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Characterization of the free radical in a plant ribonucleotide reductase

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Ribonucleotide reductase of the green alga, *Scenedesmus obliquus*, contains an organic free radical in its catalytic subunit U2 which is quenched by hydroxyurea and regenerated upon aerobic incubation in the presence of Fe^{2+}. The low-temperature EPR spectrum exhibits a doublet centered at $g = 2.0046$. This EPR signal is indistinguishable from that of the tyrosine radical in mouse cell ribonucleotide reductase, indicating that the eukaryotes possess one common enzyme apparatus for deoxyribonucleotide biosynthesis.

Ribonucleotide reductase; EPR; Hydroxyurea; (Green alga)

1. INTRODUCTION

The ribonucleoside diphosphate reductases (EC 1.17.4.1) isolated from *Escherichia coli*, mammalian cells and viruses possess an organic free radical essential for enzyme activity [1–5]. It is located on the smaller, catalytic subunit of two non-identical protein components (designated B2 in *E. coli*, or M2 in mammals) and is derived from a tyrosine in the polypeptide chain. Tyr-122 (in *E. coli* numbering), which is a conserved amino acid in the primary structures of the small subunits from different organisms [6], has been identified as the specific residue [7]. The tyrosyl radical is located close to and stabilized by a protein-bound μ-oxo binuclear iron(III) complex. It is generated in an oxygen-dependent reaction [8,9], with the consequence that deoxyribonucleotide formation and DNA synthesis may cease under anaerobiosis [10].

The tyrosine radical of ribonucleotide reductases is recognized by a characteristic doublet EPR spectrum centered around $g = 2.005$ which can be detected in enzyme preparations as well as in frozen suspensions of overproducing bacteria and mouse cells, or rapidly proliferating tumor tissues [3,4,11,12]. These EPR signals are similar but not identical, suggesting some variability in the environment of the tyrosyl residues. Variations are also observed in the half-lives of enzyme activity and EPR signal amplitude [9], reaction rates with hydroxyurea and hydroxamic acids which act as radical scavengers [13], and in the conditions required for reactivation of radical-depleted inactive enzyme samples [8,9].

The enzyme apparatus of deoxyribonucleotide synthesis in plants is not as well characterized because of the low activity and stability of the ribonucleotide reductases in plant extracts [14], and because direct detection of a reductase EPR signal is not feasible in photosynthetic cells. An active yet short-lived enzyme was recently obtained from synchronous, or from fluorodeoxyuridine-treated cultures of the unicellular green alga, *Scenedesmus obliquus* [15]. Its inactivation by hydroxyurea and aerobic reactivation [16] suggested that the algal protein belongs to the same family of radical enzymes as found in *E. coli* and animals cells. Using a more rapidly purified pro-

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tein preparation we could now characterize in detail the radical nature of the plant ribonucleotide reductase.

2. MATERIALS AND METHODS

Ribonucleoside diphosphate reductase was purified from FdU-stimulated cultures of *S. obliquus* as described [15]. For improved purification of subunit U2, the pass-through fraction from dATP-Sepharose affinity chromatography was incubated at 55°C for 20 min, the precipitate removed by centrifugation, and the enzyme subunit was recovered from the supernatant by precipitation with ammonium sulfate (60% saturation).

Enzyme assays with [5-^{3}H]cytidine diphosphate as substrate were carried out as in [15]. For reactivation of enzyme samples, phosphate buffer was replaced by 0.10 M Hepes buffer, pH 6.7, containing 1 mM dithiothreitol.

EPR first-derivative spectra were recorded on a Varian E-12 spectrometer at X-band frequency with 100 kHz field modulation. An Oxford Instrument E-9 helium flow cryostat was used for temperature control. 2,2-Diphenyl-1-picrylhydrazyl served as reference for determination of the g value.

3. RESULTS AND DISCUSSION

Previous studies of ribonucleotide reductase in *S. obliquus* had revealed the existence of a poorly resolved EPR signal in relatively crude holoenzyme preparations [15] but not in the separated subunits after several column chromatography steps. Because the catalytic subunit 2 of ribonucleotide reductases is the more stable, cofactor-like enzyme component [2,17] we have subjected algal protein U2 (which cannot be purified by affinity chromatography on dATP-Sepharose) to heat treatment at 55°C and have accomplished an additional 2-fold, rapid enrichment of this subunit. Ammonium sulfate precipitates of U2 and of holoenzyme were then used for EPR spectroscopy at liquid helium temperatures and a well-resolved radical signal could be observed in both preparations.

The characteristic doublet spectrum presented in fig.1 is centered around $g = 2.0046 \pm 0.0003$. Its

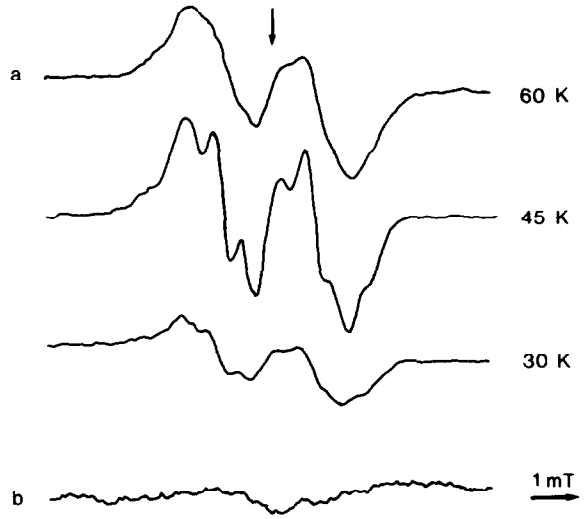

Fig.1. (a) EPR spectra of *S. obliquus* ribonucleotide reductase holoenzyme recorded at 30–60 K. (b) EPR spectrum at 45 K of an enzyme sample after incubation with 0.1 M hydroxyurea for 30 min. Identical spectra were obtained using purified subunit U2. EPR conditions: modulation amplitude, 0.32 mT; microwave power, 100 mW; scanning rate, 2.5 mT·min^{-1}; time constant, 0.5 s. The arrow indicates $g = 2.0046 \pm 0.0003$.

microwave saturation behaviour at 45 K is shown in fig.2. Both the hyperfine structure and the saturation curve of the radical signal in this algal

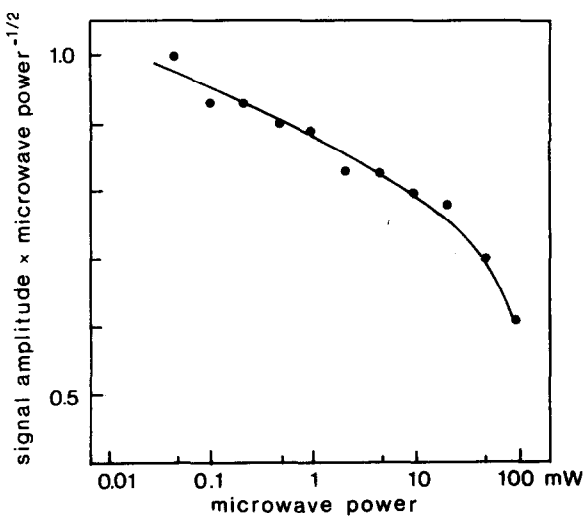

Fig.2. Microwave saturation curve for *S. obliquus* ribonucleotide reductase at 45 K.

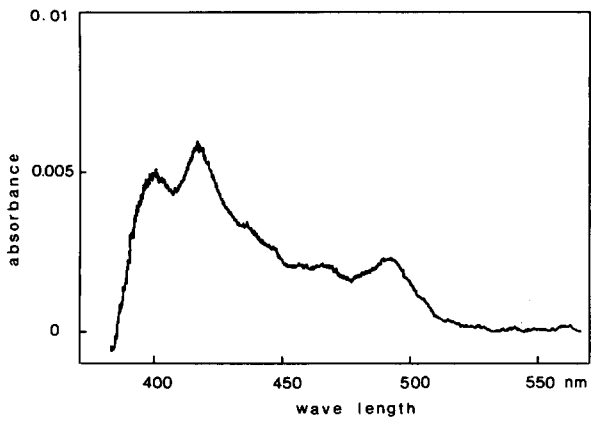

Fig.3. Difference spectrum of *S. obliquus* ribonucleotide reductase between identical enzyme samples containing native and hydroxyurea-treated protein. Conditions: 50 mM K^+ phosphate buffer, pH 6.7, containing 1 mM dithiothreitol, ± 0.1 M hydroxyurea; 20°C.

ribonucleotide reductase are very similar to those of the holoenzyme and subunit M2 found in overproducing mouse cells [4]. Enzyme activity and the EPR signal of the plant enzyme are rapidly lost in presence of 10–100 mM hydroxyurea (fig.1b), and are restored spontaneously upon aerobic incubation in the presence of 10 μM Fe^{2+} (not shown).

The organic free radical of mammalian and bacterial ribonucleotide reductase contributes to their electronic spectrum at 395 and 416 nm [18]. A difference spectrum between native and hydroxyurea-inactivated *S. obliquus* ribonucleotide reductase (fig.3) indicates absorption peaks at 400 and 417 nm in the radical-containing native algal protein. With the exception of an additional small peak at 482 nm, the difference spectra of the plant and the mouse enzymes again appear very similar.

Direct identification of the protein-bound free radical in *Scenedesmus* ribonucleotide reductase by incorporation of deuterium-labeled amino acids is not practicable in the autotrophic green algae. However, our present results ascertain that the catalytic subunit U2 of the algal protein contains a tyrosine radical like those identified in mammalian, *E. coli* and phage T_4-encoded enzymes. Unlike the diversity of ribonucleotide reductase systems found in bacteria and archaebacteria [19], animal and plant cells have a great number of similarities in cell cycle control and mechanisms of deoxyribonucleotide biosynthesis [20,21]. The identity of the EPR signals, and the spontaneous generation of the free radical in mouse and algal ribonucleotide reductase discussed above confirm that eukaryotes possess one common enzyme apparatus for DNA precursor formation.

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