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The Anaerobic (Class III) Ribonucleotide Reductase from *Lactococcus lactis*

CATALYTIC PROPERTIES AND ALLOSTERIC REGULATION OF THE PURE ENZYME SYSTEM*

(Received for publication, October 25, 1999)

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Lactococcus lactis contains an operon with the genes (*nrdD* and *nrdG*) for a class III ribonucleotide reductase. Strict anaerobic growth depends on the activity of these genes. Both were sequenced, cloned, and overproduced in *Escherichia coli*. The corresponding proteins, NrdD and NrdG, were purified close to homogeneity. The amino acid sequences of NrdD (747 residues, 84.1 kDa) and NrdG (199 residues, 23.3 kDa) are 53 and 42% identical with the respective *E. coli* proteins. Together, they catalyze the reduction of ribonucleoside triphosphates to the corresponding deoxyribonucleotides in the presence of S-adenosylmethionine, reduced flavodoxin or reduced deazaflavin, potassium ions, dithiothreitol, and formate. EPR experiments demonstrated a [4Fe-4S]⁺ cluster in reduced NrdG and a glycy radical in activated NrdD, similar to the *E. coli* NrdD and NrdG proteins. Different from *E. coli*, the two polypeptides of NrdD and the proteins in the NrdD-NrdG complex were only loosely associated. Also the FeS cluster was easily lost from NrdG. The substrate specificity and overall activity of the *L. lactis* enzyme was regulated according to the general rules for ribonucleotide reductases. Allosteric effectors bound to two separate sites on NrdD, one binding dATP, dGTP, and dTTP and the other binding dATP and ATP. The two sites showed an unusually high degree of cooperativity with complex interactions between effectors and a fine-tuning of their physiological effects. The results with the *L. lactis* class III reductase further support the concept of a common origin for all present day ribonucleotide reductases.

Lactococcus lactis is a member of the family of lactic acid Gram-positive bacteria that grow anaerobically but tolerate low concentrations of oxygen (1). Similar to all other bacteria it

produces the deoxyribonucleoside triphosphates required for DNA synthesis by reduction of ribonucleotides (2). In earlier work (3) we isolated from *L. lactis* subsp. *cremonis* a class Ib (*nrdEF*) ribonucleoside diphosphate reductase (2). This class contains a catalytically active tyrosyl radical whose generation requires molecular oxygen. Its members are not expected to function during anaerobiosis. *L. lactis* does, however, also contain *nrdDG* genes that code for a ribonucleotide reductase not depending on oxygen. The homologous proteins in *Escherichia coli* function during anaerobiosis (4). However, surprisingly, mutants in the *L. lactis* *nrdD* gene were still able to grow well under standard anaerobic growth conditions and then overproduced the NrdEF proteins (3).

There are three classes of ribonucleotide reductases that differ in their protein structure (see recent reviews in Refs. 2 and 5–8). All operate via a free radical mechanism involving protein radicals but differ in the way in which the protein radical is generated. Class I enzymes, coded either by the *nrdAB* genes (class Ia) or *nrdEF* genes (class Ib, occur in many microorganisms and all higher eukaryotes. They have an $\alpha_2\beta_2$ structure with the larger α_2 protein forming the catalytic part, responsible also for the allosteric properties of the enzyme, and the smaller β_2 protein harboring the radical-generating system, with an oxygen-linked diiron center and a neighboring free radical on a tyrosyl residue. Class II enzymes consist of only one large polypeptide, usually a dimer. Adenosylcobalamin substitutes for the β_2 protein as radical generator. This class occurs only in microorganisms. Class III enzymes consist of two proteins, NrdD and NrdG, with the larger NrdD responsible for catalysis and allosteric effects and the smaller NrdG for radical generation. *nrdD* and *nrdG* genes have been found in many different anaerobic and facultative anaerobic bacteria (2). Up to now only the class III proteins from *E. coli* (4, 9, 10) and phage T4 (11, 12) have been characterized. Class I enzymes require oxygen, and class II enzymes are independent of oxygen, and class III enzymes are poisoned by oxygen.

A special aspect of all ribonucleotide reductases concerns the regulation of their substrate specificity (2, 5, 6). Each enzyme, irrespective of class, provides all four dNTPs required for DNA synthesis. The specificity toward the reduction of one or the other substrate is directed by allosteric effectors binding to a "specificity site" on the large protein, distinct from the catalytic site. There, effectors (ATP, dATP, dGTP, or dTTP), one per polypeptide chain, bind and modify by long range interaction the conformation of the catalytic site to adapt it to a given substrate. Some class I enzymes (class Ia) contain an additional "activity site" on the large protein that regulates the overall

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catalytic activity. There, ATP acts as stimulator and dATP as inhibitor.

The *E. coli* anaerobic reductase has been the prototype of class III reductases (4). During anaerobic growth, the bacteria switch on their *nrdDG* operon and rely on the corresponding NrdD and NrdG proteins for the production of deoxyribonucleotides. The NrdD protein harbors in its active form an oxygen-sensitive glycy radical located close to the carboxyl terminus of the protein (13). Ribonucleotide reduction occurs at the triphosphate level and requires formate as hydrogen donor. The protein was found to contain two classes of allosteric sites, with one class binding dATP, dGTP, or dTTP and the other binding ATP and dATP (14).

The NrdG protein contains a [4Fe-4S] cluster that, together with *S*-adenosylmethionine, and reduced flavodoxin generates the glycy radical on NrdD (9, 10). Early experiments pointed to a stoichiometry of one cluster per NrdG dimer, but more recent evidence suggests that each NrdG polypeptide contains one [4Fe-4S] cluster (15). In many respects the anaerobic reductase resembles the pyruvate formate lyase system (16), and a recent x-ray crystallographic study (17) has added structural evidence to the hypothesis that the two enzyme systems may have a common evolutionary origin.

Several years ago one of us¹ discovered by chance that *L. lactis* subsp. *cremonis* very likely contains an *NrdDG* operon coding for an active class III ribonucleotide reductase. *L. lactis* is a Gram-positive bacterium, far removed in evolution from the Gram-negative *E. coli*. We were interested to compare the properties of the putative *L. lactis* class III enzyme with that of *E. coli* in order to find out to what extent the evolutionary divergence of the two microorganisms has affected the function and properties of their anaerobic reductases. Of particular interest was a comparison of their allosteric regulation. Results obtained now with the pure *L. lactis* enzyme show that the enzyme in many respects resembles the *E. coli* enzyme but also contains some distinct features. Its allosteric regulation is similar but not identical to that of the *E. coli* reductase.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions—*L. lactis* subsp. *cremonis* MG1363 (18) was grown at 30 °C in M17 medium (Difco, West Molesey, UK) as standing cultures or on M17 agar solidified with 1.5% agar, all supplemented with 0.5% glucose. Erythromycin (5 µg/ml) was added when required. Strict anaerobic growth of *L. lactis* was obtained after addition of 3.2 mM sodium sulfide to M17 medium as described earlier (19). *E. coli* strains were grown in TY (Difco) or LB medium at 37 °C with vigorous agitation or on TY or LB solidified with 1.5% agar. When required, 100 µg/ml ampicillin, 34 µg/ml chloramphenicol, 50–150 µg/ml kanamycin, 100 µg/ml erythromycin, or 1 mM IPTG² were used.

General DNA Techniques and Transformation—Molecular cloning techniques were by standard procedures (20). Restriction enzymes and T4 DNA ligase (Roche Molecular Biochemicals) were used according to the instructions of the supplier. Genomic DNA of *L. lactis* was isolated according to Leenhouts *et al.* (21) with the modification that the resuspended cell pellets were incubated at 55 °C for 15 min (22). *E. coli* and *L. lactis* were transformed (23) by electroporation using a Gene Pulser (Bio-Rad).

Molecular Cloning and DNA Sequencing—In previous work (24) concerning the peptidoglycan hydrolase *AcmA* of *L. lactis*, the nucleotide sequence of the 1930-bp *Sau3A/SspI* fragment of the 4137-bp insert of plasmid pAL01, containing *acmA*, was determined. We now determined the sequence of the 2207-bp *Sau3A/SspI* fragment of the insert, after subcloning into pUK21 or pBluescript SK+ (Stratagene). The 3-prime part of *nrdG* was sequenced from an overlapping clone,

pAL18. Both strands were sequenced with a T7 sequencing kit (Amersham Pharmacia Biotech), and the sequence was completed using synthetic DNA primers, synthesized with an Applied Biosystems 381A DNA Synthesizer (Applied Biosystems, Inc., Foster City, CA). DNA nucleotide and amino acid sequences were analyzed with the Clone Manager (version 5.01), the Gapped-Blast program (25), and the Wisconsin package, version 10.0, Genetics Computer Group (GCG), Madison, WI. Protein sequence alignments were performed according to the ClustalW program, version 1.7 (26).

Overexpression of NrdD and NrdG—A 3458-bp *XmnI/Eco47III* fragment of pAL18, containing both *nrdD* and *nrdG*, was subcloned into the *EcoRV* site of pUK21 under the *Plac* promoter yielding plasmid pUKDD1. This plasmid was then transformed into the *E. coli nrdDG* mutant UA6068 (19) giving *E. coli* IG016 and this strain was used for the purification of *L. lactis* NrdD. For NrdG overexpression, the *nrdG* coding sequence was amplified from pUKDD1 by PCR using the following primers: forward LIGup, 5'-ACATATGAACAATCCAAAACC-3' and reverse LIGlow, 5'-ATATGCTCGAGTGTCCATCAG-3'. These primers were designed to generate *NdeI* and *XhoI* restriction sites, respectively, at the start and the end of the PCR fragment. The amplified product (630 bp) was purified and cloned into plasmid pGEM-T (Promega), giving plasmid pIG021. After digestion with *NdeI* and *XhoI*, the *nrdG* fragment was ligated into pET24a (Novagen), and the resulting plasmid (pIG022) was transformed into *E. coli* BL21(DE3). The resulting strain IG017 was used for overproduction of NrdG.

Construction of an nrdD Interruption Mutant—An internal 478-bp *EcoRV-HindIII* fragment of *nrdD* was cloned into the integration vector pORI19 (27) using the RepA+ *E. coli* helper strain EC1000 (28). The resulting plasmid, pORIDD, was used to disrupt *nrdD* in *L. lactis* MG1363 by the method described earlier (27). Integrants were obtained by selection with erythromycin and checked by PCR using primers pALA18 (5'-GGCAAGAGCATCATGTG-3'), located in *nrdD*, and BK03AL (5'-AGCGGTAGCGCTGGAAA-3'), located in the origin of replication of pORI19. The size of the fragment (1596 bp) confirmed the successful disruption.

Expression and Purification of NrdD—*E. coli* IG016 was grown microaerophilically in 1.6-liter batches at 37 °C in LB medium containing 2% glucose, 150 µg/ml kanamycin, and 34 µg/ml chloramphenicol with continuous flow-through of 4% CO₂ and 96% N₂. When the culture had reached mid-log phase ($A_{550} = 0.6$) IPTG was added. After 2–3 h the cells were collected by centrifugation (1.6 g of packed cells) and stored frozen in liquid nitrogen. All further steps were made anaerobically and close to 4 °C. The cells were extracted (29), and clear crude extracts were obtained by high speed centrifugation and dialyzed overnight against buffer A (50 mM Tris-HCl, pH 7.5, 20 mM DTT, 1 mM phenylmethanesulfonyl fluoride) + 0.5 M KCl. The dialyzed solution was adjusted to 5 mg of protein/ml with the same buffer and added to a 2-ml column of dATP-Sepharose. The column was washed, first with 7 ml buffer A + 0.5 M KCl, followed by 4 ml of buffer A. NrdD was eluted with buffer A containing 0.5 mM ATP. Active fractions were combined and concentrated in Centricon 30 tubes, and dATP was removed by filtration through a Sephadex-G25 column equilibrated with 50 mM Tris-HCl, pH 7.5, 2 mM DTT.

Expression and Purification of the NrdG—*E. coli* IG017 was grown aerobically at 30 °C in 7 liters of LB medium until an A_{550} of 0.5. IPTG was added, and after 4 h the cells were collected by centrifugation. Purification was made in air and at 4 °C. Packed cells (15 g) were extracted after addition of streptomycin sulfate (final concentration 3%) and egg white lysozyme (final concentration 1 mg/ml) (29). After centrifugation, the clear crude extract (54 ml, 9.9 mg protein/ml) was precipitated with ammonium sulfate to 80% saturation. The precipitate was collected by centrifugation, dissolved in a small volume of buffer B (30 mM Tris-HCl, pH 7.5, 10 mM DTT, 50 mM KCl, 10 µM phenylmethanesulfonyl fluoride), and dialyzed overnight against buffer B with one change of buffer. After dilution to 5 mg/ml, 300 mg of protein was added to a 60-ml column of DE52 (Whatman) equilibrated with buffer B. The column was first washed with 60 ml of buffer B containing 0.1 M KCl and then with buffer B containing 0.2 M KCl. Active fractions were localized, precipitated with ammonium sulfate (80% saturation), and dissolved in 6 ml of buffer B containing 0.2 M KCl. Two ml (80 mg of protein) were chromatographed in a fast protein liquid chromatography machine (Amersham Pharmacia Biotech) on a Superdex-75 26/60 Prep grade column equilibrated with buffer B at a rate of 1.3 ml/min. Each fraction was analyzed enzymatically and by Phastgel electrophoresis, and NrdG was localized from its position on the gels, corresponding to a protein of ≈25 kDa. Pure fractions were pooled, concentrated in a Centriprep-10 tube to 1 ml, and finally freed from KCl by washing with

¹ G. Buist, unpublished experiments.

² The abbreviations used are: IPTG, isopropyl-β-D-thiogalactopyranoside; AdoMet, *S*-adenosylmethionine; DTT, dithiothreitol; DAF, deazaflavin; mW, milliwatts; PCR, polymerase chain reaction; bp, base pair.

30 mM Tris-HCl, pH 7.5, 10 mM DTT. The final yield from 535 mg of protein in the crude extract was 25 mg of NrdG.

Reconstitution of NrdG with Iron and Sulfide—All solutions were kept strictly anaerobic on a manifold continuously purged with argon. A solution of 5.4 mg (= 0.23 μ mol polypeptide) of NrdG (0.150 ml in two tubes) was treated for 6 h in an ice bath with 0.4 μ mol of DTT, 1 μ mol of Na₂S, and 1 μ mol of Fe(NH₄)₂(SO₄)₂, added in that order. In some instances ⁵⁹Fe (20 μ Ci) was used in this step. After addition of 3 μ mol of EDTA, the capped anaerobic tubes were transferred to an anaerobic hood, and the solution was passed through a 7-ml column of Sephadex G-25 equilibrated with 50 mM Tris-HCl, pH 7.5, 10% glycerol to remove excess reagents. After overnight dialysis in the anaerobic hood against 30 mM Tris-HCl, pH 7.5, 2 mM DTT, 0.1 M KCl, 10% glycerol, the dark brown solution containing reconstituted NrdG was either used directly for EPR experiments or stored in a closed tube under liquid nitrogen for experiments involving catalytic function.

EPR Analyses—For analyses of the FeS cluster, 0.1 ml of the anaerobic reconstituted NrdG solution (0.3 mg of protein) was mixed with an equal volume of the dialysis buffer containing 0.1 mM deazaflavin (DAF), giving a 0.06 mM solution of the NrdG polypeptide, and transferred inside the anaerobic box to an EPR tube. The tube was irradiated from a halogen lamp in a commercial projector for 60 min to reduce the FeS cluster, frozen, and analyzed by EPR. A control sample was treated identically in the absence of DAF. The oxidized form of the iron-sulfur cluster was obtained after exposure of the thawed sample to air at room temperature.

For EPR analysis of the glycy radical, 0.10 mg of NrdG was added on the manifold to a solution of 0.24 mg of NrdD in 1.5 mM *S*-adenosylmethionine (AdoMet), 5 mM DTT, 10 mM sodium formate, 30 mM KCl, 0.1 mM DAF, and 30 mM Tris-HCl, pH 7.5, final volume 0.2 ml. After irradiation at room temperature with visible light for 60 min, the solution was transferred in the anaerobic hood to an EPR tube and frozen for analysis.

EPR first derivative spectra were recorded on a Bruker ESP 300 spectrometer with X-band microwave frequency and 100-kHz field modulation frequency. Temperatures below 77 K were achieved with an Oxford Instruments continuous flow liquid helium cryostat. The 100 K measurements were accomplished by a continuous flow system for cold nitrogen gas. Spin quantitation was performed by double integration of the corresponding spectra recorded under nonsaturating conditions using a Cu-EDTA standard for calibration (30).

The EPR relaxation behavior was studied by recording spectra at different microwave powers. The parameter $P_{1/2}$, the microwave power at half-saturation, was obtained as described previously (31).

Analytical Procedures—Ribonucleotide reductase activity was routinely measured with CTP as substrate by the procedure used earlier with the *E. coli* anaerobic reductase (9). Briefly, the anaerobic mixture of NrdD and NrdG was first irradiated with light in the presence of DAF for 30 min after which time CTP reduction was started by addition of radioactive CTP. During incubation the complete mixture consisted of 30 mM Tris-HCl, pH 8.0, 0.2 mg/ml bovine serum albumin, 5 mM DTT, 20 μ M DAF, 0.6 mM AdoMet, 0.8 mM [³H]CTP, 1.5 mM ATP, 12.5 mM sodium formate, 75 mM KCl, and 15 mM MgCl₂. After 20 min the reaction was stopped with 0.5 ml of 1 M HClO₄, and the amount of dCTP formed was determined. In catalytic experiments concerning the allosteric regulation of the enzyme, [¹⁴C]ATP or [¹⁴C]GTP was substituted for CTP, and the final work-up was modified accordingly. One unit of enzyme activity is defined as 1 nmol of product formed per min. Specific activity is units per mg of protein.

Sucrose gradient centrifugations were done at 20 °C for 15 h at 35,000 rpm on 4.5 ml of 5–20% sucrose in 30 mM Tris-HCl, pH 7.5, 2 mM DTT, 10 mM MgCl₂, 30 mM KCl as described earlier (9). In some experiments 0.2 mM dATP or dGTP, 2 mM ATP or 0.01 mM AdoMet was present in the gradient.

Binding experiments were done at close to 0 °C with the previously described rapid filtration method (32). Protein was determined according to Bradford (33), with crystalline bovine serum albumin as standard. Iron analyses were as described by Fish (34), and labile sulfide was determined by a scaled down procedure described by King and Morris (35).

RESULTS

***L. lactis* Contains an *nrdDG* Operon**—During work at the University of Groningen on the gene encoding the major peptidoglycan hydrolase of *L. lactis*, one of us (G. B.) cloned and sequenced a stretch of DNA upstream of the hydrolase gene, resulting in the discovery of two open reading frames encoding

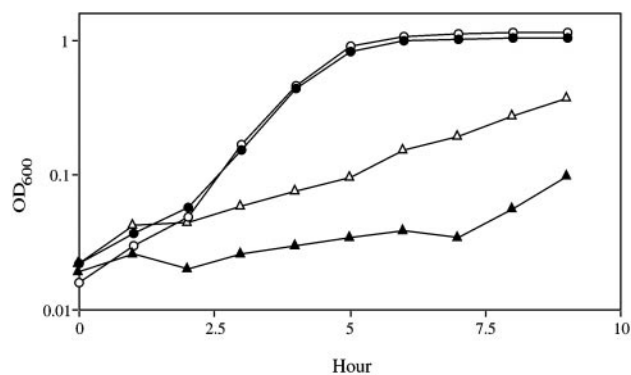


FIG. 1. Strict anaerobic growth of *L. lactis* requires the presence of active *nrdD* and *nrdG*. *L. lactis* subsp. *cremonis* MG1363, wild type, \circ , microaerobic growth; \bullet , strict anaerobic growth. *L. lactis nrdD*⁻: Δ , microaerophilic growth; \blacktriangle , strict anaerobic growth. Growth was measured in M17 with 0.5% glucose.

two putative proteins of 84.2 and 23.3 kDa, respectively, with a high degree of identity to the NrdD and NrdG polypeptides of the *E. coli* anaerobic ribonucleotide reductase. From here on we will refer to the two genes as *nrdD* and *nrdG*, as they indeed code for an active anaerobic ribonucleotide reductase. The complete determined sequence of *nrdDG* is not detailed here. It is deposited in the GenBankTM under accession number U73336.

nrdD (2,244 bp) and *nrdG* (600 bp) form one operon, with 2 bp separating the two genes. Putative ribosome binding sequences, complementary to the 3'-end of the *Lactococcus* 16 S RNA (36), were located 7 nucleotides upstream of *nrdD* (GAGGA) and 6 nucleotides upstream of *nrdG* (GGAG), the latter within the reading frame of *nrdD* indicating tight translational coupling of the two genes. Upstream of the ribosome binding sequence of *nrdD* an extended -10 sequence (TGNTA-GAAT) is present.

The deduced 747-amino acid-long NrdD protein shows an overall identity of 53% with the NrdD protein of *E. coli* NrdD. At the carboxyl-terminal end, the glycy residue in the sequence KRTCGYL, which is homologous to the sequence RRVCGYL in *E. coli*, can harbor the putative stable free radical involved in catalysis (13). The two cysteines of the active site that are conserved in all ribonucleotide reductases (6, 17) are found at positions 187 and 398. The amino-terminal sequence contains in the appropriate positions the residues required for binding of the allosteric effectors at the activity site (37). The deduced NrdG protein contains 199 amino acids and shows an overall identity of 42% with the *E. coli* NrdG protein with a particularly high degree of identity at the amino terminus where three conserved cysteines probably are involved in binding of an FeS center.

NrdDG* Is Required for Strict Anaerobic Growth of *L. lactis—The *nrdD* gene of *L. lactis* was disrupted by insertion of the integration vector pORI19. The bacteria now relied exclusively on the aerobic *nrdEF* genes. They grew well in the presence of oxygen but also to some extent under microaerophilic conditions, as is the case with *nrdD* and *nrdG* mutants of *E. coli* (19). However, when oxygen was excluded completely by inclusion of sulfide in the medium, the mutant did not grow (Fig. 1), demonstrating the importance of *nrdD* during strict anaerobiosis.

Properties of the Purified NrdD and NrdG Proteins—*E. coli* strain IG016 lacking active *nrdDG* host genes overproduces both lactococcal NrdD and NrdG proteins. NrdD was purified from this strain. On denaturing gels the final NrdD preparation gave a double band at \approx 74 and 84 kDa, similar to *E. coli* NrdD (4). This is due to truncation of the proteins at the site of the glycy radical (38). The amount of truncation varied considerably between different preparations as exemplified in Fig.

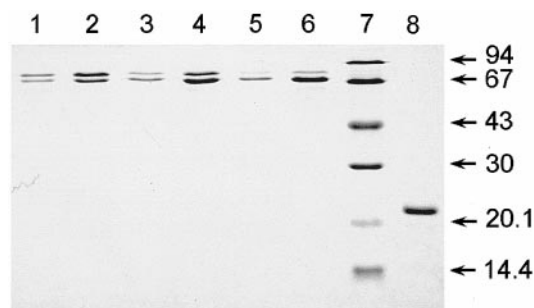


FIG. 2. Denaturing gel electrophoresis of purified NrdD and NrdG. The proteins were run on a 12% polyacrylamide gel and Coomassie-stained after the run. The three different preparations of NrdD (1 and 3 μg of each) in lanes 1–6 demonstrate the presence of varying amounts of truncated NrdD. Lane 7, molecular weight markers. Lane 8, NrdG (3 μg).

2, lanes 1–6, and greatly affected enzyme activity but not effector binding. All kinetic experiments were done with the protein shown in lanes 1 and 2, estimated to contain not more than 50% of the truncated form. Strain IG017, used for purification of NrdG, overproduces only lactococcal NrdG. The final NrdG preparation gave a single band at ≈ 25 kDa (Fig. 2, lane 8).

During aerobic purification, NrdG lost most of its ability to complement NrdD in the reductase assay. The final product contained only 0.3 atoms of Fe and 0.05 atoms of sulfide per polypeptide. After anaerobic treatment with Fe^{2+} and S^{-} , under conditions used earlier for reactivation of *E. coli* NrdG, the protein regained activity, and different preparations contained 4 to 5 Fe and 1.4 to 2 sulfide per polypeptide.

NrdD Can Form a Glycyl Radical—As isolated, NrdD showed no EPR signal. After anaerobic incubation with NrdG and irradiation in the presence of DAF and AdoMet a signal centered at $g = 2.0033$ with a principal hyperfine doublet splitting of 1.44 millitesla appeared (Fig. 3A). Under optimal recording conditions the EPR signal had an additional partially resolved hyperfine coupling (Fig. 3B). Its EPR line shape is almost identical to that of the glycyl radicals in pyruvate formate lyase (39) and ribonucleotide reductases (9, 12). Based also on the sequence similarities it seems certain that the EPR signal in Fig. 3 arises from a glycyl radical. At 100 K, the $P_{1/2}$ value of this signal was below 0.1 mW indicating that the radical is easily saturated by microwave power and hence not influenced in its relaxation by a metallo cofactor, consistent with NrdD being a metal-free protein. Spin quantitation showed ≈ 0.2 radicals per NrdD dimer, without correction for protein truncation. The free radical was relatively stable at low temperature storage and retained 70% of the unpaired spins after storage at room temperature for 5 h. It was rapidly destroyed by air, similar to other glycyl radicals.

EPR Characterization of the Iron-Sulfur Cluster of NrdG—After reconstitution, NrdG was diamagnetic under strictly oxygen-free conditions (Fig. 4A). Direct exposure to air led to a weak signal centered at $g = 2.015$, typical of a cuboidal $[\text{3Fe-4S}]^+$ cluster spectrum in a ground state with $S = 1/2$ (Fig. 4B) (40). A weak $g = 4.3$ signal of high spin ferric iron in rhombic symmetry was also observed, typical for nonspecifically bound iron (not shown). The intensity of the EPR $[\text{3Fe-4S}]$ signal decreased dramatically above 25 K. It was easily saturated by applied microwave power. Spin quantitation indicated that the species in Fig. 4B only corresponds to some percent of the NrdG dimer concentration.

Photochemical anaerobic reduction of reconstituted NrdG generated a nearly axially symmetric EPR signal in the $g = 2$ region (Fig. 4C). The signal originates from an $S = 1/2$ ground

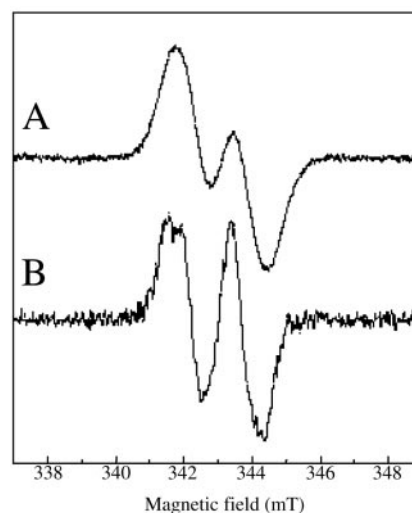


FIG. 3. EPR spectrum of the glycyl radical of activated NrdD at 10 K (A) and at 100 K (B). NrdD (7 μM) was activated as described under "Materials and Methods." The recording conditions were as follows: microwave power, 0.04 mW; modulation amplitude, 0.96 (A) and 0.19 (B) millitesla; microwave frequency, 9.62 GHz.

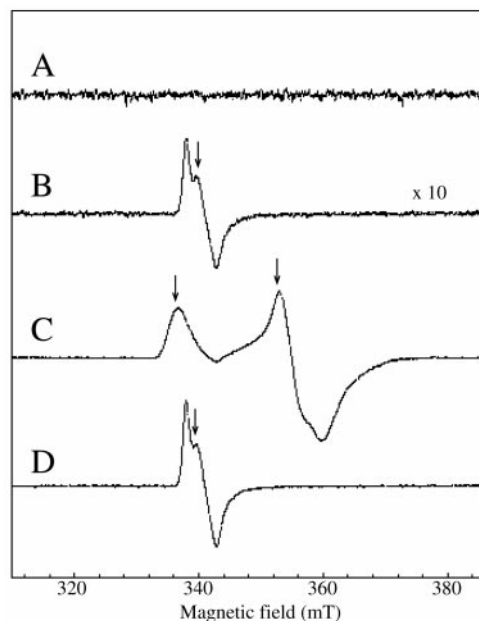


FIG. 4. EPR spectra of the iron-sulfur cluster of NrdG at 10 K. A, reconstituted NrdG protein, as isolated anaerobically; B, after direct exposure to air (10 scans); C, reduced anaerobically with DAF and light; D, after exposure of the anaerobically reduced sample to oxygen. The spectrometer conditions were as follows: microwave power: 0.2 mW in A, B, and D; 2.0 mW in C; modulation amplitude: 0.3 millitesla in A, B, and D; 0.96 millitesla in C; microwave frequency: 9.62 GHz; sweep time 168 s. The arrows indicate g values as follows: $g = 2.015$ in B and D, and $g_{\parallel} = 2.040$ and $g_{\perp} = 1.940$ in C.

state species with $g_{\parallel} = 2.040$ and $g_{\perp} = 1.940$. The optimum temperature for EPR observation was ~ 12 K, and the signal could not be detected above 40 K. It showed a fast relaxing behavior with an estimated value of $P_{1/2}$ of 14 mW at 10 K. All these properties are characteristic of a protein-bound cubanetype $[\text{4Fe-4S}]^+$ cluster (40). The maximum yield of the reduced species was close to one cluster per homodimer of NrdG. The $g = 4.3$ signal was not seen after reduction of NrdG.

When the reduced sample was exposed to air the cluster was quickly oxidized to a typical $S = 1/2$ $[\text{3Fe-4S}]^+$ species (Fig. 4D). The features of the EPR signal in Fig. 4D are similar to those in Fig. 4B, but now the spin quantitation showed 0.8 clusters

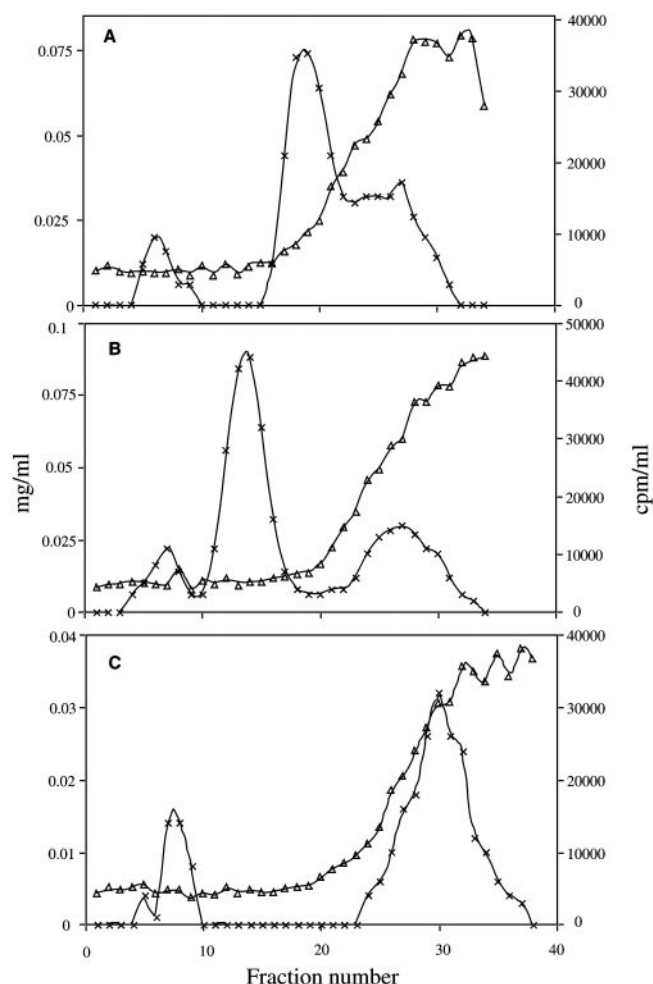


FIG. 5. Sucrose gradient centrifugations of NrdD and NrdG. A, 0.1 mg of NrdD + 0.04 mg of reconstituted ^{59}Fe -labeled NrdG + 0.017 mg of catalase were centrifuged as described under "Materials and Methods"; B, as in A, but + 0.2 mM dATP; C, as in A, but without NrdD. After centrifugation, fractions of 2 drops were collected from the bottom of the tubes and analyzed for protein (\times) and ^{59}Fe (Δ).

per dimer. A more detailed physicochemical characterization of the iron-sulfur cluster of NrdG will be reported elsewhere.³

A Stable NrdD-NrdG Complex Was Not Found—Fig. 5 illustrates the results from an experiment in which we tested by sucrose gradient centrifugation the ability of NrdD and NrdG to form a complex. In Fig. 5A roughly equimolar amounts of the two proteins were centrifuged together in a buffered salt solution; in Fig. 5B 0.1 mM dATP was included during centrifugation, and in Fig. 5C NrdG alone was centrifuged. In all cases NrdG had been reconstituted with ^{59}Fe . At the end of the run, fractions of the gradient were analyzed by determination of protein and ^{59}Fe to measure the sedimentation behavior of NrdD and NrdG. In a similar earlier experiment the corresponding *E. coli* proteins recombined to form a stable $\alpha_2\beta_2$ complex (9). This was not the case now. Under all the tested conditions the two proteins sedimented separately in the gradient when a mixture of them was centrifuged. NrdG was always positioned at an $s_{20,w}$ value of 2.9, alone (Fig. 5C), or in the presence of NrdD (Fig. 5, A and B). The position of NrdD depended on the presence of allosteric effectors. In this and other experiments the $s_{20,w}$ values ranged from 5.4 to 6.3 (Fig. 5A) in the absence of effector and increased to 8.4 to 8.8 in the presence of dATP (Fig. 5B) or dGTP. The latter values are

TABLE I

Requirements for the reduction of CTP by lactococcal reductase
Experiments were done under standard conditions in the presence of 4 μg of NrdD and 1.3 μg of NrdG.

Reaction conditions	Units ^a
Complete	0.116
-DTT	0.038
-Formate	0.035
-DTT, -Formate	0.010
-KCl	0.010
-SAM	0.002
-ATP	0.003
-CTP + CDP	0.004

^a Nanomoles of dCTP formed per min.

expected from a NrdD dimer (9). Interestingly, inclusion of ATP in the gradient did not increase the sedimentation coefficient. Inclusion of AdoMet produced no change (data not shown). After centrifugation, most of the radioactivity was present at the top of the gradients demonstrating that the FeS cluster was lost from NrdG.

Requirements for the Reduction of CTP to dCTP—Earlier results with the *E. coli* anaerobic reductase served as a guiding line for the following experiments. They show that the *L. lactis* enzyme has the same requirements for CTP reduction as the *E. coli* reductase.

The reaction required both NrdD and NrdG and occurred in two strictly anaerobic steps. During the first step NrdD was activated by AdoMet and DAF + light in a time-dependent reaction. Activation was complete after 30 min (data not shown). Reduced *E. coli* flavodoxin (produced from flavodoxin with flavodoxin reductase + NADPH) could substitute for DAF + light, but the reaction appeared to be less complete, as the final reduction of CTP to dCTP occurred only at half-rate (data not shown). In the second step the actual reduction of CTP by activated NrdD required DTT, formate, KCl, and ATP (Table I). CDP did not serve as a substrate.

Under our assay conditions the rate of dCTP formation depended on the concentrations of NrdD and NrdG. The addition of increasing amounts of NrdG to two fixed amounts of NrdD generated the results shown in Fig. 6. In both cases the system became saturated with respect to the small protein. It is not possible, however, to draw any firm conclusions about the NrdD/NrdG stoichiometry of the active enzyme from these experiments.

Allosteric Regulation of Substrate Specificity—The reduction of CTP showed an absolute requirement for ATP (Table I), suggesting that the *L. lactis* reductase is an allosteric enzyme whose substrate specificity is regulated by effectors, similar to other ribonucleotide reductases. Table II shows an overview of the effector requirements for the reduction of CTP, ATP, or GTP by the lactococcal reductase. The results are strikingly similar to those found with other reductases. Thus CTP reduction only occurred in the presence of ATP (but not dATP), and ATP reduction specifically required dGTP, and GTP reduction depended on dTTP. GTP reduction was also slightly stimulated by ATP. With CTP or ATP as substrates the effector requirement was absolute, with GTP there was a small "background" reduction also in the absence of the effector, and the dTTP-stimulated reaction was considerably slower than the corresponding reductions of CTP or ATP.

More detailed experiments revealed several interesting features of the allosteric behavior of the *L. lactis* enzyme that sets it apart from the *E. coli* class III enzyme. In repeated experiments the stimulation of CTP reduction by ATP was sigmoidal, and the sigmoidicity disappeared in the presence of 0.1 mM dGTP or dATP (Fig. 7A). ATP became then more effective, and its concentration at which the reaction occurred at $\frac{1}{2} V_{\text{max}}$

³ A. Liu and A. Gräslund, manuscript in preparation.

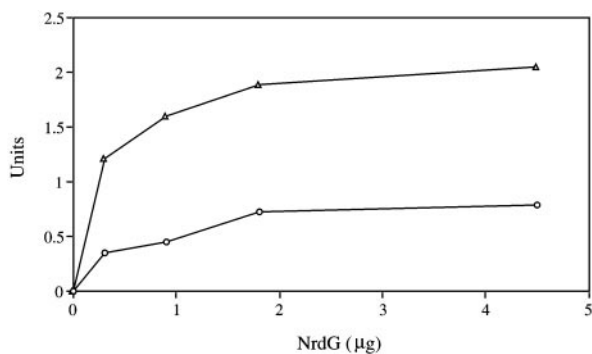


FIG. 6. Dependence of dCTP formation on the concentrations of NrdD and NrdG. Increasing amounts of reconstituted NrdG were incubated in the presence of 4 (○) or 8 (△) µg of NrdD under standard conditions, and the amount of dCTP formed was determined.

TABLE II

Allosteric regulation of substrate specificity of lactococcal reductase

The experiments were done with 3 µg of NrdD and 1.2 µg of NrdG under standard conditions, except for the allosteric effector. ATP was used at 1.5 mM concentration and dNTPs at 0.2 mM.

Substrate	Effector					
	None	ATP	dATP	dCTP	dGTP	dTTP
			<i>units</i> ^a			
CTP	0.004	0.260	0.014		0.007	0.002
ATP	0.010		0.011	0.015	0.211	0.011
GTP	0.012	0.030	0.010	0.010	0.011	0.042

^a Nanomoles of dNTP formed per min.

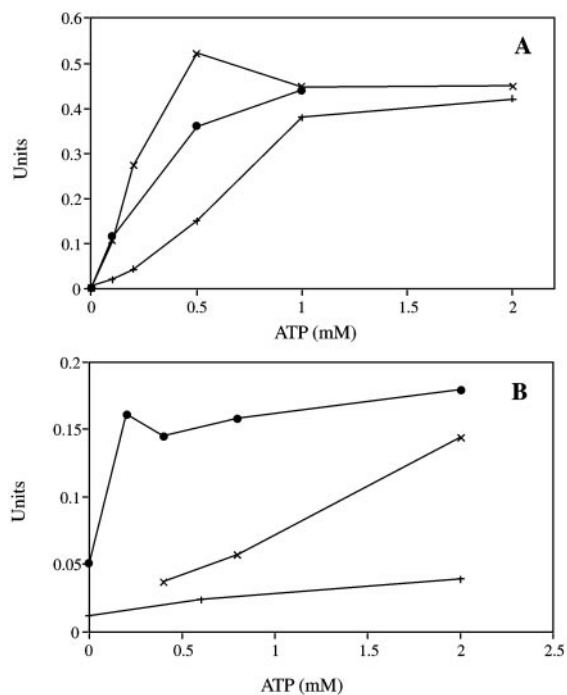


FIG. 7. Stimulation of CTP and GTP reduction by ATP. A, NrdD (3.6 µg) and NrdG (3.0 µg) were incubated under standard conditions for CTP reduction at increasing concentrations of ATP (+) or increasing concentrations of ATP + 0.1 mM dATP (●) or increasing concentrations of ATP + 0.1 mM dGTP (×). B, NrdD (3.6 µg) and NrdG (3 µg) were incubated under standard conditions for GTP reduction except for the effector. +, increasing concentrations of ATP alone; ×, increasing concentrations of ATP with 0.5 mM dTTP; ●, increasing concentrations of ATP with 0.5 mM dTTP + 0.2 mM dATP.

decreased from 0.7 to 0.2 mM. This effect disappeared at higher concentrations of dATP and dGTP, when the two effectors became inhibitory (data not shown).

The stimulation of ATP reduction by dGTP followed Michaelis-

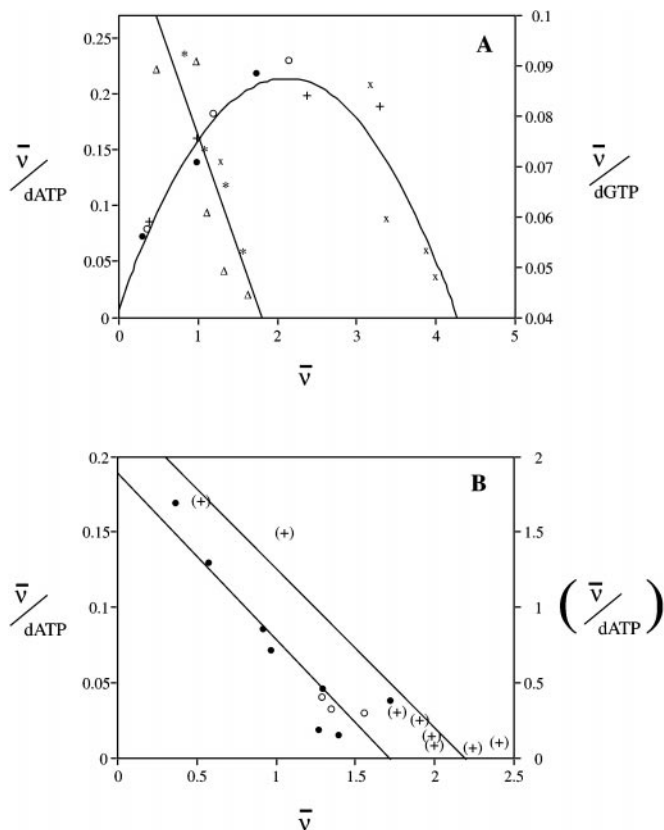


FIG. 8. A, binding of dATP and dGTP to NrdD (Scatchard plots). dATP binding (●, ○, +, ×) was measured (32) in separate experiments with four different preparations of NrdD. \bar{v} is molecules of nucleotide bound per NrdD dimer. dGTP binding (△, *) was measured with two of the four NrdD preparations. B, dATP binding in the presence of 1 mM dGTP (+) or 2 mM dGTP (●, ○). Note the differences in scales on the two ordinates (left = ATP, right = dGTP).

is-Menten kinetics with a K_m for dGTP of 3 µM (data not shown). dATP inhibited the dGTP-stimulated reaction with 50% inhibition occurring at 50 µM dATP, irrespective of the concentration of dGTP (data not shown).

The stimulation of GTP reduction by dTTP also followed Michaelis-Menten kinetics with a K_m for dTTP of 0.13 mM. ATP further stimulated the reaction more than 3-fold, and now GTP reduction reached the same level as CTP and ATP reduction (Fig. 7B). Of interest for an understanding of these complicated interactions was the finding that dATP counteracted the stimulation by ATP (Fig. 7B) and that this effect was overcome by increasing concentrations of ATP. The effects of both ATP and dATP were independent of dTTP concentration. The physical basis of these results will become clearer from the binding experiments described below.

Binding of Effector Nucleotides—Binding experiments required large amounts of protein as all effectors bound weakly to NrdD. Complete binding curves could not be obtained for ATP and dTTP. Experiments with dATP and dGTP were done during a period of more than 1 year with different preparations of the protein containing different amounts of the truncated form. This did not affect effector binding.

The Scatchard plots of Fig. 8A show a complex binding curve for dATP, whereas the curve for dGTP gave a straight line with a K_D of 15 µM. Extrapolation of the curves suggests that one NrdD dimer had the capacity to bind 4 dATP or 2 dGTP. The curve for dATP indicates a high degree of cooperativity between sites. Such a curve does not define a K_D , but one can calculate that half the sites were occupied (*i.e.* 50% binding) at 10 µM dATP.

TABLE III
Competition for binding of labeled dATP or dGTP by non-labeled nucleoside triphosphates

Binding of labeled dATP (0.1 mM) or dGTP (0.05 mM) to NrdD was measured in the presence of various non-labeled nucleoside triphosphates at the indicated concentrations.

Labeled effector	Non-labeled nucleotide	Bound effector
		<i>mol/mol</i>
		<i>NrdD</i>
		<i>dimer</i>
dATP	None	4.3
	ATP (2 mM)	1.6
	dGTP (1 mM)	1.9
	ATP (2 mM) + dGTP (0.5 mM)	0.7
	ATP (2 mM) + dGTP (1 mM)	0.3
dGTP	None	1.6
	dATP (0.05 mM)	2.3
	dATP (0.2 mM)	0.7
	dATP (0.6 mM)	0.1
	dTTP (0.1 mM)	1.4
	dTTP (0.4 mM)	0.6
	dTTP (1.0 mM)	0.0
	ATP (0.1 mM)	1.7
	ATP (2 mM)	1.8

In the presence of a large excess of dGTP, dATP binding became linear and extrapolated to only two sites per dimer (Fig. 8B), with a K_D for dATP of 1 μM . Also in the presence of ATP, dATP binding became linear and extrapolated to only two sites, but now with 10 times lower affinity ($K_D = 10 \mu\text{M}$). These two experiments suggest that dGTP and ATP each compete for 2 of the 4 dATP-binding sites. They compete for different sites as the remaining two free sites bind dATP with widely different affinity. This leads to the conclusion that dGTP and ATP bind to different sites.

This notion was corroborated by the experiments shown in Table III. There, we tested the effects of increasing concentrations of competing effectors at a constant, saturating concentration of labeled nucleotide. In one experiment with labeled dATP, addition of either dGTP or ATP blocked ≈ 2 of the 4 dATP-binding sites. When dGTP and ATP were added together all 4 dATP sites were blocked. These data suggest that dGTP competed with only 2 of the dATP sites, whereas ATP competed for the other two. In a second experiment with labeled dGTP (Table III), dATP or dTTP completely blocked dGTP binding, whereas ATP had no effect suggesting that the dGTP-binding site also binds dTTP and dATP but not ATP.

ATP binding was very weak. An additional complication is that ATP also is a substrate for the reductase. For these reasons we could not determine its binding stoichiometry. However, in view of the effects of dGTP and dATP on the sigmoidicity of the ATP curve shown in Fig. 7A, we tested the influence of dGTP and dATP on substoichiometric binding of ATP. As shown in Table IV, dGTP increased ATP binding more than 4-fold and also low concentrations of dATP stimulated slightly, whereas higher concentrations were inhibitory. These results suggest that binding of dGTP or dATP to the two sites of the NrdD dimer that do not bind ATP increased the affinity of the remaining two sites for ATP and provide an explanation for the disappearance of the sigmoidicity of the ATP curve in Fig. 7A on addition of dGTP and dATP.

DISCUSSION

L. lactis is the second organism whose anaerobic ribonucleotide reductase has been isolated in pure form and studied in some detail, the first being *E. coli*. Both bacteria depend on this enzyme during anaerobic growth but use a class I reductase during aerobiosis. *L. lactis* is a Gram-positive organism and *E. coli* is a Gram-negative organism. The two enzymes thus reside

TABLE IV
Effect of non-labeled dGTP or dATP on substoichiometric binding of labeled ATP

Binding of labeled ATP (0.03 mM) to NrdD was measured in the presence of varying concentrations of dGTP and dATP.

Non-labeled nucleotide	ATP bound
	<i>mol ATP/NrdD dimer</i>
None	0.25
dGTP (0.026 mM)	0.79
dGTP (0.065 mM)	0.83
dGTP (0.13 mM)	1.06
dATP (0.007 mM)	0.36
dATP (0.013 mM)	0.19
dATP (0.026 mM)	0.04

in two organisms that are believed to have diverged from a common ancestor more than 2 billion years ago (41). A comparison of the properties of the two enzymes defines basic features common to class III reductases, and the availability of a second class III enzyme may also in general be useful for biophysical or structural studies.

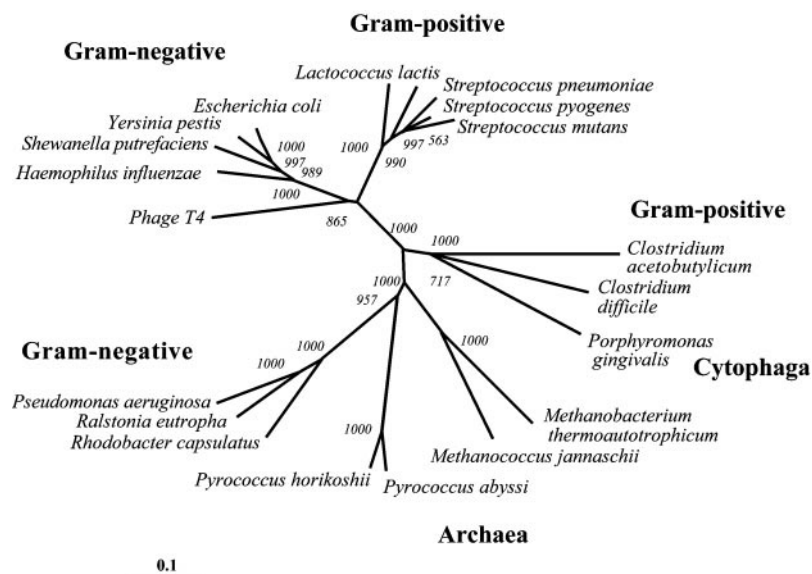
The *L. lactis* enzyme shares most of its general properties with the *E. coli* reductase. Thus both enzymes consist of a large NrdD and a small NrdG protein, and the corresponding genes form part of an operon. NrdD from each bacterium can harbor a catalytically active glycy radical close to its carboxyl terminus. NrdG contains an [4Fe-4S] cluster which, together with AdoMet and reduced flavodoxin or reduced DAF, generates the glycy radical on NrdD. The substrate specificity of both enzymes is allosterically regulated, and a given effector induces the same specificity in both enzymes.

The cluster/polypeptide stoichiometry of the *L. lactis* enzyme is not settled by our experiments. After reactivation, we always found an excess of iron over sulfide. We believe that this was caused by our inability to remove all unspecifically bound iron. The EPR experiments indicated the presence of ≈ 1 cluster/dimer together with some nonspecifically bound iron. These data are similar to early results obtained with the *E. coli* NrdG protein (9, 10) and then were interpreted to indicate the presence of one [4Fe-4S] cluster per NrdG dimer. However, more recently, experiments carried out under stricter anaerobic conditions indicated the presence of two clusters per *E. coli* NrdG dimer (15). In view of the lability of the *L. lactis* cluster suggested from the sucrose gradient centrifugation experiments (Fig. 5), it seems reasonable that also lactococcal NrdG is able to bind two clusters per dimer but that one cluster was lost during manipulations.

There are also differences between the two enzymes. In *E. coli*, NrdD and NrdG formed a tight $\alpha_2\beta_2$ complex during sucrose gradient centrifugation (9). This was not the case in corresponding experiments with the *L. lactis* enzyme. Also the FeS cluster was bound loosely and was lost during centrifugation. Finally, the association of the two polypeptide chains of the *L. lactis* NrdD dimer required the presence of effectors (dATP or dGTP) during centrifugation for its stability. This was not necessary for the *E. coli* NrdD protein. Together these data suggest a less tight association between polypeptides and between cluster and polypeptides in the *L. lactis* enzyme.

The juxtaposition of the *nrdD* and *nrdG* genes in one operon, both in *E. coli* and *L. lactis*, suggests a coordinated synthesis of the NrdD and NrdG proteins. During the generation of the glycy radical, NrdD and NrdG must be in close contact. As the two *E. coli* proteins can form an $\alpha_2\beta_2$ complex (9), we propose that glycy radical generation occurs in such a complex. The question then remains to what extent the complex dissociates during catalysis. Recent data from the *E. coli* system (15) indicate that dissociation can take place and the fact that NrdD and NrdG apparently are less tightly associated in the *L. lactis*

FIG. 9. Unrooted phylogenetic tree of the deduced amino acid sequences of the known NrdD proteins of class III ribonucleotide reductases. The tree was generated with 1000 bootstrap trials, and the bootstrap values are indicated at the nodes. All sequences were obtained from the NCBI data base (including the microbial unfinished genomes).



enzyme would suggest this to occur also in the lactococcal reductase. At the present time it is not clear if the activated NrdD protein alone can catalyze ribonucleotide reduction.

The effector binding experiments require a special discussion. Ribonucleotide reductases contain specificity sites that regulate their substrate specificity by binding ATP and dNTPs as allosteric effectors (2). Class Ia and some class II (37) enzymes contain, in addition, a second class of activity sites that only bind ATP or dATP. The enzyme dimers then have the capacity to bind 4 molecules of ATP and dATP and 2 molecules of dTTP and dGTP.

The NrdD protein of the *E. coli* class III reductase also contains two separate classes of allosteric sites (14), but in contrast to class I and II reductases, the dimer can only bind 2 ATP. The reason for this is that the sites corresponding to the specificity sites of class I do not bind ATP but only dATP, dGTP, and dTTP.

The present experiments suggest that the two allosteric sites of *L. lactis* NrdD bind the same effectors as *E. coli* NrdD. We were not able to quantitate ATP binding but had to rely on competition experiments that strongly suggested that ATP binds to sites that are different from those that bind dGTP and dTTP. (i) Only dTTP and dATP, but not ATP, blocked binding of labeled dGTP (Table III); ATP and dGTP blocked different sites in competition with labeled dATP (Fig. 8A and Table III). (ii) dGTP removed the sigmoidicity from the stimulatory effect of ATP and CTP reduction (Fig. 7A) and increased ATP binding (Table IV); ATP increased the stimulatory effect of dTTP on GTP reduction (Fig. 7B). These results are best explained by ATP binding to sites that are separate from the sites that bind dGTP and dTTP. (iii) During sucrose gradient centrifugation of NrdD, dGTP and dATP, but not ATP, promoted dimer formation. X-ray crystallography (42) has localized the specificity site to the dimer interface. The results suggest that ATP does not have access to this site.

A special feature of the allosteric regulation of the *L. lactis* enzyme, not present in other reductases, concerns the considerable "cross-talk" between binding sites. Some examples are the high cooperativity of dATP binding (Fig. 8A), the sigmoidicity of the ATP curve when this nucleotide stimulates CTP reduction, and the effects of dGTP and dATP on this stimulation (Fig. 7A) and on ATP binding (Table III). In this way the enzyme becomes sensitive to small changes in the concentrations of ATP and dATP. It should be pointed out that despite the differences in binding and cooperativity the general effect

of a given nucleotide on substrate specificity and catalytic activity is the same for class III and class Ia reductases (*cf.* Table II).

We have shown that the anaerobic reductases from a Gram-negative and a Gram-positive bacterium operate according to the same general principles, both with respect to catalytic function and allosteric regulation, despite their far evolutionary divergence. So far these two are the only class III enzymes studied at the protein level. However, we can widen the horizon to a comparison at the genomic level as by now 21 complete *nrdD* and *nrdG* sequences are known. Fig. 9 shows a tree for NrdD proteins, and a similar tree can be constructed for NrdG. It appears that class III enzymes are widely spread among anaerobic and facultative anaerobic organisms. With one exception (*Methanococcus jannaschii*), all bacteria contain in addition to a class III enzyme also one class I or class II reductase, in some cases even both. In the tree the enzymes fall in five separate groups: two of them are Gram-positive bacteria, two others are Gram-negative bacteria, and the fifth contains archaeobacteria. Surprisingly, one Gram-positive and one Gram-negative group each are closer to the archaeobacteria than to the corresponding eubacterial group.

The existence of three separate classes of ribonucleotide reductases differing greatly in their primary sequences originally raised questions concerning their origin during evolution. We have earlier argued for a common ancestor giving rise to the different classes through divergent evolution that was driven by the appearance of oxygen on the earth (2, 5). Our main argument was the highly conserved allosteric regulation of the substrate specificity of the enzymes that strongly suggested a common tertiary structure. The present results add further strength to this argument, as does the recently determined three-dimensional structure of the NrdD protein of the phage T4 class III reductase (17). As to the nature of the common ancestor, we believe it to have been most closely related to present day class III enzymes. For this speaks the anaerobic nature of the enzyme and the involvement of an iron-sulfur cluster and *S*-adenosylmethionine in the radical generating mechanism, as well as the close relation to pyruvate formate lyase (16, 17), an enzyme involved in anaerobic energy metabolism early during evolution.

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