonging to class I in which the enzyme is thought to be an αβ heterodimer. The homodimer αs is referred to as the large subunit, and the homodimer βs is referred to as the small subunit. Both subunits are required for activity. The small subunit contributes a tyrosyl free radical essential for catalysis. The large subunit contains one catalytic active site and two allosteric sites on each α polypeptide; the allosteric specificity site regulates the balance among the four dNTP pools, and the allosteric activity site regulates the total dNTP pool size by monitoring the dATP/ATP ratio (15). When the dATP pool reaches a certain level, the RNR is down-regulated by dATP feedback inhibition.

In addition to allosteric regulation, the activity of yeast RNR is controlled by binding of a protein inhibitor, Sm11, to the large RNR subunit (16, 17). The Sm11-dependent mechanism is so far unique for the yeast RNR. During S phase and after DNA damage, decreased Sm11 levels result in derepression of the RNR activity (18, 19). The decrease of Sm11 levels is caused by post-transcriptional regulation and requires Mec1/Rad53-dependent phosphorylation. Mec1 and Rad53 protein kinases are essential proteins and central players in DNA damage response in yeast (2). Deletion of SML1 rescues the lethality of a mec1 or rad53 strain, and removal of Sm11 during the S phase defines the essential function of Mec1 and Rad53 proteins (19).

The S. cerevisiae RNR system consists of the following four RNR genes: RNR1 and RNR3, which encode the polypeptides of the large subunit, and RNR2 and RNR4, which encode the polypeptides of the small subunit (20–22). The RNR genes are located on different chromosomes (V, IX, X, and VII, respectively), and expression of all four genes is induced by DNA damage (11). We have demonstrated before that, in contrast to other class I RNRs the active form of the yeast small subunit is a ββ′ heterodimer containing Rnr2 and Rnr4 (23). Rnr4, thus far a unique variant of the small subunit polypeptide, does not form a tyrosyl radical; its role is to correctly fold and stabilize

* This work was supported by the Swedish Research Council and the Medical Faculty of Umeå University and by fellowships from the Wenner-Gren Foundation and the Royal Swedish Academy of Sciences (to V. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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‡ The abbreviations used are: RNR, ribonucleotide reductase; 4-NQO, 4-nitroquinoline-1-oxide.

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Printed in U.S.A.

Received for publication, February 15, 2002
Published, JBC Papers in Press, March 12, 2002, DOI 10.1074/jbc.M201535200

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 277, No. 21, Issue of May 24, pp. 18574–18578, 2002

1 The abbreviations used are: RNR, ribonucleotide reductase; 4-NQO, 4-nitroquinoline-1-oxide.
the radical-storing Rnr2. A nucleotide sequence comparison of \textit{RNR1} and \textit{RNR3} shows 80% identities and 90% similarities of the coding sequences. However, the expression patterns of \textit{RNR1} and \textit{RNR3} are completely different (5). \textit{RNR1} is essential for mitotic viability, and its transcription is cell cycle-regulated with maximal mRNA levels present during S phase. The \textit{RNR3} transcript is nearly absent during normal growth but appears after DNA damage. Thus, during normal growth the large subunit is an \( \alpha_2 \) homodimer containing only Rnr1. Although the transcriptional regulation of the \textit{RNR3} gene has been studied in great detail, the biochemical properties and the amount of the Rnr3 protein in a yeast cell after DNA damage are unknown; also unknown is the ratio between the Rnr1 and Rnr3 proteins.

We wanted to understand the role of DNA damage-inducible Rnr3 in dNTP metabolism of yeast. In this paper we present an \textit{in vivo} and \textit{in vitro} characterization of the Rnr3 protein. After DNA damage, the levels of highly induced Rnr3 never reached more than one-tenth of the Rnr1 levels, which increased \( \sim 2 \)-fold. The Rnr3 protein showed less than 1% of the activity of the Rnr1 in an assay with the Rnr2/Rnr4 heterodimer. However, a strong synergism between Rnr1 and Rnr3 was observed (most clearly demonstrated in assays in which Rnr3 was allowed to form a complex with a catalytically inactive form of Rnr1). Interestingly, whereas the Rnr1-catalyzed CDP reduction was inhibited by the allosteric inhibitor dATP, the CDP reduction catalyzed by Rnr3 was stimulated by dATP. The reaction catalyzed by Rnr3 together with catalytically inactive Rnr1 showed an intermediate sensitivity to dATP inhibition, indicating cross-talk between the allosteric sites in Rnr1 and the catalytic site in Rnr3.

**Fig. 1.** DNA damage induction of Rnr1 and Rnr3. Crude extracts (37 \( \mu \)g of protein per lane) of yeast cells grown with and without treatment by 4-NQO were analyzed for Rnr1 and Rnr3 by immunoblotting as described under “Experimental Procedures.” \( \alpha \), lane 1, Rnr1 after 4-NQO induction; \( \beta \), lane 2, Rnr1 without induction; \( \gamma \), lane 3, Rnr3 after 4-NQO induction; \( \delta \), lane 4, Rnr3 without induction. The lower band in lanes 3 and 4 with the anti-Rnr3 antibodies is an unspecific band always observed with these antibodies.\( M \), molecular mass markers, 110 and 90 kDa. B, determination of the amounts of Rnr1 in a yeast crude extract after 4-NQO induction (lanes 3, 5, 7, 9, 11, and 13) by comparing with increasing amounts of pure recombinant Rnr1 (lane 1, 10 ng; lane 2, 20 ng; lane 4, 40 ng; lane 6, 60 ng; lane 8, 80 ng; lane 10, 120 ng; lane 12, 160 ng, and lane 14, 200 ng). Yeast extract (37 \( \mu \)g) was estimated to contain approximately 100 ng of Rnr1. C, determination of the amounts of Rnr3 in a yeast crude extract after 4-NQO induction (lanes 3, 5, 7, 9, 11, and 13) by comparing with increasing amounts of pure recombinant Rnr3 (3, 5, 7, 10, 20, 40, 60, and 100 ng). Yeast extract (37 \( \mu \)g) was estimated to contain approximately 10 ng of Rnr3.

**Fig. 2.** SDS-PAGE analyses of purified recombinant Rnr3 isolated from yeast and recombinant Rnr1-C428A isolated from \textit{E. coli}. Lane 1, molecular mass markers at 205, 116, 97.4, 66, and 45 kDa; lane 2, 6.5 \( \mu \)g of Rnr3; lane 3, 3 \( \mu \)g of Rnr1-C428A.

**Fig. 3.** Catalytic activity of Rnr3 assayed in the presence of an excess of Rnr2/Rnr4 heterodimer before and after the addition of a constant amount of Rnr1. Increasing amounts of Rnr3 were assayed for 30 min at 30°C in the presence of 5 mM ATP and 8.4 \( \mu \)g of Rnr2/Rnr4 heterodimer. The experiment was repeated as before, but 0.3 \( \mu \)g of Rnr1 was added to each reaction mixture (A→A). The middle curve (■) represents the calculated sum of activities of 0.3 \( \mu \)g of Rnr1 and increasing amounts of Rnr3 assayed separately.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification—**Rnr1 and the His\(_6\)-Rnr2/Rnr4 heterodimer were expressed in \textit{Escherichia coli} BL21(DE3) using the pET expression vector (Novagen) as described previously (23). Rnr1 was purified by ammonium sulfate fractionation and affinity chromatography on dATP-Sepharose as described (24). The co-expressed His\(_6\)-Rnr2/ Rnr4 heterodimer was purified on a nickel-nitrioltriacetic acid-agarose column (Qiagen, Valencia, CA) followed by chromatography on a Bio-scale Q10 column (Bio-Rad) (23). Rnr3 was purified from the \( rnr1 \) deletion strain Y609 (25) (MATa trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1–100 Δrnr1::HIS3) containing a 2-\( \mu \)l plasmid with the \textit{RNR3} gene (Ag' TRP1 2 \( \mu \) GAP-RNR3) under a strong and constitutive glyceraldehyde 3-phosphate dehydrogenase promoter. Yeast cells were disrupted in a Bead-Beater (Biospec Products, Bartlesville, OK), and the rest of the purification was carried out as described for the bacterially expressed Rnr1. To construct the Rnr1-C428A, the pET RNR1 expression plasmid (23) was mutagenized using the QuikChange site-directed mutagenesis kit (Stratagene) and the oligonucleotide primers 5'-ATC AAG TCA TCA AAC TTA GCC TGT GAA ATT GTT GAA TAC-3' and 5'-GTA TTC AAC ACA AAT TTC ACA GCC TAA GAA ATT GGT GAA TAC-3'. The correct sequence was confirmed by DNA sequence analysis. Expression and purification of Rnr1-C428A were carried out as described for Rnr1 (24).

**RNR Assay—**Rnr1, Rnr1-C428A, Rnr3, and mixtures of these proteins were assayed in the presence of pure Rnr2/Rnr4 heterodimer as described (23).
washed in ice-cold 50 mM Tris-HCl, pH 7.6, 2 mM dithiothreitol, and with 5 mM ATP and 0.56 for 30 min at 30°C temperatures (Rnr3 (13 Rnr2/Rnr4 heterodimer. Rnr1-C428A. was started at a density of 1/H11003 ° grown in YPDA at 30°C calibrated by the addition of catalase (23). All gradients were the absence or presence of dTTP or ATP in a 5-20% linear gradient of sucrose was performed as described previously (23). No activity was detected when Rnr3 was omitted from the reaction mixtures (f—f—B). No activity was detected when Rnr3 was omitted from the reaction mixtures (f—f—B).). Results of an assay of Rnr1 (6 μg) with 5 mM ATP and 1.9 μg of Rnr2/Rnr4 heterodimer for 10 min at increasing temperatures (v—v—v) are shown.

Sucrose Gradient Centrifugation—Sedimentation of Rnr1 or Rnr3 in the absence or presence of dTTP or ATP in a 5–20% linear gradient of sucrose was performed as described previously (23). All gradients were calibrated by the addition of catalase (s_{cat} = 11.4 S).

Determination of the Levels of Rnr1 and Rnr3 in Yeast Cells Grown in the Absence or in the Presence of 4-Nitroquinoline-1-oxide (4-NQO)—Rabbit polyclonal antibodies were made against the peptides EK-AAPIVDDEET(C) (Rnr1 from amino acid residues 852–863) and TIKEDSDKEKC (Rnr3 from amino acid residues 862–872) by Sigma. The anti-Rnr1 antibodies did not cross-react with Rnr3 or vice versa (data not shown). The yeast strain W1588Δ4C (MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1) (the W303 background) was grown in YPDA at 30°C. Treatments of yeast with 0.25 mg/liter 4-NQO was started at a density of 1 × 10^7 cells/ml. After a 3-h incubation with or without drug, 3.6 × 10^10 cells were collected by centrifugation and washed in ice-cold 50 mM Tris-HCl, pH 7.6, 2 mM dithiothreitol, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mM pepstatin, 0.6 mM leupeptin, and 2 mM benzamidine). The cells were then suspended in 20 ml of the same buffer and disrupted in a Bead-Beater. Cell debris was removed by centrifugation (30,000 × g for 20 min followed by ultracentrifugation at 200,000 × g for 2 h). The total amount of protein as determined by the Bradford assay was ~74 mg. After electrophoresis in an 8% polyacrylamide gel, immunoblotting was performed basically as described (26) with the specific anti-Rnr1 or anti-Rnr3 antibodies. The immunocomplexes were detected by incubation with goat anti-rabbit antibodies conjugated with alkaline phosphatase followed by visualization with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

RESULTS

DNA Damage Induction of the Rnr1 and Rnr3 Proteins—To understand better the contribution of Rnr3 to ribonucleotide reduction, we estimated the levels of the Rnr1 and Rnr3 proteins in yeast before and after DNA damage using Western blotting with specific Rnr1 and Rnr3 antipeptide polyclonal antibodies. The expression of RNR genes was induced by a 3-h incubation of a yeast culture in YPDA medium containing 4-NQO, and then samples were withdrawn for analyses and compared with samples from a yeast culture grown without drug. As seen in Fig. 1, the level of Rnr1 increased from about 50 to 100 ng of protein per 37 μg of yeast protein extract (equivalent to 1.8 × 10^7 cells). There was a pronounced increase also in the level of Rnr3, from undetectable levels in the uninduced cells to about 10 ng of protein per 37 μg of yeast protein extract in induced cells.

Yeast Rnr3 Has a Very Low Specific Activity Compared with Rnr1—Rnr3 was purified from the rnr1 deletion strain Y609 (25) containing a 2-μm plasmid with the Rnr3 gene under a strong and constitutive glyceraldehyde-3-phosphate dehydrogenase promoter. Using this strain ensured a complete absence of endogenous Rnr1 contamination and resulted in 1.5 mg of highly purified Rnr3 per liter of yeast culture (Fig. 2). The activity of Rnr3 in the presence of an excess of Rnr2/Rnr4 heterodimer was highly dependent on the concentration of Rnr3 (Fig. 3). However, the specific activity of the Rnr3 even when assayed at 0.5 mg/ml, which is a very high concentration for an RNR assay, was only approximately 1% of the specific activity of Rnr1 assayed at a similar concentration (data not shown). The addition of increasing amounts of Rnr3 to a series of RNR assay mixtures all containing a constant amount of Rnr1 and an excess of Rnr2/Rnr4 heterodimer demonstrated a clear synergism between Rnr1 and Rnr3 in ribonucleotide reduction (Fig. 3). Total RNR activity in these assays was up to 58% higher than the sum of the Rnr1 and Rnr3 activities assayed separately. This synergistic effect was most pronounced in assay mixtures with a very low concentration of Rnr1 and a high concentration of Rnr2/Rnr4 heterodimer.

Synergism between Rnr1 and Rnr3—The results presented above suggest that in a complex with Rnr1, Rnr3 has a much higher specific activity than when it is assayed alone. To address the contribution of the Rnr1/Rnr3 interaction in the stimulation of Rnr3 activity and exclude the contribution of Rnr1 activity, we decided to create and use a catalytically inactive form of Rnr1. We replaced cysteine 428 (corresponding to the catalytically essential cysteine 439 in the E. coli R1 protein (27)) with an alanine in the recombinant Rnr1 (Fig. 1). Rnr1-C428A was totally inactive when assayed in the presence of Rnr2/Rnr4 heterodimer (Fig. 4A); however, the addition of increasing amounts of the inactive Rnr1-C428A to assay mixtures containing a constant amount of Rnr3 and Rnr2/Rnr4 heterodimer resulted in a pronounced stimulation of RNR activity (Fig. 4). Maximal Rnr3 specific activity was obtained.
cross-talk between the allosteric sites in Rnr1-C428A and the catalytic site in Rnr3. A, reduction of CDP by Rnr1, Rnr3/Rnr1-C428A, or Rnr3 in the presence of Rnr2/Rnr4 heterodimer and increasing concentrations of ATP. Rnr1 (1.4 μg) and Rnr2/Rnr4 heterodimer (7 μg) assayed at 30 °C, dCDP formed per minute (■—■); Rnr3 (10 μg) assayed at 25 °C in the presence of 2.8 μg of Rnr2/Rnr4 heterodimer, dCDP formed during 30 min (▲—▲), and Rnr3 (10 μg/Rnr1-C428A (38 μg) assayed at 25 °C in the presence of Rnr2/Rnr4 heterodimer (2.8 μg), dCDP formed during 5 min (◇—◇). In all experiments, magnesium acetate was added in a 2.5 molar excess over ATP. B, inhibition of the ATP-stimulated CDP reduction by dATP. All reactions were incubated at 25 °C, and all mixtures contained 5 mM ATP and 20 mM magnesium acetate. Rnr3 (8.4 μg) and Rnr2/Rnr4 heterodimer (8.4 μg), dCDP formed during 30 min at increasing concentrations of dATP (■—■); Rnr3 (4.2 μg/Rnr1-C428A (4.2 μg) in the presence of Rnr2/Rnr4 heterodimer (8.4 μg), dCDP formed during 30 min at increasing dATP concentrations (▲—▲); and Rnr1 (0.8 μg) in the presence of Rnr2/Rnr4 heterodimer (8.4 μg), dCDP formed per minute at increasing dATP concentrations (◇—◇).

when the ratio of Rnr1-C428A/Rnr3 was approximately 7:1, which gave a value of 11 nmol/min per mg of Rnr3. This specific activity is approximately 5% of the maximal specific activity of wild-type Rnr1. Surprisingly, no inhibition of Rnr3 activity was observed with higher amounts of Rnr1-C428A. These data show that the presence of catalytically inactive Rnr1-C428A increases the endogenous activity of the Rnr3 by at least 10-fold. We therefore propose that Rnr3 alone has a poor ability to form an active complex with the Rnr2/Rnr4 heterodimer. Interestingly, the addition of increasing amounts of Rnr1-C428A to Rnr1-containing assay mixtures resulted in a slight stimulation of RNR activity and almost no inhibition at up to 34× molar excess of Rnr1-C428A over Rnr1 (Fig. 4B).

Rnr3 Has a Lower Temperature Optimum than Rnr1 in the RNR Assay—We compared the temperature optimum for reactions catalyzed by Rnr1, Rnr3, or the Rnr1-C428A/Rnr3 mixture in the presence of the small subunit (Fig. 5). There was a distinct difference between the various RNR complexes with the Rnr1 showing the highest temperature optimum followed by the Rnr1-C428A/Rnr3 complex and the Rnr3. These data support the notion that the Rnr1-containing RNR complex is the most stable followed in stability by the Rnr1-C428A/Rnr3- and Rnr3-containing RNRs.

Cross-talk between the Allosteric Activity Site in Rnr1-C428A and the Catalytic Site in Rnr3—It was shown earlier for the mouse RNR complex that allosteric effectors strongly influence subunit interaction (28). Reduction of CDP by yeast RNR containing the Rnr1 was stimulated by ATP with maximal activity around 5 mM ATP (Fig. 6A). The optimal ATP concentration for CDP reduction catalyzed by RNR containing Rnr1 was 15–20 mM, whereas the optimal ATP concentration for RNR containing Rnr3/Rnr1-C428A was the same as observed for Rnr1 alone (i.e. approximately 5 mM (Fig. 6A)). The Rnr1-catalyzed CDP reduction in the presence of 5 mM ATP was inhibited by dATP at concentrations above 50 μM, whereas the CDP reduction catalyzed by Rnr3 was stimulated by dATP instead. Interestingly, the CDP reduction catalyzed by Rnr3/Rnr1-C428A showed dATP inhibition typical for Rnr1 despite the fact that the catalytic site resides on Rnr3 (Fig. 6B). This result, together with the ATP optima, indicates cross-talk between the allosteric activity site on the inactive Rnr1 and the catalytic site on Rnr3.

Allosteric Effector-induced Oligomerization Is Different for Rnr1 and Rnr3—When analyzed by sucrose gradient centrifugation, Rnr1 without allosteric effectors sedimented as a mix-
DISCUSSION

The low levels of Rnr3 protein in yeast cells even after DNA damage induction, in combination with the very low catalytic activity of a complex between Rnr3 and the Rnr2/Rnr4 heterodimer, explain why an rnr3 null mutant has no phenotype under laboratory conditions. Is Rnr3 a redundant protein that has no function in yeast metabolism? Why is it induced strongly in response to DNA damage? The complex between Rnr3 and Rnr1 has a much higher catalytic activity than Rnr3 alone. The presence of such a complex in vivo is observed in a two-hybrid assay (18). If the levels of Rnr1 are limiting for ribonucleotide reduction during DNA damage, the expression of Rnr3 may increase total RNR activity. Furthermore, the resistance of Rnr3-catalyzed ribonucleotide reduction to dATP inhibition might confer a selective advantage for yeast growing in natural ecological niches under permanent DNA-damage conditions.

Why is the catalytic activity of RNR containing Rnr3 so much lower than the activity of RNR-containing Rnr1, and why is the activity improved in the presence of inactive Rnr? Studies on mouse RNR have shown that to form a complex with the small subunit, the polypeptide chains of the large subunit have to dimerize (28). The highly concentration-dependent catalytic activity shown by Rnr3 in contrast to Rnr1 (23) and the lower temperature stability of an Rnr3-containing RNR compared with an Rnr1-containing enzyme indicate that Rnr3 is much less efficient in forming a stable active complex with the Rnr2/Rnr4 heterodimer. This may be either because Rnr3 does not readily dimerize or because the Rnr3 dimer has a low affinity to the Rnr2/Rnr4 heterodimer. An Rnr3/Rnr1 complex makes a more stable RNR than Rnr3 alone. The sucrose gradient centrifugation experiments indicate that Rnr3 is less efficient in forming oligomeric structures than Rnr1, and we believe that this weakness is a major reason for the low catalytic activity of Rnr3 compared with Rnr1.

Addition of increasing amounts of inactive Rnr1-C428A to an assay mixture containing Rnr2/Rnr4 heterodimer and Rnr3 or Rnr1, to our surprise, did not result in inhibition as a consequence of competition for the small subunit. Such an inhibition is clearly observed when inactive E. coli R1-C439A protein is added to an assay mixture containing bacterial R1 and R2 proteins (29). Obviously, the inactive Rnr1-C428A protein is not very effective in competing with active Rnr1 dimers for the Rnr2/Rnr4 heterodimer. A difference between the yeast and bacterial RNR systems is that in yeast, maximally only 50% of the complexes between an Rnr1/Rnr1-C428A dimer and the Rnr2/Rnr4 heterodimer are active because Rnr4 completely lacks the catalytically essential tyrosyl radical. The initial stimulation of the catalytic activity observed when inactive Rnr1-C428A was added to Rnr1-containing RNR suggests that there is a preferential formation of enzyme complexes in which active Rnr1 is binding to the catalytically active, tyrosyl radical-containing Rnr2, while inactive Rnr1-C428A is binding to the catalytically inactive Rnr4 in the Rnr2/Rnr4 heterodimers. The mechanism for this apparent active site-dependent orientation is not known. The inability of the inactive Rnr1-C428A to compete efficiently for the small subunit may be explained by the same mechanism.

Stimulation of the Rnr3-catalyzed CDP reduction by dATP indicates that Rnr3 lacks a functional allosteric activity site. This behavior is similar to that of the mouse R1 D57N protein with a mutation in the allosteric activity site (15). Therefore, similar to ATP, dATP may only bind to the allosteric specificity site where both nucleotides induce pyrimidine nucleotide specificity. The lack of a functional allosteric activity site may explain the inefficient binding of Rnr3 to the small subunit because effector binding increases affinity between the large and small subunits in mouse RNR (28). A low affinity for nucleotide effector binding to Rnr3 is apparent from the ATP stimulation curve in Fig. 6a where the Rnr3-catalyzed reduction shows an S-shaped activity curve with increasing ATP concentrations, and Rnr1 and Rnr3/Rnr1-C428A show hyperbolic curves. A low Rnr3 affinity for effector nucleotides also explains the high concentrations of ATP/dATP required for maximal activity and the rather limited effect of ATP on the oligomerization of Rnr3 as compared with Rnr1. The unexpected observation that the allosteric regulation of Rnr3/Rnr1-C428A-containing RNR is very similar to the allosteric regulation of Rnr1-containing RNR (but not to Rnr3-containing RNR) strongly indicates that the allosteric activity site present in the catalytically inactive Rnr1-C428A cross-talks with the catalytic site in Rnr3. Such a cross-talk between two polypeptide chains of the large subunit of RNR has been suggested earlier but never before demonstrated in direct experiments. Binding of dATP to the catalytic activity site of Rnr1-C428A must signal to the catalytic site of Rnr3 through conformational changes in the two polypeptide chains.

Acknowledgments—We thank Steven Elledge, Xiaolan Zhao, and Rodney Rothstein for providing yeast strains and Elizabeth Murchison for helpful comments.

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J. Biol. Chem. 2002, 277:18574-18578.
doi: 10.1074/jbc.M201553200 originally published online March 13, 2002

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