SHORT COMMUNICATIONS

Ribonucleotide Reductase of *Pithomyces chartarum*: Requirement for B₁₂ Coenzyme

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INTRODUCTION

Ribonucleotide reductase activity has been obtained from several sources. The enzyme from *Lactobacillus leichmannii* reduces ribonucleoside triphosphates to 2'-deoxyribonucleoside triphosphates in the presence of a dithiol and coenzyme B₁₂ (Blakley, Ghambeer, Nixon & Vitols, 1965); the *Escherichia coli* enzyme reduces ribonucleoside diphosphates in a system requiring thioredoxin, thioredoxin reductase, NADPH, Mg²⁺, but no B₁₂ coenzyme (Reichard, 1962). The enzyme from mammalian sources appears to be similar to that of *E. coli* in its requirements (Millard, 1972; Moore & Reichard, 1964). No report has described ribonucleotide reductase in fungal cells; the following is a description of a coenzyme B₁₂-dependent ribonucleotide reductase from the fungus *Pithomyces chartarum*.

METHODS

Pithomyces chartarum strain C was grown in 8 l of a medium containing 0.2 % vitamin-free Casamino acids (Difco), 2 % (w/v) glucose, 0.5 mM-MgSO₄, and 0.05 M-Na K phosphate buffer (final pH 6.3). Cultures were agitated by forced aeration at 26 °C for 48 h, the mycelia were collected by filtration, washed several times with cold phosphate buffer (pH 6.0), and frozen in a dry ice-acetone bath. Cells were disrupted by a variety of methods including pressure at 1380 bar (20000 lb/in²), sonication, grinding with acid-washed alumina or sea sand, and grinding in the frozen state. The protein yields from all of these methods were low, resulting in approximately 100 ml of crude extract having 1 to 3 mg protein/ml after centrifugation (25000 g, 2 °C, 30 min) for removal of cell debris.

Reaction mixtures for ribonucleotide reductase activity routinely contained 3 mM-ATP or GTP, 5 to 20 mM-dithiothreitol (DTT) or reduced lipoic acid (the highest concentration which did not interfere with the diphenylamine test), 5 mM-magnesium acetate, 0.05 M-phosphate or acetate buffer (pH 6.0), 100 µM-coenzyme B₁₂ (5'-deoxyadenosyl cobalamin), and 1 to 3 mg fungal protein/ml. Samples were taken at zero time for controls and the mixtures were incubated at 26 °C under nitrogen in the dark for 3 to 8 h.

Production of deoxynucleotides was measured by the method of Blakley (1966) modified for maximal sensitivity: 1 ml samples were boiled with 0.5 ml chloroacetamide solution (1 M in 0.5 M-phosphate buffer, final pH 8.7) for 10 min, cooled, and mixed with 1.5 ml diphenylamine reagent (4 %, w/v, diphenylamine and 10 %, v/v, H₂SO₄ in glacial acetic acid). Colour was developed at 25 °C for 5 h and measured at 595 nm (1 cm light path); deoxyadenosine triphosphate (d-ATP) was used as a standard. In this system, 100 nmol of d-ATP produced an absorbance of about 0.67 units.

Fig. 1. Effect of B₁₂ coenzyme concentration on reduction of GTP by fungal extracts. Reaction mixtures contained: DTT, 20 mM; GTP, 3 mM; magnesium acetate, 10 mM; phosphate buffer, 50 mM; fungal protein, 2 mg/ml; and various concentrations of B₁₂ enzyme.

RESULTS AND DISCUSSION

Extracts from cultures reaching maximal growth or from cultures grown in a complex medium (Sabouraud dextrose broth) were devoid of ribonucleotide reductase. Extracts from young cultures grown in the vitamin-free medium showed little or no activity in their crude state, either dialysed or undialysed. However, precipitation with ammonium sulphate (taking that fraction which precipitated between 30 and 60 % saturation) and treatment with DNase 0.1 mg of [Sigma crystallized Type I (Sigma Chemical Co., St Louis, Missouri, U.S.A. per ml) at 0 °C for 30 min], followed by dialysis, yielded preparations which contained ribonucleotide reductase activity.

The ribonucleotide reductase was inactive with purine nucleoside monophosphates, showed barely detectable activity with purine nucleoside diphosphates, and was most active with ATP and GTP. The reaction rate with GTP was about double that with ATP, and a slight stimulation by Mg²⁺ was detected in most experiments. DTT was as effective a hydrogen donor in the system as reduced lipoic acid. The pH optimum for the enzyme was considerably lower and narrower than optima found for ribonucleotide reductase from other sources. Maximal deoxynucleotide accumulation occurred at approximately pH 5.8 (with half-maxima at pH 4.8 and 6.7) compared to optima in the pH range 7.5 to 8.1 for previously studied systems (Reichard, 1962; Blakley, 1965). The temperature optimum for the reaction was about 22 °C and probably reflects stability requirements of the enzyme during the rather lengthy incubation period.

A striking feature of this system was the absolute requirement for coenzyme B₁₂. Plots of reaction velocity versus coenzyme concentration yielded sigmoid curves which could not be described by simple first-order Michaelis-Menten kinetics. One of these plots is shown

in Fig. 1; a double reciprocal plot of the data is concave upwards. Replotting these and similar data as $1/v$ versus $1/[S]^2$ allowed fitting of straight lines with correlation coefficients averaging 0.98; from this, the K_m for coenzyme B₁₂ was estimated to be 14 μ M. These data suggest the possibility that at least two molecules of coenzyme interact with the enzyme to produce a cooperative effect where the binding of one molecule facilitates the binding of the next (see review by Stadtman, 1966). For a regulatory enzyme such as ribonucleotide reductase, a sigmoid response to an allosteric effector might be of considerable importance; Changeux (1963) has pointed out that in such a situation there exists a threshold concentration of effector below which enzyme activity is relatively insensitive to variation in effector concentration. Above that threshold, large changes in enzyme activity result from relatively small changes in effector concentration, thus providing enzyme activity highly sensitive to a narrow selected range of effector. A study of the role which coenzyme B₁₂ plays in ribonucleotide reduction by *Pithomyces chartarum* is currently hampered by the low activity obtainable; this necessitates lengthy incubation periods and precludes precise kinetic measurements. However, this dependence on B₁₂ coenzyme lends support to the suggestion (Stutzenberger & Parle, 1973) that substituted benzimidazoles inhibit fungal DNA synthesis by acting as precursors for inactive B₁₂ coenzyme analogues.

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