Ribonucleotide reductases (RNRs) catalyze the conversion of nucleotides to deoxynucleotides. Class I RNRs are composed of two types of subunits: RNR1 contains the active site for reduction and the binding sites for the nucleotide allostERIC effectors. RNR2 contains the diiron-tyrosyl radical (Y) cofactor essential for the reduction process. Studies in yeast have recently identified four RNR subunits: Y1 and Y3, Y2 and Y4. These proteins have been expressed in Saccharomyces cerevisiae and in Escherichia coli and purified to ~90% homogeneity. The specific activity of Y1 isolated from yeast and E. coli is 0.03 μmol·min⁻¹·mg⁻¹ and of (His)₆-Y2 [(His)₆-Y2·K387N] from yeast is 0.037 μmol·min⁻¹·mg⁻¹ (0.125 μmol·min⁻¹·mg⁻¹). Y2, Y3, and Y4 isolated from E. coli have no measurable activity. Efforts to generate Y1 in Y2 or Y4 using Fe₂⁺, O₂, and reductant have been unsuccessful. However, preliminary studies show that incubation of Y4 and Fe₂⁺ with inactive E. coli Y2 followed by addition of O₂ generates Y2 with a specific activity of 0.069 μmol·min⁻¹·mg⁻¹ and a Y. A similar experiment with (His)₆-Y2·K387N, Y4, O₂, and Fe₂⁺ results in an increase in its specific activity to 0.30 μmol·min⁻¹·mg⁻¹. Studies with antibodies to Y4 and Y2 reveal that they can form a complex in vivo. Y4 appears to play an important role in diiron-Y₁ cofactor assembly.

Ribonucleotide reductases (RNRs) in all organisms catalyze the reduction of nucleotides to deoxynucleotides, an essential step in DNA biosynthesis. These enzymes play a central role in maintaining a balanced pool of cellular deoxynucleotides required for fidelity of DNA replication and repair (1–3). The aerobic Escherichia coli RNR is the prototype for class I RNRs found in all eukaryotes and most prokaryotes. The class I RNRs are composed of two homodimeric proteins: RNR1 (α₂, 171 kDa) and RNR2 (β₂, 87 kDa). RNR1 contains the site where NDPs are reduced and the sites for the allostERIC effectors that govern activity and specificity. RNR2 contains a diiron-cluster-tyrosyl radical (Y) cofactor required for nucleotide reduction activity (4).

The mechanism by which the diiron-Y₁ cofactor is generated in vitro has been studied extensively (5, 6). However, the mechanism by which the cluster is assembled in vivo has remained largely unexplored. The completion of the Saccharomyces cerevisiae genome sequencing project and the identification of a large number of yeast mutants involved in iron homeostasis (7, 8) have suggested that yeast may be an excellent system to investigate diiron-Y₁ cofactor assembly and its relationship to nucleotide reductase in vivo. Recent studies have identified four genes encoding RNR subunits in S. cerevisiae: RNR1 and RNR3 (designated Y1 and Y3) and RNR2 and RNR4 (designated Y2 and Y4) (9–13). Y1 and Y3, analogues of RNR1 of E. coli RNR, share ~80% sequence identity, Y1 expression is cell cycle-regulated, and the gene is essential for mitotic viability. In contrast, Y3 is not expressed under normal vegetative conditions. However, the presence of high copy numbers of the Y3 gene has been shown to suppress a lethal mutation in the Y1 gene, suggesting that Y3 encodes a functional protein (11). Transcription of Y1 and Y3 genes is inducible by DNA damage, the latter 100-fold (11). The Y2 gene encodes a protein analogous to E. coli RNR2 and is essential for mitotic viability. The Y4 gene encodes a protein sharing 56% sequence identity with Y2, Y4, however, contains several unusual features not found in Y2 and mammalian RNR2s. These features include a deletion of 51 aa residues at its N terminus and a substitution of 6 of the 16 aa residues conserved in all class I RNR2s (12, 13). The most notable substitutions are the replacement of two histidines and one glutamate, ligands of the diiron center, by two tyrosines and an arginine. Such substitutions would be expected to alter Y4’s ability to bind iron. Y4 also appears to be important for mitotic viability, although its essentiality depends on the genetic background (12, 13). RNR subunits in yeast play not only an essential role in DNA replication but also in signal-transduction pathways sensing and responding to DNA damage and DNA replication blocks (1).

Studies in 1984 (14) and 1990 (15) from Follmann’s lab reported the partial purification of RNR(s) from S. cerevisiae and that all efforts to purify these proteins to homogeneity failed because of rapid loss of activity. As a first step in using yeast as the vessel to understand diiron-cluster assembly and allosteric regulation of RNRs, access to purified yeast RNR subunits is essential. In this paper, we report the expression, purification, and initial characterization of Y1, Y2, Y3, and Y4 from S. cerevisiae and E. coli. Preliminary studies indicate that Y4 plays an important role in the assembly of the diiron-Y₁ cofactor in Y2.

Materials and Methods

Immun-blotted poly(vinylidene difluoride) membranes (0.2-μm pore) were purchased from Bio-Rad. Kanamycin was obtained from Eastman Kodak. [¹⁴C]Cytidine 5′-diphosphate (CDP) was purchased from Moravek Biochemicals (Brea, CA). DEAE-Sephrose Fast Flow and QAE-Sephadex A-50 were purchased from Amersham Pharmacia. Talon cobalt metal affinity resin was purchased from CLONTECH. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs or Boehringer Mannheim. Competent E. coli BL21(DE3) pLYSs cells and pET vectors were obtained from Novagen. Competent DH5α cells were obtained from Life Technologies (Grand Island, NY). Pefabloc and calf intestine alkaline phosphatase were obtained from Boehringer Mannheim. E. coli thiorhodoxin (TR) (16) and thiorhodoxin reductase (TRR) (17) were isolated, and dATP-Sepharose affinity resin was prepared as described (18). Protein concentrations were determined by the Lowry method.

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Purification of ribonucleotide reductase subunits Y1, Y2, Y3, and Y4 from yeast: Y4 plays a key role in diiron cluster assembly

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The resulting 1.0-kb fragment was ligated into pSE734 as the template and introduced into the PvuII site, yielding pY2M. Site-directed mutagenesis was performed to correct this mutation, giving rise to pY2K. Sequencing of the resulting plasmid revealed a G to C transversion giving rise to a K387N mutation in the Y2 gene. The 2.7-kb Y3 gene was amplified with primers 5'-GGAATTCATATGACGTTATTAAAGAGACG-3' (NdeI) and 5'-CGGGGATCCATATGACGTACGCCTCC-3' (Ndel) and 5'-TTCGGAATTTAAGTCTTGTTAAGG-3' (BamHI) and YEp13mrn as the template. The 1.2-kb fragment was digested with Ndel and BamHI and inserted into pET24a(+), cut with the same enzymes, to give pY1A.

The 1.0-kb Y4 gene was amplified by PCR using primers 5'-TATGCTTTATATGACGTTATTAAAGAGACG-3' (NdeI) and 5'-CGGGGATCCATATGACGTACGCCTCC-3' (Ndel) and 5'-TTCGGAATTTAAGTCTTGTTAAGG-3' (BamHI) and YEp13mrn as the template. The 1.2-kb fragment was digested with Ndel and BamHI, as was pET24a(+). Ligation of these two pieces of DNA resulted in pY2K. Sequencing of the resulting plasmid revealed that it contained the 1.0-kb fragment ligated into pSE734 and the 1.2-kb fragment ligated into pET24a(+).

The 1.0-kb Y4 gene was amplified by using PCR with primers 5'-AAAATCAATATGACGTTATTAAAGAGACG-3' (NdeI) and 5'-CGGGGATCCATATGACGTACGCCTCC-3' (Ndel). Ligation of these two pieces of DNA resulted in pY2M. Sequencing of the resulting plasmid revealed that it contained the 1.0-kb fragment ligated into pSE734 and the 1.2-kb fragment ligated into pET24a(+), which was ligated into pYES2 vector cut with PnuII–BamHI, to give pY3J.

**Construction of pYES-Y1 and pHis-Y2 (pHis-Y2-K387N) for Expression in S. cerevisiae.** pYES-Y1. pY1A was cut with Ndel and filled in with E. coli DNA polymerase Klenow fragment. A second digestion with BamHI gave a 2.7-kb 5'-blunt end/3'-BamHI end Y1 fragment, which was ligated into pYES2 vector cut with PnuII–BamHI, to give pYES-Y1.

**pHis-Y2.** A double-stranded DNA linker was constructed by annealing and extending two primers: 5'-GGCCGCTTGAATATGCACCCACCATACACCACGGATGTG (DnlI) and 5'-CGGGGATCCATATGACGTACGCCTCC-3' (Ndel). The 59-bp double-stranded DNA product was then digested with DnlI and HindIII, and ligated into pYES2 cut with PnuII and HindIII. The resultant plasmid, pYES-His, was a general-purpose His-tagged expression vector that produces proteins with N-terminal 6× His sequence and a DDDDK enterokinase cleavage site. pYES-His was digested with HindIII. Blunt ends were generated with mung bean nuclease and digested with BamHI to create the 5’-blunt/3’-BamHI fragment. The 1.2-kb Y2 gene was excised from pY2K (pY2M) by successive NdeI digestion, 5’ overhang removal with mung bean nuclease, and a second digestion with BamHI. This insert was ligated into digested pYES-His to give pHis-Y2-K387N (pHis-Y2).

**Expression of Y1, Y2, Y3, and Y4 in E. coli.** An overnight culture of BL21(DE3)pLysS cells containing one of the expression plasmids (pY1A, pY2M, pY3J, or pY4J) was grown in LB medium containing 100 mg/liter kanamycin and 50 mg/liter chloramphenicol at 30 or 37°C. This culture was used to inoculate 6× 6-liter flasks containing 1.5 liter of LB medium with the same concentration of antibiotics as above (1:300 inoculum strength). When the A600 reached 0.5–0.6, the culture was chilled to 15°C before adding isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM. The cultures were grown for 12–15 h at 15°C until A600 reached 1.8–2.0. The cells (5–6 g/liter) were harvested by centrifugation, frozen in liquid nitrogen, and stored at −80°C.

**Expression of Y1 and (His)6-Y2 (pHis-Y2-K387N) in Yeast BJS5465.** The proteinase-deficient yeast strain BJS5465 was transformed with one of the expression plasmids (pYES-Y1, pHis-Y2, pHis-Y2-K387N) (20). A single colony of the transformed yeast grown on plates of yeast minimal medium minus uracil was used to inoculate 100 ml of the same medium containing 2% raffinose as the carbon source. After ~36 h at 30°C, this culture was used to inoculate 1 liter of yeast minimal medium minus uracil containing 2% w/vol raffinose. After growth at 30°C for 24 h (A600 of 1.6–1.8), solid galactose (2% w/vol) was added. Yeast cells (6–8 g/liter) were harvested ~3–4 h later by centrifugation, frozen in liquid nitrogen, and stored at −80°C.

**Purification of Y1 and Y3.** All purification steps were performed at 4°C. E. coli cells (40 g) were thawed and suspended in buffer A, which contained 50 mM HEPES, 5 mM DTT, 5 mM MgCl2, 100 mM KCl, 5% glycerol, 1 mM PMSF, and 0.6 mM Pefabloc (pH 7.0) in a final volume of 200 ml. Cells were lysed by one passage through a French press (12,000 psi; 1 psi = 6.89 kPa). Cell debris was removed by centrifugation (12,300 × g, 30 min). Streptomyacin sulfate (10% w/vol) was added dropwise to the extract to a final concentration of 1.5% w/vol, and the DNA was removed by centrifugation (12,300 × g, 30 min). Solid (NH4)2SO4 was added to the supernatant to 40% saturation (0.29 g/ml) over a period of 30 min, and the precipitate was collected by centrifugation (12,300 × g, 40 min). The pellet was dissolved in 40 ml of buffer A and desalted either by passage through a Sephadex G-25 column (5.5 × 80 cm) with buffer A as eluent or by dialysis against 2 liters of buffer A for 2–3 h. This solution was diluted with buffer A to 80 ml and loaded onto a DE52-DEAE-Sepharose column (2.5 × 4 cm). The column was washed with 200 ml of buffer A, followed by 300 ml of buffer A plus 1 mM ATP. Y1 and Y3 were eluted with 50 ml of buffer A plus 10 mM ATP followed by 50 ml of buffer A plus 20 mM ATP. Protein-containing fractions were pooled, concentrated by ultrafiltration with a PM30 membrane (Amicon) to >7 mg/ml, frozen in liquid nitrogen, and stored at −80°C. Typically ~5–7 mg of Y1 or Y3 of ~90% purity, judged by SDS/PAGE, were obtained.

**Isolation of Y2 from BL21(DE3)pLysS/pY2M.** E. coli cells (40 g) were thawed and suspended in buffer B containing 50 mM Tris (pH 7.6), 5% glycerol, 1 mM PMSF, and 0.6 mM Pefabloc to give a final volume of 100 ml. The cells were disrupted by a single passage through a French pressure cell at 12,000 psi, FeSO4 (0.1 g) and sodium ascorbate (0.1 g) were added to the cell lysate before centrifugation (19,800 × g, 30 min). Streptomycin sulfate (10% w/vol in buffer B) was added dropwise to the extract to be covalently linked to the His tag. The resulting proteins were purified as described above.
a final concentration of 1.5% wt/vol, and the DNA was removed by centrifugation (12,300 × g, 30 min). Solid (NH₄)₂SO₄ was added to the supernatant to 60% saturation (0.39 g/ml). After stirring the suspension for 30 min, the precipitated proteins were collected by centrifugation (12,300 × g, 40 min). The pellet was dissolved in 50 ml of buffer B and desalted by passage through a Sephadex G-25 column (5.5 × 100 cm) with buffer B as eluent. The protein-containing fractions were pooled, diluted with an equal volume of buffer B, and loaded onto a DEAE-Sepharose column (5.5 × 10 cm) preequilibrated with buffer B. After washing sequentially with buffer B (300 ml) and buffer B plus 50 mM NaCl (300 ml), Y2 was eluted with buffer B plus 80 mM NaCl (300 ml). Fractions (20 ml) were collected, and those containing Y2, as judged by SDS/PAGE, were pooled and concentrated by ultrafiltration (PM30 membrane) to >10 mg/ml. Y2 was frozen in liquid nitrogen and stored at −80°C. This procedure yielded ~50 mg of ~50–60% pure Y2 protein.

Isolation of Y4 from BL21(D30R)plysS7(pY4J). *E. coli* cells (40 g) were thawed and suspended in buffer C containing 50 mM Hepes (pH 7.0), 5% glycerol, 1 mM PMSF, and 0.6 mM Pefabloc in 200 ml. The work up through the desalting of the ammonium sulfate precipitate was identical to that described for isolation of Y2. The appropriate fractions were pooled, diluted with an equal volume of buffer C and loaded onto a DEAE-Sepharose column (5.5 × 7 cm) equilibrated with buffer C. The column was washed with buffer C (300 ml), buffer C plus 50 mM NaCl (300 ml), and buffer C plus 80 mM NaCl (300 ml) before Y4 elution with buffer C plus 110 mM NaCl (300 ml). Fractions containing Y4, as judged by SDS/PAGE, were pooled, diluted with an equal volume of buffer C to ~200 ml, and loaded onto a QAE-Sephadex column (3 cm × 7 cm) pre-equilibrated with buffer C plus 80 mM NaCl. After washing the column sequentially with buffer C plus 80 mM NaCl (250 ml) and buffer C plus 160 mM NaCl (250 ml), Y4 was eluted with buffer C plus 210 mM NaCl. Fractions (20 ml) were collected, and those containing high purity Y4 (by SDS/PAGE) were pooled and concentrated by ultrafiltration (PM30 membrane) to >10 mg/ml. Y4 (60 mg of ~90% purity) was frozen in liquid nitrogen and stored at −80°C.

Isolation of (His)₆-Y2 and (His)₆-Y2 K387N. Yeast cells (40 g) were thawed and suspended in buffer D containing 50 mM Hepes (pH 8.0), 10% glycerol, 300 mM (NH₄)₂SO₄, 100 mM KCl, 1 mM PMSF, and 0.6 mM Pefabloc in a final volume of 80 ml. The cells were lysed either by a Bead Beater (Biospec Products, Bartlesville, OK) or by passage through a French press (three passes at 12,000 psi). Additional buffer was added to the cell lysate to bring the total volume to ~100 ml. FeSO₄ (20 μM) and sodium ascorbate (1 mM) were added to the cell lysate before centrifugation (19,800 × g, 40 min). The extract was clarified by filtration through a glass filter (Gelman) and applied onto a Talon cobalt metal affinity column (2.5 × 3 cm) equilibrated with buffer D. The column was washed sequentially with buffer E [200 ml, buffer D minus (NH₄)₂SO₄], buffer E (300 ml) plus 10 mM imidazole, and 200 ml of buffer E adjusted to pH 7.0. The His-tagged proteins were eluted with 50 ml of buffer E (pH 7.0) plus 100 mM imidazole followed by 75 ml of buffer E (pH 7.0) plus 150 mM imidazole. Fractions (1.5 ml) containing Y2 were pooled, concentrated by ultrafiltration (PM30 or YM30 membrane) to >15 mg/ml, frozen in liquid nitrogen, and stored at −80°C. Typically, 9–12 mg of (His)₆-Y2 or 3–4 mg of (His)₆-Y2 K387N of ~90% purity based on SDS/PAGE, were obtained.

Isolation of Y1 from Yeast BJ5465/pYES-Y1. Yeast cells (40 g) were thawed and suspended in 80 ml of buffer F containing 50 mM Hepes (pH 7.0), 300 mM (NH₄)₂SO₄, 5 mM DTT, 5 mM MgCl₂, 10% glycerol, 1 mM PMSF, and 0.6 mM Pefabloc. The cells were lysed as described above. Additional buffer F was added to the cell lystate to give a final volume of 120 ml. Cell debris was removed by centrifugation (19,800 × g, 40 min). The extract was clarified by filtration through a glass filter and applied to a dATP/Sephacore column (2.5 × 5 cm) equilibrated with buffer G [buffer F minus (NH₄)₂SO₄]. The column was washed with buffer G (200 ml) and buffer G plus 1 mM ATP (250 ml). Y1 was eluted with buffer G plus 10 mM ATP (50 ml) and 20 mM ATP (50 ml). Fractions (1.5 ml) containing pure Y1 (based on SDS/PAGE) were pooled, concentrated by ultrafiltration (PM30 membrane), frozen in liquid nitrogen, and stored at −80°C. The typical yield was ~1.5 mg of ~90% pure Y1.

Enzyme Activity Assay. The combinations of yeast RNRs used in the assays were Y1/(His)₆-Y2 K387N, Y1/(His)₆-Y2, and Y2/(His)₆-Y2 K387N. In each case, the second protein component was present in 5-fold molar excess (1.25–5 μM) over the protein being assayed (0.25–1 μM). The assay mixture contained (in a final volume of 200–500 μl) 1 mM [¹⁴C]CDP (specific activity, 2.2 × 10⁹ cpm/nmol), 100 μM *E. coli* TR, 1 μM *E. coli* TRR, 2 mM NADPH, 2 mM ATP, 100 mM Hepes (pH 7.2), 20 mM MgCl₂, and 20 mM DTT. All components except CDP were incubated at 30°C for 10 min. The reaction was initiated by adding [¹⁴C]CDP. Aliquots (25–100 μl) of the reaction mixture were withdrawn at various times, 0–30 min, and quenched in a boiling water bath. After adjusting the pH to 8.5 with 2 M Tris (pH 8.5), alkaline phosphatase (10 units) was added, and the mixture was incubated at 37°C for 6 h. Products were analyzed by the method of Steeper and Steuart (21). One unit of RNR activity is defined as 1 nmol of dCDP formed in 1 min.

Electron Paramagnetic Resonance (EPR) Spectroscopy. EPR spectra were recorded by using a Bruker ESP-300 X-band (9.4-GHz) spectrometer equipped with an Oxford liquid helium cryostat. Spin quantitation of the His-tagged Y2s was achieved by double integration of EPR spectra acquired at 25 K with 1 mW of microwave power, by comparison to a sample of *E. coli* R2 whose Y- concentration had been established by UV-visible spectroscopy at 412 nm by the drop-line correction method (22).

Recombinant Y2 Activation by Y4 and Fe²⁺. All manipulations were carried out in an anaerobic wet box (M. Braun, Newburyport, MA). Deoxygenated FeSO₄ was added to deoxygenated Y4, to yield an anaerobic solution with 157 μM Y4 and 1.62 mM Fe²⁺ in 200 mM Hepes (pH 7.2). The mixture was incubated at 2–4°C for 12 h. This activated Y4 solution (0.5 ml) was then dialyzed against 500 ml of 200 mM Hepes (pH 7.2), and at various times, 100 μl aliquots were removed. At 2.5 h, 25 μl of an aliquot was used to determine an iron concentration of 150 μM by using a ferrozine assay. Deoxygenated Y2 from *E. coli* (25 μl, 30 mg/ml, 175 μM of 50% pure, as judged by SDS/PAGE) was then mixed with 25 μl of the dialyzed Y4/Fe²⁺ mixture and 50 μl of 200 mM Hepes buffer (pH 7.2), resulting in a mixture of Y2:Y4:Fe²⁺ of 40 μM (assuming 50% purity), 39 μM, and 39 μM, respectively. This mixture was incubated at 2–4°C (in the anaerobic box refrigerator) for 12 h and subsequently removed from the box and exposed to air for 1 h. Spin quantitation by 9.4-GHz EPR spectroscopy indicated that Y2 contained 18 ± 3 μM Y²⁻, and an assay indicated a specific activity of 0.061 μmol/min·mg⁻¹. A control in which Y4 was replaced with buffer under otherwise identical conditions gave Y2 with no Y⁻ and no activity.

Antibodies and Western Immunoblot Analysis. Purified Y1, Y3, Y4, and Y2 purified from *E. coli* inclusion bodies were used to generate rabbit polyclonal antibodies. *S. cerevisiae* cell-free extracts and purified RNR subunits were analyzed on SDS/PAGE. The proteins were then transferred to polyvinylidene difluoride membranes by electroblotting (3 h at 100 V) using a Semi-Phor TE70 Semi Dry Transfer unit ( Hoefer) The mem-
Table 1. Specific activity of purified yeast RNR subunits

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Expression source</th>
<th>Specific activity, nmol of dCDP-min⁻¹-mg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>As isolated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y1</td>
<td>E. coli</td>
<td>34</td>
</tr>
<tr>
<td>Y1</td>
<td>Yeast</td>
<td>24</td>
</tr>
<tr>
<td>Y3</td>
<td>E. coli</td>
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</tr>
<tr>
<td>Y2</td>
<td>E. coli</td>
<td>0</td>
</tr>
<tr>
<td>(His)₆-Y2</td>
<td>Yeast</td>
<td>37</td>
</tr>
<tr>
<td>(His)₆-Y2 K387N</td>
<td>Yeast</td>
<td>125</td>
</tr>
<tr>
<td>Y4</td>
<td>E. coli</td>
<td>0</td>
</tr>
<tr>
<td>After activating with Y4 and Fe(II)</td>
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</tr>
<tr>
<td>Y2</td>
<td>E. coli</td>
<td>69</td>
</tr>
<tr>
<td>(His)₆-Y2 K387N*</td>
<td>Yeast</td>
<td>300</td>
</tr>
<tr>
<td>(His)₆-Y2</td>
<td>Yeast</td>
<td>121</td>
</tr>
</tbody>
</table>

*Reconstitution experiments were carried out with Y4:Fe²⁺:Y2 of 153 µM: 1.5 mM: 13.2 µM. Incubation of Y4 and Fe²⁺ were under anaerobic conditions for 3 h at 4°C and this solution was then incubated with Y2 for an additional 3 h. The solution was then exposed to air for 1 h and assayed.

†Reconstitution was as above with Y4:Fe²⁺:Y2 of 90 µM: 297 µM: 9 µM. The starting specific activity of this (His)₆-Y2 was 6.

branes were blocked with a casein solution (3% casein, 50 mM Tris, pH 8.0) followed by incubation for 3 h with serum diluted in blocking buffer (1:10,000 for the anti-Y4 serum). The membranes were washed with Tris-buffered saline (TBS) (20 mM Tris, 500 mM NaCl, pH 7.5), followed by incubation with donkey anti-rabbit IgG conjugated to horse radish peroxidase for 3 h. After washing the membranes with TBS, the bands were visualized with a 1:1 mixture of TBS and ethanol containing 4-chloro-naphthol (3 mg/ml) and 0.01% H₂O₂ following the manufacturer’s instructions.

Results

Expression of Y1, Y2, Y3, and Y4. Construction of pET vectors using IPTG induction for expression of RNR subunits in E. coli and vectors using induction on galactose for expression in yeast are described in detail in Materials and Methods. Initial efforts to express the yeast RNR subunits used E. coli as the host. Under growth conditions of either 30 or 37°C, Y1, Y2, and Y3 were found as inclusion bodies with no NDP reduction activity. In the case of Y4 grown at 30°C, the protein was soluble and Y2 was isolated as the result of a mutation arising from PCR during construction of the Y2 expression vector. The stability and affinity column procedure exists for rapid isolation of RNR2s, although anion-exchange chromatography has in general been used successfully in their purification. Y2 was purified to ~50% homogeneity by using a DEAE-Sepharose column (data not shown). The fractions containing Y2, as judged by SDS/PAGE, were pooled. No nucleotide reductase activity was detected. For many RNR2s isolated from E. coli, addition of Fe²⁺, O₂, and ascorbate to the purified recombinant proteins with little or no activity results in generation of the active diferric-Y-family cofactor, and consequently, NDP reduction activity. In the case of recombinant E. coli Y2, however, this activation method was unsuccessful under a variety of conditions.

Y4 expressed in E. coli was purified by successive anion-exchange steps using DEAE-Sepharose and QAE-Sephadex A-50, to >90% homogeneity (Fig. 1, lane 8, Table 1). Y4 contained no iron, Y-family catalytic activity.

The identity of each purified RNR subunit was confirmed by N-terminal sequence analysis. In the case of recombinant Y1, Y3, and Y4 sequencing began with the N-terminal methionine, while in the case of Y2, a mixture of methionine and proline (N-1) proteins were obtained.

Purification of Y1 and (His)₆-Y2 [(His)₆-Y2 K387N] from Yeast. Our inability to obtain active nucleotide reductase activity from any combination of RNR subunits expressed in E. coli, provided the impetus for expression of these proteins in S. cerevisiae. The procedure for purifying Y1 is similar to its isolation from E. coli. However, because the expression level of Y1 in yeast is low, cell-free extract was applied directly onto the dATP-Sepharose column. Elution with ATP affords Y1 with purity and activity similar to E. coli-expressed Y1 (Fig. 1, lane 4 and Table 1).

Attempts to purify Y2 from yeast were frustrating because of low levels of expression, inability to assay, and problems with proteolysis. N-terminal (His)₆-tagged Y2s were therefore constructed and expressed in a proteinase-deficient yeast strain BJ5465. This modification allowed rapid isolation of functional, nearly homogenous Y2s within hours of cell lysis through the use of cobalt metal affinity column. Although the (His)₆-tagged tail could bind iron and prevent appropriate metal cluster assembly in Y2, this method has allowed us to obtain protein that has been successfully incubated with Y1 to obtain nucleotide reductase activity. Active Y2s [(His)₆-Y2 (wild-type) and Y2-K387N)] with a purity >90% by SDS/PAGE are obtained (Fig. 1, lanes 6 and 7).

EPR Spectroscopy of the Y-family of (His)₆-Y2 and (His)₆-Y2 K387N. The purified His-tagged Y2 proteins exhibit a Y-family signal by EPR (9.4 GHz) readily observed at <77 K and similar to the one first reported by Harder and Follmann in yeast (15). The Y-family is stable to storage at 4°C for 24 h and to repeated freeze–thaw cycles as judged by EPR intensity.

Spin quantitation of the amount of Y-family relative to the amount...
of wild-type (His)_6-Y2 revealed only 0.05 radicals per Y2. (His)_6-Y2 K387N, on the other hand, exhibits a stoichiometry of ≈0.15–0.16 radical per dimer. The radical content of purified Y2s stands in contrast to a stoichiometry of 1.2 Y· per dimer for fully active E. coli RNR2 (23). The addition of Fe^{2+} or Fe^{3+} in combination with ascorbate and O_2 to the purified proteins did not result in an increase in Y· or nucleotide reductase activity, as has been observed with many other RNR2s.

**Activities of Yeast RNR Subunits.** The assay for nucleotide reduction requires RNR1, RNR2, substrate, the appropriate allosteric effector and a reducing system to reduce the disulfide generating concomitant with dNDP formation. The yeast reducing system is most likely a TR/TRR/NADPH (24) or a glutaredoxin/glutathione reductase/NADPH (25), in common with other RNRs. In vitro, reductants such as DTT can also supply the reducing equivalents, although less efficiently. In the assays described subsequently, the combination of DTT, E. coli TR/ TRR, and NADPH, with CDP as substrate and ATP as allosteric effector, has afforded the highest activities for purified yeast RNRs.

In standard activity assays for RNRs, each subunit is assayed separately. Because (His)_6-Y2 K387N affords higher activity because of its increased Y· content, most Y1 activity assays were performed by using this mutant. Assays using the (His)_6-Y2 were carried out for comparison. Y1 expressed in E. coli and yeast exhibit similar levels of activity, typically in the range of 24–34 units/mg (Table 1). The similarity in activity and the increased expression levels of Y1 from E. coli made this source preferable for Y2 activity assays. In contrast to the Y1 from E. coli, Y3 from E. coli exhibited no detectable activity under similar assay conditions (Table 1).

The specific activities of the (His)_6-Y2 proteins were found to be concentration-dependent. This is not surprising, because the interactions between RNR1 and RNR2 are weak (K_d ≈ 0.1 μM). At high Y2 concentrations (1 μM), there was a correlation between the specific activities of the (His)_6-Y2 K387N and (His)_6-Y2 proteins (120–130 units/mg and 33–37 units/mg, respectively) and their Y· content.

Y4, as isolated from E. coli, contained no bound metal ions and exhibited no activity. Y4 was unable to substitute for Y2 in assays for Y1 activity and further inhibited the rate of dNDP formation when (His)_6-Y2 is assayed with Y1.

**Possible Role for Y4 in Cluster Assembly of Y2.** Y4 is highly homologous to Y2 but lacks several conserved residues that are ligands to the iron in the essential dифференциальный Y· cofactor. Despite these significant changes, a marR deletion is lethal (12) or conditionally lethal (13). In addition, using (myc)_6-tagged Y4 and hemagglutinin-tagged Y2 under the control of a galactose promoter, Huang and Elledge (12) showed that immunoprecipitation of proteins in crude yeast extracts with myc antibodies gave Y2 as well as Y4. These observations and recent studies on the role of Lys-7 in copper insertion into apo copper-zinc requiring superoxide dismutase (26), suggested to us that Y4 could potentially function as a chaperone for iron insertion into Y2. Several preliminary experiments have been carried out that support this proposal.

Although previous studies with apo E. coli and mouse RNR2s have shown that Fe^{2+}, O_2, and reductant are sufficient to generate dифференциальный Y· cluster (27, 28), similar experiments with Y2 have been unsuccessful. If Y4 were to function as a chaperone, then its addition might allow cluster assembly. To test this proposal, Y4 was incubated under anaerobic conditions with Fe^{2+} and was then incubated anaerobically with Y2. The entire mixture was then mixed with oxygenated buffer and assayed for nucleotide reductase activity. The results of an experiment in which Y4:Fe^{2+}:E. coli Y2 is =40 μM:40 μM:40 μM are shown in Fig. 2. The activity is now 0.061 μmol·min^{-1}·mg^{-1}, and we are able to detect Y· (18 μM) in the E. coli Y2. Under a second set of conditions in which the concentration of free Fe^{2+} relative to Y4 and Y2 was substantially higher, the activity of (His)_6-Y2 K387N was increased from 0.090 to 0.30 μmol·min^{-1}·mg^{-1}, and the E. coli Y2 now has an activity of 0.069 μmol·min^{-1}·mg^{-1} (Table 1). Control experiments with iron and no Y4 reveal no changes in activity. These preliminary studies thus suggest that Y4 is required for iron cluster assembly.

**Detection of Complex Formation Between Y2 and Y4.** For Y4 to function as a chaperone, it would need to form a complex with Y2. To probe the function of Y4 under normal growth conditions, Y4 and Y2 antibodies were generated. Control experiments show that Y4 antibodies possess no cross-reactivity with Y2 (Fig. 3, lanes 4 and 6). These antibodies were used to probe the purified (His)_6-Y2 and crude cell extracts of yeast. Y4 can be detected in yeast cell-free extracts (Fig. 3, lanes 2 and 7) and in purified Y2 (Fig. 3, lanes 4 and 6). No Y4 was observed in Y2 isolated from E. coli. Furthermore, immunoprecipitation of Y4 from crude cell extracts and probing with Y2 antibodies revealed the presence of Y2 (data not shown). These results support the earlier observations with epitope-tagged, overexpressed RNR2 and RNR4 (12). They indicate that a complex can be formed between Y2 and Y4 under normal growth conditions, a prerequisite for Y4 function as a chaperone.

**Discussion**

RNRs have been the topic of intense investigation since their discovery in the 1960s. In vitro, the mechanism of nucleotide reduction is now moderately well understood (3), as are the gross features of the allosteric regulation of these enzymes (2). The mechanism in vitro of iron cluster assembly has been pushed to the present technological limits with the wild-type proteins (6). How all of this information can be used to understand nucleic
acetylpyruvate carboxylase (ACCase) activity in the presence of normal growth conditions and under conditions of environmental stress in vivo is the next frontier.

In the past few years, elegant studies from a number of labs have begun to investigate in vivo the functions of the two large subunits of RNR1 (Y1 and Y3) and two small subunits of RNR2 (Y2 and Y4) (1, 11–13). To investigate metal cluster assembly, the possibility that this cofactor is assembled and disassembled as a control mechanism for deoxynucleotide formation, and allosteric regulation in vivo and their importance in replication and repair, we initially set out to reclone, express, and purify these proteins. Y2 and Y4 have been successfully purified to near homogeneity from E. coli. As isolated, neither have a Y, and they are consequently inactive in the nucleotide-reduction assay. The Y2 gene was therefore placed into a vector with an affinity tag and transformed into yeast in an effort to obtain an active form of Y2. This construction facilitated its isolation using metal affinity chromatography and provided the first active Y2, with iron bound and a detectable, stable Y. Active Y2 could now be used to assay Y1 for dNDP formation. Y1 was expressed in E. coli and in yeast and purified using a dATP affinity column. The activity measured with (His)_{6}-Y2 was detectable and identical from the two sources. Y3 was expressed and purified only from E. coli and using the same (His)_{6}-Y2 that successfully generated dNDPs with Y1, gave no detectable activity. Y4 had no detectable activity with either Y1 or Y3.

Y1 and Y2, as previously proposed, constitute the active RNR involved in normal nucleic acid metabolism. The activity of 0.03 μmol−1 min−1 mg−1 for Y1 and 0.30 μmol−1 min−1 mg−1 for Y2 is substantially higher than previously reported activities (13). As we still don't understand the mechanism of reconstitution of the required diferric-Y′ cofactor, the activity of Y2 and consequently Y1 will ultimately be higher.

Y3, the large subunit that is induced 100-fold in the presence of DNA-damaging agents (11), has thus far no detectable activity. It can be rapidly isolated in soluble form by the affinity column procedure that has resulted in active Y1. The absence of activity in Y3 is not understood, but given its role in DNA repair, we speculate that posttranslational modification by phosphorylation could be required for catalytic activity.

Most intriguing is Y4. Y4, as noted above, is 56% sequence identical to Y2, lacks 51 amino acids at its N terminus, and has a preponderance of acidic amino acids at its C terminus in comparison to Y2 and mouse RNR2. In Y4, the histidine coordinated to each of the ions in the Y2 diiron cluster is replaced by a tyrosine, also a possible metal ligand, and a key glutamate, the one that is most flexible, determined from a comparison to Y2 and mouse RNR2. In Y4, the histidine coordinated to each of the ions in the Y2 diiron cluster is replaced by a tyrosine, also a possible metal ligand, and a key glutamate, the one that is most flexible, determined from a comparison to Y2 and mouse RNR2.

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