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Electron paramagnetic resonance (EPR) spectroscopy of the stable-free radical in the native metallo-cofactor of the manganese-ribonucleotide reductase (Mn-RNR) of *Corynebacterium glutamicum*

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Abstract
Ribonucleotide reductases (RNR; EC 1.17.4.1) provide the 2′-deoxyribonucleotides for DNA replication of proliferating cells by a uniform radical mechanism using diverse metals. The native metallo-cofactor of the C*orynebacterium glutamicum* RNR contains manganese and is sensitive to EDTA and radical scavengers. Hybrid holoenzymes, capable of ribonucleotide reduction, were composed of the small manganese-containing (R2F) and the large catalytic subunit (R1E) from either of the two corynebacterial RNRs. A synthetic peptide deduced from the C-terminal region of the *nrdF* gene inhibited the *C. glutamicum*-RNR non-competitively and cross-reacted with the *C. ammoniagenes*-RNR. The *C. glutamicum*-R2F has a saturable organic radical signal at $g = 2.005$ detected by electron paramagnetic resonance (EPR) spectroscopy and shows a distinct absorption at 408 nm indicative of a tyrosyl-like organic radical (Y·). Quantification of the metal content revealed 0.06 mol Fe but 0.8 mol Mn per mol R2F-monomer and would thus assign two manganese atoms bound to the dimeric metallo-cofactor, while a distinct enzymatic activity ($32\ \mu mol \times mg^{-1} \times min^{-1}$) was observed in the biochemical complementation assay. Divergence of the *C. glutamicum*-RNR studied here from the prototypical *Salmonella typhimurium* class 1b enzyme and the *Chlamydia trachomatis* class Ic enzyme is discussed below.

Keywords: Corynebacterium glutamicum, *manganese ribonucleotide reductase, tyrosyl radical, electron paramagnetic resonance*

Abbreviations: *RNR, ribonucleotide reductase; R1E, large catalytic; R2F, small subunit of the RNR from* C. glutamicum *and* C. ammoniagenes*; Y·, tyrosyl radical; HU, hydroxyurea;* nrd, n*ucleotide* r*eduction; R1, large catalytic, R2, small subunit of the class I Fe-RNR; CG1, CG2 = R1E and R2F of* C. glutamicum*; CA1, CA2 = R1E and R2F of* C. ammoniagenes*; ICP-MS, inductively coupled plasma-mass spectrometry; ATCC, American Type Culture Collection; I_{50} = value of 50% inhibition; TTP, thymidine triphosphate*

Introduction

The biochemical evolution from the ancient RNA world to the present-day DNA world created an universal *de novo* route of DNA precursor biosynthesis [1,2] which is potentially the rate limiting step in DNA replication. The key enzymes are ribonucleotide reductases (RNR) which catalyse the irreversible deoxygenation of ribonucleoside 5′- di- or triphosphates to the corresponding 2′-deoxyribonucleotides concomitant with the oxidation of a pair of sulphydryl groups to disulphide in thioredoxin or other small proteins with redox-active thiols [3]. Two redox

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equivalents, in most cases delivered from NADPH, are consumed for each substrate reduction and an organic free radical is required to activate the substrate before reduction. Expression of RNR-encoding genes occurs only in proliferating cells congruent with the central role of these enzymes in DNA formation and nucleotide metabolism. The same principle of allosteric regulation exists in all RNR and a monophyletic origin of ribonucleotide reduction was suggested first by Follmann [1]. On the other hand, trace elements like Co, Fe and Mn control growth and DNA formation in different prokaryotic and eukaryotic organisms [2,4,5]. These phenomena were traced back to a diverse array of metallo-cofactors which generate the transient radical for initiation of nucleotide reduction by RNR and serve for classification of these enzymes [6].

An early described prototype of manganese-containing enzymes [7] is the RNR of *Corynebacterium ammoniagenes* [8–10] which is encoded by *nrdEF* genes [11,12]. Highly similar *nrdEF* genes were cloned from the related species *C. glutamicum* [13]. However, the metal specificity cannot be delineated merely from DNA sequence. This paper presents the first biochemical characterization of the *C. glutamicum*-RNR with a focus on the manganese metallocofactor and its stable-free radical.

Materials and methods

Bacterial strains and culture conditions

The strains *Corynebacterium glutamicum* R163 [14], a derivative of the wild type strain ATCC 13032, and *Corynebacterium ammoniagenes* ATCC 6872 were grown in a complex medium (seed medium) at large scale according to Griepenburg et al. [15]. Strain R163 has wild type characteristics with respect to the *nrd*-genes.

Chemicals

2′,5′-ADP Sepharose 4B Superose 12, UNO^{TM} Q12 and phenylsuperose were obtained from Pharmacia LKB (Freiburg, Germany). [5-^{3}H]CDP, ammonium salt (10–30 Ci/mmol) and [8-^{3}H]GDP, ammonium salt (10–15 Ci/mmol) were purchased from Amersham-Buchler (Braunschweig, Germany). Visking® dialysis tubes were obtained from Serva Feinbiochemica GmbH & Co., KG (Heidelberg, Germany).

Assays of RNR activity

Ribonucleotide reduction was assayed in cell-free extracts with tritium-labelled CDP (GDP) as substrate and dATP (TTP) as positive effector according to Willing et al. [8] and the reactions products were separated by HPLC as modified by Plönzig and Auling [16]. Assaying the activity of sub-units resulting from either spontaneous or deliberate dissociation of the holoenzyme required biochemical complementation. For this purpose appropriate amounts of the large catalytic and the small (metal- and radical-containing) sub-unit were mixed and the capability to reduce ribonucleotides was assayed as described above. Basically the same procedure was used when hybrid holoenzymes were assayed for the restoration of the capability to reduce ribonucleotides. Inactivation of the enriched R2F protein (see below) was examined by a 10 min-preincubation at 10°C in the presence of increasing amounts of hydroxyurea, *p*-methoxyphenol or EDTA and subsequent biochemical complementation as above.

Preparation of RNR sub-units

In order to demonstrate heterologous biochemical complementation sub-units of corynebacterial RNR were obtained by affinity chromatography using 2′,5′-ADP Sepharose 4B (cf. flow scheme depicted in Figure 1 of Griepenburg et al. [15]). For molecular mass determination the *C. glutamicum* R2F was further enriched from the pass-through of the affinity chromatography by hydrophobic interaction chromatography using a Phenyl Superose HR 5/5 column (Pharmacia LKB, Freiburg, Germany). Prior to loading the protein was adjusted to 1.5 M ammonium sulphate in 85 mM potassium phosphate buffer with 2 mM DTT (pH 6.6) and eluted to zero with a 10 mL-gradient (gradient programmer GP-250 Plus, Pharmacia LKB, Freiburg, Germany). The three R2F protein-containing fractions were pooled for application to a Superose^{TM} 12 column, calibrated with reference proteins and molecular mass was determined exactly as described by Griepenburg et al. [15]. For inhibition studies the *C. glutamicum* R2F was further enriched from the pass-through of the affinity chromatography by Superdex G-200 gel filtration (flow rate: 0.8 ml/min, 2 ml fractions). The R2F protein-containing fraction nos 15–19 were

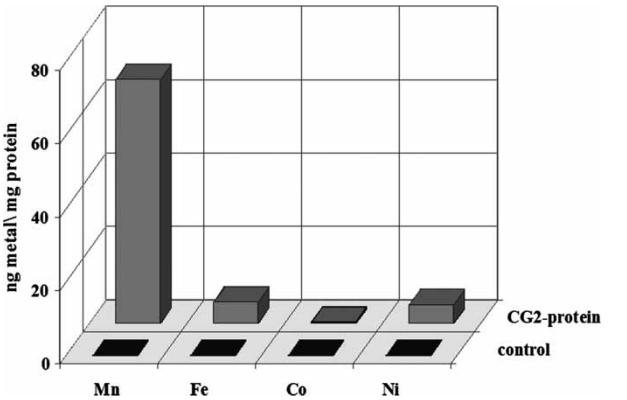

Figure 1. Metal content of the *C. glutamicum*-RNR. R2F of *C. glutamicum*, purified by gel filtration with Superdex G-200 and assayed by biochemical complementation, was analysed by ICP-MS as described by Griepenburg et al. [10].

pooled and concentrated with an Amicon-cell (PM 10 filter).

Synthetic heptapeptide and assay of its inhibitory action

The peptide Ac-EDDDWDF was obtained from SYNTEM (Nimes, France). In order to study its inhibitory effect on corynebacterial RNR the R1E protein of either *C. glutamicum* or *C. ammoniagenes* was pre-incubated in the presence of increasing concentrations of the synthetic peptide for 10 min at 0°C. Ribonucleotide reduction was assayed by biochemical complementation adding the respective R2F at 30°C as described above. Enzyme activity in the absence of the synthetic peptide was set as 100% and the value of 50% inhibition designated as I_{50}.

R2F preparation for detection of the radical signal

The active fraction from ammonium sulphate precipitation (40–60%) was dissolved in standard buffer (85 mM potassium phosphate buffer, pH 6.6 and 2 mM DTT) and the ionic strength was reduced by a 2-h dialysis against the same buffer. The resulting protein solution was applied to an UNO^{TM} Q12 column in buffer A (85 mM potassium phosphate buffer, pH 6.6, 2 mM DTT, 2 mM magnesium acetate). Unbound protein was isocratically eluted with 36 ml of buffer A. For enrichment of RNR holoenzyme a three-step linear gradient was applied: 48 ml of 10%, 36 ml of 35% and 48 ml of 65% buffer B (85 mM potassium phosphate buffer, pH 6.6, 2 mM DTT, 2 mM magnesium acetate and 1.5 M KCl). Finally, the column was re-equilibrated with 100% buffer A prior to application of new protein. The flow-rate was kept constant at 1.6 ml/min and 6 ml-fractions were collected. The active fractions eluted at 35% KCl were pooled, submitted to ammonium sulphate (70%) precipitation, concentrated by centrifugation (30 min, 40000 × g), dissolved and dialysed in buffer A as above and stored at −80°C prior to EPR measurements.

For detection of the UV/visible radical signal eluates from size exclusion chromatography (Superdex^{TM} 200, 16/60 prep grade, using 85 mM potassium phosphate buffer, pH 6.6, fractions in the range between 45–30 kDa) were pooled and submitted to a final anion exchange chromatography.

EPR spectroscopy

Freshly prepared partially purified ribonucleotide reductase (300 µl, 40 mg protein/ml) was transferred to EPR-tubes (Ilmasil-PN high purity quartz, 4.7 ± 0.2 mm outer diameter, 0.45 ± 0.05 mm wall thickness, 13 cm length, obtained from Quarzschmelze Ilmenau GmbH, Langewiesen, Germany) and immediately frozen and stored in liquid nitrogen. EPR spectra were recorded with an EMX-6/1 X-band spectrometer (Bruker, Karlsruhe, Germany) with a standard TE102 rectangular cavity and a variable temperature ESR-900 helium flow cryostat (Oxford instruments, Oxford, UK). Data acquisition was done with the software supplied by Bruker (WINEPR, version 2.3.1), data manipulation (determination of *g*-values, subtraction, baselining, integration and conversion to ASCII files for use with Microsoft EXCEL) was done with the WINEPR program version 2.11. For *g*-value determination the microwave frequency was measured with the built-in ER-041-1161 counter. The minor offset of the magnetic field as measured by the EMX-032T Hall probe was corrected using a strong pitch standard ($g = 2.0028$). A solution of 10 mM $CuSO_4$ in 2 M $NaClO_4$ and 10 mM HCl was used as standard for spin integration. Further EPR conditions are given in the legend to the Figure 4.

Other methods

Manganese and other transition metals of the R2F protein were determined by ICP-MS as described by Griepenburg et al. [10]. In the holoenzyme, enriched by UNO^{TM} Q12 anion exchange chromatography, the iron content was determined spectroscopically using the phenantroline method in comparison with a Fe standard (Merck Darmstadt), the Mn content by oxidation to MnO_4^- according to Jander et al. [17] and the UV/vis spectrum (380–450 nm) was recorded at 10°C in an Ultrospec^{TM} 3300pro (Amersham Biosciences) using quartz cuvettes (Suprasil®, Hellma, Mülheim, Germany). Protein concentrations were determined according to Lowry et al. [18].

Results

The RNR of C. glutamicum is a manganese enzyme and enzymatically active hybrid holoenzymes can be composed with the C. ammoniagenes Mn-RNR

From available DNA sequences [11–13] of the related bacterial species *C. ammoniagenes* and *C. glutamicum* similar corynebacterial RNR would be expected, i.e. holoenzymes with a large catalytic (R1E) and a small metal- and radical-containing sub-unit (R2F). In order to determine whether the metallo-cofactor of the *C. glutamicum*-RNR contains manganese or iron its R2F protein was prepared by affinity chromatography and gel filtration. A high manganese content was found by ICP-MS, whereas the level of iron was close to that of the buffer control (Figure 1). The molecular mass of the R2F protein was determined to 34 kDa ± 5 kDa matching the 38 kDa-value calculated for the 334 amino acid residues derived from its *nrdF* gene.

A specific heptapeptide corresponding to the C-terminus of the small sub-unit of the RNR of *C. glutamicum* was synthesized. This heptapeptide

distinctly inhibited the activity of the *C. glutamicum*-RNR when the concentrations of both sub-units (R1E and R2F) were held constant while concentrations of the CDP substrate and heptapeptide were varied. Double-reciprocal plots of velocity vs total CDP at five levels of heptapeptide yielded a family of lines intersecting on the ordinate (Figure 2). The pattern is consistent with a non-competitive type of inhibition. For a test of functional similarity the Mn-RNR of *C. ammoniagenes* was also assayed with the C-terminal *C. glutamicum*-R2F heptapeptide. A distinct cross-inhibition albeit at a reduced level (I_{50}-value: 37 μM vs 94 μM) was observed.

An additional proof for similarity of the two corynebacterial RNRs was to generate hybrid enzymes by heterologous biochemical complementation. For this purpose, large and small sub-units of the two corynebacterial RNRs were deliberately separated by affinity chromatography with 2′, 5′-ADP Sepharose 4B and combined to yield heterologous holoenzymes. Remarkably, these hybrid enzymes were capable to reduce ribonucleotides (Figure 3). Compared to the value from homologous complementation the highest activity was observed when R2F of the *C. glutamicum*-RNR was complemented with R1E of the *C. ammoniagenes*-RNR.

The radical in the metallo-cofactor of the C. glutamicum-RNR

It was of interest to examine if the enzyme from *C. glutamicum* would be a target for known inhibitors of the *C. ammoniagenes* Mn-RNR, namely EDTA [8] and the radical scavengers *p*-methoxyphenol [10] and hydroxyurea [11,15]. Compared to the *C. ammoniagenes* enzyme the *C. glutamicum*-RNR was significantly more sensitive to EDTA and *p*-methoxyphenol and had a similar sensitivity towards hydroxyurea (Table I). The remarkable sensitivity towards the

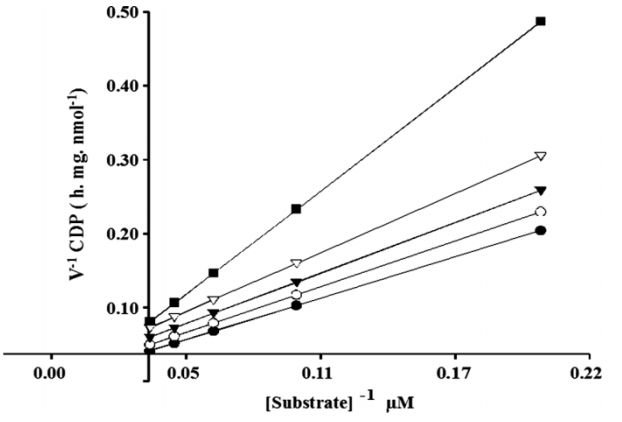

Figure 2. Double-reciprocal plot of velocity versus CDP concentration (5, 10, 15, 20, 25 μM) during cytidine nucleotide reduction catalysed by *C. glutamicum* RNR in the standard biochemical complementation assay (30°C, pH 6.6, 0.1 mM dATP, 6 mM dithithreitol) in the presence of increasing heptapeptide concentrations: 0 μM (●), 5 μM (○), 25 μM (▼), 35 μM (△), 50 μM (■).

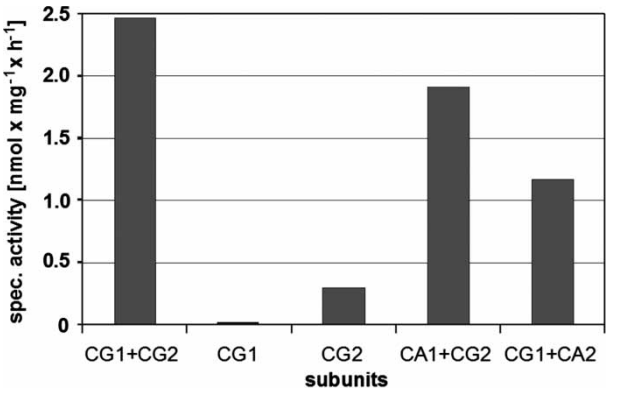

Figure 3. Specific activity of hybrid ribonucleotide reductase holoenzymes upon heterologous biochemical complementation of the sub-units prepared from *C. glutamicum* and *C. ammoniagenes*. RNR sub-units alone are not ribonucleotide reduction proficient. Abbreviations: CG1, CG2 = R1E and R2F of *C. glutamicum*; CA1, CA2 = R1E and R2F of *C. ammoniagenes* (specific activity of 3.6 nmol $\times mg^{-1} \times h^{-1}$ as holoenzyme).

radical scavengers prompted us to search for a putative radical by EPR. However, detection of a radical signal was a challenge for two reasons: (I) the radical in the metallo-cofactor of the *C. glutamicum*-RNR appeared to be less stable than its counterpart in the related species *C. ammoniagenes* [15] with its reported half-life time of only 1.5 h, and (II) a wild type strain containing an estimated amount of RNR less than 1/1000th of its total protein content is currently the only available enzyme source. As lengthy chromatographic procedures would reduce the actual spin concentration it was decided to apply active protein from the ammonium sulphate precipitation (40–60%) to a trimethylammonium based anion exchange column (UNO^{TM} Q12). A sample of holoenzyme enriched as described in the Materials and methods section allowed to detect an EPR signal of the *C. glutamicum* RNR (Figure 4, traces recorded at various temperatures and at non-saturating conditions) for the first time. The spin concentration of this clear organic radical signal at $g = 2.005$ was determined to 2 μM. Unlike the tyrosyl radical in various binuclear iron centres containing RNR, the signal here had an isotropic appearance and did not show partially resolved proton-hyperfine coupling in X-band EPR at various experimental conditions (i.e. field modulation of 0.3 mT, 10–150 K). The low concentration of the radical species did not allow extensive EPR characterization by multi-frequency or pulsed experiments. This EPR signal is saturable (not

Table I. Efficacy of specific inhibitors towards RNRs from *Corynebacterium* species.

Inhibitors	I_{50}- *C. glutamicum*	I_{50}- *C. ammoniagenes*
Hydroxyurea	1.7 mM	2.5 mM [8]
p-methoxyphenol	0.5 mM	5.3 mM [10]
EDTA	2.4 mM	10.0 mM [8]

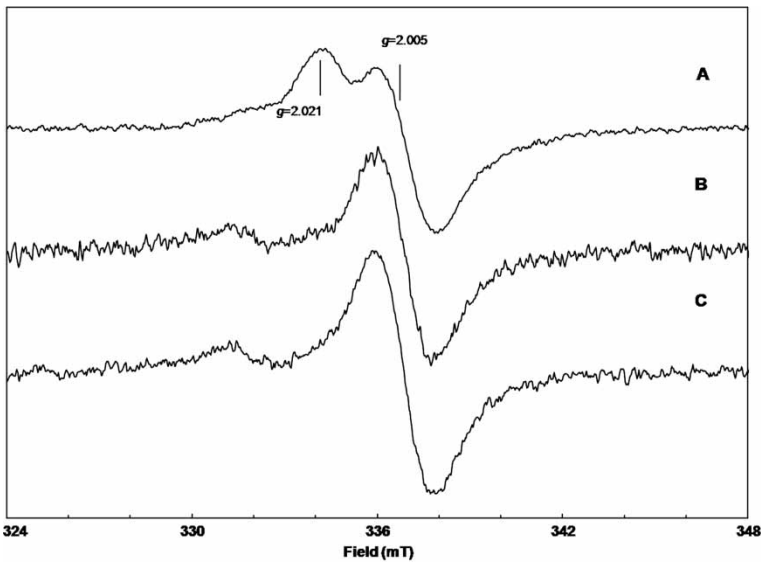

Figure 4. EPR spectra of the organic radical of *C. glutamicum* RNR (holoenzyme, 40 mg/ml, equivalent to 2.5 μM R2F monomer) in 85 mM potassium phosphate buffer, pH 6.6, containing 2 mM DTT at different temperatures and microwave power. EPR parameters: modulation amplitude, 1.2 mT; modulation frequency, 100 kHz; microwave frequency 9458 ± 1 MHz. The temperature and (non-saturating) microwave power was 10 K, 0.80 μW (trace A); 35 K, 12.7 μW (trace B); 80 K, 50.5 μW (trace C), respectively. For comparison the amplitudes were corrected to trace C (gain, number of scans, microwave power and Curie Law).

shown) and the microwave power at half-saturation at 80 K was ≈ 0.3 mW. The blurred lineshape and saturation behaviour indicate the presence of a nearby paramagnetic metal ion (Mn^{2+}), a coupled binuclear centre with paramagnetic ground state or a weakly coupled binuclear centre with thermally populated paramagnetic excited state(s) which appear(s) as binuclear Mn^{2+}. Since no other EPR signals could be detected the type of coupling partner remains to be elucidated. Further purification of the active fractions by anion exchange chromatography (to be described elsewhere) allowed us to detect a sharp visible signal at 408 nm (Figure 5). Its absorption maximum is characteristic of tyrosyl radicals in other ribonucleotide reductases [19]. The purified *C. glutamicum*-R2F had a high ribonucleotide reductase activity ($32\ \mu mol \times mg^{-1}$ protein $\times\ min^{-1}$) in the biochemical complementation assay. Chemical analysis revealed a low iron (0.08 μg Fe/mg R2F), but a high manganese (1.16 μg Mn/mg R2F) content in the purified metallo-cofactor.

Discussion

Corynebacterium glutamicum has been intensively studied with respect to large-scale production of amino acids and its underlying metabolism [20], while the metal dependence of its DNA precursor biosynthesis is completely unknown. The work presented here (Figure 1) suggests that we were studying a manganese-containing ribonucleotide reductase in *C. glutamicum* with a high manganese but a low iron content as reported for the *C. ammoniagenes* R2F [10]. Thus, the RNRs from both corynebacteria contain manganese and lack iron when obtained as native enzymes from the original source.

The above data and sequence information [11–13] place the two corynebacterial RNRs side-by-side. However, an amino acid exchange between the two corynebacterial R2F proteins [11] would explain the lower efficacy of the synthetic heptapeptide, while similar efficacy [21] is due to sequence identity of the C-terminal region of *C. glutamicum* and *M. tuberculosis* (Table II). Our data in Figure 2 are consistent with other reports on competition of synthetic peptides for R2 binding in RNRs [22–25]. Furthermore,

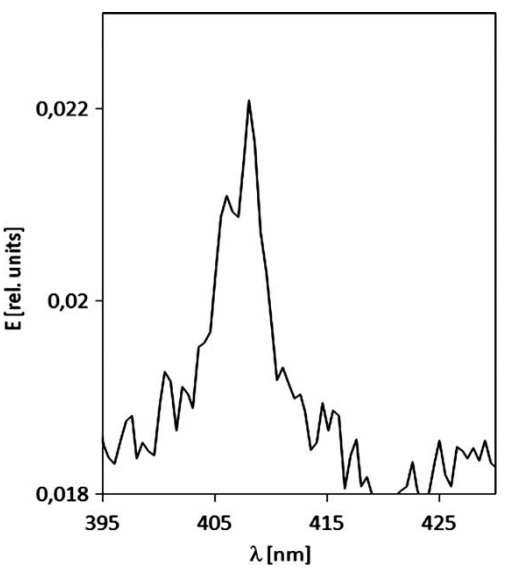

Figure 5. The 408 nm tyrosyl-like signal of the *C. glutamicum*-Mn-RNR in the UV/visible difference spectrum (absorption of HU-treated protein subtracted from native sample) from the purified R2F sample.

Table II. Inhibition of RNRs from high-GC Gram-positive bacteria (Actinobacteria) by a specific heptapeptide derived from the C-terminal region of the *C. glutamicum nrdF* gene.

C-terminal sequence	I_{50}	Source of target RNRs	Reference
E-D-D-D-W-D-F	37 μM	*C. glutamicum*	This work
T-D-D-D-W-D-F	94 μM	*C. ammoniagenes*	This work
E-D-D-D-W-D-F	20 μM	*M. tuberculosis* (R2-2)	[21]

the non-competitive mode of inhibition of the *C. glutamicum*-RNR points to a common binding region of R2F and its terminal heptapeptide distant from the active centre of R1E and suggests a long-range electron transfer from the radical-bearing R2F to the catalytic R1E well known from the class Ia RNR [19].

The tendency of RNRs to dissociate into large catalytic and small metal-containing sub-units facilitates generation of hybrid enzymes and evaluation of their capability for reduction of ribonucleotides. The successful biochemical complementation of R1E and R2F from *C. glutamicum* or *C. ammoniagenes* to active holoenzymes (Figure 3) confirmed the similarity of the two corynebacterial RNRs. It is noteworthy that previous attempts for generation of hybrid enzymes composed of sub-units from the *C. ammoniagenes* Mn-RNR and the *E. coli*-Fe-RNR failed [8]. This indicates diversity of the latter enzymes in spite of an analogous sub-unit structure.

On the other hand, the two corynebacterial Mn-RNRs have many properties in common, e.g. inhibition by EDTA and the radical scavengers hydroxyurea and *p*-methoxyphenol. EPR spectroscopy revealed a saturable organic radical in the *C. glutamicum* RNR. The $g_{average}$-value is in the range reported for tyrosyl radicals in other RNRs (Table III). However, the use of an average *g*-value to prove the nature of the radical-bearing moiety has limitations, especially since the presence of a nearby coupling partner or a second underlaying radical species could shift the *g*-value. It is known that the *g*-anisotropy and linewidth of radicals strongly depend on the local environment (i.e. hydrophobicity, hydrogen bonds, β-methylene dihedral angles) [26,27]. A factor which complicates the definitive assignment to a tyrosyl radical is the variable relaxation behaviour of tyrosyl radicals in RNRs; i.e. *E. coli* RNR has a $P_{1/2}$ of 12 mW (77 K), *S. typhimurium* RNR (Fe-substituted) of 3.7 mW (95 K), *C. glutamicum* RNR of 1.3 mW (95 K), *M. tuberculosis* RNR of 0.72 mW (77 K) and *C. ammoniagenes* RNR of 0.5 mW (77 K). Differences in the relative orientation of the tyrosyl radical with respect to the binuclear centre and in the coupling constants of the binuclear centres cause this variation.

Table III. Values of $g_{average}$ factors of selected tyrosyl radicals in biological systems.

Tyrosyl radical sources	$g_{average}$ value	References
R2, *E. coli*	2.0047*	[30]
	2.0053	[31]
R2F, *S. typhimurium*	2.0047	[32]
	2.0051	[33]
R2F, *C. ammoniagenes*	2.004	[15]
R2F, *C. ammoniagenes*	2.005	[34]
R2F, *C. glutamicum*	2.005	This work
R2F, *M. tuberculosis*	2.0053	[26]
	2.0056	[35]

*In this first report on a tyrosyl radical of RNR a doublet centred at $g = 2.0047$ was reported as the only signal detected.

As for tyrosyl radicals of RNRs from Gram-positive bacteria [15], the radical of the *C. glutamicum* enzyme shows a saturation behaviour which is characteristic of a weak magnetic interaction. Both the visible 408 nm Y·-radical signal observed here (Figure 5) and the conserved Y_{120} suggest that a tyrosyl radical exists in the *C. glutamicum*-R2F. Assignment of the featureless EPR signal (Figure 4) to a tyrosyl radical broadened by coupling is tempting, but will have to await site-directed mutagenesis of Tyr_{120} or multiple frequency EPR studies using R2F with enriched spin concentration from homologous expression. Noteworthily, our results were obtained with the wild type of *C. glutamicum* grown in complex medium without any perturbation of trace element nutrition. Thus, the manganese detected here by different methods is intrinsic to the *C. glutamicum* RNR. Chemical quantification of the metal content in the purified sample revealed 0.06 mol Fe but 0.8 mol Mn per mol R2F-monomer and would thus assign two manganese atoms bound to the dimeric metallo-cofactor of the *C. glutamicum* RNR. Options which are compatible with this observation are: (1) R2F contains a mononuclear manganese centre, (2) our preparations contain apo-protein and the metallo-cofactor is a binuclear Mn centre and (3) R2F has a binuclear centre with Mn and a second metal ion other than Mn or Fe.

The corresponding *nrdEF* genes represent the only RNR in the genome of *C. glutamicum* ATCC 13032 [28]. Therefore, this genomic information and our finding of a Mn-type of RNR exclude a class Ia RNR in *C. glutamicum* since the type of metal centre defines the class of the RNR [6].

Finally, the *C. glutamicum* metallo-cofactor is also remarkably different from the recently described unconventional manganese(IV)/iron(III) cofactor in the *Chlamydia trachomatis* ribonucleotide reductase

[29] due to the presence of a tyrosyl radical (Figures 4 and 5).

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