Bleomycin-Iron Complex and Oxygen Activate Algal Ribonucleotide Reductase

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Ribonucleotide reductase of green algae *(Scenedesmus obliquus)* is a radical-containing enzyme which rapidly loses activity under anaerobic conditions. Reactivation in the presence of air is enhanced by 10 μM iron(II)-bleomycin chelate. The reaction lends new biochemical potential to the antibiotic and should be valuable in mechanistic studies of ribonucleotide reduction.

Introduction

Ribonucleoside diphosphate reductases (EC 1.17.4.1), which produce the 2′-deoxyribonucleotides essential for DNA replication, require sulfhydryl groups, a binuclear non-heme-iron center, an organic free radical, and aerobic conditions for activity [1–3]. These properties make the isolated proteins very vulnerable to inactivation by a great number of radical scavenging and/or metal chelating compounds. In the study of novel such inhibitors [4] and of a new eukaryotic enzyme system characterized in green algae [5, 6] we also tested for an effect of the DNA-degrading glycopeptide antibiotic bleomycin, which combines metal binding capacity with oxygen activation and the production of radicals [7–9], and which should therefore be capable of interfering with ribonucleotide reduction. Unusual sensitivity to bleomycin of mouse cells with an altered ribonucleotide reductase had in fact been reported [10]. Contrary to our expectation, the drug did not inhibit freshly prepared enzyme solutions to any measurable extent. At low bleomycin concentration, however, we found a marked activating effect on aged enzyme preparations suggesting bleomycin complexes as model catalysts for ribonucleotide reductase radical generation.

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Materials and Methods

Ribonucleotide reductase of the unicellular green freshwater algae *Scenedesmus obliquus* was prepared and determined as described [5, 6]. Holoenzyme fractions of approximately 5 $nmol \cdot h^{-1} \cdot mg^{-1}$ protein specific activity were used for the experiments. Assays contained 2 μCi [5-^{3}H]cytidine diphosphate (spec. activity, 0.3 Ci/mmol) and 15 mM dithiothreitol as substrates in a total volume of 0.30 ml 0.05 M K-phosphate buffer pH 6.7; the enzyme does not require exogenous metal ions or effector nucleotides under these conditions. Incubations were for 30 min at 30 °C after which time the reaction was terminated by 3 min boiling and the reaction mixtures subjected to liquid chromatographic product/substrate separation. Anaerobic enzyme assays were performed in a glove box under 99.99% nitrogen atmosphere. All necessary solutions were deaerated in vacuum and flushed with nitrogen repeatedly before use.

Bleomycin sulfate from *Streptomyces verticillus* (a clinical mixture composed of 55–70% A_2 and 25–32% B_2) was a generous gift from Dr. R. Roth, Fa. Mack, Illertissen, Germany. The concentration of the iron complex, prepared *in situ* from $(NH_4)_2Fe(SO_4)_2$ and an approximately three-fold excess of the antibiotic (mean molecular weight, 1450), is defined by the metal content.

Results and Discussion

The recently isolated first plant ribonucleotide reductase [5, 6] resembles the enzymes from mammalian sources [11–13] in substrate requirements, content of a free radical, and inhibition by hydroxyurea but its study is hampered by the low intracellular amount and other plant-specific experimental problems. For example, in photosynthetic cells the ESR signal of a ribonucleotide reductase tyrosyl radical [1] cannot be measured directly. We sought additional evidence for correlating enzyme activity and radical character in the behaviour of the enzyme system under various environmental conditions. When kept in solution at 0–5 °C, purified algal ribonucleotide reductase (like any enzyme of its kind) was completely inactivated within a few days and could not be recovered, most probably owing to unspecific denaturation of the non-identical protein subunits. Lifetime was greatly prolonged in frozen solutions at −80 °C where the half-life of holoenzyme prepara-

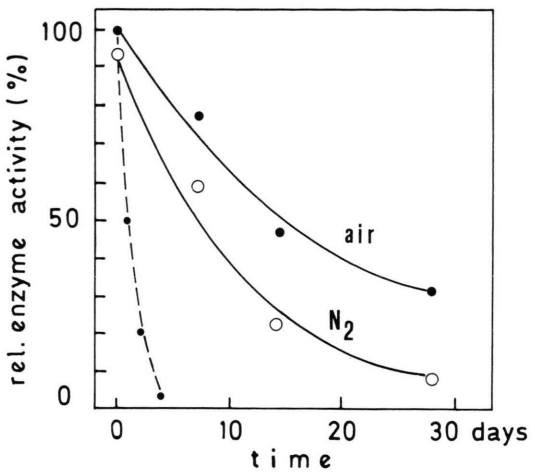

Fig. 1. Activity of ribonucleotide reductase from *Scenedesmus obliquus* after storage of holoenzyme solutions at −80 °C, determined in standard assays (30 min at 30 °C; cytidine diphosphate as substrate). ●: Sample stored and assayed under normal, aerobic conditions. ○: Anaerobic storage and assay of the same enzyme preparation under nitrogen atmosphere. Dashed line: Activity decay of an enzyme solution stored at 5 °C under aerobic or anaerobic conditions.

tions is 2–3 weeks. Under these conditions inactivation was twice as fast when a deaerated protein sample was stored and assayed under nitrogen atmosphere (Fig. 1). Freshly prepared enzyme samples were not adversely affected by anaerobiosis during a 30 or 60 min assay period. This suggests that the slow activity loss of algal ribonucleotide reductase at low temperature is to a large extent due to decay of its organic radical which is stabilized or continuously regenerated by oxygen, in analogy to the situation observed in enzyme preparations from calf thymus [14]. Oxygen dependence of DNA precursor biosynthesis thus appears universal among animals, plants, and aerobic bacteria.

Some limited reactivation occured in the presence of up to 0.1 mM ferrous ions when aged ribonucleotide reductase preparations were exposed to air under standard assay conditions, but these effects were difficult to reproduce in the complex system. Thus, inactivated enzyme may also have suffered irreversible denaturation after more than 2 weeks; enzyme stability and activity at ambient temperature are linear only for about 90 min incubation; and divalent metal ions inhibit the enzyme [6] so that the potentially catalytic effect of Fe^{++} ions will be masked. At the best, 1.5-fold reactivation was reached under such conditions, and the activities did hardly exceed those which are maintained anyway during aerobic storage as shown in Fig. 1.

Significantly better, reproducible activation of algal ribonucleotide reductase was observed, however, when bleomycin plus ferrous ions were added to aerobic assay mixtures (Table I). The stimulating effect of bleomycin-iron chelate manifests itself only in a narrow concentration range, between 10 and 15 μM, whereas the metal-free antibiotic and higher (up to 1 mM) concentrations of the iron complex were ineffective. In no case have we noted inhibition of substrate reduction. Freshly prepared enzyme solutions were stimulated by about one third while aged (frozen) preparations increased at least twofold in activity and could regain up to 70% of the original value. We interpret these observations as catalytic function of the bleomycin : iron(II) : O_2 complex which can produce phenoxy radicals from phenols [8] and should likewise be capable of oxidizing the highly conserved, essential tyrosine residue of eukaryotic ribonucleotide reductases [15]. The enzyme assays also contain a thiol (dithiothreitol) necessary to reduce and recycle oxidized bleomycin : iron(III) species [9].

It is not very likely (although not impossible) that the catalytic role of activated bleomycin described here is of physiological significance besides its cytotoxic action *via* radical-induced DNA strand scission [7]. The increased bleomycin sensitivity of

Table I. Aerobic activation of algal ribonucleotide reductase holoenzyme. Enzyme activity was measured under standard assay conditions (30 min at 30 °C).

Conditions	Relative enzyme activity
Fresh enzyme (2 days old), aerobic	100%[a]
+ 10 μM – 1 mM metal-free bleomycin	98–102
+ 10 μM bleomycin:iron(II)	131
+ 0.5 mM bleomycin:iron(II)	100
Aged enzyme (frozen 2 weeks), anaerobic	22
sample reaerated	36
+ 5 μM bleomycin:iron(II)	33
+ 10 μM bleomycin:iron(II)	46
+ 15 μM bleomycin:iron(II)	70
+ 30 μM bleomycin:iron(II)	37

[a] Specific activity, 4.25 nmol CDP · h^{-1} · mg $protein^{-1}$.

mouse cell mutants having altered deoxyribonucleotide pools [10], which had not been correlated with *in vitro* enzyme measurements, may not be linked to ribonucleotide reduction itself in view of the lack of ribonucleotide reductase inhibition by bleomycin. This conclusion appears safe despite the different organisms under study because the ironcontaining enzymes resemble each other in their response to various classes of inhibitors [2, 4, 16] and because the plant enzyme, by its oxygen dependence discussed above and by many other properties [5, 6] is a typical eukaryotic ribonucleotide reductase. The new activating effect of bleomycin: Fe (II): O_2 complex, however, could be a valuable practical means for stabilizing the sensitive radical subunits of ribonucleotide reductases. It should also provide a simple model reaction for the oxygen-dependent enzymatic formation of the protein radical which in *E. coli* requires several protein components [17] but is still not understood in mechanism.

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