Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 12892–12896, December 1994
Biochemistry

A second class I ribonucleotide reductase in *Enterobacteriaceae*: Characterization of the *Salmonella typhimurium* enzyme

(deoxyribonucleotide synthesis/glutaredoxin/tyrosyl radical/iron center)

ALBERT JORDAN*†, ELISABET PONTIS*, MOHAMED ATTA‡, MARIA KROOK§, ISIDRE GIBERT†, JORDI BARBÉ†, AND PETER REICHARD*

Department of *Biochemistry I, Medical Nobel Institute and §Chemistry I, MBB, Karolinska Institutet, S-17177 Stockholm, Sweden; †Department of Genetics and Microbiology, Faculty of Sciences, Autonomous University of Barcelona, Bellaterra, 08193 Barcelona, Spain; and ‡Department of Biophysics, Stockholm University, S-10691 Stockholm, Sweden

Contributed by Peter Reichard, September 19, 1994

ABSTRACT **The *nrdA* and *nrdB* genes of *Escherichia coli* and *Salmonella typhimurium* encode the R1 and R2 proteins that together form an active class I ribonucleotide reductase. Both organisms contain two additional chromosomal genes, *nrdE* and *nrdF*, whose corresponding protein sequences show some homology to the products of the genes *nrdA* and *nrdB*. When present on a plasmid, *nrdE* and *nrdF* together complement mutations in *nrdA* or *nrdB*. We have now obtained in nearly homogeneous form the two proteins encoded by the *S. typhimurium nrdE* and *nrdF* genes (R1E and R2F). They correspond to the R1 and R2 proteins. Each protein is a homodimer. Together they catalyze the reduction of CDP to dCDP, using dithiothreitol or reduced glutaredoxin, but not thioredoxin, as an electron donor. CDP reduction is strongly stimulated by low concentrations of dATP, presumably acting as an allosteric effector. Protein R2F contains an antiferromagnetically coupled dinuclear iron center and a tyrosyl free radical. The *E. coli* and *S. typhimurium* chromosome thus have maintained the information for a potentially active additional class I ribonucleotide reductase, whose role *in vivo* is as yet unknown. The allosteric regulation of this enzyme differs from that of the normally expressed reductase.**

Ribonucleotide reductases catalyze the synthesis of deoxyribonucleoside triphosphates (dNTPs) required for DNA synthesis. At least three separate classes of enzymes are known, each with a distinct protein structure but all requiring a protein radical for catalysis (1). The long-studied aerobic *Escherichia coli* enzyme is the prototype for class I enzymes, also present in all higher organisms and some other microorganisms. *E. coli* genes *nrdA* and *nrdB* encode the α and β polypeptide chains, respectively, that form the R1 (α_2) and R2 (β_2) proteins that constitute the enzyme (2).

Each protomer of the R1 dimer (2 × 85.7 kDa) contains one substrate-binding site with redox-active thiols involved in the reduction of the substrate ribonucleoside diphosphate, and two separate types of allosteric sites: one, the activity site, controls the overall activity of the enzyme, with ATP as a positive effector and dATP as a negative effector; the other, the substrate-specificity site, controls the specificity of the enzyme, with ATP and dATP favoring pyrimidine reduction, dTTP favoring GDP reduction, and dGTP favoring ADP reduction (3).

The R2 dimer (2 × 43.4 kDa) contains two dinuclear iron centers with associated stable tyrosyl free radicals, located at Tyr-122 of the polypeptide chain (4). The drug hydroxyurea scavenges this radical and thereby inactivates the enzyme. Class II and III enzymes lack the tyrosyl radical. Class II enzymes, with the *Lactobacillus leichmanni* enzyme as a prototype, employ adenosylcobalamin as a radical generator, whereas class III enzymes use *S*-adenosylmethionine together with iron for this purpose.

Salmonella typhimurium contains an active class I enzyme with amino acid sequences 96.5% and 98.4% identical to the *E. coli* R1 and R2 proteins, respectively (A.J., unpublished results). Recent genetic evidence involving complementation of *nrd* mutants of *E. coli* suggested the presence in *S. typhimurium* of the genes, *nrdE* and *nrdF*, coding for a second class I enzyme (5). These genes are also present on the chromosome of *E. coli* but are, under standard growth conditions, expressed only when present on a plasmid. The amino acid sequences deduced for the corresponding proteins showed a limited identity with other class I enzymes but contained many of their catalytically important residues.

We have purified and characterized the two proteins encoded by the cloned genes *nrdE* and *nrdF* from *S. typhimurium*. Each protein is a homodimer. Together they catalyze the reduction of CDP with the glutaredoxin system as hydrogen donor. The reaction is strongly stimulated by dATP, which probably acts as a positive allosteric effector. The NrdF protein contains an oxygen-linked dinuclear iron center and a tyrosyl radical.

MATERIALS AND METHODS

Materials. *E. coli.* UA5018, an H1491 (MC4100 *aroB nrdB*::Mud1, Ap^r) derivative (6) harboring plasmid pUA346 was used for protein purification. pUA346 is a kanamycin-resistant derivative of pK184 (7) carrying a 3.45-kb *Not* I–*Acc* I fragment from pUA338 (5) containing the *S. typhimurium nrdEF* structural genes under the control of the *lac* promoter of the vector. Thioredoxin, thioredoxin reductase, glutaredoxins 1–3, and glutathione reductase were gifts from A. Holmgren and F. Åslund (Karolinska Institute). The R1 and R2 proteins of the aerobic ribonucleotide reductase were gifts from B.-M. Sjöberg (University of Stockholm).

Enzyme Purification. UA5018 was grown aerobically to midlogarithmic phase (OD_{600} of 1.5) in Luria broth with ampicillin and kanamycin (both at 50 μg/ml) at 30°C in a New Brunswick fermentor and harvested by centrifugation at 4°C, resulting in 30–45 g (wet weight) of bacteria. In a typical experiment 7.5 g of bacteria was extracted with 13 ml of 50 mM Tris·HCl, pH 7.5/50 mM KCl/1 mM phenylmethanesulfonyl fluoride/10 mM dithiothreitol (DTT) containing lysozyme at 0.6 mg/ml (8). All operations were done at 4°C. After centrifugation, 3.5 ml of a 10% (wt/vol) solution of streptomycin sulfate was added slowly to the supernatant solution. The turbid solution was centrifuged again and proteins were precipitated by addition of solid ammonium sulfate to 70% saturation over a period of 1 hour. The

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: DTT, dithiothreitol.

precipitate was collected by centrifugation, dissolved in 50 mM Tris·HCl/10 μM phenylmethanesulfonyl fluoride/10 mM DTT (buffer A), and dialyzed overnight against buffer A. The protein was then adsorbed to a 67-ml column of DEAE-Sepharose and eluted with a linear KCl gradient (450 ml; 0–0.4 M KCl in buffer A) at a rate of 0.35 ml/min. Fractions (7 ml) were collected and analyzed for protein and reductase activity. The procedure separated two proteins which together are required for enzyme activity (Fig. 1). From this point on, the two proteins, named R1E and R2F, were purified separately.

Chromatographic fractions containing R1E activity were pooled, dialyzed against buffer A containing 20 mM DTT, and concentrated by ultrafiltration in Centriprep 30 (Amicon) tubes to a final volume of 4.5 ml. R1E activity was lost if this concentration step was carried too far. $MgCl_2$ was added (15 mM) and the material was then adsorbed to a 2-ml column of dATP-Sepharose (9). The column was first washed with 4 ml of buffer A containing 20 mM DTT and 15 mM $MgCl_2$, and enzyme activity was then eluted with 1 mM dATP in the same buffer. Solutions of R1E were divided into appropriate aliquots, frozen immediately, and kept at −80°C before use.

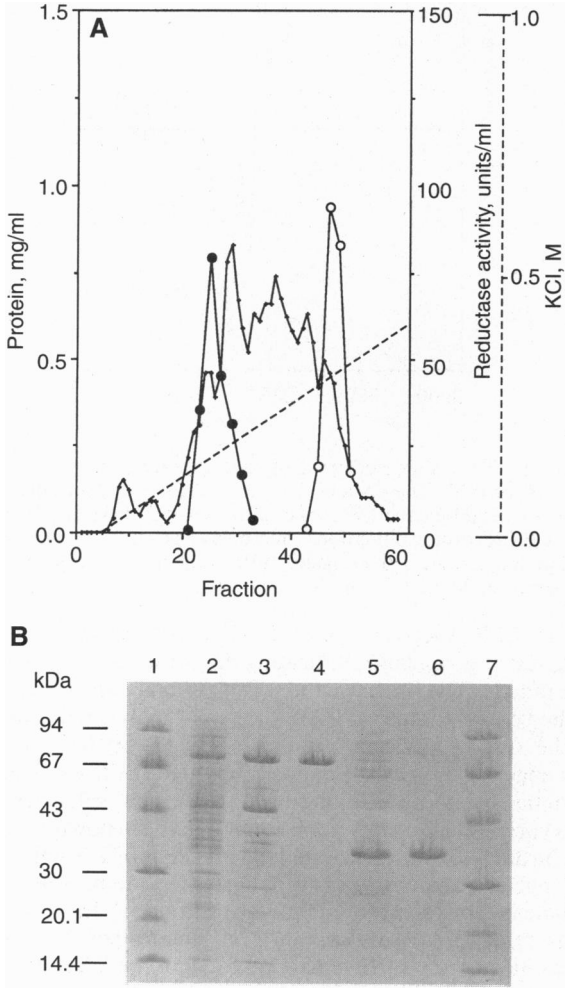

FIG. 1. (*A*) Separation of R1E and R2F by chromatography on DEAE-Sepharose. The protein (161 mg) after precipitation with ammonium sulfate was chromatographed on a column of DEAE-Sepharose (see *Materials and Methods*). +, Protein concentration; ●, R1E activity; ○, R2F activity. (*B*) Protein patterns obtained by SDS/polyacrylamide gel electrophoresis. Lanes: 1 and 7, size markers; 2, crude extract; 3, R1E after DEAE-Sepharose chromatography; 4, R1E final product; 5, R2F after DEAE-Sepharose; 6, R2F final product.

Fractions after DEAE-Sepharose chromatography containing R2F activity were dialyzed against 30 mM Tris·HCl (pH 7.5) (buffer B) and concentrated by centrifugation in Centriprep 10 tubes to a final volume of 2 ml. The material was adsorbed to a Mono Q HR 10/10 column equilibrated with buffer B on a Pharmacia FPLC machine. The enzyme eluted with a 0–1 M KCl gradient in buffer B and concentrated by centrifugation in Centriprep 10 tubes. Final purification of R2F was achieved by chromatography of the protein on a Superdex 200 HR 10/30 FPLC column with buffer B containing 0.2 M KCl.

Enzyme Assays. Under standard conditions each protein was measured in the presence of an excess of the other protein in 0.5 mM [^{3}H]CDP (specific activity, 25 cpm/pmol)/0.3 mM dATP/20 mM DTT/10 mM $MgCl_2$/50 mM Tris·HCl, pH 8. Final volume was 0.05 ml and incubation was at 37°C for 20 min. The amount of dCDP formed was determined as described (10). One enzyme unit is the activity that produces 1 nmol of dCDP during 1 min under these conditions.

N-Terminal Amino Acid Sequence and Amino Acid Analysis. N-terminal amino acid sequences were obtained with an Applied Biosystems gas-phase sequencer (model 470A) or a Milligen solid-phase sequencer (model 6600), both fitted with on-line analysis. Amino acid compositions were determined with an LKB Pharmacia Alpha Plus instrument after hydrolysis at 110°C for 24 hr in 6 M HCl/0.5% phenol.

RESULTS

Proteins Encoded by *nrdE* and *nrdF* Have Ribonucleotide Reductase Activity. *E. coli* H1491, a strain that contains an insertion in the *nrdB* gene, does not grow aerobically. *E. coli* UA5018, which is H1491 harboring plasmid pUA346 with the *S. typhimurium nrdE* and *-F* genes, does grow aerobically. We found that crude extracts from this strain reduce CDP to dCDP in the standard ribonucleotide reductase assay. Furthermore, analysis by SDS/polyacrylamide gel electrophoresis demonstrated overproduction of two proteins with apparent molecular masses of 70 and 36 kDa (Fig. 1*B*), suggesting that these two proteins together are responsible for reductase activity.

Purification of the R1E/R2F Ribonucleotide Reductase. The two proteins R1E and R2F responsible for the reductase activity were purified to near homogeneity (see *Materials and Methods*). Each protein alone lacked activity. With one protein in excess the amount of dCDP formed in the standard assay became proportional to the amount of the second protein (Fig. 2). This formed the basis for the assays of the two proteins during their purification, which is summarized in Table 1. R1E was purified 11-fold over the activity in the crude extract, with a final yield of 37%, whereas R2F was purified 32-fold, with a 39% yield. The final specific activities of the two proteins were 20–50% of those found routinely for pure preparations of R1 and R2, the two proteins that constitute the previously known aerobic *E. coli* reductase.

Preliminary Characterization of R1E and R2F. The names R1E and R2F derive from our finding that many of the properties of the two proteins resemble those of R1 and R2. On SDS/polyacrylamide gel electrophoresis (Fig. 1*B*) each protein gave one major band with mobilities corresponding to molecular masses of 70 kDa (R1E) and 36 kDa (R2F), in reasonably good agreement with the expected values for the products of the *nrdE* (80.5 kDa) and *nrdF* (36 kDa) genes.

N-terminal sequences were ATTTPERVMQXTMD (R1E) and MKLSRXXA (R2F). Whereas the R2F sequence starts at the methionine codon predicted from the nucleotide sequence of *nrdF*, the R1E sequence starts with an alanine, 8 residues upstream of the predicted *nrdE* methionine codon (5). This suggests that the TTG codon preceding the alanine codon was read as methionine and that this amino acid was

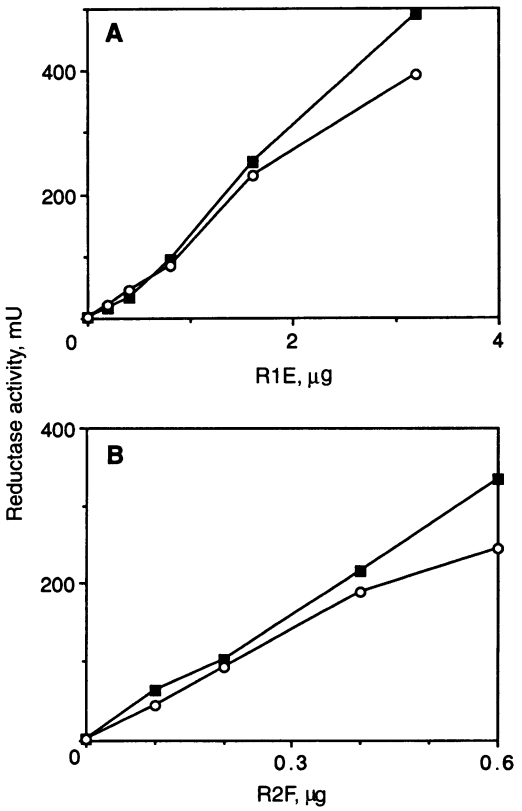

FIG. 2. Dependence of CDP reduction on both R1E and R2F. Incubations were under standard conditions with various amounts of R1E in the presence of 1 μg (○) or 5 μg (■) of R2F. (*A*) or with various amounts of R2F in the presence of 2 μg (○) or 10 μg (■) of R1E (*B*). mU, milliunits.

removed by N-terminal processing. Analyses of the complete amino acid composition of R1E and R2F preparations were in good agreement with the compositions predicted from the nucleotide sequence of the genes (data not shown).

The electronic spectrum of R2F between 300 and 550 nm (Fig. 3) closely resembles that of R2. As is the case with R2, a sharp peak at 408 nm signals the presence of a tyrosyl radical, whereas the broader bands at 370 and 325 nm derive from a μ-oxo bridged dinuclear iron center. The molar extinction coefficients at 370 and 410 nm were 11,000 and 7040 $M^{-1}\cdot cm^{-1}$, respectively, compared with values of 8700 and 6600 $M^{-1}\cdot cm^{-1}$ for R2 (11). For this calculation, as well as for the following determination of the iron content of R2F, the concentration of R2F was obtained from total amino acid analysis.

Table 1. Purification of the R1E and R2F proteins.

Step	Protein, mg	Reductase units × 10^3		Specific activity, units/mg	
		R1E	R2F	R1E	R2F
Bacterial extract	189	4.9	4.9	26	26
Streptomycin	170	4.7	4.2	25	22
Ammonium sulfate	161	4.2	4.5	22	24
DEAE-Sepharose (R1E)	30	2.7		90	
dATP-Sepharose (R1E)	6.6	1.8		280	
DEAE-Sepharose (R2F)	14		3.9		280
Mono Q (R2F)	2.9		2.9		850
Superdex (R2F)	1.9		1.9		830

The data summarize the results from an experiment starting with 7.5 g of bacteria. The two proteins were purified together up to the DEAE-Sepharose step. From there on the two proteins were purified separately (see *Materials and Methods*).

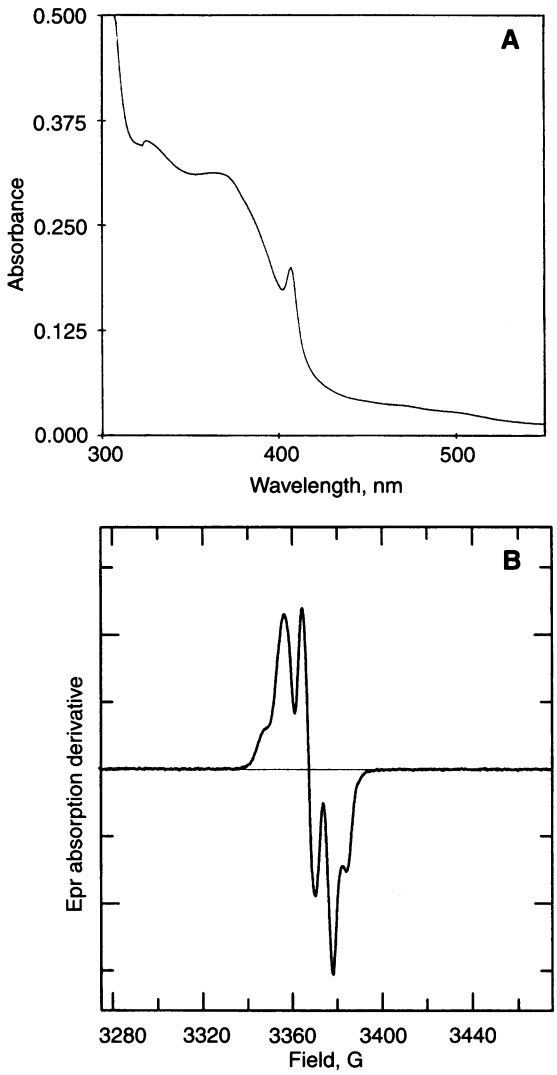

FIG. 3. Electronic spectrum (*A*) and first-derivative EPR spectrum (*B*) of R2F. The protein (2.05 mg/ml for the electronic spectrum, 10 mg/ml for the EPR spectrum) was in 50 mM Tris·HCl (pH 7.5). EPR recording conditions: microwave power, 0.02 mW; modulation amplitude, 1.6 G; gain, 10^4; frequency, 9.452799 GHz; temperature, 30 K.

The EPR spectrum of R2F (Fig. 3*B*) shows a signal centered at $g = 2.0047$, indicating the presence of an organic free radical. The fine structure of this signal differs from that of the tyrosyl radical of R2 but is remarkably similar to that of the tyrosyl radical of photosystem II. Hydroxyurea, a scavenger of the tyrosyl radical, inhibited the activity of the reductase, with 50% inhibition occurring at ≈0.5 mM hydroxyurea under assay conditions (data not shown).

Iron analysis of R2F showed the presence of 1.8 mol of iron per mol of polypeptide chain, supporting the presence of a dinuclear iron center. The concentration of the unpaired spins relative to iron content was determined by double integration of the EPR signal and found to be 0.26 spin/iron atom—i.e. one radical per dimer enzyme.

Characterization of the Catalytic Activity. The highly purified reductase required CDP as substrate, with a K_m of 16 μM for the nucleotide with dATP as allosteric effector (data not shown). CMP and CTP showed no activity. The reaction was dependent on magnesium and showed a pH optimum at 8.0 (data not shown).

CDP reduction was strongly stimulated by dATP (Fig. 4), which presumably acts as an allosteric effector. From Fig. 4

one can calculate an apparent K_m of 3 μM for dATP. In contrast to the reaction catalyzed by protein R1, ATP and dTTP gave only a small stimulatory effect. The same effect was seen with dGTP, whereas dCTP was completely inactive. Concentrations of dATP up to 1 mM did not inhibit the reaction. In the R1-catalyzed reaction dATP is a positive modulator up to 1 μM but then becomes a strong inhibitor (3).

Under the assay conditions DTT functions as a reductant and substitutes for the physiological electron donor. Two small proteins with redox-active thiols, thioredoxin and glutaredoxin, supply this function for the R1/R2 reductase (12). We now tested the function of the two proteins for the R1E/R2F reductase. First, we investigated whether addition of either of the two proteins would affect the dependence of CDP reduction on the concentration of DTT. We found that optimal CDP reduction requires up to 40 mM DTT (Fig. 5*A*). Both glutaredoxin 1 and thioredoxin stimulated the enzyme reaction at low concentrations of DTT, but the effect was much more pronounced with glutaredoxin (Fig. 5*A*). In this case the small amount of DTT present in the preparation of R1a to protect the labile protein sufficed to give a strong reaction.

Fig. 5*B* shows concentration curves for glutaredoxin 1 and thioredoxin that were reduced enzymatically in the absence of added DTT. CDP reduction occurred only with glutaredoxin 1. Glutaredoxins 2 and 3 (13) showed no effect. The K_m determined for glutaredoxin 1 from this experiment is 6 μM, much higher than the K_m (0.13 μM) reported (12) for glutaredoxin with the R1/R2 enzyme of *E. coli*.

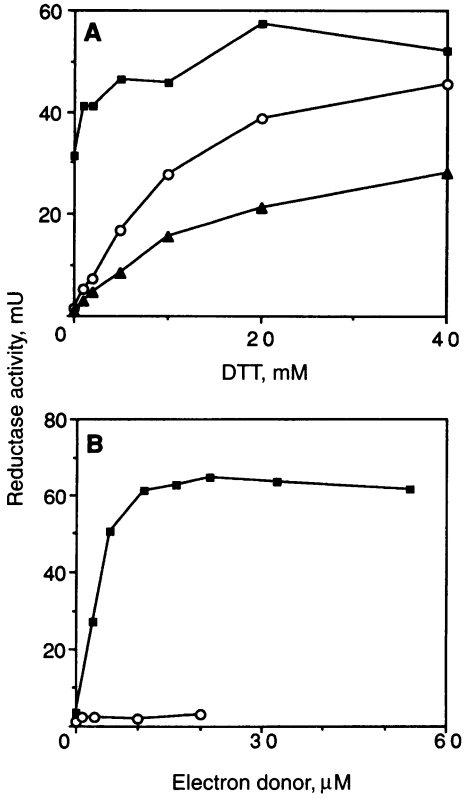

FIG. 5. Effects of glutaredoxin 1 and thioredoxin on CDP reduction. (*A*) Stimulation by glutaredoxin 1 and thioredoxin at various concentrations of DTT. R1E (0.2 μg) and R2F (1 μg) were incubated under standard conditions except for the concentration of DTT shown on the abscissa. Where indicated, 5 μM glutaredoxin 1 or 5 μM thioredoxin was added. (▲) No addition; ■, with glutaredoxin 1; ○, with thioredoxin. (*B*) Concentration curves for glutaredoxin 1 and thioredoxin. R1E (0.2 μg) and R2F (1 μg) were incubated under standard conditions, but without DTT, with various concentrations of glutaredoxin 1 plus 1 mm NADPH, 4 mM glutathione, and 0.5 μg of glutathione reductase (■) or with various concentrations of thioredoxin plus 1 mM NADPH, and 0.5 μg of thioredoxin reductase (○). mU, milliunits.

DISCUSSION

Together, the cloned *nrdE* and *nrdF* genes of *S. typhimurium* complement mutations in either of the *nrdA* and *nrdB* genes encoding the R1 and R2 ribonucleotide reductase proteins of *E. coli*. Since the amino acid sequences of the proteins encoded by *nrdE* and *nrdF* contained characteristic features of the proteins encoded by *nrdA* and *nrdB*, it appeared likely that *nrdE* and *nrdF* also encoded a class I ribonucleotide reductase. Our present work establishes that this is the case. *E. coli* carrying a multicopy plasmid containing the *S. typhimurium nrdE* and *nrdF* genes overproduces two proteins, named R1E and R2F, that functionally and structurally correspond to R1 and R2, respectively. The R1E/R2F reductase reduces CDP to dCDP with roughly the same efficiency as the R1/R2 reductase. That R2F corresponds to R2 appears from the finding that R2F contains stoichiometric amounts of a tyrosyl radical and a dinuclear iron center. Spectroscopic evidence suggests that the two iron atoms are linked by a μ-oxo bridge. Evidence for the correspondence of R1E and R1 is more indirect. It rests on the requirement of the reaction for allosteric activation and on the finding that DTT and reduced glutaredoxin function as hydrogen donors.

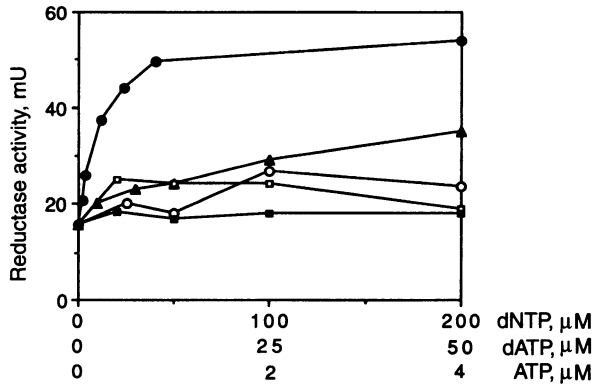

FIG. 4. Allosteric stimulation of CDP reduction. Incubation under standard conditions with 0.4 μg of R1E and 1 μg of R2F. The standard concentration of 0.3 mM dATP was replaced by the concentrations of dATP (●), ATP (○), dTTP (▲), dGTP (□), and dCTP (■) indicated on the abscissa. mU, milliunits.

There are also differences between the R1/R2 reductase and the R1E/R2F reductase. One is that the R1E/R2F system does not use reduced thioredoxin as hydrogen donor. More striking is that dATP was by far the best allosteric activator of CDP reduction, whereas it is a strong inhibitor of the R1/R2 reductase at the concentrations used here.

The finding of a potentially active second ribonucleotide reductase gene in both *S. typhimurium* and *E. coli* is highly surprising. Several independent mutants in the *nrdA* and *-B* genes of *E. coli* showing temperature or oxygen-sensitive phenotypes have been described (6, 14–16). This strongly suggests that the chromosomal *nrdE* and *-F* genes are silent under normal circumstances. They occupy corresponding positions on the chromosomes of both bacteria, next to the *proU* operon. The activity of this operon is affected by supercoiling, and a similar control seems possible for *nrdE* and *nrdF*. We do not understand the functional significance of *nrdE* and *-F*. Since its potential activity has been maintained by both *S. typhimurium* and *E. coli*, we presume that the enzyme is active under specific physiological circumstances. What little we have learned about the regulation of the R1E/R2F reductase shows important differences vis à vis

the "normal", R1/R2 enzyme. The stimulatory effect of dATP for CDP reduction is reminiscent of the phage T4-encoded reductase (17).

Clues to the physiological functions of the two enzymes may come from an analysis of the environmental factors which affect the expression of both types of genes. A more complete comparison of the allosterical properties of the two enzymes might also be illuminating. A further question is whether genes equivalent to *nrdE* and *nrdF* are present in other organisms.

This study was supported by grants from the Swedish Medical Research Council and the European Community (P.R.) by a predoctoral grant from the Direccio General Universitat de la Generalitat de Catalunya and the European Molecular Biology Organization (A.J.).

1. Reichard, P. (1993) *Science* **260,** 1773–1777.
2. Fontecave, M., Nordlund, P., Eklund, H. & Reichard, P. (1992) *Adv. Enzymol.* **65,** 147–183.
3. Brown, N. C. & Reichard, P. (1969) *J. Mol. Biol.* **46,** 39–55.
4. Larsson, Å. & Sjöberg, B.-M. (1986) *EMBO J.* **5,** 2037–2040.
5. Jordan, A., Gibert, I. & Barbé, J. (1994) *J. Bacteriol.* **176,** 3420–3427.
6. Hantke, K. (1988) *Arch. Microbiol.* **149,** 344–349.
7. Jobling, M. G. & Holmes, R. K. (1990) *Nucleic Acids Res.* **18,** 5315–5316.
8. Eliasson, R., Pontis, E., Fontecave, M., Gerez, C., Harder, J., Jörnvall, H., Krook, M. & Reichard, P. (1992) *J. Biol. Chem.* **267,** 25541–25547.
9. Berglund, O. & Eckstein, F. (1972) *Eur. J. Biochem.* **28,** 492–496.
10. Thelander, L., Sjöberg, B.-M. & Eriksson, S. (1978) *Methods Enzymol.* **51,** 227–237.
11. Petersson, L., Gräslund, A., Ehrenberg, A., Sjöberg, B.-M. & Reichard, P. (1980) *J. Biol. Chem.* **255,** 6706–6712.
12. Holmgren, A. (1989) *J. Biol. Chem.* **264,** 13963–13966.
13. Åslund, F., Ehn, B., Miranda-Vizuete, A., Pueyo, C. & Holmgren, A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9813–9817.
14. Fuchs, J. A., Karlström, H. O., Warner, H. R. & Reichard, P. (1972) *Nature (London) New Biol.* **238,** 69–71.
15. Jamison, C. S. & Adler, H. I. (1987) *J. Bacteriol.* **169,** 5087–5094.
16. Taschner, P. E. M., Verest, J. G. J. & Woldrinch, C. L. (1987) *J. Bacteriol.* **169,** 19–25.
17. Berglund, O. (1972) *J. Biol. Chem.* **247,** 7276–7281.