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IDENTIFICATION OF A FREE RADICAL AND OXYGEN DEPENDENCE OF RIBONUCLEOTIDE REDUCTASE IN YEAST

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Ribonucleotide reductase is a key enzyme for DNA biosynthesis. The enzymes isolated from animal and plant cells possess a stable tyrosyl free radical which is essential for catalysis. Fungal ribonucleotide reductases are little known; the partially characterized enzyme from yeast cells proved exceptionally shortlived, and a free radical could not as yet be demonstrated. We here show that a doublet ESR signal centered at $g = 2.0046$ can be measured below 60°K in rapidly purified protein samples which is very similar to the ESR spectra of the tyrosine radicals present in other eukaryotic ribonucleotide reductases in structure, microwave saturation, and quenching by hydroxyurea. Because generation of these radicals requires oxygen, anaerobic yeast cultures were also studied. No change in ribonucleotide reductase was observed at 50 ppm residual oxygen in the gas phase, but cell proliferation ceased entirely under complete anaerobiosis.

KEY WORDS: ESR Spectroscopy, hydroxyurea, oxygen, ribonucleotide reductase, *Saccharomyces cerevisiae*, tyrosyl radical, yeast.

INTRODUCTION

An increasing number of free radical reactions is being uncovered in biochemical catalysis mechanisms.[1] One of the better known cases is enzymatic ribonucleotide reduction which provides the deoxyribonucleotides for DNA replication during the S phase of a cell cycle.[2-4] The ribonucleoside diphosphate reductases (EC 1.17.4.1) catalyze an irreversible elimination of the 2′-hydroxyl group from purine and pyrimidine ribonucleotides and its replacement by hydrogen derived from dicysteine proteins (thioredoxins or glutaredoxins). The enzymes isolated from *E.coli*, mouse cells, and unicellular green algae[5-7] bear a tyrosyl free radical in their smaller protein subunit (Tyr-122 in *E.coli* enzyme numbering), which is located in close proximity to a protein-bound binuclear μ-oxo-iron(III) center and is thought to participate in the hydrogen transfer chain. Generation of the tyrosyl radical is an oxygen-dependent process.[8] Although variations exist in the behaviour of the various enzymes towards radical-scavenging inhibitors like hydroxyurea and hydroxamic acids[9], it was concluded that all the eukaryotes possess one type of ribonucleotide reductase, which has also been acquired by their enteric bacteria.

On the other hand, ribonucleotide reduction is catalyzed by quite different, coenzyme B_{12}-requiring or manganese-containing enzymes in most bacteria[2-4] and

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additional, anaerobic ribonucleotide reductases appear to exist in the methanogenic archaebacteria and in oxygen-sensitive *E.coli* strains.[10,11] Therefore, the uniformity of eukaryotic enzyme structures should not *a priori* be taken for granted, in particular in the lower eukaryotes with biochemical, ecological, and phylogenetic anomalies. The eukaryotic algae *Euglena gracilis*, which contains a B_{12}-dependent ribonucleotide reductase,[13] is such an exception. Ribonucleotide reduction in fungi has found very little attention. An S phase-specific ribonucleotide reductase was demonstrated in baker's yeast (*Saccharomyces cerevisiae*)[13,14] and this enzyme later was partially purified.[15,16] The yeast protein showed unusual features: It was inherently shortlived in solution ($t_{1/2}$ = 10 h at 0°C), but at the same time yeast extracts contained much higher activity than mammalian or plant cells. High concentrations of hydroxyurea were required for inactiviation (I_{50} = 5 mM). No specific metal or cofactor requirement could be demonstrated with the partially purified reductase, and a free radical could not be detected by ESR spectroscopy. We reasoned that yeast might utilize a special type of enzyme adapted to growth under both aerobic or anaerobic conditions. On the other hand, a gene coding for the small reductase subunit Y2 (RNR2) was recently identified and shown to contain a conserved tyrosine residue, Tyr-182, equivalent to Tyr-122 in *E.coli*.[17,18] However, although the enzyme subunit (RNR2 protein) has been purified, these genetic studies were not correlated with enzyme activity determinations.

We have previously succeeded to measure the ESR spectrum of ribonucleotide reductase from green algae, where the reductase signal is masked by tyrosyl radical intermediates of the photosynthetic apparatus and special purification measures had to be taken[7]. In view of the central importance of ribonucleotide reduction, and its paradoxical appearance in yeast, we have reexamined the radical nature of the fungal enzyme.

MATERIALS AND METHODS

Yeast cells (Moormann TS Hefe, aneuploid cells) were grown at 30°C in a standard medium containing 0.67% Bacto Yeast Nitrogen Base, 2% glucose, 1% succinic acid, and 0.60% NaOH under addition of 80 μg 5-fluorouracil/ml as described before.[15] The cells were suspended in 0.1 M K-phosphate buffer, pH 6.7, containing 2 mM dithiothreitol, broken with glass beads in a refrigerated Vibrogen cell mill (4 × 4 min), the cell debris was removed by centrifugation at 24,000 × g, and the homogenate was then centrifuged for 90 min at 130,000 × g in the TST 28.3 rotor of a Centrikon T-2060 ultra centrifuge. Inhibitory cell constituents were removed in this step. The clear supernatant was made 60% saturated in ammonium sulfate, and the precipitate was collected.

For ESR measurements, the protein pellet was suspended in a K-phosphate buffer-glycerol (1:1) mixture and transferred into 3 mm quartz tubes. For activity determination, the precipitate was dissolved in a small volume of buffer (K-phosphate, pH 6.7, as above, or HEPES, pH 7.5) and the sample was dialyzed against the same buffer for 4 × 1 hour.

Anaerobic yeast cultures were maintained in 2 l-round bottom flasks equipped with airtight seals. The above culture medium (4% glucose) was supplemented with Na-thioglycolate (0.5 g/l), cysteine (0.5 g/l), Tween 80 (2 ml/l), ergosterol (2 mg/l), and contained resazurin (1 mg/l) as redox indicator. A slow stream of nitrogen (99.995%)

was passed over the culture. Alternatively, the medium was stirred for 5 h in the closed flask for chemical reduction of residual oxygen, and then inoculated.

Ribonucleotide reductase assays were carred out as before,[15] containing 0.2 mM [5-^{3}H] cytidine diphosphate (spec. activity, 7.5 mCi/mmol), 15 mM dithiothreitol, 2.5 mM ATP, and 5 mM Mg^{++} in the above buffers; incubation was for 30 min at 30°C.

ESR spectra were recorded on a Varian E-12 spectrometer at X-band frequency with 100 Hz field modulation. An Oxford Instruments E-9 helium flow cryostat was used for temperature control.

The monoclonal antibody YL 1/2[19] was a generous gift of Dr. J.V. Kilmartin, MRC, Cambridge, England. All other reagents and chemicals were commercial products of the highest purity available.

RESULTS AND DISCUSSION

Ribonucleotide reductase was rapidly extracted from fluorouracil-treated, over-producing yeast cultures by the modified procedure described in the Experimental Section, and ammonium sulfate precipitates were studied by ESR spectroscopy at low temperature. The doublet signal shown in Figure 1 could only be observed below 60°K. The well-resolved spectrum is centered around $g = 2.0046$ and is very similar to the ESR spectra of mouse cell and algal ribonucleotide reductase. Under the same experimental conditions a doublet signal could also be measured in packed cells of a growing yeast culture but not in stationary cells (not shown). The microwave saturation behavior of the radical signal is depicted in Figure 2. Again, this curve closely resembles the data observed with the other eukaryotic enzymes[6,7] indicating closely comparable structure and environment.

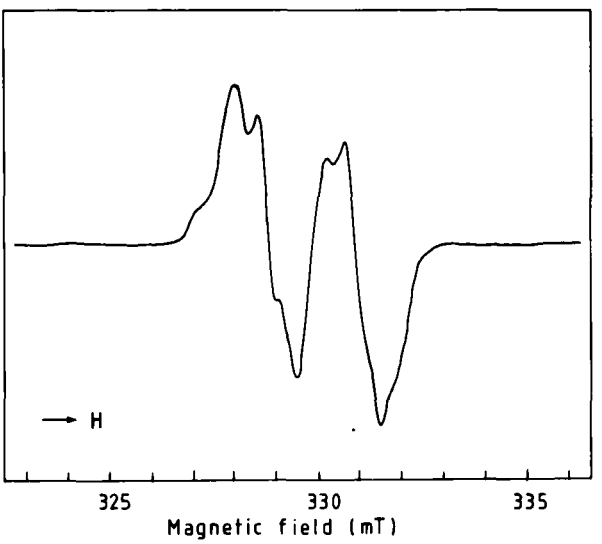

FIGURE 1 ESR spectrum of yeast ribonucleotide reductase (ammonium sulfate precipitate) measured at 30°K. Conditions: Modulation amplitude, 0.16 mT; microwave power, 1 mW; scanning rate, 2.5 mT min^{-1}; time constant, 1 s; receiver gain, 4,000.

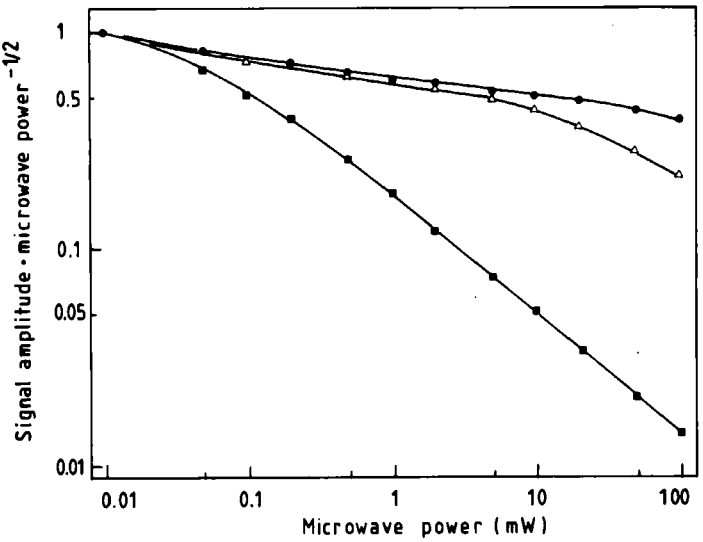

FIGURE 2 Microwave saturation at 40°K (●), 30°K (△), and 20°K (■).

As noted earlier,[15] yeast ribonucleotide reductase undergoes spontaneous inactivation in solution. Loss of enzyme activity is accompanied by a decrease of the ESR signal amplitude (Figure 3a); at ambient temperature the half-life of both is only 2 hours. All attempts have failed to stabilize the enzyme and the radical by addition of ATP, EDTA, or Mg^{++} ions, or in presence of antipain, chymostatin, leupeptin, and pepstatin for inhibition of proteolysis. In enzyme samples treated with hyd-

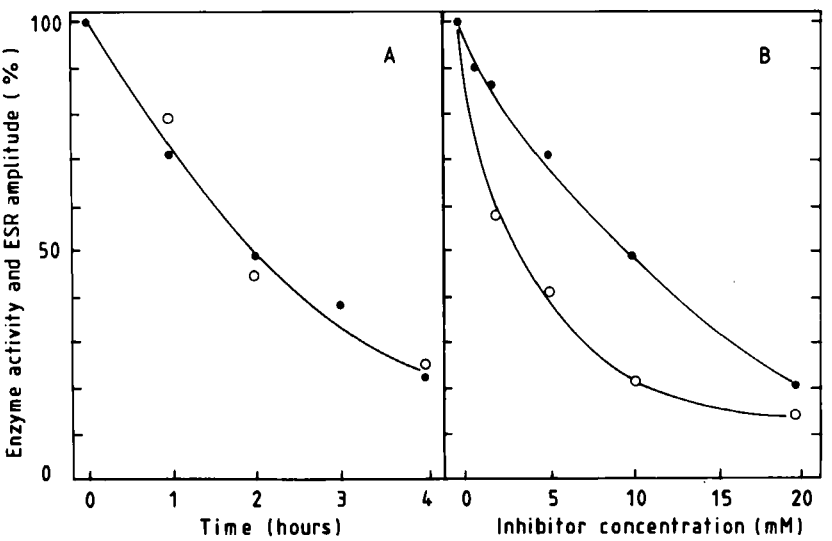

FIGURE 3 A: Stability of enxyme activity (●) and ESR signal amplitude (○) of yeast ribonucleotide reductase during storage of 25°C. B: Inhibition of enzyme activity (●) and ESR signal amplitude (○) in presence of hydroxyurea. The 100% value corresponds to an enzyme activity of 4.2 nmol CDP reduction $\cdot h^{-1} \cdot mg^{-1}$ protein.

roxyurea (Figure 3b) the ESR signal was initially quenched more strongly than substrate reduction, suggesting an excess of the radical-bearing subunit Y2 in holoenzyme preparations; non-stoichiometric subunit composition has been observed in other reductases, too.[20]

The ESR spectrum described above and the presence of a conserved tyrosine residue in Y2 demonstrate that yeast ribonucleotide reductase contains a tyrosyl free radical. Because generation of tyrosyl radical in the eukaryotic enzymes requires oxygen[9] we sought to establish conditions under which anaerobic yeast cultures would lose the regular ribonucleotide reductase and possibly switch to an anaerobic type of enzyme as observed in anaerobic bacteria.[10,11] In extracts from yeast cells grown in an anoxic medium under nitrogen containing $\leqslant$ 50 ppm oxygen the small subunit Y2 could still be detected in Western blots with a monoclonal antibody, YL 1/2, which recognizes the protein's C-terminus[19] and low, hydroxyurea-sensitive reductase activity was still measureable. In a sealed, strictly anaerobic culture flasks cell proliferation ceased entirely; Y2 was no longer detectable in such cells and no ribonucleotide reductase activity of any type (assayed under various conditions) was found.

These results are in accordance with early reports about the inability of baker's and brewer's yeast to proliferate under complete exclusion of oxygen,[21,22] whereas 2 ppm oxygen in the gas phase were sufficient to maintain yeast cultures in a chemostat.[23] Oxygen-dependent activation of the ribonucleotide reductase free radical would be an obvious process to limit DNA synthesis and cell proliferation in yeast. Evidence for this type of cell cycle control has also been found in anoxic ascites tumor cells.[24] Unfortunately, nothing is known about the intracellular O_2 concentration necessary to oxidize a specific tyrosine residue in an enzyme.

In conclusion, we have established by ESR and enzyme activity measurements that yeast contains the common eukaryotic type of ribonucleotide reductase, confirming the genetic studies.[17,18] The organism does not appear to possess a second system for *de novo* deoxyribonucleotide formation under anaerobiosis. An understanding of the special properties of yeast ribonucleotide reductase (high cellular concentration, inherent instability, and low sensitivity towards hydroxyurea) requires more detailed knowledge of the native structures of both enzyme subunits and of the cellular control mechanisms.

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