NrdH-redoxin is a representative of a class of small redox proteins that contain a conserved CXXC motif and are characterized by a glutaredoxin-like amino acid sequence and thioredoxin-like activity profile. The crystal structure of recombinant *Escherichia coli* NrdH-redoxin in the oxidized state has been determined at 1.7 Å resolution by multiwavelength anomalous diffraction. NrdH-redoxin belongs to the thioredoxin superfamily and is structurally most similar to *E. coli* glutaredoxin 3 and phase T4 glutaredoxin. The angle between the C-terminal helix α3 and strand β4, which differs between thioredoxin and glutaredoxin, has an intermediate value in NrdH-redoxin. The orientation of this helix is to a large extent determined by an extended hydrogen-bond network involving the highly conserved sequence motif EWSG, which is unique to this subclass of the thioredoxin superfamily. Residues that bind glutathione in glutaredoxins are in general not conserved in NrdH-redoxin, and no glutathione-binding cleft is present. Instead, NrdH-redoxin contains a wide hydrophobic pocket at the surface, similar to thioredoxin. Modeling studies suggest that NrdH-redoxin can interact with *E. coli* thioredoxin reductase at this pocket and also via a loop that is complementary to a crevice in the reductase in a similar manner as observed in the *E. coli* thioredoxin-thioredoxin reductase complex.

Thioredoxins (Trx) and glutaredoxins (Grx) are two classes of redox active proteins, which are members of the thioredoxin superfamily (1–3). Characteristics of this family are the common fold and a conserved sequence motif, CXXC, which contains the redox active cysteine residues. A distinctive feature between thioredoxins and glutaredoxins is the pattern of residues linking these two cysteine residues, i.e. CGPC for thioredoxin and CPYC for most glutaredoxins (3–5). These two ubiquitous small redox proteins (molecular mass, 9–12 kDa) have been identified in numerous organisms and are, in their dithiol form, the major disulfide reductases in cells (2, 6). They perform a large number of functions in cell growth, such as redox control of transcription factors (7), electron transport to ribonucleotide reductase (8), or defense against oxidative stress and apoptosis (6). Glutaredoxins are specifically reduced by glutathione (GSH), whereas thioredoxins depend on thioredoxin reductase (TrxR) for reduction.

Recently, members of a new class of small redox proteins, glutaredoxin-like proteins, NrdH-redoxin (NrdH), have been found in *Escherichia coli* and several other organisms, in particular organisms lacking GSH (9, 10). NrdH proteins typically show sequence identities in the range of 34–85% and are homologous to glutaredoxins. For instance, the *Lactococcus lactis* NrdH redoxin has 27% sequence identity to *E. coli* glutaredoxin-3 and 17% identity to glutaredoxin-1 (9). NrdH-redoxin also contains the active site motif CXXC as in Trx and Grx, but the intervening residues (valine and glutamine) are different from both these classes. Although NrdH is related in amino sequence to glutaredoxins, it behaves functionally as a thioredoxin. It is not reduced by GSH but by thioredoxin reductase, it has a low redox potential, and it can reduce insulin disulfides; these biochemical features are characteristic of thioredoxin (10). The *in vivo* function of NrdH is not completely clear, but it can act as the functional hydrogen donor for class Ib ribonucleotide reductase and is part of a *nrdHIEF* operon (9–11). Recently, *E. coli* *nrdHIEF* mRNA levels were shown to be strongly enhanced in cells treated with oxidant (12).

To elucidate the structural basis of the thioredoxin-like activity profile of the glutaredoxin homologue NrdH, we have solved the crystal structure of *E. coli* NrdH to 1.7 Å by multiwavelength anomalous diffraction. Comparison of the three-dimensional structure of NrdH with that of other redoxins suggests a hydrophobic pocket at the surface and a loop between helix α2 and strand β3 as common determinants in the recognition and binding of Trx and NrdH to thioredoxin reductase. The architecture of this hydrophobic pocket is completely different from the glutathione binding cleft in glutaredoxins, thus preventing binding of glutathione.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—Plasmid pUA625, containing the gene for *E. coli* NrdH (10) was transformed into BL21-CodonPlus (Strategene), and the cells containing pUA625 were grown as described (10). To produce the NrdH selenomethionine derivative, *E. coli* BL21-CodonPlus cells with the expression vector pUA625 were grown at 37°C in 1-liter flasks in M9 medium (13) containing 50 μg/ml kanamycin. After 4–5 h, (A578 = 0.6), methionine biosynthesis was suppressed using an amino acid mixture as described (14) including l-selenomethionine (50 mg liter⁻¹). After 15 min the culture was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1.0 mM. The cells were harvested after 4 h by centrifugation, and the pellet (2.4 g) was stored at −20°C.

Purification of *E. coli* NrdH followed a protocol described elsewhere.
TABLE I

| Data collection and phasing statistics |
|---------------------------------------|
| λ1 peak                  | λ2 inflection | λ3 remote | Native |
|---------------------------|---------------|-----------|--------|
| Wavelength (Å)            | 0.9786        | 0.9787    | 0.9184 | 1.0292 |
| Beaml ine                 | BM14          | BM14      | BM14   | I711 Lund |
| X-ray source              | ESRF          | ESRF      | ESRF   | MaxII |
| Resolution (Å)            | 25.0–2.9      | 25.0–2.9 | 25.0–2.9 | 25.0–1.7 |
| R cryst (%)               | 6.1           | 6.1       | 5.3    | 5.8    |
| (R I/Io)                  | 12.9          | 12.8      | 14.5   | 17.5   |
| Completeness (%)          | 95.3 (70.5)   | 95.5 (71.0) | 99.1 (98.4) | 98.4 (88.7) |
| Total reflections         | 26702         | 26252     | 26144  | 113655 |
| Unique reflections        | 5578          | 5594      | 5785   | 8503   |
| Phasing power (iso/ano)   | 2.5/3.9       | 1.5/3.4   | 2.9    |        |
| R cryst (iso/ano)         | 0.59/0.49     | 0.85/0.60 | 0.64   |        |

(24). Structure comparison and structural alignments were carried out with the programs TOP and MAPS using default parameters (25). The figures were produced using the programs Grasp (26), O (20), and Bobscript (27). The coordinates and structure factors have been submitted to the Protein Data Bank, accession code 1H75 and r1H75ef, respectively.

RESULTS

Overall Structure—The refined model of NrdH at 1.7 Å resolution contains residues 1–76 of the protein and 82 water molecules. The polypeptide chain is well defined in the electron density map (Fig. 1), except for the last five residues, which were not visible in the electron density map, probably because of disorder. The side chain of Arg35 has been modeled in two different conformations, with relative occupancies of 0.7 and 0.3, respectively.

NrdH is a compact molecule with overall dimensions of 18 × 24 × 37 Å and a surface of 5141 Å². The structure consists of a four-stranded β-sheet with topology –1x, +2x, +1 and three flanking helices, a fold that is characteristic of the thioredoxin superfamily (3) (Fig. 2). Helix α2 runs roughly perpendicular to helices α1 and α3. A cis-proline (Pro)28, found at the beginning of strand β3, is highly conserved among proteins of the thioredoxin family.

A NrdH-specific Sequence Motif—In NrdH-like proteins the chain comprising the end of β4, the following loop, and the beginning of helix α3 contains a highly conserved sequence motif, \( \text{WSGFRP}^{14} \) (Fig. 3A). A pattern search in Swiss-Prot and TrEMBL showed that this pattern is unique to NrdH proteins. The position of helix α3 relative to the remaining part of the molecule is to a large extent determined by an intricate network of hydrogen bonds formed by the WSGFRP(D/E) motif, two water molecules, and surrounding protein residues (Fig. 3). A central role is played by a water molecule, which forms...
hydrogen bonds to atom Ne1 of Trp61, Oγ1 of Thr17, and main chain oxygen and nitrogen atoms of Gly63 and Arg65, respectively. Another water molecule interacts with the main chain oxygen of residue Ser62 and NH1 of Arg65. The latter residue forms a salt-bridge with Asp67 at the entrance to helix 1. The main chain nitrogen atom of Phe64 forms a hydrogen bond with side chain atom Oγ of Gln13, a residue that is part of the active site.

Similarities to Other Members of the Thioredoxin Superfamily—The structurally most similar proteins to NrdH in the PDB data base are E. coli Grx3 (28), T4 Grx (29), glutathione S-transferase from Arabidopsis thaliana (30), E. coli Trx (31), and human Trx (32) (Table III). These proteins all belong to the thioredoxin superfamily and share the thioredoxin fold. Although NrdH does not bind GSH, the overall structure is most similar to Grx and is less similar to Trx (Table III).

A comparison of NrdH with the structures of Grx and Trx from E. coli shows that despite the high overall similarity, all three proteins have distinctive features, which are typical for their subclass (Fig. 4). The N-terminal part is structurally most similar among all three proteins. Strands β1, β2, and β3, as well as helix α1 and the N termini of the helices α2 align well among the three proteins (Fig. 4). The C-terminal part is structurally less conserved. The most striking difference between these structures is the orientation of helix α3 in relation to strand β4 (Fig. 4B). Trx has a rather long connection between strand β4 and helix α3, and α3 is almost antiparallel to the strand. In Grx the chain instead immediately adopts helix conformation after strand β4 and this helix is then approximately perpendicular to the helix in Trx. In NrdH the connecting loop is longer than in Grx but shorter than in Trx resulting in a helix orientation in between that of Trx and Grx3 (Fig. 4B).

Trx contains one additional β-strand and one helix at the N terminus, which are not present in NrdH and Grx (29, 33). Trx also has a longer helix α1 and a shorter helix α2 than NrdH and Grx. The loop from helix α2 to strand β3 in Trx (residues 70–75) occupies a complementary groove on the surface of thioredoxin reductase (24) upon binding. The longer helix α2 of NrdH shifts the position of this loop compared with its location in Trx (Fig. 4).
Strand β4 and the following loop to helix α3 are more similar between NrdH and Trx than between NrdH and Grx3 (Fig. 4B). At the tip of strand β4 is a glycine residue (position 63 in NrdH, and 92 in E. coli Trx), which is conserved in the superfamily (34). This residue has been shown to be important in binding of Trx to other molecules, such as T7 DNA polymerase, TrxR (35), or in the assembly of filamentous phages (36).

Archaebacteria contain small redox proteins of as yet unknown function, which are similar in sequence to the glutaredoxins (19–30% sequence identity), but do not react with glutathione (37, 38). These redoxins are also related in amino acid sequence to NrdH proteins, with about 20% sequence identities, and it has therefore been suggested that they may form a common subgroup in the thioredoxin superfamily (38). Comparison of the three-dimensional structure of NrdH with the solution structure of the reduced form of an archaebacterial redoxin, Mj0307 from Methanococcus jannaschii (38), reveals that the structures are not as similar as one might expect for members of the same subgroup. In fact, superposition gives a r.m.s. of 2.0 Å with 45 equivalent Ca atoms, significantly worse than superposition of NrdH with Grx or Trx (Table III). Furthermore, these archaebacterial sequences do not contain the $^6$WSGFRP(D/E)$^97$ sequence motif typical of the mesophilic NrdH redoxins. These differences suggest that the two proteins are not members of one subgroup but represent different subgroups of the thioredoxin family.

The Active Site—The active site of E. coli NrdH is located at the beginning of helix α1. The electron density map clearly shows formation of a disulfide bond between residues Cys$^{11}$ and Cys$^{14}$, and the structure of NrdH described here is thus that of the oxidized protein.

The N-terminal cysteine of the CXXC motif provides the reactive thiolate in the reduced protein (2, 39, 40) and is more solvent accessible whereas the C-terminal cysteine side chain is buried. Although the active sites of NrdH, Trx, and Grx are quite similar, NrdH is more similar to Trx than to Grx. In particular, the $\phi$, $\psi$, and $\chi$ angles for the first cysteine of the active site are more similar to Trx than to Grx (Table IV). Compared with Grx the $\phi$ angle shows a difference of about 40° but only 18° difference compared with Trx. The $\psi$ angle of NrdH is 108° and is similar to E. coli Trx (108°), whereas the glutaredoxins have angles about 86–96°. Also in the $\chi$ angle, NrdH and Grx differs about 18–24°, whereas NrdH and E. coli Trx have an identical 164° $\chi$ angle. In all compared redoxins, the bond angles of the second active site cysteine are more similar to each other than the bond angles of the first cysteine (Table IV). We find no obvious correlation between the conformational angles of the first cysteine and the reduction potential. T4 Grx with $E^\circ = -240$ mV has the most similar reduction potential to NrdH ($E^\circ = -248.5$ mV), but differs most in the active site cysteine angles. However, T4 Grx, like NrdH, has a valine adjacent to the first cysteine residue in the CXXC motif. This is consistent with observations that the residues between the two active site cysteines are important determinants of the redox potential (41).

Table III

| Molecule                              | Equivalent residues | Identical residues | r.m.s.d. |
|---------------------------------------|---------------------|--------------------|----------|
| E. coli Glutaredoxin 3 (1FOV)         | 66                  | 14                 | 1.54     |
| T4 Glutaredoxin (1AAZ)                | 62                  | 17                 | 1.49     |
| A. thaliana glutathione S-transferase (1GNW) | 53                  | 14                 | 1.22     |
| E. coli thiorodoxin (2TRX)            | 55                  | 7                  | 1.36     |
| human thiorodoxin (1AUC)              | 46                  | 7                  | 1.36     |

Fig. 4. A, structure-based sequence alignment of NrdH with Grx and Trx from E. coli. The β-strands are shown as arrows and α-helices as boxes. Shaded areas denote structurally equivalent residues. Residues involved in glutathione binding are shown in bold letters. B, superposition of the Ca traces of NrdH (red), glutaredoxin-3 (blue), and thiorodoxin (gray) from E. coli.
dues, Arg<sup>8</sup>, Arg<sup>44</sup>, and Arg<sup>49</sup>, form a positively charged surface around the cleft. A pocket with similar location and width is also found in Trx, formed by Phe<sup>12</sup>, Phe<sup>27</sup>, Phe<sup>81</sup>, and Leu<sup>58</sup> (Fig. 5). The hydrophobic pocket of Trx is not surrounded by as many positively charged residues as observed in NrdH, only one positively charged surface residue, Arg<sup>73</sup>, is structurally conserved and aligns to Arg<sup>49</sup> in NrdH. Instead of a hydrophobic pocket, Grx has a narrow groove where GSH is binding. Tyr<sup>13</sup>, Thr<sup>50</sup>, Thr<sup>51</sup>, Val<sup>52</sup>, and Arg<sup>40</sup> form the walls of a bent cleft, which contains the glutathione binding site (42, 43). As in the hydrophobic pocket of NrdH, arginines, Arg<sup>16</sup>, Arg<sup>40</sup>, and Arg<sup>49</sup> (Fig. 5) surround the channel. In NrdH, side chains block part of this cleft, in particular by Gln<sup>50</sup>. Residues interacting with glutathione in Grx are in general not conserved in NrdH (Fig. 4a).

**Interaction of NrdH with E. coli TrxR—Superposition of NrdH on E. coli Trx in the Trx-TrxR complex (24) gives a model that shows only few steric clashes between the two molecules. These occur in the area of the loop that connects helix a2 and strand β3 in NrdH, and are because of the longer helix a2 in NrdH compared with Trx. However, a small rotation of NrdH will dock this loop in a groove on TrxR and at the same time allow interaction of TrxR with the hydrophobic pocket in NrdH, analogous to the Trx-TrxR interaction (Fig. 6). Phe<sup>141</sup> and Phe<sup>142</sup> of TrxR fit very well into this hydrophobic pocket. The distance between Cys<sup>11</sup> of NrdH and Cys<sup>138</sup> of TrxR in this docking model is 4.3 Å but as in the Trx-TrxR complex, the loop containing Cys<sup>11</sup> could bend toward TrxR, enabling the cysteine residues to form a disulfide bond.**

**DISCUSSION**

Structural analysis of NrdH has shown that it lacks the glutathione binding site seen in the glutaredoxins. The architecture of the surface, which in glutaredoxins defines the binding site of GSH, is completely different, and residues interacting with the electron donor are also not conserved between the two subfamilies of redoxins. NrdH is thus unable to accept GSH as a substrate.

The interaction of thioredoxin with thioredoxin reductase is characterized by three major features: (i) the docking of two phenylalanine residues from TrxR into a hydrophobic pocket of Trx close to the redox active cysteine, (ii) binding of the loop between helix a2 and strand β3 (nomenclature according to Fig. 4) of Trx into a groove on the surface of TrxR and (iii) interaction of the redox active cysteines enabling transfer of reducing equivalents (24). A striking observation is the pres-
ence in NrdH of a large hydrophobic pocket, at the same position as the corresponding pocket in Trx. Modeling experiments showed that it is possible to dock NrdH onto TrxR in a very similar way as observed in the TrxR-Trx complex (24), with the essential features of redoxin-reductase interactions preserved. It thus appears that the architecture of the hydrophobic pocket and the protruding loop, formed mainly by residues 43–55 at the surface of NrdH, are the major determinants of the substructure of this protein and at least in part define the thioredoxin-like activity profile.

NrdH is part of the NrdHIEF operon, where NrdEF codes for a class Ib ribonucleotide reductase (9). The NrdEF genes appear not to be expressed under standard growth conditions (44). However, recent results show that basal transcript levels of the NrdHIEF mRNA are strongly enhanced depending on growth medium and phase (12). Furthermore, addition of oxidants also results in strong up-regulation of NrdHIEF transcription suggesting functions under stress conditions. NrdH is a better hydrogen donor for the NrdEF ribonucleotide reductase than for the NrdAB enzyme. The class Ib RNR is efficient at low oxygen levels (9) and it has been suggested that this additional aerobic enzyme could function when the oxygen availability is too low to activate class Ia but high enough to inactivate class III (11). Under microaerophilic conditions most GSH is conjugated with spermidine (45) and not available for the cell. NrdH lacks a glutathione binding site and instead has the surface of NrdH, are the major determinants of the substructure of this protein and at least in part define the thioredoxin-like activity profile.

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