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Detection of a Stable Free Radical in the B2 Subunit of the Manganese Ribonucleotide Reductase (Mn-RRase) of *Corynebacterium ammoniagenes*

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Ribonucleotide reductases catalyze the irreversible reductive formation of 2'-deoxyribonucleotides required for DNA replication and cell proliferation, and a radical mechanism was assumed to be involved in this reaction. In order to search for a radical in the aerobic manganese ribonucleotide reductase (Mn-RRase) by electron paramagnetic resonance (EPR) the native metal-containing 100 kDa B2 subunit was deliberately prepared from the wild type strain *Corynebacterium ammoniagenes* ATCC 6872. Enrichment by 2'5'-ADP Sepharose 4B affinity chromatography, fast protein liquid chromatography (FPLC) with Superose^{TM}12 and concentration by vacuum evaporation allowed for the first time the detection of a stable free radical by EPR spectroscopy at 77 K. The EPR spectrum exhibits an easily saturable doublet of 1.8 mT splitting and a line width of 1.3 mT at g = 2.0040. The EPR signal intensity showed a clear correlation with the enzymatic activity upon long-time storage at ambient temperature (294 K) and inactivation by the specific RRase inhibitor hydroxyurea (HU). This leads to the assumption of a protein-linked radical, with functional significance, in the metal-containing 100 kDa B2 subunit of the Mn-RRase of *Corynebacterium ammoniagenes*.

Key words: manganese ribonucleotide reductase, *Corynebacterium ammoniagenes* ATCC 6872, protein-linked radical, hydroxyurea, electron paramagnetic resonance

INTRODUCTION

From a biochemist's viewpoint the RRases (EC 1.17.4.1) are a unique class of enzymes due to the diversity of metal catalysis[1] and because they were the first enzymes shown to have a stabilized free radical in the active enzyme involved in the reaction mechanism.[2] The reduction of a ribonucleotide to a deoxyribonucleotide is a chemically difficult reaction and is initiated generally by an

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organic free radical. Surprisingly this is done in different ways in different ribonucleotide reductases.[3] Recently the crystallographic structures of both subunits of the iron ribonucleotide reductase (Fe-RRase) of *Escherichia coli* have been made available.[4,5] It has been proposed that the actual nucleotide reduction in this enzyme occurs on the R1-subunit using the tyrosyl radical of the R2-subunit as a radical chain initiator and communication between subunits being affected by a specific long-range electron transfer pathway within the holoenzyme.[6,7]

The Mn-RRase of *Corynebacterium* (formerly *Brevibacterium*) *ammoniagenes* has a number of characteristics which may at a first view allow to consider it as a manganese analogue of the well-studied *E. coli* Fe-RRase, viz., substrate specificity, sensitivity to HU, and dimeric ($a\beta_2$) subunit structure.[8] Thus, sample preparation in our laboratory for radical detection in the Mn-RRase was designed initially[9] under the assumption that an easily detectable radical would be present in analogy to the tyrosyl radical of the *E. coli* Fe-RRase which is stable for weeks[10] at 4°C. Although a conspicuous EPR signal was recorded at g=4 in crude extracts of *C. ammoniagenes*, this disappeared on further purification. Additionally, the detection of any radical signal at g=2.00 where organic radicals are usually centered was impaired by the presence of free Mn^{2+} ions displaying the characteristic sextet of the manganо-(II)-hexaqua-complex. Extensive dialysis with 2 mM EDTA of a crude Mn-RRase fraction, precipitated with 40 to 60% ammonium sulfate, did not allow the detection of a radical signal. Even enriched B2 subunit from 2′,5′-ADP Sepharose 4B affinity chromatography and additional Sephadex G-100 gel filtration did not display a radical signal. Further experiments (either biochemical complementation with the catalytic B1 subunit to the Mn-RRase holoenzyme or addition of ribonucleotide substrates) failed to generate a detectable organic radical.[9]

This paper describes EPR evidence for an organic radical linked to the metal-containing B2 subunit of the Mn-RRase obtained by fast protein liquid chromatography.

MATERIALS AND METHODS

Chemicals

2′,5′-ADP Sepharose 4B and Superose™12 were obtained from Pharmacia LKB (Freiburg, FRG). [5-^{3}H] CDP, ammonium salt (0.37–1.1 TBq/mmol, 10–30 Ci/mmol) and [8-^{3}H] GDP, ammonium salt (370–550 GBq/mmol, 10–15 Ci/mmol) were purchased from Amersham-Buchler (Braunschweig, FRG). Visking® dialysis tubes were obtained from Serva Feinbiochemica GmbH & Co. KG (Heidelberg, FRG).

Bacteria and Growth Conditions

Corynebacterium (formerly *Brevibacterium*) *ammoniagenes* ATCC 6872 which is a member of the high-GC-branch of the Gram-positive bacteria and exploited for industrial nucleotide fermentation[8] was the source of our enzyme preparation. This wild type strain had previously been used for the purification of the Mn-RRase holoenzyme.[11] Strain ATCC 6872 was grown in seed medium[12] at 27°C in a 14 l fermentor with an aeration of 4 l/min and an agitation of 250 r. p. m. using a Fermentor Drive Assembley (New Brunswick Scientific Co. Inc. NJ., USA). Because Mn-RRase (as a cell-cycle controlled enzyme) is present only in proliferating cells and previous investigations had shown highest activity of the Mn-RRase at the late logarithmic phase of the growth curve[13] *C. ammoniagenes* was grown for 10 hs and harvested at an optical density at 578 nm of 20.

Assays for Determination of Enzymatic Activity for Ribonucleotide Reduction

Ribonucleotide reduction was assayed with CDP (GDP) as (radioactive) substrate and dATP (TTP) as positive effector and the reaction products were separated by HPLC as described previously.[11] In

order to determine the activity of the B2 subunit after deliberate dissociation of the holoenzyme into subunits (see below) a biochemical complementation assay was carried out by mixing suitable amounts of the B1 and the B2 subunit. For the EPR-experiment in Figure 5b the free B2 subunit was preincubated on ice for 5 minutes in the presence of increasing concentrations of HU and then assayed as usually. Radioactivity was determined using a Wallac 1410 liquid scintillation counter (Pharmacia LKB (Freiburg, FRG) after mixing of aliquots eluted by HPLC with 10 ml of a scintillation cocktail (Quicksafe A, Zinsser Analytik GmbH, Frankfurt, FRG). Protein was determined according to Bradford[14] using BSA as standard.

Preparation of the B2 Subunit

All enzyme procedures were carried out at 4°C as summarized in the scheme of Figure 1. Basically the preparation of partially purified native B2 subunit followed a previously described protocol.[11] However, to obtain enzyme solutions with higher concentrations the following modifications were necessary: (I) Wet cells (30 g), suspended in 45 ml of 0.1 M HEPES buffer pH 6.6, containing 2 mM dithiothreitol (DTT) were pre-treated with a Branson B_{12}-sonifier (6 × 20′ at 80 W, macrotip). The final cell disrupture was achieved by a threefold passage through a french pressure cell (40 ml, SLM Instruments, Inc., Urbana, USA) at 1500 psi. (II) The Mn-RRase activity was enriched from crude extract by fractionated (40–60%) ammonium sulfate precipitation. (III) 3 ml (120 mg total protein) of an enriched Mn-RRase fraction were twice applied to 2′, 5′-ADP Sepharose 4B affinity chromatography (column 15 × 120 mm, 5 g) for deliberate separation of the subunits by specific retention of the B1 subunit. The initial conventional gel filtration on Sephadex G-100 (30 × 900 mm) with self-packed columns and the ultrafiltration with an Amicon-cell (PM 10 filter, Amicon GmbH, Witten, FRG) was carried out as described previously.[9]

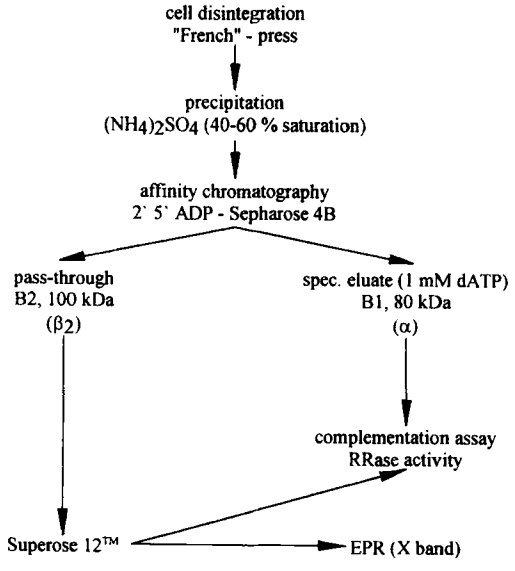

FIGURE 1 Flow scheme of FPLC purification and assay of the radical-containing B2 subunit of the Mn-RRase of *C. ammoniagenes* for EPR spectroscopy.

For FPLC the B2 subunit and the other proteins which eluted from the 2′5′ ADP Sepharose 4B column (16 × 200 mm) with 20 mM potassium phosphate buffer, containing 2 mM DTT, pH 6.6, were concentrated four-fold by vacuum-evaporation (Genevac SF 50, Biometra, Göttingen, FRG) to a final protein concentration of 20 mg/ml and applied to the gel filtration column Superose™12 (HR 10/30), equilibrated with 85 mM potassium phosphate, containing 2 mM DTT, pH 6.6. For FPLC a flow rate of 0.3 ml/min was used and 3 min-fractions were collected. The chromatography, fraction collection and recording of column runs were controlled automatically (gradient programmer GP-250 Plus, Pharmacia LKB Freiburg, FRG). The Superose™12 column was calibrated with reference proteins of known molecular weight; 158 kDa: aldolase, 45.5 kDa: ovalbumin, 35 kDa: β-lactoglobulin, and 12.3 kDa: cytochrome C (Pharmacia). The three fractions containing the 100 kDa dimeric B2 subunit were immediately identified by biochemical complementation with the B1 subunit for recovery of the enzymatic activity. To each complementation assay 36 μg B1 eluted from the 2′5′ ADP Sepharose 4B affinity column were routinely added. The B2-containing fractions (no. 8–10, Figure 2) of 15

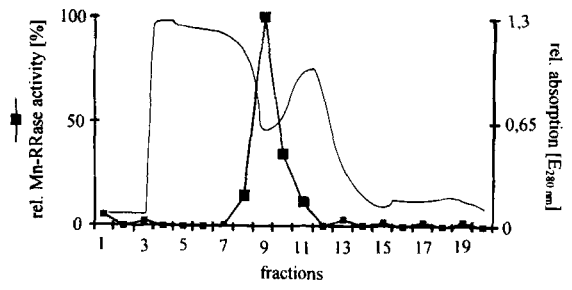

FIGURE 2 Superose™12-elution profile (solid line) and identification of the B2 subunit of Mn-RRase of *C. ammoniagenes* by complementation assay (—■—) as described in Materials and Methods.

repeated column runs were pooled and concentrated as above to yield a final volume of 2 ml with a concentration of 4 mg total protein per ml. This protein solution was dialyzed against 8.5 mM potassium phosphate buffer (pH 6.6) containing 0.2 mM DTT for 2 hours and then tenfold concentrated as above.

Removal of Unspecific Mn^{2+} Ions for EPR Sample Preparation

In order to avoid any traces of soluble Mn^{2+} buffer solutions were prepared with water from reversed osmosis only (3 module E-pure D4632 240 VAC Barnstead, Dubuque, IA, USA) using plastic beakers rinsed with 2 mM EDTA. All columns used for chromatography were first rinsed with 2 mM EDTA (threefold column volume) and then equilibrated with the above potassium phosphate buffers. Protein samples from each purification step were dialyzed first against buffers with and then without 2 mM EDTA (1 h, hundredfold volume).

EPR Spectroscopy

The fractions of B2 with the highest available concentration were transferred into 3 mm-quartz glass tubes (Spintech, No. 707-SQ) to yield a final volume of 177 µl containing 8 mg total protein (filling height of 2.5 cm). EPR spectra were recorded at 77 K using a quartz finger cryostat with an X-band EPR spectrometer (Bruker ESP300E) with 1 mW microwave power and 0.5–1 mT modulation amplitude. Microwave saturation has been measured at 77 K with 1 mT modulation and microwave power from 1 µW to 100 mW.[15] Spin concentrations were determined by double integration of the first derivative EPR spectra of the sample and comparison with a reference (gift from A. Gräslund, University of Stockholm) containing the R2 subunit of the aerobic *E. coli* RRase with a known concentration (102 µM) of tyrosyl radicals.

RESULTS

Because our initial trials failed to detect a radical in the Mn-RRase it was concluded that the postulated radical was less stable than originally expected and that the chromatographic procedures applied for sample preparation were too slow leading to an actual spin concentration below the detectability of EPR.

Therefore, we decided to examine samples of the B2 subunit enriched by *fast* protein liquid chromatography. (The Mn-RRase holoenzyme consists of the monomeric 80 kDa catalytic subunit B1 and the dimeric (2 × 50 kDa) metal-containing B2 subunit[11]). When the B2-containing pass-through from the 2',5'-ADP Sepharose 4B column (cf. flow scheme Figure 1) was submitted to Superose™12 gel filtration, the B2 subunit of the *C. ammoniagenes* Mn-RRase eluted as a 100 kDa protein as expected. The B2-containing fractions were shown to reduce ribonucleotides (Figure 2) when complemented with the B1 subunit as described in Materials and Methods. The specific activity in the peak fraction (no. 9) was determined as 53 nmol × mg^{-1} × h^{-1}. The observed capability for ribonucleotide reduction confirmed that we were still dealing with the native B2 subunit suspected to contain an organic radical necessary for the enzymatic activity of the holoenzyme. With respect to B2 the pooled material from our

FPLC procedure was seventyfivefold enriched over the crude extract and the enrichment protocol reproducibly led to a protein solution containing a fraction of roughly 15 to 20% native B2 subunit referred to the value reported by Willing *et al.*[11] for the specific activity of the purified holoenzyme (273 nmol × mg^{-1} × h^{-1}). Thus, a sample concentration of approximately 100 μM B2 subunit for the first time allowed the detection of an EPR signal from an organic radical of the *C. ammoniagenes* Mn-RRase as described below.

The EPR spectrum recorded at 77 K is characterized by a doublet splitting of 1.8 mT and a line width of 1.3 mT with g = 2.0040 (Figure 3). This EPR signal is easily saturable; the microwave saturation curve exhibits a half-saturation at 0.5 mW at 77 K (Figure 4) indicating to a free radical. The spin concentration of this EPR signal was determined as to 13 μM using the R2-subunit of the aerobic *E. coli* RRase with a known concentration of tyrosyl radicals as a reference. In order to confirm that the EPR signal was functionally correlated with the native B2 subunit of the Mn-RRase we used two approaches of kinetic experiments concerning a correlation of EPR signal intensity with the biochemical activity of B2: (I) long-term storage, and (II) addition of increasing amounts of

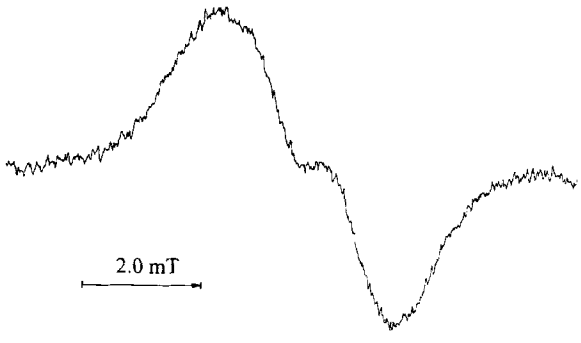

FIGURE 3 EPR spectrum of the B2 subunit of Mn-RRase of *C. ammoniagenes* at 77 K. Modulation amplitude 0.5 mT; microwave power: 1 mW; sweep time 5.6 min; time constant: 0.6 s). The spin concentration was estimated by double integration as 13 μM using a standard (R2 from *E. coli*). The concentration of the native B2 subunit in this sample was calculated as 100 μM based on the determination of both the total protein concentration and the enzymatic activity for ribonucleotide reduction by biochemical complementation as described in Materials and Methods.

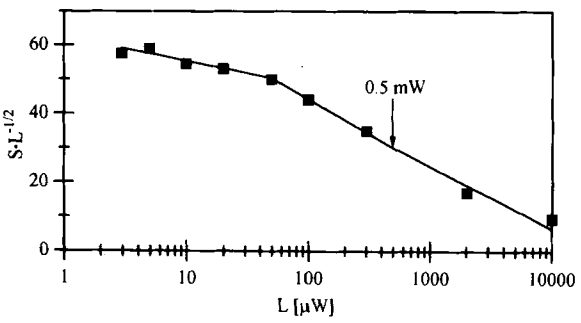

FIGURE 4 Microwave saturation behaviour of the EPR signal of the B2 subunit of Mn-RRase of *C. ammoniagenes* at 77 K. Modulation amplitude 1 mT. S = amplitude of the EPR signal (maximum to minimum) according to Fig. 3; L = microwave power.

the inhibitor HU. The results are shown in Figure 5. At room temperature, the signal intensity of the radical and the activity of B2 for ribonucleotide reduction (when complemented with B1) decreased to 50% within the first one and a half hours. This indicates to a rather stable radical (1.5 hs at room temperature), i.e., a stable free radical linked to a protein. The comparison of the slowly decreasing (enzymatic) activity of B2 with time and the change of the amplitude of the EPR signal at 294 K revealed that catalytic activity and EPR signal are correlated (Figure 5a). The obviously biphasic kinetics might be explained by a superimposition of a rapid rate of radical decay and a slow rate of aerobic radical reactivation, less pronounced in the initial phase. Reactivation of the catalytic activity of the holoenzyme from inhibition by HU proceeds only in the presence of oxygen.[11] Here, the progressive inactivation of B2 in the presence of HU was again closely correlated to the decrease of the EPR signal in B2 (Figure 5b). A 50% reduction of both the EPR signal intensity and the catalytic activity of B2 required 1.4 mM and 1.6 mM HU, respectively.

DISCUSSION

The delivery of the deoxyribonucleotide building blocks for DNA chain elongation by ribonucleotide reduction is generally assumed to require a

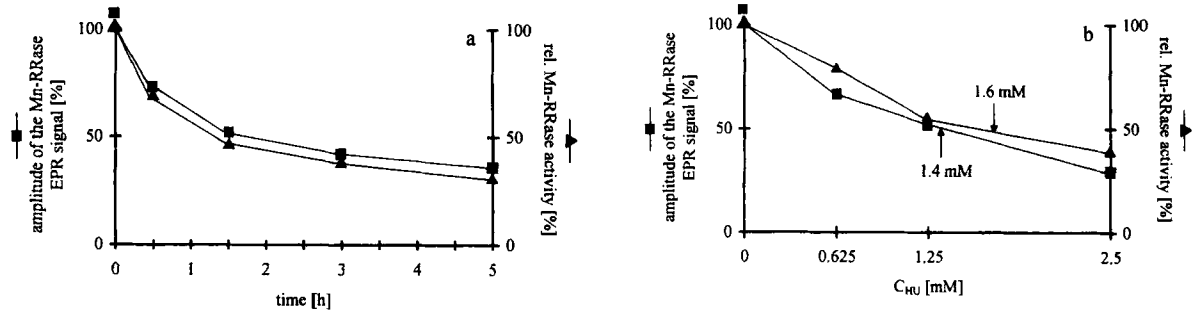

FIGURE 5 Kinetics of inactivation of the protein radical (—■—) and the enzymatic activity (—▲—) of the B2 subunit of the Mn-RRase at room temperature (a) and in the presence of increasing concentrations of hydroxyurea (b). The enzymatic activity of B2 was assayed by biochemical complementation as described in Materials and Methods. The relative enzymatic activity was normalized to 100% for the untreated sample. The EPR spectra were recorded at 77 K after incubation of the B2 subunit at room temperature for different time intervals and subsequent freezing. The relative radical concentration was estimated from the amplitude of the doublet signal and normalized to 100% for the untreated sample.

radical mechanism.[3,16,17] Although the ribonucleotide reductase of *C. ammoniagenes* is sensitive to the radical scavenger HU and inactivated enzyme solutions recover nearly to full activity when incubated aerobically,[11] no EPR signal of an organic free radical was detected at 77–113 K in the holoenzyme of the Mn-RRase thus far. Nevertheless, the existence of a stable tyrosyl radical in the smaller subunit of the Fe-RRase from *E. coli* suggested the presence of an organic radical in the smaller subunit of the Mn-RRase as well. Considering the facile dissociation of the enzyme from *C. ammoniagenes* resulting in substantial loss of activity during purification, the strategy here was based on deliberate dissociation of the subunits under controlled conditions by 2′, 5′-ADP Sepharose 4B affinity chromatography followed by selective chromatographic enrichment of the metal- and radical-bearing B2 subunit through gel filtration chromatography.

Although this work has shown that a stable free radical exists in the Mn-RRase, its demonstration required extreme efforts because no system for overexpression is available for this enzyme. Thus far, presence of a protein-linked organic radical has been reported for few metal-containing ribonucleotide reductases only (Table 1). The half-life of the radical in the Mn-RRase is distinctly shorter

TABLE 1 Radical stability of microbial metal-containing ribonucleotide reductases

source of protein	metal center	radical half-life (ambient temperature)	reference
E. coli R2*	Fe	days	10
E. coli mutants* (hydrophobic pocket near Y122)		minutes or seconds	19
yeast R2*	Fe	2 hours	18
Corynebacterium ammoniagenes B2	Mn	1.5 hours	this work

*Expression system available

than in the Fe-RRase from *E. coli* but comparable to that of the Fe-RRase in baker's yeast (*Saccharomyces cerevisiae*).[18] However, the enzyme activity in baker's yeast is high enough for radical detection in a rapidly purified sample (precipitate with 40% ammonium sulfate) whereas the enzyme activity of the Mn-RRase in the *C. ammoniagenes* wild type strain was too low for this approach.

Our observation was that the initial experiments had shown a trend towards decreasing the specific activity of the B2 subunit with increasing protein concentration. This indicated the failure of conventional Sephadex G-100 gel filtration chromatography to maximize the concentration of both the native B2 subunit and the postulated radical. Gel filtration with pre-packed Superose™12 columns greatly improved the reproducibility and led to a steeper elution profile. Thus, more enriched pools of the B2 subunit were provided from the same amount of crude protein. However, in the light of the limited half-life (1.5 h) of the organic radical of the *C. ammoniagenes* Mn-RRase (Figure 5a) any acceleration of the biochemical sample preparation clearly would increase the probability of its EPR detection. The FPLC procedure used here reduced the time required for elution of the B2-containing fraction from four hours to fourty minutes. Likewise, it appeared as an important improvement to concentrate the protein solutions by vacuum evaporation throughout due to the high loss of activity (more than 30%) during the ultrafiltration procedure, applied initially. Nevertheless, even the improved procedure for the preparation of the highly enriched B2 sample required roughly forty hours from cell disruption to the frozen EPR sample. This may explain why the spin concentration observed for the protein-linked radical (13 μM) is distinctly lower than the concentration of the B2 subunit (100 μM) and thus deviating from expected stoichiometry.

The central message reported here is that the EPR signal intensity in and the biochemical activity of the smaller subunit of the Mn-RRase are linked closely together. However, B2 alone (even when conserved in its native state) is not enzymatically active. Therefore measurements of its capability for ribonucleotide reduction require a biochemical complementation with B1. Thus all our conclusions depend on the reliability of the latter assay. The general difficulty with this kind of assays is that the subunits have to be delivered in the state and stoichiometric amounts as present in the enzymatically active holoenzyme. Our finding that similar concentrations of HU were required to inhibit the activity for ribonucleotide reduction by 50% regardless whether the holoenzyme[11] or B2 (present paper) were assayed, clearly confirmed the reliability of our approach.

Our EPR studies at 77 K reveal the existence of an organic radical in the smaller B2 subunit of the Mn-RRase as concluded from the g-factor of 2.0040 and the easy saturation at 0.5 mW. This EPR signal is not detectable in a comparable fraction of nonfunctional apo-B2 prepared from manganese-depleted cells (Griepenburg and Laßmann, unpublished results). Clearly, a catalytically essential radical has been demonstrated in the B2 subunit of the *C. ammoniagenes* ribonucleotide reductase because the EPR signal intensity is strictly proportional to the enzymatic activity as both decrease in parallel upon long-time storage as well as after treatment with the common radical scavenger hydroxyurea.

This is the first report on a functional competent radical in the Mn-dependent RRase, the third class of aerobic RRases[16,17] or the fourth class of RRases in the view of Reichard,[3] e.g. a radical enzyme using Mn-catalysis. It is reasonable to assume that the radical is protein-linked because its signal intensity is strictly correlated to the catalytic activity for ribonucleotide reduction. The elucidation of the type and site of radical remains open and needs further spectroscopic studies with higher radical content, eventually the use of deuterated amino acids and site-directed mutants, respectively as well as higher resolving EPR techniques (Q-band or ENDOR). The ability of hydroxyurea to quench the radical in Mn-RRase and the line shape observed (1.8 mT-doublet) may probably indicate to a tyrosyl radical similar to the

situation known from the Fe-RRases of *E. coli*, mammalia, and herpes-virus.[20,21] However, in recent spectroscopic studies further types of protein-linked radicals were reported which also exhibit EPR doublet spectra with a comparable splitting of about 1.8 mT, e.g. tryptophanyl radicals in the *E. coli* mutant R2-Y122F,[22] and glycinyl radicals in pyruvate formate lyase[23] and in the anaerobic *E. coli* RRase.[24]

Finally, the saturation behaviour of the radical in the B2 subunit of the Mn-RRase of *C. ammoniagenes* indicates to a weaker magnetic metal interaction than in the Fe-RRase of *E. coli*. The half-saturation recorded here at 77 K (0.5 mW) is lower than observed in the Fe-RRase of *E. coli*[25] (about 10 mW at 77 K) where the phenoxyl oxygen of the tyrosyl radical at Y122 in the R2-subunit exhibits a distance of 5 Å to the closest of the two antiferromagnetically coupled iron (III) atoms.[4] In another protein (lysine 2,3-aminomutase) the saturation of an EPR signal (1.8 mT) at microwave power of 5 mW was also traced back to a significant magnetic metal interaction.[26] Beside the distance between a protein-linked radical and a paramagnetic metal site the spin-state of the metal is essential for the magnetic interaction. Thus, the type of the Mn-complex of the Mn-RRase and its spin-state which is under current investigation will be described elsewhere (Blasczyk and Auling, unpublished results).

Acknowledgements

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