RIBONUCLEOTIDE REDUCTASES (RNRs) are key enzymes in living cells that provide the precursors of DNA synthesis. The three characterized classes of RNRs differ by their metal cofactor and their stable organic radical. We have purified to near homogeneity the enzymatically active Mn-containing RNR of Corynebacterium ammoniagenes, previously claimed to represent a fourth RNR class. N-terminal and internal peptide sequence analyses clearly indicate that this C. ammoniagenes RNR is a class Ib enzyme. In parallel, we have cloned a 10-kilobase pair fragment from C. ammoniagenes genomic DNA, using primers specific for the known class Ib RNR. The cloned class Ib locus contains the nrdHIEF genes typical for class Ib RNR operon. The deduced amino acid sequences of the nrdE and nrdF genes matched the peptides from the active enzyme, demonstrating that this C. ammoniagenes RNR is composed of R1E and R2F components, typical of class Ib. We also show that the Mn-containing RNR has a specificity for the NrdHI-redoxin and a response to allosteric effectors that are typical of class Ib RNRs. Electron paramagnetic resonance and atomic absorption analyses confirm the presence of Mn as a cofactor and show, for the first time, insignificant amounts of iron and cobalt found in the other classes of RNR. Our discovery that C. ammoniagenes RNR is a class Ib enzyme and possesses all the highly conserved amino acid side chains that are known to ligate two ferrous ions in other class I RNRs evokes new, challenging questions about the control of the metal site specificity in RNR. The cloning of the entire NrdHIEF locus of C. ammoniagenes will facilitate further studies along these lines.

RIBONUCLEOTIDE REDUCTASE OF CORYNEBACTERIUM AMMONIAGENES IS A CLASS IB ENZYME

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The manganese-containing ribonucleotide reductase of Corynebacterium ammoniagenes is a class Ib enzyme.*

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Ribonucleotide reductases (RNRs) catalyze the reduction of ribonucleotides providing 2'-deoxyribonucleotides for DNA replication and repair. Three well-characterized classes of RNRs, with limited sequence similarities, have been described. They differ in their overall protein structure and cofactor requirement but have in common an allosteric regulation and the use of an organic radical to initiate catalysis through free radical chemistry (1, 2). Apart from the similarity in mechanism, the radical chain initiator and the accompanying metal cofactor differ between the three classes. Class I enzymes (αβ) contain a stable tyrosyl radical and a dinuclear iron center. Class II enzymes (α and α¡) use adenosylcobalamin as cofactor and cleave it to produce a 5'-deoxyadenosyl radical (3, 4). The anaerobic class III enzymes (αβ) possess a stable glycol radical and an iron-sulfur cluster (5). Moreover, the different RNRs require their specific physiological reductants thioredoxin, glutaredoxin, and formate, respectively (6–8). At the beginning of the 1990s, only these three classes of RNR were known, and they were found to cover all major branches of the tree of life. However, additional types of RNRs may remain to be discovered, and questions about non-exhaustively characterized atypical RNRs have to be answered.

During the last few years, an additional operon, in practice silent under normal laboratory growth conditions, coding for a new type of RNR, was found in Salmonella typhimurium and Escherichia coli (9–11). These enzymes share with class I enzymes the subunit composition and distinct sequence similarity, including all highly conserved residues, such as the iron ligands, the tyrosyl radical, and active site cysteines. Thus, the discovery of these enzymes led to the division of the class I RNR in two subclasses, classes IA and IB (12). The class IA reductase is encoded by the nrdA and nrdB genes, coding for the homodimeric proteins R1 and R2, respectively, and the class IB reductase is encoded by the nrdE and nrdF genes, coding for the homodimeric proteins R1E and R2F, respectively. In E. coli and S. typhimurium, the low expression of the nrdE and nrdF genes of class IB cannot support aerobic growth, and these bacteria are totally dependent on class IA (11). Moreover, the physiological role of these "silent" enzymes is still unknown. However, the Lactococcus lactis RNR was found to be a functional...
Mn-containing Ribonucleotide Reductase of *C. ammoniagenes*

**EXPERIMENTAL PROCEDURES**

**Materials, Strains, and Plasmids—**Wild type *C. ammoniagenes* (ATCC 6872), obtained from the collection of A. N. Bach (Institute of Biochemistry, Russian Academy of Sciences, Moscow, Russia) and *E. coli* DH5αF’ (CLONTECH) strains were used. Plasmid vectors used were pBlueScript SK (+) (pBSK, Stratagene) for subcloning and sequencing and pGEM-T (Promega Corp.) for cloning of PCR-generated fragments. Oligonucleotide primers were from MWG-Biotech (Germany). Restriction endonucleases and other enzymes were from Boehringer Mannheim. [5-3H]CDP was obtained from Amersham Corp.

**PCR Amplification of Partial nrdF Gene—**For PCR amplification of the nrdF gene of *C. ammoniagenes*, two primers were designed from conserved R2F peptide sequences (GYKYK and NHDDFFS, respectively; indicated in Fig. 3) and CoryFup, 5’-GGCTACAGATTACAG-3’, and CoryFlow, 5’-AACCAGACTCTTTC-3’ (antisense). Genomic DNA (0.2 μg) was used as template in a 50-μl PCR amplification reaction with 50 pmol of each primer, all dNTPs (0.2 mM each), 5 μl of 10× PCR buffer (Boehringer Mannheim), 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 50 °C, and 1 min at 72 °C; and (c) 7 min at 72 °C. The amplification product was purified from an ethidium bromide, 3% Nusieve-agarose gel by melting the band in 6 M NaCl at 50 °C and using the Wizard DNA Clean-up system (Promega Corp.).

**Construction and Screening of a Chromosomal *C. ammoniagenes* λ Phage Library—**The library of *C. ammoniagenes* ATCC 6872 genomic DNA consisted of a mixture of partially digested DNA. Freshly prepared genomic DNA (15 μg) was partially digested with ScaI. Fragments of 6–11 kb were pooled, and restriction-generated ends were filled in with A and G nucleotides by incubation at 37 °C for 30 min with 10 units of Klenow DNA polymerase (Boehringer Mannheim). Lambda GEM-12 vector (Promega) was prepared by filling in XhoI-generated ends with T and C nucleotides. After ligation of insert DNA to vector, the library was packaged using Packgene extracts (Promega).

**Statistical calculations (28) indicate that about 3900 recombinant phages would cover the entire *C. ammoniagenes* genome with a probability of 99.99% when an insert length of 11 kb and a genome size of 3 Mb (29) are assumed. A library with a titer of 1.8 × 1010 plaque-forming units was obtained and screened by phage-DNA hybridization after blotting to Hybond-N nylon membranes (Amersham Corp.) by using the DIG DNA labeling and detection kit from Boehringer Mannheim and following the supplier’s recommendations. Phase A DNA was isolated as described by Sambrook et al. (27).

**Fermentation and Purification of RNR—**RNR of *C. ammoniagenes* ATCC 6872 was inoculated from a slant (1% yeast extract, 1% glucose, 1% yeast nitrogen base, Difco) grown at 30 °C for 24 h in fermentable medium (2% glucose, 1% peptone, 1% yeast extract, 0.3% NaCl, 0.05 mg/ml biotin) and cultivated at 30 °C overnight. The overnight culture was used to inoculate several 1-liter batches of minimal fermentation medium (21), and cultivation was continued in 5-liter flasks at 30 °C and 220 rpm. After 10 h of growth, 10 μM MnCl2 was added to the medium, and 1 h after Mn repletion, cells were harvested by centrifugation.

**Growth Conditions and General Recombinant DNA Techniques—**
applied on a MemSep column HP1500 (DEAE-cellulose) equilibrated with Buffer A. The separation was performed by ConSep system at a flow rate of 20 ml/min. After a washing step with 200 ml ofBuffer A, the elution was continued with 400 ml of 0.15 ± 0.0 NaCl in Buffer A followed by a 0.15–0.4 M NaCl linear gradient in Buffer A in a total volume of 500 ml of buffer at a flow rate of 10 ml/min. The fractions were collected and concentrated by ultradialysis (Sartorius; cutoff, molecular weight of 12,000) in Buffer A and stored at ~8 °C for further purification.

The concentrated enzyme solution was loaded onto a Superdex 200 column (30 × 1.3 cm) previously equilibrated in Buffer A containing 10% glycerol at 0 °C temperature. Proteins were eluted from the column at a flow rate of 0.5 ml/min. Active fractions were pooled and concentrated at 4 °C in Centricon 30 (Amicon) and stored at ~8 °C.

The concentrated protein was then adsorbed to a 1-m1 MonoQ-anion exchange column run at room temperature. After a first washing of the column by 5 ml of Buffer A containing 10% glycerol and 0.28 ± 0.0 NaCl, the proteins were eluted with a linear NaCl gradient at a flow rate of 1 ml/min (25 ml of 0.28–0.7 ± 0.0 NaCl in Buffer A containing 10% glycerol). Fractions (0.5 ml) were collected in tubes immersed in an ice bath, pooled according to the UV absorption profile, concentrated at 4 °C in Centricon 30, and analyzed for protein concentration and reductase activity. The procedure separated two protein components that together are required for enzyme activity. The purified components were stored at ~8 °C.

Enzyme Activity Assay—RNR activity was assayed in 50-μl mixtures containing 120 m mM potassium phosphate buffer, pH 7.0, 1 mM dATP, as a positive effector, 1 mM magnesium acetate, 10 mM DTT, 13 mM 2-thioglycerol, 5–20 μl of the concentrated protein solution. The reaction was started by addition of [3H]CDP (specific activity, 60,000–80,000 cpm/nmol) to a final concentration of 0.5 μM. Assay mixtures were incubated for 20 min at 30 °C and stopped by addition of 0.5 ml of ice-cold 1 M perchloric acid. One unit of enzyme activity corresponds to 1 nmol of dCDP formed per min of incubation (30).

SDS-PAGE and Protein Blotting—To obtain partial peptide amino acid sequences, SDS-PAGE was used. Protein samples (50 μg of total protein) were first denatured in a mixture of 125 mM Tris-HCl, pH 6.8, 2.5% SDS, 10 mM DTT, 15% glycerol, and 0.01% bromphenol blue. After boiling for 2–3 min and cooling to room temperature, the incubation was continued with 20 ml of ice-cold acetic acid for another 20 min in darkness at room temperature. Reduced and alkylated protein samples were separated on 7.5% SDS-polyacrylamide gel, stained with 0.1% Coomassie Brilliant Blue R-250 in 50% methanol, and destained in 50% methanol, 10% acetic acid. Protein bands corresponding to the α- and β-polypeptide chains of RNR were excised from the gel and used for subsequent proteolytic digestions.

For N-terminal sequence analysis, protein samples were treated as described above, but the alkylation step was omitted. After separation by SDS-PAGE as above, nonstained protein bands were blotted from the gel onto a nitrocellulose membrane (Fluorotrans; pore size, 0.22 μm). After blotting in a blotting buffer containing 23% formic acid, 192 mM glycin, 20% methanol, and 500 mM NaCl, an overnight blotting at 200 mA in a cold room, the proteins were visualized by staining with Coomassie Brilliant Blue R-250 (0.1% in 50% methanol) for 2 min and destaining in several changes of 50% methanol, 10% acetic acid, followed by rinsing with MilliQ water. Membrane pieces were subjected to automated Edman degradation in a Perkin-Elmer Applied Biosystems Model 494A sequencer, operated according to the manufacturer’s instructions.

Proteolytic Digestion and Amino Acid Sequence Analysis—The two excised gel bands containing the alkylated α- and β-polypeptides, respectively, were treated for in-gel digestion to prepare internal peptides for amino acid sequence analysis. Briefly, the gel pieces were washed with Tris-HCl/acetoneitrile to remove SDS and the Coomassie dye and to put the gel pieces in the appropriate digestion environment. After complete drying of the gel pieces, a solution containing 0.5 μg of Achromobacter tytocus prostea Lys-C (Wako Chemicals GmbH, Neuss, Germany) was allowed to absorb into the gel pieces. Rehydration with digestion buffer was continued until the gels were soaked, and incubation was carried out overnight at 30 °C. After acifying the incubation mixture, generated peptides were extracted from the gels and subjected to automated Edman degradation on a Perkin-Elmer-Applied Biosystems Model 494A sequencer, operated according to the manufacturer’s instructions.

Spectroscopic Methods—EPR spectra at 9.36 GHz measured at 77 K were recorded on a Bruker ESP 300 spectrometer using a cold finger Dewar flask for liquid nitrogen. Subtractions were performed using the ESP 300 software. Denaturation was done by adjusting the sample to pH 1 by addition of 1 M nitric acid. Buffer from the flow-through of the centricon concentration step prior to the EPR analysis was used as background control for the native sample. For the denatured sample, the same amount of nitric acid as added to the protein sample was added to the background control sample. Background spectra were recorded under conditions identical to those for the native and denatured protein and thereafter subtracted from the total spectrum to give the spectra presented in Fig. 7.

PCR products were made on a Perkin-Elmer Z3030 graphite furnace. Calibrations for each metal were made by the use of several solutions of known metal concentration in the same buffer as used for the sample.

Other Methods—Protein concentration was determined either by the modified Lowry method (32) or the Bradford method (33) using bovine serum albumin as standard. Analytical protein gel electrophoresis was done by the Phast gel system (Pharmacia) in 7.5% or 10–18% denaturing polyacrylamide gels with Coomassie or silver staining.

RESULTS

PCR Isolation of an Internal Fragment of the C. ammonigenes nrdF Gene—The deduced amino acid sequences of all known RNR class Ib nrdF genes contain some highly conserved regions that allow the design of NrdF-specific oligonucleotides for PCR amplification. Primers CoryFup and CoryFlow (see “Experimental Procedures”) were designed from the R2F conserved regions GKYKYQ and NHDFFS, respectively, according to the Corynebacterium codon usage (34) and used for PCR amplification of selected parts of genomic DNA extracted from C. ammonigenes. A single 297-bp product, which was of the expected size range, was amplified, cloned in pGEM-T plasmid DNA, and sequenced in both directions. The sequence of the amplified and cloned product corresponded to a nrdF gene fragment according to its high homology to the S. typhimurium nrdF gene (60.7% identity at the nucleotide sequence level).

The cloned fragment was used as a probe for screening a genomic C. ammonigenes library.

Cloning of the C. ammonigenes nrdEF Genes—Our cloning strategy assumed that the nrdE and nrdF genes would be located in close proximity to each other in the C. ammonigenes genome as in all bacterial nrdF operons studied thus far (9, 11, 12, 14–16). The amplified nrdF fragment was used as a hybridization probe for screening a Phage genomic C. ammonigenes library enriched for 6–11 kb fragments (see “Experimental Procedures”). Several positive phage plaques were purified, and their DNA was extracted and checked by restriction endonuclease analysis and Southern hybridization and found to contain the nrdF gene. Several fragments derived from the endonuclease digestion of these λ phage DNA clones were cloned into pBSK(+) and sequenced from both extremes to localize the nrdE and nrdF genes. A 10 kb SacI fragment from one of these positive plaques was assumed to contain both genes and was subcloned into SacI-digested pBSK(+) resulting in plasmid pUA728.

Southern hybridization was performed to confirm that the cloned SacI fragment originated from C. ammonigenes genomic DNA and was not hybridizing with some other bacterial chromosomes (data not shown). Plasmid pUA728 was then used for DNA sequencing. To obtain the full-length sequence, a combination of fragment subcloning and generation of progressive unidirectional nested deletions for both strands were applied. A sequence of 6054 bp, covering the nrdHIEF genes of C. ammonigenes (Fig. 1), has been deposited into the GenBank data base.

Analysis of the nrdHIEF Gene Sequence—Five different open reading frames are present in the nucleotide sequence obtained from plasmid pUA728 (Fig. 1). Four of them correspond to the previously reported genes nrdH (228 bp), nrdI (435 bp), nrdE (2
163 bp), and \( nrdF \) (990 bp). The fifth putative open reading frame (714 bp), located between \( nrdE \) and \( nrdF \), would be transcribed in the opposite direction to the \( nrd \) genes. The function of this open reading frame still remains unknown, although comparison with the current data bases shows the highest homologies to several bacterial transcription regulatory proteins of similar size.

The G+C contents of the \( nrd \) genes (\( nrdH \), 53.5%; \( nrdI \), 50%; \( nrdE \), 51.5%; and \( nrdF \), 48.5%), as well as their codon usage, are in accordance with those described for genes of corynebacterial origin (34). The putative translational start codon of genes \( nrdE \), \( nrdF \), and \( nrdI \) is GTG; that of \( nrdH \) is ACG. Putative RBS sequences complementary to the 3’ end of the 16S rRNA of \( B. \) subtilis (35) are located 14 nucleotides upstream of \( nrdE \) (GAAGAG), 13 nucleotides upstream of \( nrdF \) (AGGAGG), 14 nucleotides upstream of \( nrdH \) (AAAGAG), and 10 nucleotides upstream of \( nrdI \) (AAAGAGG).

When we searched for a hypothetical promoter region, we found a putative TATA box (TATAAG) 111 bp upstream of the \( nrdH \) gene. Sixteen base pairs upstream of the TATA box, a GTG promoter (990 bp), a putative TATA box (TATAGT) 111 bp upstream of the predicted molecular masses of both proteins, 81.2 kDa for R1E (720 residues) and 37.9 kDa for R2F (329 residues), are in accordance with other known class Ib proteins. As expected for class Ib proteins, only limited similarities exist between the \( C. \) ammoniagenes RNR proteins and the class Ia enzymes; the percentages of similarity to the \( E. \) coli R1 and R2 proteins are 35 and 37%, respectively. The corresponding similarities for class Ia and Ib proteins within one species are on the same order (38). Interestingly, all residues that are functionally important in the class I proteins are also present in the deduced \( C. \) ammoniagenes RNR proteins.

Purification of Active RNR from \( C. \) ammoniagenes—To correlate our genetic results with previously published biochemical observations, we essentially followed the published strategy (18) for cell growth and the first steps of enzyme purification. Cells grown in Mn-deficient medium lost their colony-forming ability after about 10 h of fermentation, but addition of 10 \( \mu \)M MnCl, at that time fully preserved the viability of the cells. The cells were harvested 1 h after manganese repletion and used as a starting material for purification of enzymatically active RNR.

Purification of the holoenzyme (described in detail under “Experimental Procedures”) involved three major steps: precipitation by dialysis of cell-free extract against low salt buffer, chromatography on a weak anion exchanger, and size fractionation by Superdex 200 gel filtration. At this stage, the specific enzyme activity was 6.5 units/mg, and the overall yield was 35% (Table I). Separation of the R1E and R2F components was achieved by fast protein liquid chromatography anionic chromatography (Fig. 4), resulting in preparations of 70 and >90% purity, respectively (Fig. 5). Mixing of the two components resulted in a specific activity of 34 units/mg. In general, the specific activities obtained by us in the different purification steps are approximately an order of magnitude higher than those reported earlier (18).

The RNR activity eluted from the gel filtration column at a volume corresponding to an apparent molecular mass of 160 kDa, according to a calibration of the column with gel filtration standard protein. Considering the theoretical molecular mass of...
of NrdE and NrdF polypeptides as deduced from nucleotide sequence analyses, this would fit with an αβ2 subunit composition for the C. ammoniagenes RNR. A previous study also reported an αβ2 composition according to gel filtration and sucrose gradient centrifugation experiments (18). Such a quaternary structure is, however, in contrast to the α2β2 composition of other class Ib RNRs (39) and is not very likely to be the true in vivo composition. A lower than expected molecular mass may be explained by a high dissociation constant, low protein concentration, and/or the absence of positive allosteric effector nucleotides. Further characterization of this particular point has to await the overexpression of cloned material.

Identification of the Active RNR as a Class Ib Enzyme by Amino Acid Sequence Analyses—The enzyme preparation after DEAE-cellulose chromatography contained several protein bands when analyzed by SDS-PAGE, but after Superdex 200 chromatography, the two most prominent bands were of the expected sizes for RNR α- and β-polypeptides (Fig. 5). Material from the 80- and 40-kDa bands was subjected to partial amino acid sequence analyses using the techniques described under "Experimental Procedures." N-terminal sequences, as well as partial internal peptide sequences, were obtained for both subunits (Table II). The peptides were analyzed by comparison to the deduced NrdE (deposited in GenBank) and NrdF (Fig. 3) sequences of C. ammoniagenes and found to match perfectly in all positions of the internal peptides. The N-terminal peptides obtained from the blotted α- and β-polypeptide bands were also in accordance with the gene sequences. The N-terminal se-
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The table summarizes the averaged purification result from three different purifications using the procedure described under “Experimental Procedures,” starting with 8 liters of culture (approximately 30 g of wet cells).

| Fraction          | Protein mg | Total activity units | Specific activity units mg⁻¹ | Recovery % | Purification factor |
|-------------------|------------|----------------------|-----------------------------|------------|---------------------|
| Cell-free extract | 983        | 24                   | 0.024                       | 100        | 1                   |
| Low-salt precipitate | 132      | 35                   | 0.26                        | 146¹¹      | 11                  |
| DEAE-cellulose chromatography | 5.1 | 16                   | 3                           | 67         | 125                 |
| Superdex 200      | 1.3        | 8.5                  | 6.5                         | 35         | 271                 |
| MonoQ             | 0.045      | 1.5                  | 34²                        | 6          | 1417                |

¹ A unit is defined as 1 nmol of CDP reduced per min.
² Increase was probably due to the removal of inhibitory compounds from the cell extract.
³ Data reported on this line concern only the R2F purified fraction, not a holoenzyme complex.
⁴ Activity was measured by complementation with the R1E fraction, but the specific activity was calculated considering only R2F protein concentration.

DISCUSSION

To date, three different classes of RNR have been described in detail. Suggestions had been put forward as to the existence of a fourth, manganese-dependent class, based on the presence of metal ion and the radical signal in C. ammoniagenes RNR (18, 24). This enzyme was, however, shown to have certain features (e.g. hydroxyurea sensitivity and polypeptide sizes) in common with the well characterized class I RNR of eukaryotes and bacteria (18, 23). Our purpose was to establish whether the manganese-dependent RNR really is a new class that could be fitted into the evolutionary pattern described by the other three classes. We therefore purified the active RNR of C. ammoniagenes to obtain partial amino acid sequence results of its components and to clone the genes for this enzyme. We also wanted to establish whether C. ammoniagenes has the widespread (in bacteria) class Ib RNR.
In this report, we show that the active Mn-containing RNR of *C. ammoniagenes* is of the class Ib type and that the *nrd* genomic region contains the same open reading frames as previously seen for the class Ib operon in enterobacteria and *L. lactis* (11, 12). These are the two genes for R1E and R2F, as well as the gene for a thioredoxin-like protein called NrdH-redoxin and a fourth open reading frame of unknown function called *nrdI*. The *nrdH* gene is not present in all *nrdEF* clusters; it is absent in *B. subtilis* and in *Mycoplasma* species (14–16).

As in other class Ib systems, we found that the species-specific NrdH-redoxin was the preferred reductant for the *C. ammoniagenes* RNR. The *nrdI* gene is present in all known *nrdEF* loci, and preliminary studies with the *S. typhimurium* system have shown that the NrdI protein stimulates the NrdEF-dependent CDP reduction in the presence of NrdH-redoxin (37).

In Fig. 2, the gene organization of the *nrd* locus of *C. ammoniagenes* is homologous to the ones present in enterobacteria, *L. lactis*, *B. subtilis*, and *Deinococcus radiodurans* and to *M. tuberculosis* (in which the two *nrdF* genes are less closely linked to the rest of the operon). A different organization is found in

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**Table II**

Peptide sequences of *C. ammoniagenes* R1E and R2F

| Source | Origin | Sequence |
|--------|--------|----------|
| R1E N | terminus | 1X(T)Qg(Q)LGKTVAEPVK14 |
| R1E Internal peptide 1 | | 3KQFDK |
| R1E Internal peptide 2 | | 226KHIENQSSGI235 |
| R2F N | terminus | 1X(S)NEYDEYIANHTDPVKAIN |
| R2F Internal peptide 2 | | 44KIPVSNDIQSWNK56 |
| R2F Internal peptide 3 | | 56KMTPQEQLATMRV68 |
| R2F Internal peptide 4 | | 112RYSNIF118 |

*X* denotes unidentified residue with corresponding residue deduced from nucleotide sequencing in parenthesis; lowercase indicates differences from nucleotide sequencing results with corresponding residue deduced from nucleotide sequencing in parenthesis; superscript numbers refer to numbering of protein products as deduced from nucleotide sequencing of the corresponding genes (see GenBank accession no. Y09572 for R1E sequences and Fig. 3 for R2F sequences).

The Lys in front of all internal peptides was not obtained from the sequencing results but was introduced based on the known specificity of Lys-C protease. The correctness of this assumption is evident from a comparison with the corresponding gene sequence.

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**Table III**

Atomic absorption metal ion analysis of nitric acid-denatured R2F purified from *C. ammoniagenes*

| Metal ion | Metal:R2F stoichiometry | mol/mol |
|-----------|-------------------------|---------|
| Manganese | 0.45                    |         |
| Iron      | 0.08                    |         |
| Cobalt    | 0                       |         |

*Calculated with a known R2F concentration of 12 μM in the sample and the corresponding metal molecular weight. Numbers given are expressed as number of metal ions per R2F polypeptide chain.*
MycoPlasma species, in which the nrdF gene is located upstream from the nrdI and nrdE genes.

The deduced NrdEF proteins from C. ammoniagenes are currently most closely related to the R1E and the active R2F protein of M. tuberculosis. Both species belong to the phylogenetic group of Gram-positive eubacteria with a high G+C content. It was recently reported that M. tuberculosis contains a second nrdF gene, which is inactive (48). We have not been able to find a second C. ammoniagenes nrdF gene by PCR amplification or Southern blotting. The identification of the active RNR from C. ammoniagenes as belonging to class Ib helps to replace the initial idea, based on the enterobacterial loci, that nrdE genes are generally silent. As exemplified in the phylogenetic tree of R2F proteins (Fig. 2), class Ib enzymes are widely spread among eubacteria, and the completely sequenced genomes of B. subtilis, Mycoplasma genitalium, and M. pneumoniae code only for class Ib RNRs (15, 16, 49).

The specific activity of the Mn-containing RNR of C. ammoniagenes obtained by us, even if improved at least an order of magnitude compared with previous studies (18, 23), is only 12 and 18% of the specific activities described for class Ib RNR from S. typhimurium and L. lactis, respectively (10, 12). There are some obvious reasons for the low enzyme activity obtained by us. First, our preliminary studies indicate that inclusion of species-specific NrdH-redoxin will increase the C. ammoniagenes RNR activity at least 2-fold. Second, the substoichiometric amount of metal ion per R2F polypeptide observed after the four-step purification procedure may lead to substoichiometric levels of organic free radical.

Atomic absorption analysis of the isolated C. ammoniagenes R2F protein showed about 0.5 mol/mol Mn/R2F polypeptide chain. Because of the homology with the well known diiron-RNRs, 2 metal ions per R2F was expected. The EPR analysis suggests that the manganese ions may be magnetically coupled, but the substoichiometric amount of metal ion does not allow a definitive conclusion about the structure of the metal center at this point. However, our EPR and atomic absorption analyses clearly confirm earlier published observations (18) that the active C. ammoniagenes RNR contains manganese, and as we show here, in essence, it lacks iron. The strong amino acid sequence homology between active Mn-containing RNR from C. ammoniagenes and class Ib RNRs is thus in many respects remarkable: (a) all previously described class I enzymes are diiron proteins, including the class Ib enzyme from S. typhimurium (10); (b) all iron binding residues in the Fe-RNRs (class Ia and Ib) are conserved in the C. ammoniagenes RNR (Fig. 3); and (c) even though both E. coli class Ia R2 and mouse R2 can bind manganese at their metal centers, Mn substitutions have invariably led to nonactive enzymes (41, 42).

Our results bring a series of new fascinating questions to the field of RNR research, in particular concerning metal specificity and diversity despite high sequence similarities. The metal ion content of the class Ib enzymes has currently only been investigated for the recombinant S. typhimurium (10) and native C. ammoniagenes enzymes. Even though the S. typhimurium R2F has a diiron center, it is not known whether it can also work with manganese. Likewise, it is not yet known whether the C. ammoniagenes enzyme will work with iron. A clear definition of the metal ion dependence of the C. ammoniagenes RNR will have to await the design of an overproducing system. In addition, manganese activation experiments should be performed with other class Ib enzymes. Interestingly, the R2F sequences in the two Mycoplasma species both lack 3 of the metal ligating residues conserved in the rest of the class I enzymes. However, because it is not known which metal ions are present in other class Ib reductases, neither the deduced C. ammoniagenes NrdF amino acid sequence nor the phylogenetic tree can yet be used for predictions about metal ion specificity. Specific three-dimensional features in the vicinity of the metal site may have to be identified to explain a Mn dependence.

Some other enzymatic systems are known to use, alternatively, iron or manganese and have similar or identical metal binding residues (43). In the superoxide dismutase family, the enzyme from Propionibacterium shermanii is functional with either Fe or Mn, i.e. cambilastic, whereas other superoxide dismutases are strictly manganese- or iron-dependent. Comparisons of their three-dimensional structures revealed that the metal ligands are the same in all three types and that differences are localized to the second coordination sphere of the metal center (44). A similar phenomenon seems to occur among extradiol-cleaving catechol dioxygenases. All members of this family are iron enzymes except the 3,4-dihydroxyphenylacetate 2,3-dioxygenase from Arthrobacter globiformis, which contains manganese instead of iron (45). Comparison using the structure of one iron enzyme, sequence alignment, and site-directed mutagenesis of the 3,4-dihydroxyphenylacetate 2,3-dioxygenase suggests that differences can be seen only in the second coordination sphere and that all direct ligands of the two metal ions are the same. These observations suggest a major role for the residues of the second coordination sphere in determining the metal specificity. The hypothesis may also apply to the metal specificity in RNR, because the well known diiron-binding site of E. coli class Ia R2 is intrinsically capable of binding manganese, albeit without activating the protein (41). Perturbations of the second coordination sphere might modify the redox properties of such a Mn center and lead to an active enzyme. One striking difference between prokaryotic class Ia and Ib R2 proteins is the substitution of Gln-43 and Ser-114, which form hydrogen bonds to the iron ligand His-241 in E. coli R2, for hydrophobic counterparts in the class Ib NrdF sequences. However, a preliminary modeled structure of the C. ammoniagenes R2F protein, based on the E. coli R2 structure, highlights only differences between class Ia and class Ib but none that are specific to the C. ammoniagenes RNR and absent from the other NrdF sequences. 3

The characterization of the C. ammoniagenes RNR as a class Ib enzyme evokes new, challenging questions. The cloning of the NrdHIEF locus will facilitate future studies on this RNR, whereby new insights in the design and fine-tuning of metal-active sites may be gained.

Note Added in Proof—Preliminary experiments indicate that binding of manganese ions to S. typhimurium apo R2F protein results in enzymatically inactive protein (P. Reichard, personal communication) and that cloned and overproduced C. ammoniagenes R2F can bind either manganese or ferrous ions and generate a characteristic tyrosine radical EPR signal.

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The Manganese-containing Ribonucleotide Reductase of *Corynebacterium ammoniagenes* Is a Class Ib Enzyme

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