THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 8, Issue of February 20, pp. 4329–4337, 1998
Printed in U.S.A.

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

(Received for publication, October 10, 1997)

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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 the *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 *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 NrdH-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 ferric 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 reductases (RNRs)[1] 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 ($\alpha_2\beta_2$) contain a stable tyrosyl radical and a dinuclear iron center. Class II enzymes (α or α_2) use adenosylcobalamin as cofactor and cleave it to produce a 5′-deoxyadenosyl radical (3, 4). The anaerobic class III enzymes ($\alpha_2\beta_2$) possess a stable glycyl 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 func-

* This work was supported by grants from the Swedish Cancer Society, the Swedish Research Council for Engineering Sciences and the Swedish National Board for Technical Development (to B.-M. S.); from the Spanish DGICYT (PB94-0687) and the CUR de la Generalitat de Catalunya (GRQ93-2049) (to I. G. and J. B.); and from Stiftelsen Lars Hiertas Minne, Magn. Bergvalls stiftelse, and Jeanssonska stiftelserna (to M. K.).

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y09572.

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** Supported by a predoctoral fellowship from Direcci-General d'Universitats de la Generalitat de Catalunya.

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[1] The abbreviations used are: RNR, ribonucleotide reductase; DTT, dithiothreitol; EPR, electron paramagnetic resonance; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s).

tionally active reductase of the class Ib type (12), and the purified enzyme from *Mycobacterium tuberculosis* also turned out to belong to this class (13, 48). Class Ib genes have also been described in *Bacillus subtilis, Mycoplasma genitalium,* and *M. pneumoniae* (14–16).

The isolation and characterization of a unique manganese-dependent RNR activity in *Corynebacterium* (formerly *Brevibacterium*) *ammoniagenes* was reported in the 1980s (17, 18). The specific Mn requirement of *C. ammoniagenes* was first observed in the 1960s during studies of factors controlling nucleotide overproduction (19, 20). Mn-starved cells showed so-called "unbalanced growth death" because they were arrested in DNA synthesis (17) due to inhibition of DNA precursor synthesis (21). Upon addition of manganese ions to the medium, DNA synthesis and growth were rapidly restored to the level of a nonstarved culture. The main target of Mn starvation was suggested to be RNR activity, which was very low in a Mn-depleted culture but was increased when manganese ions were supplied *in vivo* (17). Similar correlations between RNR activity and Mn-starvation conditions have been demonstrated in other coryneform bacteria, such as *Arthrobacter citreus*, *A. globiformis*, and *A. oxydans*, and in *Micrococcus luteus*, (17, 21, 22).

The partially purified *C. ammoniagenes* RNR was suggested to consist of two subunits (18, 23), a nucleotide-binding component called B1 (in this report renamed R1E) and a metal-containing component called B2 (in this report renamed R2F). The presence of Mn was suggested on the basis of specific ^{54}Mn incorporation into the R2F subunit, as well as appearance of a characteristic Mn six-line EPR spectrum after denaturation of a protein preparation containing R2F. Recently, a novel type of stable organic free radical signal was reported for partially purified *C. ammoniagenes* RNR (24). However, the radical has not been characterized in detail. Generally, a new metal center and a novel organic radical would be enough to define a new class of RNR. However, other properties, such as the sensitivity to hydroxyurea and the polypeptide sizes of this *C. ammoniagenes* RNR, suggest a similarity with the well known class I. An intriguing question is therefore whether the *C. ammoniagenes* RNR is a prototype of a new class of RNR or a subtype of one of the existing classes.

In this study, we report that the Mn-containing RNR of *C. ammoniagenes* is of the class Ib type. We have followed two parallel approaches: identification of class Ib genes in the *C. ammoniagenes* genome by PCR and purification to homogeneity of the active RNR from *C. ammoniagenes*, followed by N-terminal and internal peptide amino acid sequence analysis of the α- and β-polypeptide chains. The amino acid sequences obtained from the enzyme proper matched the cloned and sequenced class Ib genes *nrdE* and *nrdF*. In addition, the sequence of the neighboring genes *nrdH* and *nrdI* is reported.

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-^{3}H]CDP was obtained from Amersham Corp. *E. coli* thioredoxin was purified from SK3981 (25). *C. ammoniagenes* NrdH-redoxin was obtained from an overproducing strain carrying a recombinant vector with the *nrdH* gene.[2]

Growth Conditions and General Recombinant DNA Techniques—C. ammoniagenes ATCC 6872 and *E. coli* strains were grown aerobically in LB medium at 30 and 37 °C, respectively. Ampicillin was added at 50 μg/ml when selecting for plasmid-containing clones. Genomic DNA from *C. ammoniagenes* was extracted as described (26) and purified by ultracentrifugation on a cesium chloride gradient. ExoIII deletions were constructed by using the double-stranded nested deletions kit (Pharmacia Biotech Inc.) following the supplier's instructions. DNA sequencing was carried out using the dideoxynucleotide sequencing method with fluorescent universal primers (M13 direct and reverse) and the Automated Laser Fluorescent DNA sequencer (Pharmacia). Other general DNA manipulations and Southern hybridizations were done by standard procedures (27). Sequence analyses were made with the University of Wisconsin Genetics Computer Group package (version 9.0 for UNIX).

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 (GYKYQ and NHDFFS, respectively; indicated in Fig. 3): CoryFup, 5′-GGCTACAAGTACCAG-3′, and CoryFlow, 5′-AACCACGACTTCTTCTC-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 NaI at 50 °C and using the Wizard DNA Clean-up system (Promega Corp.), and cloned in pGEM-T according to the manufacturer's protocol. This fragment was labeled with the DIG DNA labeling and detection kit (Boehringer Mannheim).

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 *Sau*3A. 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 *Xho*I-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 1.8×10^4 plaque-forming units/ml 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. Phage λ DNA was isolated as described by Sambrook *et al.* (27).

Fermentation and Purification of RNR—C. ammoniagenes ATCC 6872 was inoculated from a slant (1% yeast extract, 1% glucose, 1% $CaCO_3$, 2% agar; Difco) grown at 30 °C for 24 h in 100 ml of inoculate 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 $MnCl_2$ was added to the medium, and 1 h after Mn repletion, cells were harvested by centrifugation. The cell paste was washed with Buffer A (85 mM potassium phosphate buffer, pH 7.0, 2 mM DTT), frozen on dry ice, and stored at −80 °C.

Unless otherwise indicated, all purification procedures were carried out at 4 °C. In a typical experiment, 24 g of wet weight frozen cells were disrupted through a X-press (BIOX). The disintegrated cells were homogenized and extracted with 3 volumes (per wet weight cells) of Buffer A by stirring for 45 min and then centrifuged for 30 min to remove cell debris. Nucleic acids were precipitated by dropwise addition of streptomycin sulfate to a final concentration of 1.5%. After stirring for 30 min, the precipitate was removed by centrifugation. The supernatant was dialyzed in SpectraPor membrane tubing (cutoff, molecular weight of 3500; Spectrum Medical Industries, Inc.) against 10 mM potassium phosphate buffer, pH 7.0, 2 mM DTT for 1 h. Precipitated proteins were removed by centrifugation, and the supernatant was further dialyzed against the same buffer overnight. Precipitated proteins (called low salt fraction) were collected by centrifugation, dissolved in a minimal volume (15–25 ml) of Buffer A, and stored at −80 °C for further purification.

Aliquots of the low-salt protein fraction (≤100 mg of protein) were

[2] E. Torrents, unpublished data.

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 of Buffer A, the elution was continued with 400 ml of 0.15 M NaCl in Buffer A followed by a 0.15–0.4 M NaCl linear gradient in Buffer A in a total volume of 800 ml. Fractions of 10 ml were collected, and RNR activity was eluted between 0.15–0.25 M NaCl. RNR-containing fractions were pooled and concentrated by ultradialysis (Sartorius; cutoff, molecular weight of 12,000) in Buffer A and stored at −80 °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 room temperature. Proteins were eluted with the same buffer 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 −80 °C.

The concentrated protein was then adsorbed to a 1-ml 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 M 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 M 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 −80 °C.

Enzyme Activity Assay—RNR activity was assayed in 50-μl mixtures containing 120 mM potassium phosphate buffer, pH 7.0, 1 mM dATP as a positive effector, 1 mM magnesium acetate, 10 mM DTT, 13 μM *E. coli* thioredoxin, 5–20 μl of the concentrated protein solution. The reaction was started by addition of [^{3}H]CDP (specific activity, 60,000–80,000 cpm/nmol) to a final concentration of 0.5 mM. 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 mM iodoacetamide 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 prewet (100% methanol) polyvinylidene difluoride membranes (Fluorotrans; pore size, 0.22 μM; Pall Filtron) in a blotting buffer containing 23 mM Tris base, 192 mM glycine, 20% methanol. After 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/acetonitrile 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 lyticus* protease 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 acidifying the incubation mixtures, generated peptides were extracted from the gels and isolated by narrow-bore reversed phase liquid chromatography on a μRPC C2/C18 SC 2.1/10 column operated in the SMART System (Pharmacia). A full description is found elsewhere (31). Of the collected peptides, some were selected for automated Edman degradation in 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.

Atomic absorption measurements 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 10–15% denaturing polyacrylamide gels with Coomassie or silver staining.

RESULTS

PCR Isolation of an Internal Fragment of the C. ammoniagenes 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 GYKYQ and NHDFFS, respectively, according to the *Corynebacterium* codon usage (34) and used for PCR amplification of selected parts of genomic DNA extracted from *C. ammoniagenes*. 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. ammoniagenes* library.

Cloning of the C. ammoniagenes nrdEF Genes—Our cloning strategy assumed that the *nrdE* and *nrdF* genes would be located in close proximity to each other in the *C. ammoniagenes* genome as in all bacterial *nrdEF* operons studied thus far (9, 11, 12, 14–16). The amplified *nrdF* fragment was used as a hybridization probe for screening a λ phage genomic *C. ammoniagenes* 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 *Sac*I fragment from one of these positive plaques was assumed to contain both genes and was subcloned into *Sac*I-digested pBSK(+), resulting in plasmid pUA728.

Southern hybridization was performed to confirm that the cloned *Sac*I fragment originated from *C. ammoniagenes* 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. ammoniagenes* (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

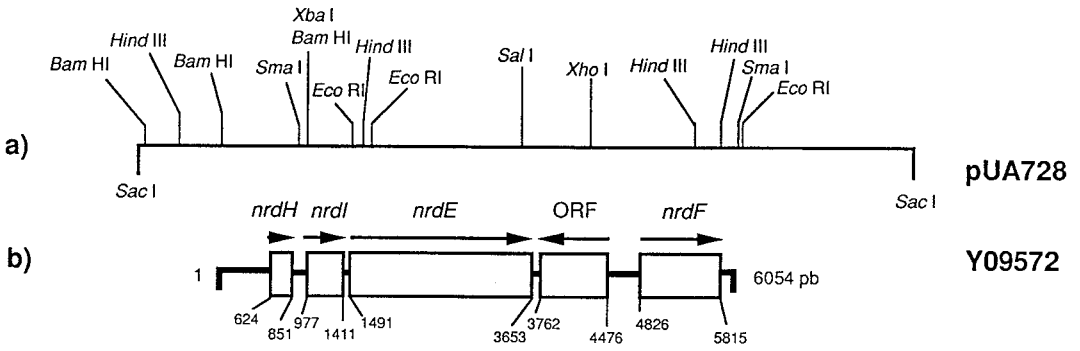

FIG. 1. *a*, restriction map of the *C. ammoniagenes* 10-kb *Sac*I fragment obtained from the λ phage library and used for DNA sequencing. *pAU728* denotes the resulting pBSK(+) derivative. *b*, gene organization of all open reading frames found in the 6054 bp deposited in the GenBank data base under the accession number Y09572.

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 ATG. Putative RBS sequences complementary to the 3′ end of the 16S rRNA of *B. subtilis* (35) are located 14 nucleotides upstream of *nrdE* (GAAAGG), 13 nucleotides upstream of *nrdF* (AGCAGGG), 14 nucleotides upstream of *nrdH* (AAAGG), and 10 nucleotides upstream of *nrdI* (AAAGGAGG).

When we searched for a hypothetical promoter region, we found a putative TATA box (TATAGT) 111 bp upstream of the *nrdH* gene. Sixteen base pairs upstream of the TATA box, a −35 promoter sequence (TTGCAG) was identified by its resemblance to the consensus promoter sequence from *C. glutamicum* (36). No promoter sequences were identified upstream of the *nrdF* gene. Nevertheless, because there exists a large intergenic region between *nrdE* and *nrdF* (1.2 kb), more evidence is needed to confirm that the *nrdHIEF* genes form an operon with a unique polycistronic mRNA, as occurs in the previously characterized *nrdHIEF* operons of Enterobacteriaceae (11) and *nrdIEF* from *B. subtilis* (14). In addition, no putative transcriptional terminator could be clearly identified, although two weak stem loops with ΔG (25 °C) of −10.2 and −12.5 kcal/mol can be found downstream from the *nrdE* and *nrdF* genes.

The hypothetical product encoded by the *nrdH* gene (75 residues, 8.3 kDa) corresponds to the previously described NrdH-redoxin from *E. coli* (37). The NrdH product has been found to be a specific electron donor for the class Ib enzyme of *S. typhimurium* and *L. lactis* (12, 37). The deduced NrdI product comprises 144 amino acid residues and has a predicted molecular mass of 15.8 kDa. The *nrdI* gene is conserved in all known *nrdEF* operons (Fig. 2), but its function remains to be clarified. A preliminary study has shown its stimulatory effect on the activity of the *S. typhimurium* NrdEF system (37) .

The deduced amino acid sequences of *C. ammoniagenes nrdE* and *nrdF* strongly resemble previously sequenced class Ib proteins. The percentages of identical amino acids are 70% for the *C. ammoniagenes* and *E. coli* R1E proteins and 66% for the R2F proteins (compare Figs. 2 and 3). Nevertheless, when comparing all known R1E and R2F sequences, the *C. ammoniagenes* proteins are more closely related to the *M. tuberculosis* proteins (13, 48) than to any other known class Ib proteins (Fig. 2). Also, the predicted molecular masses of both proteins, 81.2 kDa for R1E (720 residues) and 37.9 kDa for R2F (329 residues), are in agreement 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. Among others, in the R1E protein, there are five cysteine residues known to be involved in catalysis and enzyme turnover in *E. coli* R1, and in the R2F protein, there is the potential radical harboring residue Tyr-115, as well as residues forming a hydrophobic pocket around the tyrosyl radical in *E. coli* R2 (Fig. 3). In addition, all residues postulated to participate in radical transfer between R1 and R2 during catalysis are preserved in the deduced *C. ammoniagenes* R1E and R2F proteins. Most striking is that all six residues that act as ligands for the μ-oxo-bridged diiron site in the *E. coli* R2 protein also occur in equivalent positions in the deduced *C. ammoniagenes* R2F sequence (Fig. 3).

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 μM $MnCl_2$ 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

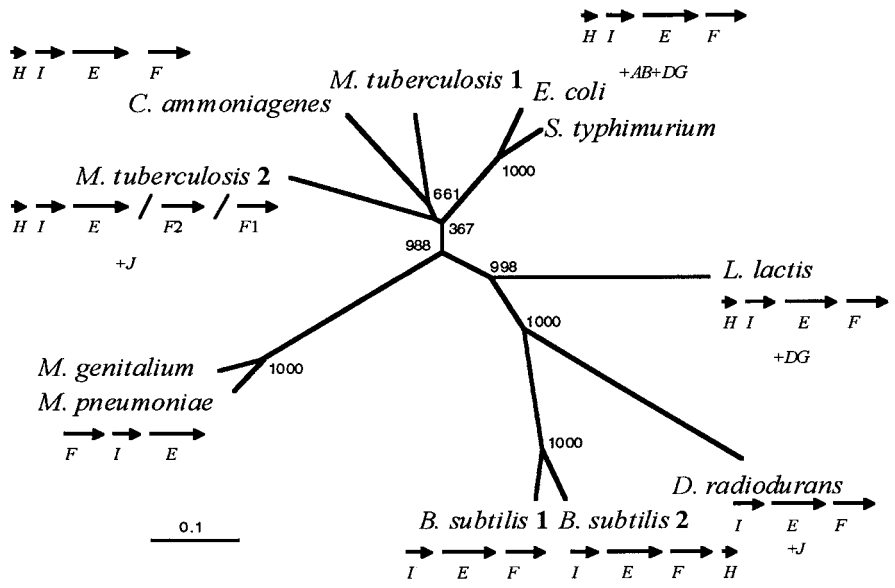

FIG. 2. **Phylogenetic tree of all available NrdF proteins.** The following protein sequences from the Swiss-Prot data base were used: RIR4_ECOLI (*E. coli*), RIR4_SALTY (*S. typhimurium),* RIR2_BACSU (*B. subtilis*), RIR2_MYCGE (*M. genitalium*), RIR2_MYCPN (*M. pneumoniae*), and RIR2_MYCTU (*M. tuberculosis 1*). NrdF2 sequence from *B. subtilis* was from Ref. 49 and NrdF sequences from *L. lactis*, *M. tuberculosis* 2, and *D. radiodurans* were from G. Buist (personal communication), H. Rubin (48), and The Institute for Genomic Research (personal communication), respectively. The CLUSTAL W (version 1.7) program (46) was used for sequence alignment. The tree was generated with 1000 bootstrap trials; the bootstrap values are indicated at the nodes. For each species, a schematic representation of the gene organization in the *nrdEF* locus, including the *nrdH* and *nrdI* genes, is shown, and when known, the presence of *nrdAB*, *nrdJ*, or *nrdDG*, encoding class Ia, class II, or class III RNRs, respectively, is shown.

```
                                                                      60
NrdF E.c. ------------------MKLSRISAINWNKISDDKDLEVWNRLTSNFWLPEKVPLSNDIP
NrdF S.t. ------------------MKLSRISAINWNKIQDDKDLEVWNRLTSNFWLPEKVPLSNDIP
NrdF C.a. --------SNEYDEYIANHTDPVKAINWNVIPDEKDLEVWDRLTGNFWLPEKIPVSNDIQ
NrdB E.c. AYTTFSQTKNDQLKEPMFFGQPVNVARYDQQKYDIFEKLIEKQLSFFWRPEEVDVSRDRI
                                . :.. .::    :   :: ::  . ** **:: :*.*
                                                                     120
NrdF E.c. AWQTLTVVEQQLTMRVFTGLTLLDTLQNVIGAPSLMPDALTPHEEAVLSNISFMDAVHAR
NrdF S.t. AWQTLSAAEQQLTIRVFTGLTLLDTIQNIAGAPSLMADAITPHEEAVLSNISFMDAVHAR
NrdF C.a. SWNKMTPQEQLATMRVFTGLTLLDTIQGTVGAISLLPDAETMHEEAVYTNIAFMESVHAK
NrdB E.c. DYQALPEHEKHIFISNLKYQTLLDSIQGRSPNVALLPLISIPELETWVETWAFSETIHSR
           :: :.  *:   :  :.  ****::*.     :*:.     . *:   . :* *::*::
                                                                     180
NrdF E.c. SYSSIFSTLCQTKDVDAAYAWSEENAPLQRKAQIIQQHYR------------GD------
NrdF S.t. SYSSIFSTLCQTKEVDAAYAWSEENPPLQRKAQIILAHYV------------SD------
NrdF C.a. SYSNIFMTLASTPQINEAFRWSEENENLQRKAKIIMSYYN------------GD------
NrdB E.c. SYTHIIRNIVNDPSV--VFDDIVTNEQIQKRAEGISSYYDELIEMTSYWHLLGEGTHTVN
          **: *: .: .  .:  .:     *  :*::*: *  :*             .:
                                                                     240
NrdF E.c. --------DPLKKKI-----ASVFLESFLFYSGFWLPMYFSSRGKLTNTADLIRLIIRDE
NrdF S.t. --------EPLKKKI-----ASVFLESFLFYSGFWLPMYFSSRGKLTNTADLIRLIIRDE
NrdF C.a. --------DPLKKKV-----ASTLLESFLFYSGFYLPMYLSSRAKLTNTADIIRLIIRDE
NrdB E.c. GKTVTVSLRELKKKLYLCLMSVNALEAIRFYVSFACSFAFAERELMEGNAKIIRLIARDE
                  ****:     :   **:: ** .*  .: ::.*  : ..*.:**** ***
                                                                     300
NrdF E.c. AVHGYYIGYKY---QKNMEKISLGQ-REELKSFAFDLLLELYDNELQYTDELYAE---TP
NrdF S.t. AVHGYYIGYKY---QIALQKLSAIE-REELKLFALDLLMELYDNEIRYTEALYAE---TG
NrdF C.a. SVHGYYIGYKY---QQGVKKLSEAE-QEEYKAYTFDLMYDLYENEIEYTEDIYDD---LG
NrdB E.c. ALSLTGTQHMLNLLRSGADDPEMAEIAEECKQECYDLFVQAAQQEKDWADYLFRDGSMIG
          ::*     :     :   .. .  :  ** *    **: :  ::*  ::: :: :
                                                                     358
NrdF E.c. WADDVK-AFLCYNANKALMNLGYEPLFPAEMAEVNPAILAALSPNADENHDFFSGSGSSY
NrdF S.t. WVNDVK-AFLCYNANKALMNLGYEALFPPEMADVNPAILAALSPNADENHDFFSGSGSSY
NrdF C.a. WTEDVK-RFLRYNANKALNNLGYEGLFPTDETKVSPAILSSLSPNADENHDFFSGSGSSY
NrdB E.c. LNKDILCQYVEYITNIRMQAVGLDLPFQTRSNPIPWINTWLVSDNVQVAPQEVEVS--SY
            .*:   :: * :*  :  :* :  * .    :        :* *.:   : .. *  **
                                 377
NrdF E c. VMGKAVETEDED--WNF--
NrdF S.t. VMGKTVETEDED--WNF--
NrdF C.a. VIGKAEDTTDDD--WDF--
NrdB E.c. LVGQIDSEVDTDDLSNFQL
          ::*:  .  * *   :*
```

FIG. 3. **Sequence alignment of the R2F from *C. ammoniagenes* with some other class Ib R2F (NrdF) proteins and class Ia R2 (NrdB) from *E. coli*.** Comparison of the predicted amino acid sequence of the *C. ammoniagenes* (*C.a*) NrdF product with the NrdF sequences from *E. coli* (*E.c.*; Swiss-Prot: RIR4_ECOLI), and *S. typhimurium* (*S.t.*; Swiss-Prot: RIR4_SALTY) and NrdB from *E. coli.* (*E. c.*; Swiss-Prot: RIR2_ECOLI). The numbering refers to the *E. coli* NrdB sequence. *Asterisks* (*) mark identical amino acid residues in all four proteins; *colons* (:) mark high similarity residues; and *periods* (.) mark low similarity residues. The alignment was made using multiple sequence alignment program CLUSTAL W (version 1.7) (46). *Underlined* residues correspond to the peptides identified in the peptide sequence analyses (Table II). Peptides used for the design of NrdF-specific PCR primers (see "Experimental Procedures") and present in all three NrdF sequences are *boxed*. Conserved residues corresponding to the iron ligands and tyrosyl radical in *E. coli* R2 protein (47) are shown with *black* background.

of NrdE and NrdF polypeptides as deduced from nucleotide sequence analyses, this would fit with an $\alpha\beta_2$ subunit composition for the *C. ammoniagenes* RNR. A previous study also reported an $\alpha\beta_2$ composition according to gel filtration and sucrose gradient centrifugation experiments (18). Such a quaternary structure is, however, in contrast to the $\alpha_2\beta_2$ 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-

TABLE I

Purification of RNR from C. ammoniagenes ATCC 6872

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	Total activity	Specific activity	Recovery	Purification factor
	mg	*units*[a]	*units · mg*$^{-1}$	*%*	
Cell-free extract	983	24	0.024	100	1
Low-salt precipitate	132	35	0.26	146[b]	11
DEAE-cellulose	5.1	16	3	67	125
Superdex 200	1.3	8.5	6.5	35	271
MonoQ[c]	0.045	1.5	34[d]	6	1417

[a] 1 unit is defined as 1 nmol of CDP reduced per min.

[b] Increase was probably due to the removal of inhibitory compounds from the cell extract.

[c] Data reported on this line concern only the R2F purified fraction, not a holoenzyme complex.

[d] Activity was measured by complementation with the R1E fraction, but the specific activity was calculated considering only R2F protein concentration.

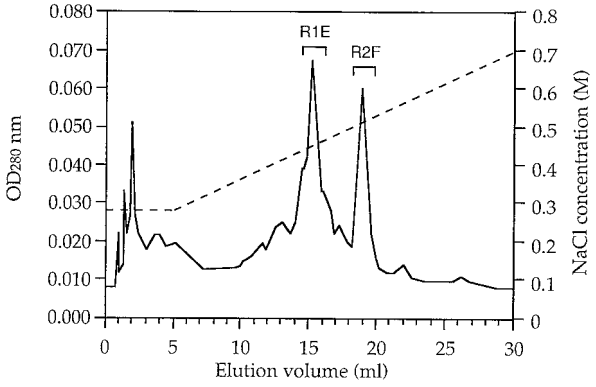

FIG. 4. **Separation of the R1E and R2F by weak anion exchange chromatography.** The RNR protein fraction after Superdex 200 gel filtration step was chromatographed on a MonoQ column (see "Experimental Procedures"). *Solid line*, absorbance at 280 nm; *dashed line*, NaCl gradient. No enzyme activity was found in isolated fractions, but it was obtained by combining the two pools containing R1E and R2F (Table II), as demonstrated by SDS-PAGE analysis (Fig. 5).

quencing results indicate that the initiator methionines have been processed during protein maturation, confirming that both genes, as suggested from the nucleotide sequence results, start with a GTG initiator codon that is read as methionine instead of valine.

Preliminary Characterization of C. ammoniagenes RNR—The nucleoside triphosphates ATP, dTTP, and dATP were found to be positive allosteric effectors for CDP reduction. At low concentrations of effector, dATP was more effective than ATP (Fig. 6*a*). Optimal activity with dATP was obtained at nucleotide concentrations as low as 0.02 mM, and no significant inhibition was seen even with 1 mM dATP. When ATP was used, a concentration of at least 0.12 mM was needed for optimal activity (Fig. 6*a*). This type of allosteric regulation is typical of class Ib enzymes and differs from that of class Ia enzymes (10, 12) .

During the entire purification procedure of *C. ammoniagenes* RNR, high levels of both DTT and *E. coli* thioredoxin were included as potential reductants, because we were not able to detect any species-specific "redoxin"-like activity in the supernatant fraction after the low-salt precipitation. With the *C. ammoniagenes* RNR obtained after the gel filtration step, we

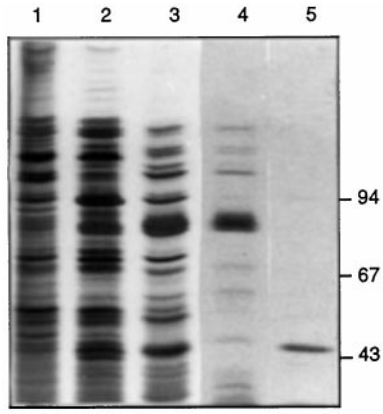

FIG. 5. **SDS-PAGE after each step of purification of *C. ammoniagenes* ribonucleotide reductase (see "Experimental Procedures").** *Lane 1*, low salt precipitate: *lane 2*, after DEAE-cellulose chromatography; *lane 3*, after Superdex 200 gel filtration; *lane 4*, R1E pool after MonoQ chromatography; *lane 5*, R2F pool after MonoQ chromatography. The electrophoretic mobilities of low molecular weight markers (Pharmacia) have been indicated.

then characterized the reductant requirement (Fig. 6*b*). Homogeneous *C. ammoniagenes* NrdH-redoxin obtained from an overproducing strain[2] was an effective reductant even at a low DTT concentration, whereas *E. coli* thioredoxin did not have any significant effect. The distinct requirement of NrdH-redoxin further supports the idea that RNR from *C. ammoniagenes* behaves as a typical class Ib enzyme.

The enzyme activity was sensitive to hydroxyurea in a concentration-dependent manner (Fig. 6*c*). This behavior is typical of class I RNRs, in which the stable tyrosyl radical essential for activity is sensitive to radical scavenging. The degree of sensitivity observed for *C. ammoniagenes* RNR is comparable to that observed earlier for *E. coli* class Ia RNR (40) .

The Purified C. ammoniagenes R2F Protein Contains Bound Manganese Ions—EPR analysis of the active R2F component obtained after the MonoQ purification step showed no signal corresponding to an organic free radical or a metal center (Fig. 7*a* and data not shown). However, upon denaturation of R2F by nitric acid, a 6-line EPR spectrum typical of Mn^{2+} in solution ($S = 5/2$) was observed. This shows that the native R2F protein contained EPR-silent Mn bound to the polypeptide chain. Preliminary atomic absorption spectroscopic analysis of the nitric acid-denatured R2F protein showed that it contained approximately 0.5 mol of manganese ions/mol of R2F polypeptide (Table III). In contrast, the content of iron in the R2F preparation was close to that of the buffer control, and essentially no cobalt was found, confirming that we have purified the previously described Mn-containing RNR of *C. ammoniagenes*.

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.

TABLE II
Peptide sequences of C. ammoniagenes R1E and R2F

Source	Origin	Sequence[a,b]
R1E	N terminus	^{1}*X*(T)Qg(Q)LGKTVAEPVKN^{14}
R1E	Internal peptide 1	^{35}KIQFDK^{40}
R1E	Internal peptide 2	^{226}KHIENQSSGI^{235}
R2F	N terminus	^{1}*X*(S)NEYDEYIANHTDPVKAIN*X*(W)NVIPDEKDL^{29}
R2F	Internal peptide 2	^{44}KIPVSNDIQSWNK^{56}
R2F	Internal peptide 3	^{56}KMTPQEQLATMRV^{68}
R2F	Internal peptide 4	^{112}KSYSNIF^{118}

[a] *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).
[b] 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.

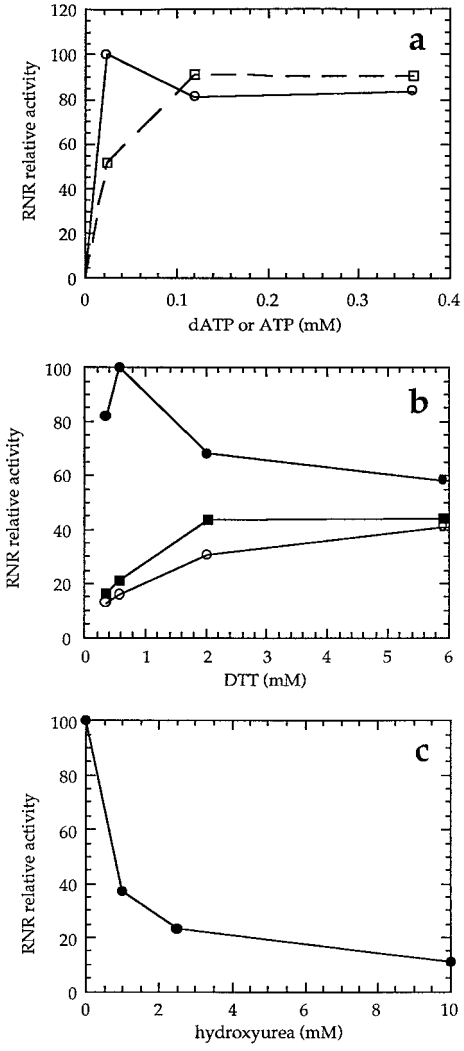

FIG. 6. *a*, effect of ATP and dATP on CDP reduction. Incubations were as described under "Experimental Procedures," except that 1 mM dATP was replaced with the indicated concentrations of either ATP (□) or dATP (○). *b*, effect of DTT and redoxin on CDP reduction. Incubations were as described under "Experimental Procedures" except for the concentration of DTT, which is shown on the *abscissa*; ○, without redoxin; ■, with 13 μM Trx; ●, with 13 μM NrdH-redoxin. *c*, hydroxyurea-dependent inhibition of CDP reduction. Aliquots of *C. ammoniagenes* RNR were incubated for 30 min at 4 °C with the indicated concentration of hydroxyurea, diluted into the assay mixture, and incubated as described under "Experimental Procedures." 100% activity corresponds to 4 (*a*), 110 (*b*), or 27 (*c*) milliunits.

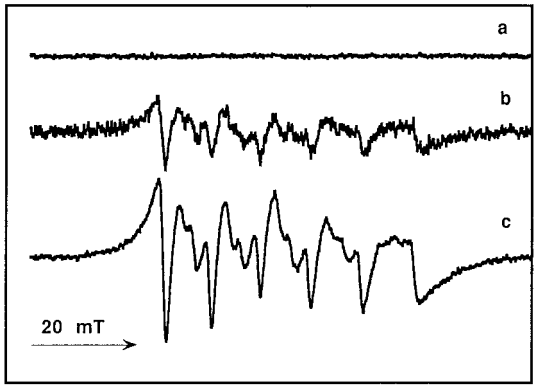

FIG. 7. **X-band EPR spectra at 77K of native (1 scan) (*a*) and denatured (16 scans) (*b*) R2F from *C. ammoniagenes*, compared with a standard of 30 μM $MnCl_2$ (6 scans) (*c*).** The R2F protein sample and the $MnCl_2$ standard were in 85 mM potassium phosphate buffer containing 10% glycerol. Spectra *a* and *b* were obtained after subtraction of background (see "Experimental Procedures"). Recording conditions were as follows: microwave frequency, 9.36 GHz; modulation amplitude, 0.5 millitesla; sweep width, 100 millitesla; time constant, 82 ms; sweep time, 167 s; microwave power, 1 mW.

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

Metal ion	Metal:R2F stoichiometry[a]
	mol/mol
Manganese	0.45
Iron	0.08
Cobalt	0

[a] 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.

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). As shown 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

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 *nrdEF* 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.* cambialistic, 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.

Acknowledgments—We are grateful to Margareta Sahlin for help with the EPR analyses and evaluations and for constructive discussions and to Agneta Slaby for help with purification of *E. coli* thioredoxin. We thank the Institute for Genomic Research for availability of sequence data prior to publication.

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J. Biol. Chem. 1998, 273:4329-4337.
doi: 10.1074/jbc.273.8.4329