RIBONUCLEOTIDE REDUCTASE FROM *SACCHAROMYCES CEREVISIAE**

E. Vitols, V.A. Bauer and E.C. Stanbrough

Department of Biochemistry
Scripps Clinic and Research Foundation
La Jolla, California 92037

Received August 21, 1970

Summary. Partially purified ribonucleotide reductase from *Saccharomyces cerevisiae* was unstable in solution; however, enzyme activity could be maintained by quick-freezing and storage at low temperatures. The reduction of CDP was stimulated by ATP/Mg^{++}, and maximal activity was obtained with *E. coli* thioredoxin as a reductant, but not with dithioerythritol.[1] Enzyme activity was inhibited by dATP, hydroxyurea, and moderately high salt concentrations; addition of 5'-deoxyadenosylcobalamin or of iron salts was without effect. Gel filtration of the yeast extract yielded a fraction highly inhibitory to enzyme activity.

Ribonucleotide reductase, which in the presence of certain dithiols [$R(SH)_2$] catalyzes the overall reaction:

$$\text{Ribonucleotide} + R(SH)_2 \xrightarrow[\text{cofactors}]{\text{allosteric effectors}} \text{deoxyribonucleotide} + RS_2 + H_2O$$

has previously been studied in two bacterial species, *Escherichia coli* and *Lactobacillus leichmannii*, and in some mammalian tissues and tumors (cf. 1-3). In the present investigation yeast was selected as a source of ribonucleotide reductase because this organism occupies a position intermediate between bacterial and animal cells, both with regard to its growth rate and to its complexity of intracellular organization. Furthermore, yeast has an advantage over mammalian systems in that it can be grown under varying, but strictly

* This investigation was supported by a research grant (AM 12931) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, and by Special Grant No. 439 and a Dernham Senior Fellowship in Oncology, both from the California Division of the American Cancer Society.

1. Abbreviation used: DTE, dithioerythritol.

controlled conditions, including synchronous division of cells.

Two strains of *S. cerevisiae* have been used in the present study: a "wild" strain of Fleischmann's yeast obtained commercially, and a leucine/adenine auxotroph XT300-4A (4). The cells were grown on a peptone-yeast extract-glucose-salts medium (5) with vigorous aeration and, after rapid cooling, harvested and washed by centrifugation. A suspension (50%, v/v) of packed cells in 0.05 M potassium phosphate, pH 7.5, was disrupted by ultrasonication; the mixture was then diluted (to reduce viscosity) with 0.025 M potassium phosphate, pH 7.0, and centrifuged twice (10 min, and 30 min) at 40,000 x g. The crude cell-free extract was treated with streptomycin sulfate (0.8%); protein was precipitated with ammonium sulfate (0.3 g/ml), dissolved in 0.025 M potassium phosphate, pH 7.0, and then desalted by dialysis against the same buffer to give Fraction I. Further purification was achieved by stepwise elution of the enzyme from a DEAE cellulose column. The material which was eluted by 0.15 M phosphate buffer, then concentrated and desalted (Fraction II), contained about 2% of the total crude extract protein.

The standard assay mixture for ribonucleotide reductase contained: 10 mM potassium phosphate, pH 7.0; 0.4 mM CDP-2-^{14}C (1 mC/mmole); 10 mM DTE; 7 µM *E. coli B* thioredoxin; 2.5 mM ATP; 5 mM Mg^{++} (acetate); and 10 mM KF in a total volume of 0.4 ml. The mixture was incubated at 30° for 30 min. After addition of perchloric acid to stop the reaction and to precipitate protein, and following the addition of carriers, the cytidine and deoxycytidine nucleotides were hydrolyzed to the monophosphates and separated on Dowex-50 (H^+) as described by Reichard (6). The concentration of the isolated dCMP was measured spectrophotometrically and its radioactivity was determined by counting aliquots in a Beckman LS-233 liquid scintillation counter. Results are expressed in nmoles of dCMP formed/mg protein.

In our initial experiments, ribonucleotide reductase activity could not be detected in crude cell-free yeast extracts because of the interference of catabolic enzymes. Both substrate and product were degraded by deamination to

uridine compounds and also by breakage of the glycosidic bonds. This difficulty was partially overcome by purification (to the stage of Fraction I) and by the addition of fluoride to inhibit nucleotide phosphorylase. When, under certain conditions, some residual dCMP deaminase activity was still evident in Fraction I, the ribonucleotide reductase activity was calculated by determining and correcting for the amount of dUMP formed. Fraction II from the DEAE cellulose column was essentially free from the above degradative enzymes, and the specific ribonucleotide reductase activity in this fraction was relatively constant ($\pm$ 15%) from one preparation to another, provided that the cells were grown under identical conditions. Consequently, it was possible to determine and compare the amounts of enzyme present in the two strains of yeast at different stages of growth. Maximal enzyme activity from the "wild" strain was obtained by harvesting cells in mid-log phase (i.e., at the stage of most rapid cell division). From the mutant strain (XT300-4A), however, enzyme preparations with a 4-fold higher specific activity were obtained when adenine sulfate was added to these cultures at early stationary phase, and the cells harvested shortly thereafter. Apart from this, no qualitative differences such as the requirements of reaction components or the effects of stimulators and inhibitors could be detected between the enzymes from the two strains. The results presented below have been obtained with enzyme preparations from the mutant.

Isolation of the yeast ribonucleotide reductase was difficult because of its extreme instability in solution, the half-life of activity being less than 24 hrs. at 0°. Enzyme stability was not significantly improved by purification (to Fraction II) nor by the addition of thiols, EDTA, or magnesium and iron salts which have been used with varying degrees of success as stabilizers for the isolation of ribonucleotide reductases from other sources. The yeast enzyme activity could be preserved for periods of up to several weeks, however, by quick-freezing the preparation in liquid nitrogen and subsequent storage at -20°.

Under the optimal assay conditions described above, the enzyme prepara-

tions (both Fractions I and II) catalyzed the reaction at a relatively linear rate over a period of one hour (Fig. 1). The reduction of CDP was completely

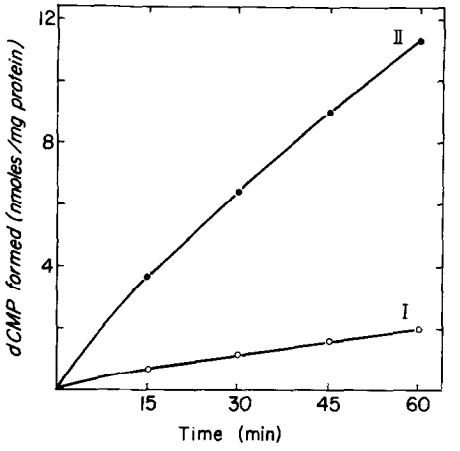

Fig. 1. Reduction of CDP by Fractions I and II as a function of time. Standard assay conditions (see text), incubated for periods of time indicated.

---

dependent upon the addition of a reducing substrate; *E. coli* thioredoxin (chemically reduced by DTE) was a far more effective reductant than DTE alone (Fig. 2). The relation of enzyme activity to thioredoxin concentration

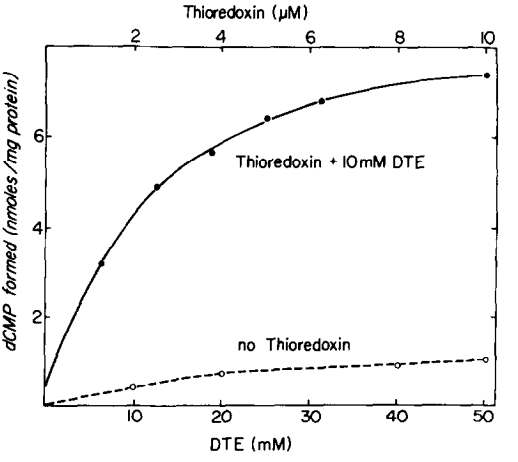

Fig. 2. Effect of DTE and *E. coli* thioredoxin concentration on CDP reduction by Fraction II. (--o--), activity at varying DTE concentrations (bottom scale) in the absence of thioredoxin; (—●—), activity at varying concentrations (top scale) of thioredoxin, chemically reduced in the presence of 10 mM DTE.

followed simple Michaelis-Menten kinetics, the apparent K_m being about 3 μM. Previously, the dependence upon thioredoxin for maximal activity was observed only with the *E. coli* enzyme, for which thioredoxin, either native or that recently purified from yeast (7), is a better reductant than simple dithiols. In contrast, the enzymes from *L. leichmannii* (8) and from different mammalian sources (3,9) exhibit maximal activity in the presence of such dithiols alone.

Maximal CDP reduction by the yeast enzyme depended upon the presence of ATP and Mg^{++}, whereas low concentrations of dATP produced significant inhibition even in the presence of 2.5 mM ATP (Table I). Addition of either

TABLE I

Effect of Reaction Components on the Activity of Yeast Ribonucleotide Reductase

	Activity*
Complete System (Fraction II)	7.20
Omission	
DTE	0.00
Thioredoxin	0.44
Mg^{++}	1.08
ATP	0.86
Addition	
0.1 mM dATP	4.03
0.5 mM dATP	3.67
2.5 mM Hydroxyurea	5.04
5.0 mM Hydroxyurea	3.89
0.05 M Phosphate, pH 7.0	9.36
0.2 M Phosphate, pH 7.0	1.44
0.2 M NaCl	0.77
0.2 M $(NH_4)_2SO_4$	0.00

* nmoles dCMP/mg protein

5'-deoxyadenosylcobalamin or iron salts did not stimulate activity, and iron chelating agents, such as 8-hydroxyquinoline and orthophenanthroline (1 mM),

did not inhibit activity when added either directly to the assay mixtures or preincubated with the enzyme for one hour at 0°. Preincubation or dialysis against the chelators for longer periods (10) was not feasible, because of the instability of the enzyme. Activity was inhibited by hydroxyurea and by moderately high salt concentrations (Table I). It appeared that the salt inhibition might be due to ionic strength, rather than to the presence of any particular ion. Some stimulation of enzyme activity by the addition of 0.05 M phosphate (Table I) may have been due to the blocking of CDP dephosphorylation to the inactive monophosphate; however, higher phosphate concentrations were inhibitory. A low molecular weight compound which acts as a highly potent inhibitor of enzyme activity was isolated by gel filtration of the ammonium sulfate precipitate used to prepare Fraction I. Not yet identified, this compound had a spectrum reminiscent of certain nucleotides and appeared in preliminary experiments to be at least 100-fold more inhibitory than dATP, presuming that its molar extinction coefficient is comparable with that of nucleotides

Thus, some of the properties of the yeast enzyme appear similar to the *E. coli* ribonucleotide reductase (i.e., the requirement of thioredoxin for maximal activity and, probably, possession of a tightly-bound iron) whereas its extreme instability is more akin to the mammalian enzymes, particularly those from tumor cells (3). The sensitivity of the yeast ribonucleotide reductase to high ionic strength also bears some resemblance to the mammalian tumor enzymes, but is in sharp contrast to the bacterial ribonucleotide reductases which are fully active in the presence of up to 1 M concentrations of certain salts (11, 12). It seems unlikely that the rapid loss of the yeast enzyme activity at 0° was due to contaminating proteolytic enzymes, since the reaction rate was almost linear at 30° for one hour (Fig. 1), and stability did not improve during successive steps of enzyme purification. Because of the observed differences in the level of enzyme activity at varying stages of cell growth and because of the probable involvement of a highly effective (as yet unidentified) endogenous inhibitor, the yeast ribonucleotide reductase seems likely to provide a useful

system for studying the manner in which the control of reductase activity may be related to cell division.

ACKNOWLEDGMENTS

The authors are indebted to Dr. J.A. DeMoss for a culture of *S. cerevisiae* strain XT300-4A, and to Dr. F.M. Huennekens for valuable comments and helpful discussion during the course of this work.

REFERENCES

1. Larsson, A. and Reichard, P., in *Progress of Nucleic Acid Research and Molecular Biology*, J.N. Davidson and W.E. Cohen, Eds., Vol. 7, Academic Press, New York, 1967, p. 303.
2. Moore, E.C., in *Methods in Enzymology*, S.P. Colowick and N.O. Kaplan, Eds., Vol. XII (A), Academic Press, New York, 1967, p. 155.
3. Blakley, R.L. and Vitols, E., Ann. Rev. Biochem. 37, 201 (1968).
4. Manney, T.R., Duntze, W., Janosko, N. and Salazar, J., J. Bacteriol. 99, 590 (1969).
5. Halvorson, H.O. and Spiegelman, S., J. Bacteriol. 64, 207 (1952).
6. Reichard, P., Acta Chem. Scand. 12, 2048 (1958).
7. Porque, P.G., Baldesten, A. and Reichard, P., J. Biol. Chem. 245, 2363 (1970).
8. Vitols, E. and Blakley, R.L., Biochem. Biophys. Res. Commun. 21, 466 (1965).
9. Larsson, A., European J. Biochem. 11, 113 (1969).
10. Brown, N.C., Eliasson, R. Reichard, P. and Thelander, L., Biochem. Biophys. Res. Commun. 30, 522 (1968).
11. Brown, N.C., Canellakis, Z.N., Lundin, B., Reichard, P. and Thelander, L., European J. Biochem. 9, 561 (1969).
12. Jacobsen, D.W. and Huennekens, F.M., Biochem. Biophys. Res. Commun. 37, 793 (1969).