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The Active Form of the R2F Protein of Class Ib Ribonucleotide Reductase from *Corynebacterium ammoniagenes* Is a Diferric Protein*

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Corynebacterium ammoniagenes **contains a ribonucleotide reductase (RNR) of the class Ib type. The small subunit (R2F) of the enzyme has been proposed to contain a manganese center instead of the dinuclear iron center, which in other class I RNRs is adjacent to the essential tyrosyl radical. The *nrdF* gene of *C. ammoniagenes*, coding for the R2F component, was cloned in an inducible *Escherichia coli* expression vector and overproduced under three different conditions: in manganese-supplemented medium, in iron-supplemented medium, and in medium without addition of metal ions. A prominent typical tyrosyl radical EPR signal was observed in cells grown in rich medium. Iron-supplemented medium enhanced the amount of tyrosyl radical, whereas cells grown in manganese-supplemented medium had no such radical. In highly purified R2F protein, enzyme activity was found to correlate with tyrosyl radical content, which in turn correlated with iron content. Similar results were obtained for the R2F protein of *Salmonella typhimurium* class Ib RNR. The UV-visible spectrum of the *C. ammoniagenes* R2F radical has a sharp 408-nm band. Its EPR signal at g = 2.005 is identical to the signal of *S. typhimurium* R2F and has a doublet with a splitting of 0.9 millitesla (mT), with additional hyperfine splittings of 0.7 mT. According to X-band EPR at 77–95 K, the inactive manganese form of the *C. ammoniagenes* R2F has a coupled dinuclear Mn(II) center. Different attempts to chemically oxidize Mn-R2F showed no relation between oxidized manganese and tyrosyl radical formation. Collectively, these results demonstrate that enzymatically active *C. ammoniagenes* RNR is a generic class Ib enzyme, with a tyrosyl radical and a diferric metal cofactor.**

Deoxyribonucleotides are synthesized in all organisms from ribonucleotides, in a reaction catalyzed by the enzyme ribonucleotide reductase (RNR).[1] Common to all RNRs is that they proceed via a radical mechanism (1). However, based on amino acid sequence similarities and polypeptide compositions (2), as well as metal and radical cofactors, three well-characterized RNR classes (I–III) can be distinguished. Class I RNRs consist of two homodimeric components, the larger R1 protein and the smaller R2 protein. Protein R1 contains the active site, and protein R2 contains a stable tyrosyl radical in close proximity to a diferric center. Class II RNRs are monomeric or homodimeric proteins that bind and utilize a vitamin B_{12} cofactor (deoxyadenosine cobalamin). Class III RNRs consist of two proteins. The larger protein contains in its active form a stable glycyl radical close to the active site. The smaller protein generates this radical with the aid of a 4Fe-4S cluster and *S*-adenosylmethionine. The class I RNRs are further subdivided into class Ia and class Ib based on amino acid sequence similarities (3, 4), allosteric properties, and dependence on different physiological reductants (5). Among prokaryotes several different classes and subclasses of RNR may be encoded in the same genome. In class Ia RNRs, the R1 protein is encoded by the *nrdA* gene and the R2 protein by the *nrdB* gene (1). In class Ib RNRs, the *nrdE* gene codes for the R1E protein and the *nrdF* gene codes for the R2F protein (3–6).

The three-dimensional structures of proteins R1 and R2 of class Ia RNR from *Escherichia coli* are known (7, 8). Four carboxylate residues and two histidines ligate the di-iron site in protein R2. In the native, enzymatically competent form, the two ferric ions are bridged by a μ-oxo bridge and one of the carboxylates (7). The iron ligands and the stable tyrosyl radical residue are conserved among all class I sequences (1). Recently, the three-dimensional structure of the class Ib R2F protein from *Salmonella typhimurium* was solved (9). The ligand sphere is slightly different in *S. typhimurium* R2F as compared with *E. coli* R2.

In this study we characterize the radical and metal cofactor of enzymatically competent R2F protein of a class Ib RNR from *Corynebacterium ammoniagenes.* It has been suggested that R2F from *C. ammoniagenes* binds and is activated by manganese instead of iron, because manganese is needed for growth of the orga-

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[1] The abbreviations used are: RNR, ribonucleotide reductase; apoR2(F), R2 proteins lacking a metal site; DTT, dithiothreitol; Fe-R2F, R2F protein reconstituted with iron; IPTG, isopropyl-1-thio-β-D-galactopyranoside; metR2(F), R2 protein with a metal site but lacking a tyrosyl radical; Mn-R2(F), R2 protein purified from bacteria grown in the presence of manganese or manganese-reconstituted apoR2; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

nism (10, 11). We and others have isolated manganese-containing R2F proteins with poor enzymatic activity from *C. ammoniagenes* cells, which have been starved for manganese and subsequently recovered for some time in manganese-supplemented growth medium (6, 11–13). We have now constructed an efficient expression system for the enzyme by cloning the *C. ammoniagenes nrdF* gene in *E. coli* and characterized the overproduced R2F protein. Surprisingly, its enzyme activity and its tyrosyl radical content showed a dependence for iron but not for manganese. Comparative studies on the R2F protein from *S. typhimurium* (4, 5) gave similar results.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Materials—*E. coli* DH5α (CLONTECH) was used for routine maintenance and isolation of plasmid DNA, and JM109(DE3) (Promega) was used for protein expression. *E. coli* strains were grown in LB medium at 37 °C supplemented with 200 μg/ml carbenicillin and 30 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Roche Molecular Biochemicals) when necessary. Wild type *C. ammoniagenes* (ATCC 6872) was obtained from the American Type Culture Collection and grown in LB medium at 37 °C. Plasmid vectors used were pBluescript SK(+) (pBSK, Stratagene) for subcloning, pGEM-T (Promega Corp.) for cloning of polymerase chain reaction (PCR)-generated fragments and pET21 (Novagen) for protein expression. *S. typhimurium* class Ib ribonucleotide reductase components R1E and R2F were purified as described (4). Radioactive $^{54}MnCl_2$ was obtained from Amersham Pharmacia Biotech and used after dilution with nonlabeled $MnCl_2$ to a final specific activity of 9000 cpm/nmol.

Cloning of the C. ammoniagenes nrdF Gene in an Expression Vector—Primers CoryFup (5′-AAA**CATATG**TCGAATGAATATGACGAG-3′; the *Nde*I site is *underlined* and the start codon is in *boldface*) and CoryFlow (5′-ACAGATCGTACATAAGGTCG-3′) were used to amplify the 5′ extreme of the *nrdF* gene. A *C. ammoniagenes* genomic library clone, pUA728 (6) (0.1 μg), was used as a template in a 50-μl PCR amplification reaction with 50 pmol of each primer, all dNTPs at 0.2 mM each, 5 μl of 10× PCR buffer (Roche Molecular Biochemicals), and 1.5 units of *Taq* polymerase. The reaction was run with the following program: (*a*) 3 min at 94 °C; (*b*) 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C; and (*c*) 7 min at 72 °C. The amplified 717-base pair product was purified from ethidium bromide containing 2% Nusieve-agarose gel by melting the band in 6 M NaI at 55 °C and using the Wizard DNA Clean-up system (Promega), and cloned in pGEM-T vector according to the manufacturer's protocol, resulting in plasmid pIG053. This plasmid was sequenced in both directions to assure lack of mutations generated during PCR amplification. An *Eco*RI fragment from pUA728 containing the *nrdF* gene was cloned into pBSK giving plasmid pIG054. The entire *nrdF* gene with a *Nde*I restriction site at the 5′ end was obtained by ligating a *Sac*I-*Bst*XI fragment of the pIG053 to a *Sac*I-*Bst*XI deletion of pIG054, generating pIG055. A *Nde*I-*Sal*I digestion of pIG055 was cloned into pET21, downstream of a strong ribosome-binding site preceded by a T7 promoter giving pIG056. General cloning procedures were carried out by standard methods (14).

Growth and Purification of Overexpressed C. ammoniagenes R2F Protein—JM109(DE3) cells containing the pIG056 plasmid were grown in LB medium at 37 °C in the presence of 200 μg/ml carbenicillin to mid log phase (A_{640} = 0.5) and subsequently induced to overproduce R2F by addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to 0.5 mM final concentration for 3–3.5 h. Three different growth conditions were used: LB without supplement of metal ions, LB supplemented with manganese (2 mM $MnCl_2$), and LB supplemented with iron (2 mM $FeSO_4$). Cells from the culture grown without metal supplement and from the manganese-supplemented culture were harvested and stored at −80 °C for further purification of the *C. ammoniagenes* R2F protein. Protein from the cells grown in ordinary LB medium is denoted R2F, and protein purified from the manganese-supplemented cells is denoted Mn-R2F below.

Frozen cells were disintegrated by X-press (Biox AB) and extracted in 50 mM Tris-HCl, pH 7, after which nucleic acids were precipitated with 1.5% streptomycin sulfate, as described (6). The R2F sample had 80% purity according to SDS-polyacrylamide gel electrophoresis (PAGE) (*cf.* Fig. 1*B*, below) and a yield of approximately 300 mg of R2F from 7.6 g of wet cells. The Mn-R2F sample gave only one band on SDS-PAGE (*cf.* Fig. 1*C*, below), indicating a high level of purity and a yield of approximately 300 mg from 7.7 g of wet cells. Prior to experiments, samples were further purified with ion exchange chromatography (MonoQ) as described by Fieschi *et al.* (6).

Reconstitution of C. ammoniagenes R2F with Iron for Correlation of Iron Content, Tyrosyl Radical, and Enzyme Activity—392 mg of $(NH_4)_2Fe(SO_4)_2$ and 396 mg of sodium ascorbate were dissolved in 10 ml of argon-flushed 50 mM Tris-HCl, pH 7.6, resulting in a 100 mM ferrous solution with 200 mM ascorbate. To 500 μl of 0.58 mM R2F (containing approximately 1 Fe/R2F) solution (0.29 μmol) was added 11.6 μl of the ferrous solution (1.16 μmol). The mixture was incubated for 5 min at 25 °C and then transferred to ice. The reaction mixture was desalted on a G-25 column (NAP-10) equilibrated with 50 mM Tris, pH 7.6, purified on MonoQ as described earlier (6), and concentrated by ultradialysis to 250 μl of R2F and a concentration of 0.14 mM.

Preparation of S. typhimurium apoR2F by Reduction of R2F—The following procedure was done at 4 °C in an anaerobic box. Inside a collodium bag, 6.2 mg (71 nmol) of R2F in 0.25 ml of 50 mM Tris-HCl, pH 7.5, was treated with 0.03 ml of 1 mM methylviologen and 0.05 ml of 10 mM dithionite. The bag was immersed for 5 h in 10 ml of a stirred solution of 50 mM Tris-HCl, pH 7.5, 50 mM EDTA, 50 mM 8-hydroxyquinoline, 1 mM dithiothreitol (DTT) to remove iron. The material inside the bag was then passed through a 3-ml column of Sephadex G-25 equilibrated with 50 mM Tris-HCl, pH 7.5, 10% glycerol (buffer A). The protein present in the void volume was filtered a second time through a similar Sephadex G-25 column to remove the last traces of interfering material. The final preparation (apoR2F) was stored frozen.

Reactivation of S. typhimurium apoR2F with Iron—The procedure was essentially as described earlier (15). Briefly, apoR2F (0.53 mg, 6.1 nmol, in 0.28 ml of buffer A) was treated anaerobically with 7.5 μl of 10 mM $Fe(NH_4)_2(SO_4)_2$, 100 mM ascorbate for 5 min, followed by passage through a 3-ml column of Sephadex G-25 equilibrated with buffer A. After concentration in a Centricon 30 tube, the reconstituted protein, Fe-R2F (1.6 mg/ml, 18 μM), was used for experiments.

Preparation of S. typhimurium Mn-R2F—ApoR2F (1.7 mg, 19 nmol, in 0.65 ml of buffer A) was treated for 5 min with 24 μl of 10 mM $^{54}MnCl_2$ followed by passage through a 10-ml column of Sephadex G-25 in buffer A. Fractions (0.5 ml) were analyzed for protein and radioactivity. A first radioactive peak coincided with the protein peak, clearly separated from a second larger radioactive peak. All protein fractions showed the same specific radioactivity. After concentration, the solution (3.2 mg/ml, 36 μM) was analyzed for enzyme activity and its EPR spectrum was recorded.

Preparation of S. typhimurium-mixed Fe/Mn-R2F—ApoR2F (0.6 mg, 6.9 nmol, in 0.33 ml) was treated anaerobically with a mixture of 9 μl of 10 mM $^{54}MnCl_2$ and 9 μl of 10 mM $Fe(NH_4)_2(SO_4)_2$, 100 mM ascorbate, followed by passage through a 5-ml column of Sephadex G-25 equilibrated with buffer A. The amount of iron and manganese were determined in the purified protein and its enzyme activity was determined.

Activity Assays—The enzyme activity of *C. ammoniagenes* R2F proteins was measured as described in Fieschi *et al.* (6), with 0.5 mM CDP as substrate, 1 mM dATP as allosteric effector, and 10 mM DTT as reducing agent. Unless otherwise indicated excess of R1E, purified as described in Fieschi *et al.* (6), was included in the assays.

The enzyme activity of *S. typhimurium* R2F proteins was determined in the presence of an excess of protein R1E and with 20 mM DTT as hydrogen donor, CDP as substrate, and dATP as allosteric effector (4). The activity found in this way amounted to approximately one third of the full activity observed with glutaredoxin or the reduced NrdH-protein.

Specific activity is expressed as units/mg of protein, where one unit corresponds to the reduction of 1 nmol of CDP/min; *C. ammoniagenes* RNR was assayed at 30 °C and *S. typhimurium* RNR at 37 °C.

Analytical Methods—Protein (16) and iron (15, 17) were determined by standard methods. In spectrophotometry experiments, *C. ammoniagenes* protein concentration was calculated, using an extinction coefficient at 280 nm for R2F, ϵ = 136,000 M^{-1} cm^{-1}. Manganese was determined by atomic absorption spectroscopy or calculated from the radioactivity of the reconstituted protein and the determined specific activity of $^{54}MnCl_2$.

EPR Spectroscopy —EPR spectra at 77 K were obtained directly on thick slurries of harvested cells or on purified protein samples (0.25–0.77 mM R2F). X-band EPR spectra were measured either on a Bruker ESP 300 spectrometer at 77 K with the sample placed in a cold finger liquid nitrogen Dewar or on a Bruker Elexsys system flowing nitrogen gas at 95 K through a Dewar flask placed in the cavity. Spectra were evaluated using Xepr 2.0 software from Bruker. The microwave saturation curves were evaluated as described previously by the formula $DI_{rel} = 1/(1 + P/P_{½})^{(b/2)}$ (18), where DI_{rel} is the normalized double integral of the EPR signal, P the microwave power in milliwatts (mW), $P_{½}$ the power of half saturation, and b the inhomogeneity factor. The value of b varies between 1, for the completely inhomogeneously broadened line, and 3, for an homogeneously broadened line.

Light Absorption Spectroscopy—The UV-visible spectra were re-

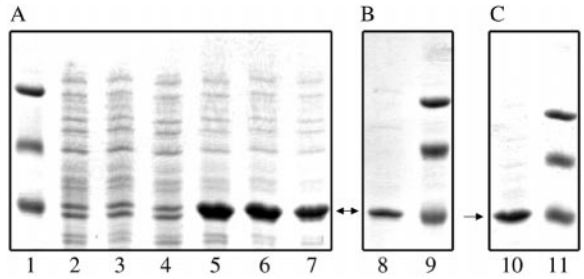

FIG. 1. **SDS-PAGE of *E. coli* overproducing the *C. ammoniagenes* R2F and of the purified protein.** In *A*, cells harvested before and after 3.5 h of induction. In *B* and *C*, *C. ammoniagenes* R2F purified from cells grown in LB medium (*B*) and grown in LB medium supplemented with 2 mM $MnCl_2$ (*C*). *Lanes 1*, *9*, *11*, marker proteins (Amersham Pharmacia Biotech LMW marker kit; 14,400, 20,100, and 30,000 Da); *lanes 2–4*, overproducing cells grown in LB, LB plus 2 mM $FeSO_4$, and LB plus 2 mM $MnCl_2$, respectively, and harvested before induction; *lanes 5–7*, overproducing cells grown in LB, LB plus 2 mM $FeSO_4$, and LB plus 2 mM $MnCl_2$, respectively, and harvested after induction; *lane 8*, protein purified from cells grown in LB; *lane 10*, protein purified from cells grown in LB plus 2 mM $MnCl_2$.

corded on a Perkin-Elmer λ2 spectrophotometer. Radical content was determined from the drop line absorption at 408 nm; the absorption deviation of the 408-nm peak from a theoretical value was calculated from the linear slope between 420 and 402 nm. The extinction coefficient was determined to $\epsilon = 3400\ M^{-1}\ cm^{-1}$ after correlation of radical content determined by EPR and light absorption spectrum.

Radical Scavenging and Oxidation of the Metal Center in R2F Proteins—During these experiments 10–30 μl of *C. ammoniagenes* R2F or Mn-R2F (9–22 μM) was diluted with 50 mM Tris-HCl, pH 7.6, to a final volume of 500 μl in a quartz cuvette. The stability of the tyrosyl radical in Mn-R2F and R2F was studied by addition of a hydroxyurea crystal to the protein directly in the cuvette and followed by UV-visible spectroscopy. Iron reconstitution was performed with R2F or Mn-R2F and 2, 4, and 6 Fe^{2+} per dimer. Manganese reconstitution was done with 1, 4, and 10 Mn^{2+}/R2F, with and without oxygen flush of the solution. To try to oxidize the manganese sites chemically, different redox active molecules were titrated to Mn-R2F. H_2O_2 oxidation of the metal center was made by equimolar amounts of the oxidant and metal center and followed by UV-visible spectroscopy. The Mn-R2F protein was treated with ascorbate (1–7 reducing equivalents per metal center). The ascorbate reaction was studied by X-band EPR at 77 K and UV-visible spectroscopy. KIO_4 oxidation of Mn-R2F (with a 2:1 stoichiometry) was followed by EPR.

RESULTS

Cloning and Overproduction of C. ammoniagenes R2F in E. coli

The plasmid pIG056 contains the *C. ammoniagenes nrdF* gene under the control of the T7 promoter. Plasmid-containing *E. coli* strain JM109(DE3), which carries the T7 RNA polymerase gene under control of the Lac promoter, produced massive amounts of *C. ammoniagenes* R2F after IPTG induction. Three different conditions were used: LB medium, LB medium supplemented with manganese, and LB medium supplemented with iron. All three conditions resulted in similar overproduction of R2F according to SDS-PAGE analysis (with Coomassie Blue staining) (Fig. 1*A*). The different cell suspensions were examined by EPR. A prominent free radical signal was detected in the sample grown in LB (Fig. 2*A*, *upper spectrum)*. Twice as much radical was detected when cells were grown in iron-supplemented medium (Fig. 2*A*, *lower spectrum)*. In contrast, the culture with manganese-supplemented medium contained no radical EPR signal, but showed Mn(II) with octahedral coordination (Fig. 2*B*). Fig. 2*B* covers the same magnetic field as in Fig. 2*A* and shows the three middle lines of the octahedrally coordinated Mn^{2+}. The vertical scales differ by a factor 0.62 (*A*/*B*), which is in agreement with an assignment of the Mn^{2+} in Fig. 2*B* to unbound Mn^{2+} in the medium hiding low amounts of radical features in the g = 2.00 region. The additional splittings from the forbidden transitions of the nuclear spins are not seen in the middle line. Subtraction of approximately 0.3 times the signal in Fig. 2*A (upper spectrum)* gives a spectrum like the Mn^{2+} standard, indicative of a small amount of tyrosyl radical also in the sample from the manganese-supplemented medium. Cultures grown in ordinary or manganese-supplemented media were used for purification of R2F protein in high yield (Fig. 1 and Table I).

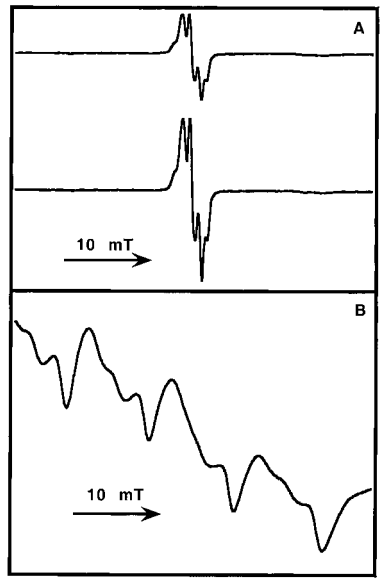

FIG. 2. **EPR spectra at 77 K on intact *E. coli* cells overproducing the *C. ammoniagenes* R2F after growth in medium with or without metal ion supplement.** *A*, cells grown in LB *(upper spectrum)* and in LB plus 2 mM $FeSO_4$ *(lower spectrum)*. *B*, cells grown in LB plus 2 mM $MnCl_2$. EPR conditions were: 1 scan, 0.9-mW microwave power, 0.5-mT modulation amplitude.

Characterization of Purified C. ammoniagenes R2F Proteins

Radical Content and Enzymatic Activity—The R2F sample purified from cells grown in rich medium without metal ion supplement had a prominent EPR signal at g = 2.005 arising from a major splitting of 0.9 millitesla (mT) and additional hyperfine couplings at 0.7 mT (Fig. 3*A*). The EPR spectrum can be superimposed on that from the tyrosyl radical from the *S. typhimurium* R2F (*cf.* Fig. 3*B*) (4, 19). The spectra in Fig. 3 differ significantly from the EPR spectrum of the tyrosyl radical of the *E. coli* class Ia R2 protein (20). The latter has a major splitting from one of the β-protons of 2 mT giving a clearly resolved doublet spectrum with additional splitting from the 3,5 ring protons of 0.7 mT (20, 21). The EPR spectrum of purified *C. ammoniagenes* R2F in Fig. 3*A* thus excludes contamination of ribonucleotide reductase from the *E. coli* host cells.

Protein R2F purified from Mn-supplemented cells, Mn-R2F, displays a complex multiline spectrum, superimposed on a broad signal often found in dimanganese proteins (22). A small amount of free radical signal is also seen, but the concentration is lower in this sample compared with the *C. ammoniagenes* R2F sample without manganese addition (Fig. 4*A* and Table I).

The R2F sample, purified from cells grown without supplement of metal ions, had a UV-visible spectrum with a 408-nm peak, characteristic of a tyrosyl radical (20). The absorption spectrum obtained by us differs from the previously published *C. ammoniagenes* RNR band at 437 nm (23). The radical was sensitive to hydroxyurea, and the difference spectrum between before and after hydroxyurea scavenging (Fig. 5) agrees well with the spectrum of a tyrosyl radical (24). It is different from

TABLE I
Metal ion content, radical content, and specific activity of Corynebacterium ammoniagenes R2F proteins

	Fe/monomer	Mn/monomer	Tyr·/monomer	Specific activity
				nmol mg^{-1} min^{-1}
Mn-R2F	0.5	1.6	0.03	9
R2F	0.5	0.09	0.05	36
Fe-R2F[a]	1.5	ND[b]	0.2	48[c]

[a] Iron-reconstituted R2F.
[b] ND, not determined.
[c] These assays contained ca. 0.2 μM R1E and 0.05 or 0.2 μM R2F.

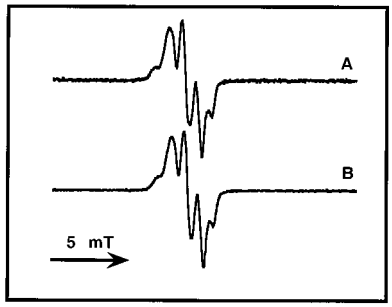

FIG. 3. **EPR spectra at 77 K on purified R2F proteins.** *A*, *C. ammoniagenes* R2F purified from overproducing *E. coli* cells grown in LB medium (16 scans). *B*, *S. typhimurium* R2F reconstituted in the presence of ferrous ions (1 scan). EPR conditions were: 0.2-mW microwave power, 60-db receiver gain, 0.1-mT modulation amplitude.

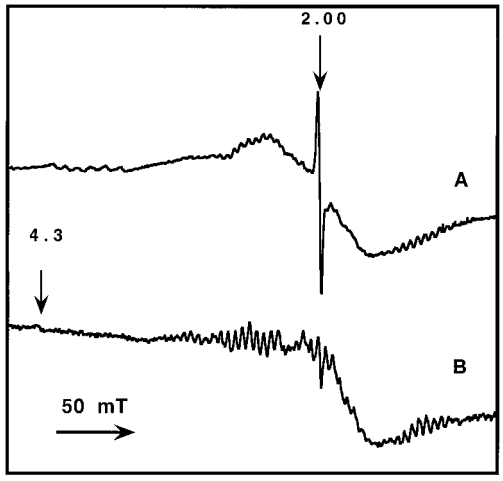

FIG. 4. **EPR spectra on purified Mn-R2F proteins.** *A*, *C. ammoniagenes* R2F purified from overproducing *E. coli* cells grown in LB medium plus 2 mM manganese and measured at 77 K. *B*, *S. typhimurium* R2F reconstituted in the presence of manganese ions. Measured at 95 K, the coupling signals in the high field part of the spectrum are more clearly seen. The corresponding spectrum recorded at 77 K has the same features, although the noise levels signals were higher. EPR conditions: *A*, 1 scan, 1.97-mW microwave power, 60-db receiver gain, 1-mT modulation amplitude; *B*, 2 scans, 1.9-mW microwave power, 63-db receiver gain, 0.5-mT modulation amplitude.

the spectrum obtained earlier by others from a similar experiment (23), but it agrees with tyrosyl radical spectra from other class I RNRs (20, 24).

Activity measurements of the two preparations showed that the R2F sample had a 4-fold higher specific activity than the Mn-R2F sample and that even higher specific activity was obtained after reconstitution of the protein in the presence of iron (Table I).

Metal Content and EPR Characteristics of the Metal Sites—The iron content of the R2F sample was 0.5 Fe/monomer, whereas the manganese content was only 0.09 Mn/monomer (Table I). The Mn-R2F sample, however, contained 1.6 Mn/

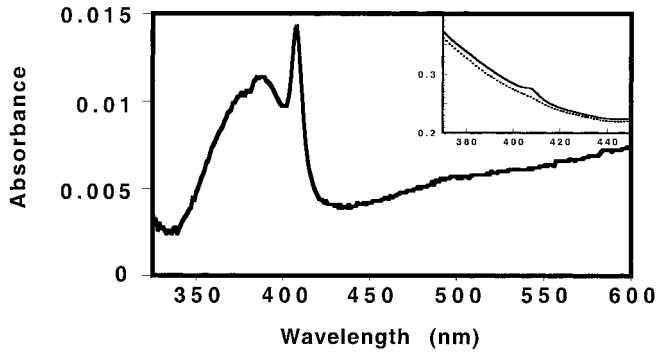

FIG. 5. **UV-visible spectrum of radical in *C. ammoniagenes* R2F (approximately 100 μM).** Subtraction of spectra obtained before (*inset*: *solid line*) and after (*inset*: *dotted line*) hydroxyurea quenching of the radical signal. For better resolution of individual spectra in the *inset*, the sample after hydroxyurea treatment was not corrected for dilution.

monomer (Table I), suggesting that some protein molecules contained dinuclear manganese sites. Interestingly, addition of manganese to the growth medium did not affect the amount of iron in the *C. ammoniagenes* R2F. Iron reconstitution of the R2F sample, which was low in metal, led to a Fe-R2F containing 1.5 Fe/monomer.

To evaluate the metal states, the features of the EPR signals were further characterized. The EPR spectrum at 77 K of the Mn-R2F sample gave rise to more than 30 resolved lines on a broad signal. The resolved lines are separated by approximately 4.6 mT and grouped into three groups of 11 lines partly overlapping with intensities 1:2:3:4:5:6:5:4:3:2:1 centered at g 2.64, 2.31, and 1.69. A splitting of 4.6 mT (*cf.* Fig. 4*A*) is half of the value (9.5 mT) observed for mononuclear Mn^{2+} and is typical of a coupled dimanganese center in the Mn^{2+} state. Dimanganese Mn(II) is also found in arginase and manganese catalase (22, 25–28) and the hyperfine pattern observed in manganese-reconstituted *C. ammoniagenes* R2F resembles arginase, where the manganese center (29) is bridged by two carboxylates and one oxygen (water or hydroxyl).

The presence of an oxidized free radical signal in the Mn-R2F sample suggests that some antiferromagnetically coupled diferric center ($S = 0$), hence not detectable by EPR, should also be present in this sample. Because no unspecific bound iron is seen at g = 4.3 by EPR, the iron present is likely to bind specifically to the metal center in this way. In the R2F sample only the free radical signal is seen but no specific manganese features (Fig. 3*A*).

Metal Reconstitution Studies with C. ammoniagenes R2F

The metal site of R2F was reconstituted with iron or manganese and studied by UV-visible spectroscopy. Iron was shown to form an oxidized metal center concomitantly with tyrosyl radical formation, because the characteristic bands from the oxidized iron center and the tyrosyl radical peak at 408 nm evolved together. It was not possible to generate an oxidized manganese center and/or tyrosyl radical by addition of manganese to the aerobic R2F.

The results presented so far suggest that the enzyme activity of *C. ammoniagenes* R2F correlates with the presence of a tyrosyl radical, which in turn correlates with the presence of a di-iron center. However, R2F from *C. ammoniagenes* has earlier been assumed to have a metal center different from other R2F proteins (6, 11–13). We therefore attempted to reconstitute the well-characterized class Ib R2F protein from *S. typhimurium* with iron and manganese.

TABLE II
Metal ion content, radical content, and enzyme activity in Salmonella typhimurium R2F proteins

	Fe/monomer	Mn/monomer	Tyr·/monomer	Specific activity
				nmol mg^{-1} min^{-1}
R2F	1.4	ND	0.5	660
ApoR2F	0.05	ND	ND	2
Fe-R2F	1.6	ND	0.2	325
Mn-R2F	ND	1.6	≤0.01	3
Fe/Mn-R2F	0.7	1.1	ND[a]	41

[a] ND, not determined.

Metal Reconstitution Studies with S. typhimurium R2F

The conventional procedure for the removal of iron from class Ia ribonucleotide reductase involves dialysis of the R2 protein against a solution of 8-hydroxyquinoline in 1 M imidazol buffer (15). This method was not suitable to remove iron from *S. typhimurium* R2F, as the protein precipitated. Instead, we dialyzed the protein after reduction of its iron-center against EDTA, as described under "Materials and Methods." This gave an inactive apoR2F protein, free of iron. Treatment of R2F with either iron or manganese resulted in close to stoichiometric amounts of each metal incorporated (Table II).

Reconstituted Fe-R2F contained slightly more iron than the original R2F preparation but was only half as active. It gave an EPR spectrum typical of *S. typhimurium* R2F tyrosyl radical (Fig. 3*B*) but with less than half the amount of spins per iron (4, 20). The lower activity suggests that the protein during preparation of apoR2F was partly denatured and had lost the full ability to form the free tyrosyl radical. During manganese treatment the metal was incorporated with the same stoichiometry as during iron treatment. However, Mn-R2F was completely inactive and EPR spectroscopy showed no sign of tyrosyl radical. Instead, the EPR spectrum suggests the presence of a dinuclear Mn(II) center with similar, but somewhat different, coupling pattern to that obtained for *C. ammoniagenes* Mn-R2F (Fig. 4*B*). In this case four different multiplets of 11 lines are resolved and the background for the excited states is different from that of *C. ammoniagenes* Mn-R2F (Fig. 4*A*). A small amount of unspecifically bound mononuclear Fe^{3+} is seen at $g = 4.3$.

Treatment of apoR2F with equimolar amounts of iron and manganese gave a product that had incorporated roughly equal amounts of each ion. The reconstituted protein showed some activity, amounting to 13% of the iron-reconstituted enzyme. The low activity suggests the existence of inactive protein dimers containing both iron and manganese.

Saturation Behavior of the Tyrosyl Radicals in the C. ammoniagenes and S. typhimurium R2F Proteins

To distinguish between different environments around the tyrosyl radical, the microwave saturation properties of radical-containing R2F samples of *C. ammoniagenes* and *S. typhimurium* were compared with that of the R2 protein from *E. coli.* The normalized double integral of the EPR signals are plotted *versus* microwave power (Fig. 6). $P_{½}$, or the power of half saturation, gives a measure on the magnetic interaction between the tyrosyl radical and the metal center. At 95 K, the tyrosyl radical in Fe-R2F from *C. ammoniagenes* has a value $P_{½} = 1.3$ mW ($b = 1$). The corresponding value for *S. typhimurium* is $P_{½} = 3.7$ mW ($b = 1$). The signal from *C. ammoniagenes* is thus saturating at lower power, indicating a slightly weaker coupling. Still, these signals saturate at the same order of magnitude of microwave power. At 95 K the $P_{½}$ for the class Ia *E. coli* R2 tyrosyl radical was determined to 91 mW, which is almost two orders of magnitude larger than for *C. ammoniagenes* R2F, indicating a much faster relaxation of the tyrosyl

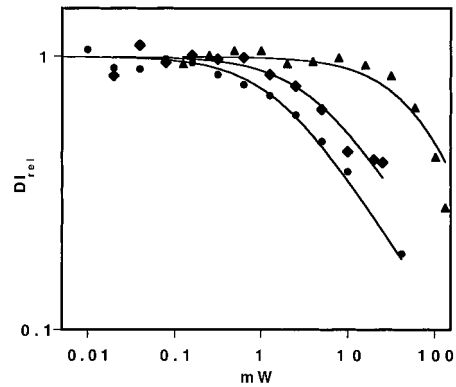

FIG. 6. **Saturation behavior of tyrosyl radical in purified R2 proteins at 95 K.** Curves from *C. ammoniagenes* (●) and *S. typhimurium* (◆) R2F were fitted with an inhomogeneity factor of $b = 1$, and *E. coli* R2 (▲) was fitted to $b = 2$.

radical due to interaction with the neighboring diiron site. However, also the b value has changed to $b = 2$, indicative of changed relaxation processes at higher temperatures. *E. coli* has typically $b = 1.3$ and $P_{½} = 12$ mW at 77 K, and an isolated tyrosyl radical has $P_{½} = 0.2$ mW ($b = 1.0$) at 77 K and $P_{½} = 0.36$ mW ($b = 1.0$) at 93 K (30). The determined values of $P_{½}$ for *C. ammoniagenes* and *S. typhimurium* clearly show that they are interacting with the neighboring diiron center. Whether the observed difference is the result of different magnetic properties of the di-iron site and/or different geometry of the radical-diiron site will have to await magnetic susceptibility measurements and structural determinations.

Attempts to Form a Tyrosyl Radical by Oxidation of the Metal Centers in C. ammoniagenes R2F

Because the EPR spectrum of the *C. ammoniagenes* R2F sample showed a coupled Mn(II)-Mn(II) signal, we explored several different conditions to oxidize the metal chemically. It has been shown previously that H_2O_2 is capable of generating the tyrosyl radical in *E. coli* metR2, containing a diferric metal site but lacking the radical (17, 31). Addition of H_2O_2 to Mn-R2F oxidized the metal center as seen with UV-visible spectroscopy but did not generate a radical (data not shown), suggesting that oxidation of the manganese site and formation of the tyrosyl radical are uncoupled processes. Another well characterized manganese enzyme is Mn-catalase. Its dinuclear Mn(II) center changes oxidation state transiently during the enzyme reaction, but higher valency states can also be obtained chemically, *e.g.* by addition of KIO_4 (25, 32). Addition of KIO_4 to Mn-R2F generated a radical but the Mn(II)-Mn(II) EPR signal was not changed, also suggesting that generation of radical is not related to oxidation of the manganese site. Addition of the reducing agent ascorbate to *C. ammoniagenes* Mn-R2F generated some radical and an UV-visible absorption indicative of an oxidized metal site. Again, EPR analysis of the oxidized sample showed an increase of radical but no change in the feature of the manganese center. Presumably, ascorbate did not interact directly with the metal sites but reduced ferric iron to ferrous, thereby making generation of an active metal site with radical possible.

DISCUSSION

More than two decades of literature have emphasized the typical manganese requirement of some Gram-positive bacteria (10, 33, 34). Manganese starvation studies have shown that the primary lesion in these bacteria is impaired replication (33). Growth arrest caused by starvation of manganese is reversible, and addition of manganese can resume normal

TABLE III
Radical content and specific activity of E. coli, S. typhimurium, and C. ammoniagenes R2/R2F proteins with iron or manganese metal centers

Type of protein	Metal center	Fe/monomer	Mn/monomer	Tyr·/monomer	Specific activity[a]
					nmol mg^{-1} min^{-1}
E. coli R2	Fe[b]	1.4–1.5	ND[c]	0.5	4000–6000
	Mn[d]	ND	1.8–1.9	0	ND
S. typhimurium R2F	Fe	1.4–1.6	ND	0.2–0.5	325–660
	Mn	ND	1.6	0	3
C. ammoniagenes R2F	Fe	0.5–1.5	0.09	0.05–0.2	36–48
	Mn	0.5	1.6	0.03	9

[a] Specific activities were obtained at 25 °C for *E. coli*, at 30 °C for *C. ammoniagenes*, and at 37 °C for *S. typhimurium*.
[b] References 1, 24, and 38.
[c] ND, not determined.
[d] Reference 37.

growth. This is not the case when iron is added to the manganese-starved bacteria (34). Thus iron and manganese are not interchangeable *in vivo.* Based on these observations, the nature of the *C. ammoniagenes* RNR and in particular its metal cofactor have attracted much attention, and it has been claimed that the *C. ammoniagenes* RNR constitutes a separate class of manganese-containing RNRs (35, 36).

The primary amino acid sequence of the *C. ammoniagenes* R2F protein is 67% identical to that of the *S. typhimurium* R2F protein. All the amino acid residues identified in the three-dimensional structure of the *S. typhimurium* protein that are metal ligands (9) are also found in *C. ammoniagenes* R2F (6). The metal site of *S. typhimurium* R2F can be considered to be a model for the *C. ammoniagenes* site, and we therefore investigated whether the naturally occurring diiron site in *S. typhimurium* R2F could be substituted by manganese and if the manganese protein would show activity. Reconstitution of apoR2F with manganese gave an inactive protein, whereas reconstitution with iron restored activity. Reconstitution with a mixture of iron and manganese resulted in partial, but low activity. From this it is a reasonable assumption that only the *S. typhimurium* R2F dimers with a full complement of four iron ions were enzymatically active. In analogy, we suggest that the low activity of R2F isolated from *C. ammoniagenes* after exposure to manganese also resided in a few molecules of R2F-retaining diiron sites.

Hitherto studied samples of *C. ammoniagenes* R2F have all been prepared from cells grown under manganese starvation and subsequently recovered in manganese-supplemented medium prior to harvest. These are of course not critical growth conditions for establishing the nature of the metal site. In preliminary experiments, we observed that a crude extract of *C. ammoniagenes* RNR prepared from a manganese-starved culture showed the same specific activity as a crude extract prepared after recovery of the cells in manganese-supplemented medium.[2] However, several attempts to obtain highly purified R2F from *C. ammoniagenes* grown in rich media without metal ion additions were unsuccessful in our hands.[3] We therefore used the cloned *C. ammoniagenes nrdF* gene to construct an efficient expression system for *C. ammoniagenes* R2F in *E. coli.*

Both *in vivo* studies in the *E. coli* overproduction system and *in vitro* studies on overproduced R2F favor the idea that enzymatically active *C. ammoniagenes* R2F is an iron protein. *In vivo*, a high content of a typical tyrosyl radical signal was generated in iron-supplemented cultures, but not in manganese-supplemented cultures. Likewise, by studying the relation between metal content and enzyme activity in the overproduced purified protein, we can conclude that an enzymatically active iron center is self-assembled in *C. ammoniagenes* R2F. Formation of the manganese center, on the other hand, does not seem to correlate with enzyme activity. It was possible to reconstitute the R2F sample to higher radical content and specific activity in the presence of ferrous ions. Similar results were also obtained for the class Ib R2F protein from *S. typhimurium.*

Enzymatically active *C. ammoniagenes* R2F has a typical tyrosyl radical UV-visible absorption spectrum with a sharp 408-nm band and an EPR spectrum identical to that of *S. typhimurium* R2F (19, 20). The radical is susceptible to the radical scavenger hydroxyurea, and the saturation behavior of the tyrosyl radical suggests that the radical is in magnetic interaction with an antiferromagnetically coupled diferric center. Collectively, these results show that active *C. ammoniagenes* R2F is a typical class Ib RNR with a diferric metal cofactor coupled to a stable tyrosyl radical.

The EPR signal of the manganese-containing R2F from *C. ammoniagenes* has not been seen in ribonucleotide reductase before. When the class Ia R2 from *E. coli* is loaded with manganese, it shows a broad EPR singlet at 77 K centered at $g = 2.0$ (37). The manganese-reconstituted R2F from *S. typhimurium* used in this study showed similar features as R2F from *C. ammoniagenes*. Thus, the coupling between manganese atoms seems to be a property of class Ib R2F.

It has been claimed that the metal center in the class Ib enzyme of *C. ammoniagenes* is a mononuclear manganese center (13). The most convincing argument against a mononuclear center is the conservation of the metal ligands. Sequence alignment of *C. ammoniagenes* and *S. typhimurium*, whose structure has been determined, argues for a preserved structure of the site. We have shown that R2F from *C. ammoniagenes* and *S. typhimurium* can bind at least 1.6 manganese/monomer. The class Ia R2 from *E. coli* (37) can also bind approximately 2 manganese/monomer (Table III). The different class I enzymes have conserved metal ligands, and the amino acids in a sphere with a 9-Å radius around the metal center are the same (9). This means that very fine differences could regulate the properties of the metal center. As seen in Table III the enzyme activity of class Ib is lower than that of class Ia. Most likely, we have not yet found the optimal reaction conditions for class Ib. For instance, DTT is used as a reductant in the activity assay instead of the class Ib NrdH-redoxin, which gives higher activity (6). However, because many species have more than one type of ribonucleotide reductase, class Ib might be expressed under specific conditions where a lower activity is sufficient. In addition, the enzyme activity of *C. ammoniagenes* class Ib RNR was measured either at a suboptimal amount of iron or a suboptimal amount of R1E. Our previous sequence homology studies have shown that *C. ammoniagenes* RNR is a class Ib enzyme (6). Our current demonstration that *C. ammoniagenes* R2F is active with iron corroborates its classification as a true class Ib enzyme.

[2] L. Tulokhonova and B.-M. Sjöberg, unpublished result.
[3] Y. Huque, unpublished results.

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The Active Form of the R2F Protein of Class Ib Ribonucleotide Reductase from *Corynebacterium ammoniagenes* Is a Diferric Protein

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