Characterization of Escherichia coli NrdH

A GLUTAREDOXIN-LIKE PROTEIN WITH A THIOREDOXIN-LIKE ACTIVITY PROFILE*

(Received for publication, February 25, 1997, and in revised form, May 12, 1997)

Albert Jordan‡§, Fredrik Åslund¢, Elisabet Pontis, Peter Reichard, and Arne Holmgren‡

From the Medical Nobel Institute for Biochemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institute, S-171 77 Stockholm, Sweden and the Department of Genetics and Microbiology, Faculty of Sciences, Autonomous University of Barcelona, Bellaterra, 08193 Barcelona, Spain

Ribonucleotides are converted to deoxyribonucleotides by ribonucleotide reductases. Either thioredoxin or glutaredoxin is a required electron donor for class I and II enzymes. Glutaredoxins are reduced by glutathione, thioredoxins by thioredoxin reductase. Recently, a glutaredoxin-like protein, NrdH, was isolated as the functional electron donor for a NrdEF ribonucleotide reductase, a class Ib enzyme, from Lactococcus lactis. The absence of glutathione in this bacterium raised the question of the identity of the intracellular reductant for NrdH. Homologues of NrdH are present in the genomes of Escherichia coli and Salmonella typhimurium, upstream of the genes for the poorly transcribed nrdEF operon, separated from it by an open reading frame (nrdI) coding for a protein of unknown function. Overexpression of E. coli NrdH protein shows that it is a functional hydrogen donor with higher specificity for the class Ib (NrdEF) than for the class Ia (NrdAB) ribonucleotide reductase. Furthermore, this glutaredoxin-like enzyme is reduced by thioredoxin reductase and not by glutathione. We suggest that several uncharacterized glutaredoxin-like proteins present in the genomes of organisms lacking GSH, including archae, will also react with thioredoxin reductase and be related to the ancestors from which the GSH-dependent glutaredoxins have evolved by the acquisition of a GSH-binding site. We also show that NrdI, encoded by all nrdEF operons, has a stimulatory effect on ribonucleotide reduction.

Ribonucleotides are reduced to deoxyribonucleotides by ribonucleotide reductases, which are radical containing enzymes that may be divided into three main classes (1, 2).

The electrons for this reaction are supplied by small redox-active proteins such as thioredoxin (Trx) or glutaredoxin (Grx) in the case of class I and II ribonucleotide reductase (3, 4), whereas formate fulfills this function for the anaerobic class III enzyme (5). Thioredoxin and glutaredoxin both contain two redox-active cysteine thiols in their reduced form, which by dithiol-disulfide interchange change reduce an acceptor disulfide in the active center of ribonucleotide reductase. The active site sequences of thioredoxins and glutaredoxins are conserved among species, being Cys-Gly-Pro-Cys for thioredoxin and Cys-Pro-Tyr-Cys for glutaredoxin (3, 4). The disulfide in oxidized thioredoxin is regenerated to a dithiol by thioredoxin reductase (TR) and NADPH, whereas oxidized glutaredoxin is reduced by 2 mol of GSH with the formation of GSSG, which is reduced by glutathione reductase (GR) and NADPH.

Escherichia coli contains three different glutaredoxins (called Grx1, -2, and -3 (6)), which, like all glutaredoxins from other species, show high activity as general GSH-disulfide oxidoreductases in a coupled system with GSH, NADPH, and glutathione reductase (7). Three-dimensional structures for thioredoxins (8) and glutaredoxins (9, 10) show that they have essentially completely unrelated amino acid sequences but a similar overall fold (often referred to as the thioredoxin fold), consisting of a central four-stranded β-sheet flanked by three helices in the order βββββ (the alignment in Fig. 1 includes the location of secondary structure elements in glutaredoxins). Within the thioredoxin superfamily of proteins (for review, see Ref. 11) two distinct subtypes are the thioredoxins proteins and the glutaredoxin proteins, the latter having a conserved GSH-binding site (boxed in Fig. 1), which has been experimentally determined by NMR (12) for E. coli Grx1.

E. coli and Salmonella typhimurium contain the genetic information for two different ribonucleotide reductases belonging to class I, NrdAB (class Ia) and NrdEF (class Ib), with a limited sequence similarity (13) and differences in their allosteric regulation (14). The expression of NrdEF is repressed and insufficient to allow growth of NrdAB-defective cells under aerobic conditions, unless the expression of NrdEF is increased by the presence of additional copies of the nrdEF genes either on the chromosome or on a plasmid (13, 15). Characterization of the proteins encoded by the S. typhimurium nrdEF genes showed a fully functional ribonucleotide reductase that could use E. coli Grx1 (but not thioredoxin) as a hydrogen donor with an apparent $K_m$ of 5 μM (16) (cf. 0.15 μM for NrdAB; Ref. 17).

Recently, it was shown by enzyme fractionation that the functional ribonucleotide reductase in the Gram-positive bacterium Lactococcus lactis grown under microaerophic conditions is an NrdEF type of enzyme and that its hydrogen donor protein (NrdH, 72 amino acids) with the active site sequence Cys-Met-Gln-Cys shows appreciable homology to glutaredoxins polymerase chain reaction; T4-Grx, phage T4 glutaredoxin; TR, thioredoxin reductase; NTA, nitrolotriacetic acid.
Characterization of \textit{E. coli} NrdH

(18). Coding sequences for proteins homologous to NrdH are present upstream of the \textit{nrdEF} genes in \textit{E. coli} and \textit{S. typhimurium} (81 amino acids), separated by an open reading frame (Orf2; 136 amino acids). Since we have found that this latter protein seems to be involved in reduction of ribonucleotides, the protein will henceforth be referred to as NrdI. An alignment of NrdH and NrdI proteins from different species are presented in Figs. 1 and 2. The four genes, \textit{nrdH-nrdI-nrdE-nrdF}, constitute a unique transcriptional unit (15). On the assumption that the gene organization was the same in \textit{L. lactis}, it was possible to clone the analogous operon for the \textit{L. lactis} NrdEF enzyme including the gene (\textit{nrdH}) for the hydrogen donor protein and \textit{nrdI} (18). Since \textit{L. lactis} has no GSH and the NrdH proteins also appeared to lack the typical GSH-binding site identified in Grx1 (12), we wondered how NrdH is reduced. To solve this question we have cloned and expressed the NrdH protein from \textit{E. coli}, an organism with a high content of GSH. Characterization of the NrdH protein showed that it lacked activity with GSH but was a substrate for thioredoxin reductase. Additionally, and with the aim of completing the understanding of the function of all the genes that constitute the conserved \textit{nrdEF} operon, we have overexpressed the NrdH protein from \textit{S. typhimurium} and investigated its influence on the activity of the NrdEF reductase.

\textbf{EXPERIMENTAL PROCEDURES}

\textbf{Materials}—Plasmids \textit{pET-24a} and \textit{pET-15b} were from Novagen, and \textit{pGEM-T} was from Promega Corp. \textit{E. coli} DH5\textalpha\ (CLONTECH) was used for general cloning procedures. BL21(DE3) was from Novagen. \textit{Taq} DNA polymerase and T4 DNA ligase were from Pharmacia Biotech Inc. Oligonucleotide primers were from the Department of Cell and Molecular Biology, Karolinska Institute. Sephadex G-50 resin was from Pharmacia, and \textit{DEAE}-cellulose (DE-32) anion exchanger was from Whatman. \textit{NdeI}-\textit{NTA} agarose was from Qiagen. \textit{E. coli} Grx1, Trx, and TR were obtained as described previously (3, 19, 20), and \textit{S. typhimurium} NrdH proteins (R1E and R2F) were purified as described (16). \textit{E. coli} NrdAB RR subunits (R1 and R2) were available from this laboratory. The University of Wisconsin Genetics Computer Group (GCC) package (version 8.0, Open VMS) was used for sequence analysis.

\textbf{Overexpression of \textit{E. coli} NrdH}—The entire gene for \textit{E. coli} NrdH was amplified by PCR using plasmid pUA523 (15) as a template and a series of PCR primers containing restriction sites for \textit{NdeI} and \textit{BamHI}, respectively (underlined): 5′-ATAGGATATAGGGCGAGTTATTTA-3′ and 5′-ACACGGATTTCTACTGTGAGCCCGGCGG-3′ (antisense). Thirty cycles of amplification were used with annealing at 55 °C. The amplified DNA fragment was gel-purified and cloned into vector \textit{pGEM-T} according to the manufacturer’s protocol, giving rise to plasmid pUA624. To ascertain that no \textit{Taq} polymerase-induced mutations were introduced, the cloned fragment was sequenced with fluorescent \textit{pUC/M13} universal primers, and the sequence was determined using an automated laser fluorescent DNA sequencer (Pharmacia). pUA624 was digested with \textit{NdeI} and \textit{BamHI}, and the \textit{nrdH} coding sequence was cloned into the expression vector \textit{pET-24a} digested with the same restriction enzymes, downstream from the inducible T7 promoter and a strong ribosome binding site. Plasmid (pUA625) from one clone unambiguously confirmed to code for NrdH was transformed into BL21(DE3) cells, which carry an IPTG-inducible T7 RNA polymerase gene.

\textbf{Overexpression of \textit{S. typhimurium} NrdH}—The \textit{S. typhimurium} \textit{nrdH} gene was amplified using plasmid pUA335 (13) as a template and two primers containing restriction sites for \textit{NdeI} and \textit{BamHI}, respectively (underlined): 5′-GACGATATAGGGCGAGTTATTTA-3′ and 5′-CCGCGATTTCTGTGAGCCCGGCGGC-3′ (antisense). The same general procedure described above was used for cloning the \textit{nrdH} gene into vectors \textit{pGEM-T} (pUA626) and \textit{pET-15b} (pUA627), consecutively. The cloning into \textit{NdeI-BamHI} digested \textit{pET-15b} introduces a sequence coding for six His codons upstream the \textit{nrdH} gene, facilitating purification of the recombinant protein on \textit{Ni2+}-\textit{NTA}-agarose.

\textbf{Purification of Overexpressed \textit{NrdH} Protein}—\textit{E. coli} BL21(DE3) cells harboring the plasmid pUA625 were grown in LB medium at 37 °C in the presence of 50 µg/ml of ampicillin to an optical density of 600 nm of 0.5 and subsequently induced with 0.4 mM IPTG for 3 h. After centrifugation and extraction by a combination of lysozyme (0.1 mg/ml) and sonication in 5 volumes of binding buffer (20 mM Tris-HCl, pH 8.0, 0.5 mM NaCl, 5 mM imidazole, 0.5 mM CHAPS, 20% glycerol, 1% Nonidet P-40, 10 mM \textit{b}-mercaptoethanol), the soluble crude extract was loaded into a \textit{Ni2+}-NTA agarose column according to the manufacturer’s protocol. The column was washed with the same buffer containing 60 mM imidazole, and NrdH was eluted with 300 mM imidazole. Prior to use, the sample was dialyzed to remove NaCl, imidazole, and \textit{b}-mercaptoethanol.

\textbf{Purification of Overexpressed \textit{NrdI} Protein}—\textit{E. coli} BL21(DE3) cells harboring the plasmid pUA267 were grown in LB medium at 30 °C in the presence of 50 µg/ml of ampicillin to an optical density of 0.5 and subsequently induced with 0.4 mM IPTG for 3 h. After centrifugation and extraction by a combination of lysozyme (0.1 mg/ml) and sonication in 5 volumes of binding buffer (20 mM Tris-HCl, pH 8.0, 0.5 mM NaCl, 5 mM imidazole, 0.5 mM CHAPS, 20% glycerol, 1% Nonidet P-40, 10 mM \textit{b}-mercaptoethanol), the soluble crude extract was loaded into a \textit{Ni2+}-NTA agarose column according to the manufacturer’s protocol. The column was washed with the same buffer containing 60 mM imidazole, and NrdI was eluted with 300 mM imidazole. Prior to use, the sample was dialyzed to remove NaCl, imidazole, and \textit{b}-mercaptoethanol.

\textbf{Reduction of insulin disulfides}—Reduction of insulin disulfides

(2) Reduction of insulin disulfides—Reduction of insulin disulfides

\begin{align*}
\text{Reduction of insulin disulfides} &= \text{Reduction of insulin disulfides} \\
\text{Reduction of insulin disulfides} &= \text{Reduction of insulin disulfides}
\end{align*}
Characterization of E. coli NrdH

FIG. 1. Alignment of glutaredoxin-like enzymes of the NrdH type with classical glutaredoxins. The sequences were obtained from the following sources: NrdH from E. coli, S. typhimurium, and L. lactis (18); Grx from phage T4 (45); Grxl from E. coli (34); Grx3 from E. coli (27). The secondary structure of Grx3 determined by NMR (27) is shown at the bottom of the alignment (dark lines), and the predicted secondary structure of E. coli NrdH obtained as described (26) is shown at the top of the alignment (stippled lines). The sequences have been aligned so that the predicted secondary structure elements of E. coli NrdH match the determined secondary structure elements of E. coli Grx3. The residues that constitute the GSH-binding site of E. coli Grx3 are boxed. Residues conserved both in NrdH proteins and in glutaredoxins are on a gray background.

(50 μM final concentration) was performed in parallel for Grx1, NrdH, T4-Grx, and Trx using a final concentration of 5 μM of each enzyme in the presence of 1 mM DTT in 100 mM potassium phosphate, 1 mM EDTA, pH 6.0. The reaction was followed as the increase of absorbance at 600 nm due to the precipitation of insulin when reduced to A and B chains (25).

The activity of NrdH, T4-Grx, and Trx (final concentration of each enzyme, 5 μM) as reductants of insulin disulfides (100 μM final concentration) was also determined in the presence of 200 μM NADPH, 0.1 μM thioredoxin reductase in 100 mM potassium phosphate, 1 mM EDTA, pH 7.0. The activity was in this case monitored as the decrease in absorbance of NAPDH at 340 nm.

GSH-Disulfide Oxidoreductase Assays—GSH-disulfide oxidoreductase assay were performed as described (17), measuring the reduction of β-hydroxyethyl disulfide by GSH at the expense of NAPDH as monitored at 340 nm. A standard of purified E. coli Grx1 was used in each experiment as a positive control.

RESULTS

Overexpression and Purification of the E. coli NrdH Protein—The gene for E. coli NrdH was amplified by PCR using primers with designated restriction sites. The PCR product was cloned into the T7 RNA polymerase-dependent expression vector pET-24a. Using this system, the NrdH protein was expressed to levels of around 30% of total soluble protein, as judged by densitometry scans of SDS-polyacrylamide gels. The $M_r$ of the overexpressed polypeptide (9 kDa) was in accordance with the expected size for NrdH. The protein was purified to homogeneity but showed poor solubility. Thus, to avoid precipitation of the protein when concentrated, the final steps of the purification were done in a buffer containing 10% glycerol and 0.5 M NaCl. Purified NrdH protein was subjected to N-terminal amino acid sequence determination, confirming the homogeneity of the protein and also showing an unprocessed initiator Met residue as expected.

The sequence homology between NrdH proteins and glutaredoxins has been noted, with E. coli Grx3 being the closest glutaredoxin homologue (18). A prediction of the secondary structure elements of E. coli NrdH using the PHD program (26) predicted a similar pattern of secondary structure elements (with the exception of β4) as experimentally determined for Grx1 and Grx3 (9, 27). Fig. 1 shows a refined alignment where the predicted secondary structure elements of E. coli NrdH have been matched against the known secondary structure elements of E. coli Grx3 (27). The presence of a proline (Pro-52 in E. coli NrdH) in the NrdH proteins in the same relative position as the conserved cis-proline in glutaredoxins reemphasizes the structural similarity between NrdH proteins and glutaredoxins. The conclusion that NrdH proteins lack the conserved GSH-binding site in glutaredoxins is still valid in this refined alignment. It is noteworthy that E. coli NrdH may be aligned with E. coli Grx3 with essentially no introduction of gaps. This could provide a basis for further studies of E. coli NrdH intended to introduce a GSH-binding site by changing the relevant amino acids of NrdH to the corresponding ones found in Glx3 of Bacillus subtilis (29), one should suspect a function for this unknown protein in the ribonucleotide reduction reaction. Searching for functional similarities of NrdH in the protein sequence data bases was unsuccessful. Fig. 2 shows the predicted amino acid sequence alignment of all known nrdI products. To investigate the effect of NdrI in the in vitro assay of S. typhimurium NdrE ribonucleotide reductase, we overexpressed and partially purified the recombinant NrdI protein of this bacterium.

After cloning the PCR-amplified nrdI into the vector pET-15b, the His-tagged NrdI protein was expressed to levels of around 30% of total soluble protein. Only a small fraction of the overexpressed recombinant protein was found in the soluble fraction of the crude extract. This soluble NrdI material was purified by one-step chromatography on Ni$_2$-NTA resin, resulting in material that was about 50% pure. The size of the recombinant protein was higher (17 kDa) than the size of the expected NrdI polypeptide (15 kDa) due to the His tag. Any attempt to increase the

2. K. Nordstrand, unpublished data.
Characterization of E. coli NrdH

The enzymes showed Michaelis-Menten kinetics with overall similar $V_{\text{max}}$ values (except for Trx, which was inactive with NrdEF). Since the NrdH curves (Fig. 3A) are slightly sigmoidal, the $K_m$ values should only be considered as half-saturation concentrations obtained from a biased analysis.

| Protein | $V_{\text{max}}$ with NrdAB | $V_{\text{max}}$ with NrdEF |
|---------|-----------------------------|-----------------------------|
| NrdH (in the presence of TR and NADPH) | 1.25 | Inactive |
| NrdH (in the presence of TR and NADPH) | 4.2 | 1.2–1.6 |
| NrdH (in the presence of DTT) | ND* | 0.5–0.6 |
| NrdH (in the presence of GSH, GR, and NADPH) | Inactive | Inactive |
| Grx1 (in the presence of GSH, GR, and NADPH) | 0.15* | 1.2–5* |

a Data from Ref. 17.

b ND, not determined.
c Data from Ref. 16.

Activity of NrdH and NrdI with the NrdAB and NrdEF Ribonucleotide Reductases—The activity of NrdH was compared with that of E. coli Grx1 and Trx as reductants of the NrdAB enzyme from E. coli and the NrdEF enzyme from S. typhimurium. NrdH was found to be a functional hydrogen donor for both enzymes in the presence of either 1 mM DTT or thioredoxin reductase/NADPH but not in the presence of GSH/glutathione reductase/NADPH. The $V_{\text{max}}$ of NrdH (in units/µg of the R1 or R1E subunits) was similar to that of Grx1 and Trx with the NrdAB enzyme and similar to the $V_{\text{max}}$ of Grx1 with the NrdEF enzyme. As shown in Table I and Fig. 3A, NrdH showed a lower $K_m$ value with the S. typhimurium NrdEF enzyme than with the E. coli NrdAB enzyme, whereas the opposite was found to be the case for E. coli Grx1 (Fig. 3B). This tendency for NrdH was even more pronounced when the assays were performed in the presence of 1 mM DTT instead of thioredoxin reductase/NADPH. Thus, the apparent $K_m$ value of NrdH as a hydrogen donor for NrdEF was repeatedly found to be lower (0.3–0.6 µM) in the presence of 1 mM DTT than in the presence of NADPH/thioredoxin reductase ($K_m = 1.2$ µM). This finding is hard to reconcile with the finding that NrdH is efficiently reduced by thioredoxin reductase (see below). Nevertheless, the results clearly demonstrate that NrdH is a more specific hydrogen donor for NrdEF than for NrdAB, whereas the opposite is the case for Grx1. E. coli Trx was a hydrogen donor for NrdAB but not for NrdEF (Fig. 3C) as observed previously (16).

solubility of the recombinant NrdI during growth or extraction (e.g. by decreasing the growth temperature or IPTG concentration or by additions of NaCl, glycerol, detergents, or reducing agents such as DTT) was unsuccessful. NrdI could be purified in the presence of guanidinium hydrochloride or urea, but the protein precipitated when the denaturing agent was removed by dialysis.

Activity of NrdH with Thioredoxin Reductase—Having established that NrdH is a substrate for thioredoxin reductase, we next chose to characterize how NrdH performed as a substrate for this enzyme compared with the other known substrates, Trx and T4-Grx. Using the standard DTNB reduction assay in the presence of NADPH/thioredoxin reductase at pH 8.0, we found that the three enzymes had similar $K_m$ values in this assay and that the $V_{\text{max}}$ for Trx was only somewhat higher than for NrdH or T4-Grx (Table II).

Since NrdI stimulated both the activity of NrdH plus NrdEF and that of Trx plus NrdAB in the presence of NADPH/thioredoxin reductase, we tested whether NrdI would affect the reduction of NrdH or Trx by thioredoxin reductase in the DTNB assay. No effect by NrdI was seen.

Redox Potential of the NrdH Enzyme—The ability of NrdH to be reduced efficiently by thioredoxin reductase allowed the determination of its redox potential by assessing the equilibrium constant with NADP+/NADPH. Calculated from a standard state redox potential for NADP+/NADPH of $-315$ mV, a redox potential of $-248.5 \pm 1.5$ mV was obtained for NrdH.

General Disulfide Reduction Capacity of NrdH—Reduction of insulin disulfides is a classical assay for thioredoxin (25). As shown in Fig. 5, NrdH was almost as potent a reductant as Trx in this system and was much more potent than Grx1 and T4-Grx. The same relative order of activity among Trx, NrdH, and T4-Grx was also obtained when thioredoxin reductase/NADPH was used instead of DTT.

In contrast to glutaredoxins, the NrdH protein lacked detectable activity in the GSH-disulfide oxidoreductase assay, where glutaredoxins show a high activity (data not shown).

DISCUSSION

The NrdH protein was originally discovered as the hydrogen donor for L. lactis NrdEF, a class Ib enzyme that is the active ribonucleotide reductase under aerobic conditions in this organism (18). The juxtaposition of nrdH and nrdI upstream of nrdEF in a conserved operon suggests a specific involvement of NrdH and NrdI in the ribonucleotide reduction process. Since the NrdEF enzyme from E. coli and S. typhimurium serves as an excellent model system for class Ib enzymes, we have now extended the characterization of the proteins encoded by this operon to include NrdH and NrdI. We have found NrdH to be an efficient hydrogen donor for ribonucleotide reductase, with higher specificity for the NrdEF enzyme than for the NrdAB enzyme. This reaction was stimulated modestly by the addition of NrdI by an as yet unknown mechanism. Furthermore, we show that NrdH is a good substrate for thioredoxin reductase and that it has no detectable activity with NrdAB or NrdEF in the presence of GSH. A summary of the biochemical properties of NrdH in comparison with other redox active proteins from E. coli and phase T4 is presented in Table III.

Functionally, NrdH thus behaves like the classical thioredoxin (3, 30) of E. coli, sharing some of its biochemical properties including a low redox potential and the ability to reduce insulin disulfides. However, the sequences of the two proteins
are not related. Instead, NrdH shows sequence homology with *E. coli* glutaredoxins (Fig. 1). Glutaredoxins function as dithiol shuttles during ribonucleotide reduction. Their distinction is that they contain a glutathione binding site and that glutathione reduces their active cysteines. With one known exception, they are not reduced by thioredoxin reductase, the exception being the glutaredoxin induced by phage T4, which for this reason originally was classified as a thioredoxin (31). This protein was later renamed T4-Grx (32), since thioredoxin reductase can be fully substituted by GSH (33) and also because of the sequence homology with *E. coli* Grx1, which became apparent upon the determination of the primary structure of the latter (34). The three-dimensional structure of T4-Grx is also more related (10) to the known structure of *E. coli* Grx1 (9) than to thioredoxin.

In addition to NrdH, the known substrates for *E. coli* thioredoxin reductase are Trx and phage T4-Grx. The sequence homology between these three proteins is, however, restricted (21%) between NrdH and phage T4-Grx and essentially insignificant between NrdH (or T4-Grx) and Trx. This prevents us from making any predictions of the residues that are involved in the interaction with thioredoxin reductase. However, given the predicted high structural similarity (Fig. 1) between in particular *E. coli* Grx3 and NrdH it might be possible to mutate Grx3 with the aim of making it a substrate for thioredoxin reductase. The identity of the residues to be changed, however,
is not obvious and will require careful analysis of three-dimensional structures.

Conceivably, the inability of NrdH to use GSH could be an effect of the redox potential of the protein being too low to allow an efficient reduction by GSH. We have found that *E. coli* NrdH has a redox potential of $-248$ mV at pH 7.0 compared with $-270$ mV for thioredoxin (23). Since this number is quite similar to the $-240$ mV determined for T4-Grx (31, 35), which is relatively efficiently reduced by GSH, the lack of activity of NrdH with GSH can best be explained by the protein lacking the residues needed to interact with GSH. Furthermore, NrdH did not show any activity in the general assay for glutaredoxins using the artificial disulfide $\beta$-hydroxyethyl disulfide as a substrate, supporting our conclusion that the protein does not catalyze GSH-dependent disulfide reductions.

Where does this leave the NrdH protein? It lacks a glutathione binding site, and its active site cysteine residues are not reduced by glutathione. It was actually discovered in an organism that lacks glutathione. Therefore, it may not functionally be classified as a glutaredoxin, despite the sequence homology (Fig. 1). In the phylogenetic tree (Fig. 6), it is apparent that NrdH proteins form a separate group, on the same branch as glutaredoxins but definitely separated from thioredoxins. Since the two groups are easily distinguished by the presence of several typical conserved residues (8, 10, 11), we chose to refrain from classifying NrdH as a thioredoxin despite its thioredoxin-like activity profile. *E. coli* contains only one known thioredoxin so far, but yeast has two isoforms (36) and plants many organisms lacking GSH, including archae (38, 39). Glutaredoxins are the simplest members of the thioredoxin superfamily, and the glutaredoxin fold is present in all of the members (here including GSH-peroxidases and glutathione S-transferases (11). A reasonable evolutionary scenario could actually be that NrdH and similar glutaredoxin-like proteins are related to the progenitors of the thioredoxin superfamily, from which the other members evolved by divergent evolution (the glutaredoxins simply by the acquisition of a GSH-binding site).

![Fig. 6. Phylogenetic tree of glutaredoxin-like proteins including NrdH and thioredoxins constructed using the following protein sequences obtained from the SwissProt database: Trub_Clopa, VpG66_Bmp5, Glr1_Ecoli, Glr3_Ecoli, Ye65_Yearst, GlrX_Yearst, Glrx_Human, Glrx_Bovin, Glrx_Varv, Glrx_Vace, Thio_Bpt4, Thio_Strci, Thio_Bacu, Thiz_Borld, Thiz_Corne, Thiz_Arath, Thiz_Yearst, Thiz_Yearst, Thiz_Yearst, Thiz_Yearst, Thiz_Yearst, Thiz_Yearst, Thiz_Yearst, Thiz_Yearst, Thiz_Yearst, and Glrx_Metth. The following proteins have been obtained from a different source: L. lactis, E. coli and S. typhimurium NrdH (translation of the *nrdH* gene; Refs. 15 and 18); GRX O. sativa (44), and GRX2 phage T4 (45). The CLUSTAL W program was used for sequence alignments (46). To estimate the relationships among these aligned sequences, the neighbor-joining method was applied to a matrix of pairwise distances; any gaps in the alignment were excluded from all pairwise comparisons, and the distance values were corrected for multiple hits by the empirical method of Kimura (47).[313x325 to 558x531]
Characterization of E. coli NrdH

The solubility of the protein is, however, a factor to consider for the lack of detection of NrdH protein in extracts of E. coli. This paper demonstrates that the NrdH gene located upstream of the nrdEF genes in E. coli codes for a protein that has a higher specificity as a hydrogen donor for NrdEF than for NrdAB. By analogy with the situation in Lactococcus lactis, it would thus seem that most NrdEF enzymes use NrdH as the functional in vivo hydrogen donor. However, the recently completed sequence determination of the M. genitalium genome (28) shows the presence of an NrdEF type of ribonucleotide reductase but no NrdH-like protein. This could provide an example of an NrdEF enzyme that interacts with, for example, thioredoxin for which M. genitalium contains a coding sequence. Thus, care should be taken not always to associate NrdEF with NrdH, as the situation in Lactococcus lactis, and S. typhimurium would suggest.

The mechanism of the stimulatory effect of NrdI on the activity of NrdH with NrdEF and on the activity of Trx with NrdAB remains elusive. Since the presence of nrdI in the known nrdEF loci is more conserved than that of nrdH, we believe that the NrdI protein has an important in vivo function for the activity of NrdEF. It is surprising that all of the genes products encoded by the nrdEF operon are fully functional proteins also in E. coli and S. typhimurium, since this operon seems to be poorly transcribed and knock-out mutants of the nrdEF genes have no phenotype (15). The conservation of the operon does, however, suggest an important in vivo function that at present is not understood.

Acknowledgment—We are indebted to Isidre Gibert for suggestions and contributions to the phylogenetic analysis of protein sequences.

REFERENCES
1. Reichard, P. (1993) Science 260, 1773–1777
2. Reichard, P. (1997) Trends. Biol. Sci. 22, 81–85
3. Holmgren, A. (1985) Annu. Rev. Biochem. 54, 237–271
4. Holmgren, A. (1989) J. Biol. Chem. 264, 13963–13966
5. Mulliez, E., Ollagnier, S., Fontecave, M., Eliasson, R., and Reichard, P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8759–8762
6. Åslund, F., Ehn, B., Miranda, Vizuet, A., Pueyo, C., and Holmgren, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9813–9817
7. Holmgren, A., and Åslund, F. (1995) Methods Enzymol. 252, 283–292
8. Eklund, H., Gleason, F. K., and Holmgren, A. (1991) Proteins 11, 13–28
9. Sodano, P., Xia, T. H., Bushweller, J. H., Björkberg, O., Holmgren, A., Billetier, M., and Withrich, K. (1991) J. Mol. Biol. 221, 1311–1324
10. Eklund, H., Ingelman, M., Soderberg, B. O., Uhlén, T., Nordlund, P., Nikkola, M., Sonnerstrom, U., Jornvall, H., and Pettersson, K. (1992) J. Mol. Biol. 228, 596–618
11. Martin, J. L. (1995) Structure 3, 245–250
12. Bushweller, J. H., Billetier, M., Holmgren, A., and Withrich, K. (1994) J. Mol. Biol. 235, 1585–1597
13. Jordan, A., Gibert, I., and Barbe, J. (1994) J. Bacteriol. 176, 3420–3427
14. Eliasson, R., Pontis, E., Jordan, A., and Reichard, P. (1996) J. Biol. Chem. 271, 26582–26587
15. Jordan, A., Aragall, E., Gibert, I., and Barbe, J. (1996) Mol. Microbiol. 19, 777–780
16. Jordan, A., Pontis, E., Atta, M., Krook, M., Gibert, I., Barbe, J., and Reichard, P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12892–12896
17. Holmgren, A. (1979) J. Biol. Chem. 254, 3672–3678
18. Jordan, A., Pontis, E., Åslund, F., Hellman, U., Gibert, I., and Reichard, P. (1996) J. Biol. Chem. 271, 8779–8785
19. Björkberg, O., and Holmgren, A. (1991) Protein Expression Purif. 2, 287–295
20. Dyson, H. J., Holmgren, A., and Wright, P. E. (1989) Biochemistry 28, 7074–7087
21. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
22. Thelander, L., Eriksson, S., and Sjöberg, B.-M. (1978) Methods Enzymol. 51, 227–237
23. Krause, G., Lundstrom, J., Barea, J. L., de la Cuesta, C. P., and Holmgren, A. (1991) J. Biol. Chem. 266, 9494–9500
24. Moore, E. C., Reichard, P., and Thelander, L. (1964) J. Biol. Chem. 239, 3445–3452
25. Holmgren, A. (1979) J. Biol. Chem. 254, 9627–9632
26. Rost, B., and Sander, C. (1993) J. Mol. Biol. 232, 584–599