An Intestinal Parasitic Protist, *Entamoeba histolytica*, Possesses a Non-redundant Nitrogen Fixation-like System for Iron-Sulfur Cluster Assembly under Anaerobic Conditions*

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We have characterized the iron-sulfur (Fe-S) cluster formation in an anaerobic amitochondrial protozoan parasite, *Entamoeba histolytica*, in which Fe-S proteins play an important role in energy metabolism and electron transfer. A genomewide search showed that *E. histolytica* apparently possesses a simplified and non-redundant NIF (nitrogen fixation)-like system for the Fe-S cluster formation, composed of only a catalytic component, NiFS, and a scaffold component, NiFU. Amino acid alignment and phylogenetic analyses revealed that both amebic NiFS and NiFU (EhNiFS and EhNiFU, respectively) showed a close kinship to orthologs from e-proteobacteria, suggesting that both of these genes were likely transferred by lateral gene transfer from an ancestor of e-proteobacteria to *E. histolytica*. The EhNiFS protein expressed in *E. coli* was present as a homodimer, showing cysteine desulfurase activity with a very basic optimum pH compared with NiFS from other organisms. EhNiFU protein existed as a tetramer and contained one stable [2Fe-2S]$^+$ cluster per monomer, revealed by spectroscopic and iron analyses. Fractionation of the whole parasite lysate by anion exchange chromatography revealed three major cysteine desulfurase activities, one of which corresponded to the EhNiFS protein, verified by immunoblot analysis using the specific EhNiFS antibody; the other two peaks corresponded to methionine γ-lyase and cysteine synthase. Finally, ectopic expression of the EhNiFS and EhNiFU genes successfully complemented, under anaerobic but not aerobic conditions, the growth defect of an *E. coli* strain, in which Fe-S proteins are necessary and sufficient for Fe-S clusters of non-nitrogenase Fe-S proteins to form under anaerobic conditions. This is the first demonstration of the presence and biological significance of the NIF-like system in eukaryotes.

Iron-sulfur (Fe-S)$^+$ clusters are cofactors of proteins probably present in all living organisms. The Fe-S clusters play various important roles in electron transfer, redox regulation, nitrogen fixation, and sensing for regulatory processes (1). Despite the importance of Fe-S proteins, little is known about the biochemical mechanisms of Fe-S cluster assembly in vivo. Recent studies using genetic and biochemical methods have unveiled the complex mechanism of the assembly in vitro and in vivo (2–8). These studies led to the identification of three distinct systems, namely nitrogen fixation (NIF), iron-sulfur cluster (ISC), and mobilization of sulfur (SUF). Two to six components have been shown to participate in the formation of Fe-S clusters, depending upon the system. For instance, in the NIF system, two genes (niFS and niFU) and their encoded proteins have been shown to be involved in the assembly of Fe-S clusters of the nitrogenase proteins in *Azotobacter vinelandii* (2, 3). NiFS is a homodimer of two identical subunits of a pyridoxal-5'-phosphate (PLP)-dependent cysteine desulfurase, which catalyzes the formation of L-alanine and elemental sulfur from L-cysteine. The catalysis is initiated by the formation of a Schiff base between the amino group of cysteine and PLP cofactor (3) and nuclocephalic attack of the reactive cysteine by a conserved histidine residue near the active site (9), which in turn mobilizes elemental sulfur. NiFU is a scaffold protein for the transient assembly of Fe-S clusters, which are transferred to target apoproteins (10). The NiFU protein possesses two distinct types of iron-binding sites (10). One of these binding sites, located in the central third of the NiFU protein, binds a stable permanent [2Fe-2S]$^{2+}$ cluster per subunit as shown in *A. vinelandii* (11). The second type of site, located within the amino-terminal third of the NiFU protein, binds a labile mononuclear iron and/or labile cluster (11). Site-directed mutagenesis of the cluster-ligated cysteine residues of NiFU from *A. vinelandii* has shown that the permanent [2Fe-2S]$^{2+}$ clusters play an essential role in the maturation of nitrogenase (12). The NIF system has been identified in only nitrogen-fixing bacteria and non-diazotrophic e-proteobacteria including *Campylobacter jejuni* and *Helicobacter pylori*, and appears to be involved in Fe-S

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1 The abbreviations used are: Fe-S, iron-sulfur; NIF, nitrogen fixation; ISC, iron-sulfur cluster; SUF, mobilization of sulfur; PLP, pyridoxal-5'-phosphate; ORF, open reading frame; rEhNiFS, recombinant EhNiFS; DTT, dithiothreitol; GSH, glutathione S-transferase; CS, cysteine synthase; MGL, methionine γ-lyase.
assembly of the nitrogenase proteins in bacteria that belong to the former group and of non-nitrogenase Fe-S proteins in the latter (13). In contrast to the NIF system, the ISC system is well conserved from bacteria to a wide range of eukaryotes including Saccharomyces, Arabidopsis, Caenorhabditis, Drosophila, and Homo, and thus, assumed to play more general housekeeping roles for Fe-S cluster assembly. The components of the ISC system are more complex than those of the NIF system: at least six proteins, encoded in a single operon (isc-SUA-hscBA-fdx) in prokaryotes, e.g. Escherichia coli, are involved in the process (4, 6, 7, 14–16) and well conserved from bacteria to a wide range of eukaryotes (13). In contrast to the NIF system, the ISC system is comprised of at least six proteins, encoded in a single operon (isc-SUA-hscBA-fdx) in prokaryotes, e.g. Escherichia coli, and is involved in the process (4, 6, 7, 14–16) and well conserved from bacteria to a wide range of eukaryotes (13).

IscA is closely related to its NIF counterpart (NifIscA) and shares function as a scaffold for intermediate Fe-S clusters. IscA is closely related to its NIF counterpart (NifIscA) and shares function as a scaffold for intermediate Fe-S clusters. IscA is closely related to its NIF counterpart (NifIscA) and shares function as a scaffold for intermediate Fe-S clusters.

IscU is similar to the amino-terminal domain of NifU and shares function as a scaffold for intermediate Fe-S clusters. IscU is similar to the amino-terminal domain of NifU and shares function as a scaffold for intermediate Fe-S clusters.

IscU from E. histolytica has also been demonstrated (18, 20). HscA and HscB are chaperones that belong to the DnaK and DnaJ protein families, respectively; but their roles in Fe-S cluster biogenesis remain unclear (19). Protein-protein interaction between each of the components of the ISC system has also been demonstrated (18, 20). The SUF system, a third bacterial system for the assembly of Fe-S clusters, is encoded in the suf operon (sufABCDSE) and widely present in Eubacteria, Archaea, and plastids (8). Disruption of the E. coli suf operon alone did not cause any major defect, whereas lethal was observed when both the isc and suf operons were inactivated (8). It has also been shown that the SUF system plays a role for Fe-S cluster assembly and/or repair under oxidative stress conditions (21, 22) and iron starvation (23). In addition, the SUF system has been shown to be necessary for virulence of Gram-negative bacterium Erwinia chrysanthemi, causing soft-rot disease in plants (21, 24). In addition to the catalytic component SufS, the SUF system requires at least five additional proteins including SufA, a scaffold component, SufC, an unorthodox ATPase of the ABC superfamily, SufB and SufD, which are associated with SufC (24), and SufE, which interacts with SufS and stimulates its cysteine desulfurase activity (25).

Recent studies have demonstrated that a mitochondrial IscS homolog in yeasts (Nf1) is involved in Fe-S assembly of aconitase and succinate dehydrogenase and thus is essential for mitochondrial function (26). IscS homologs are produced in the cytosol and transported to mitochondria (27) and the nucleus in yeasts and mammals (28, 29). An independent study also revealed that mitochondria play a crucial role in the cluster formation of extramitochondrial Fe-S proteins (30). Thus, the assembly of Fe-S clusters is more complex in eukaryotes than prokaryotes and apparently occurs in mitochondria, cytoplasm, and nucleus, suggesting organelle-specific Fe-S cluster assembly in eukaryotes (31, 32). Thus, the presence or absence, i.e. ubiquity and specificity, of these three distinct systems for Fe-S assembly among organisms, together with their specific function in each organism, remains largely unknown, especially in parasitic protozoa.

IscS has been demonstrated in an aerobic protozoan parasite, Plasmodium falciparum (33), and two anaerobic protozoa, Giardia lamblia and Trichomonas vaginalis (34). The latter two parasites belong, together with another enteric parasitic protist Entamoeba histolytica, to a group of amitochondrial eukaryotes. Ami tochondrial eukaryotes can be divided into two metabolic types (35). Type I organisms including G. lamblia and Entamoeba lack organelles involved in core energy metabolism. Instead, Fe-S protein (i.e. pyruvate:ferredoxin oxidoreductase)-mediated metabolism of pyruvate, substrate-level phosphorylation, and ATP synthesis takes place in the cytosol. In contrast, type II organisms including Trichomonas, some
ciliates, and chytrid fungi harbor a double-membrane limited organelle, hydrogenosome, which represents a site of the above-mentioned core energy metabolism. The fact that the scaffold component IscU is also present in Trichomonas and Giardia (36) supported the premise that the machinery for Fe-S cluster assembly in these amitochondrial anaerobic protists shares common features with the ISC system in other organisms. In contrast, the machinery for the Fe-S cluster assembly in E. histolytica is largely unknown. The present study demonstrates that E. histolytica possesses the NIF-like system as a sole pathway for the biosynthesis of Fe-S clusters. We describe here for the first time the molecular identification of Nifs and NifU from E. histolytica and provide evidence for the possible horizontal transfer of these genes from an ancestor of e-proteobacteria. We also show biochemical properties distinct from those of other organisms including bacteria and mammals. In addition, we demonstrate that the amebic Nifs and NifU are necessary and sufficient for the Fe-S cluster formation under anaerobic conditions by heterologous complementation of an nisuf-lacking mutant of E. coli.

**Experimental Procedures**

**Chemicals and Reagents—**All chemicals of analytical grade were purchased from Wako (Tokyo, Japan) unless stated otherwise. l-cysteine, O-acetyl cysteine, N-acetyl cysteine, N-homocysteine, n-cysteine, sodium sulfide, O-phenanthrolin, hydroxylamine, N,N-di methyl-p-phenylenediamine sulfate, ferrous ammonium sulfate, PLP, 2-(N-morpholino) ethanesulfonic acid, HEPES, N-[tris(hydroxymethyl) methyl]-3-amino propanesulfonic acid, 3-(cy clohexylamino) 1-propanesulfonic acid, ampicillin, and carbencillin disodium salt were purchased from Sigma-Aldrich.

**Microorganisms and Cultivation—**Trophozoites of E. histolytica strain HM-1:IMSS cl-6 (37) were maintained axenically in Diamond's BS-33 medium (38) at 35.5 °C. Trophozoites were harvested in the late-logarithmic growth phase 2–3 days after inoculation of 1/12 to 1/6 of the total culture volume and washed twice with ice-cold phosphate-buffered saline, pH 7.4, at 4 °C. E. coli strains BL21 (DE3) and DH5α were purchased from Novagen (Madison, WI) and Invitrogen, respectively.

**Search of the E. histolytica Genome Database—**The E. histolytica genome databases (contigs and singletomes) at The Institute for Genomic Research2 and Sanger Institute3 were searched using the TBLASTN algorithm with protein sequences corresponding to the catalytic component of Fe-S cluster formation (Nifs, IscS, and SufS) from a variety of species. We also searched for homologs of the Nif- or Isc-specific scaffold component (NifU or IscU, respectively) from A. vinelandii and H. pylori and components shared by both the Isc and Suf systems (IscA and SufA) of E. coli, A. vinelandii, and P. falciparum, or components unique to the Suf system (SufS, C. D. and E) from E. coli, Bacillus subtilis, Methano- coccus jannaschii, Mycobacterium tuberculosis, and P. falciparum.

**Amino Acid Alignments and Phylogenetic Analysis—**The sequences of Nifs, IscS, NifU, IscU, and related proteins showing similarity in amino acid sequence to EhNifs and EhNifU were obtained from the National Center for Biotechnology Information4 using the BLASTP search. Alignment and phylogenetic analysis were performed with CLUSTAL W version 1.81 (39) using the neighbor-joining method with the Blossom matrix. A rooted or an unrooted neighbor-joining tree composed of the amino acid sequences of 25 Nifs/IscS or 20 NifU/IscU from various organisms was constructed using 352 or 120 shared amino acid positions, respectively, after removing gaps. The most distal members of Nifs/IscS that belong to group II including E. coli cysteine sulfinate desulfurase (CsdA) and SufS (also known as CsdB or selenocysteine lyase) were used as the out-group to obtain a rooted tree. Trees were drawn by Tree ViewPPC version 1.6.6 (40). Branch lengths and bootstrap values (1000 replicates) were derived from the neighbor-joining analysis.

**Cloning of E. histolytica Nifs and NifU—**On the basis of the nucleotide sequences of the protein-encoding region of the putative amebic nifs and nifU genes (EhNifs and EhNifU) in the genome database, two sets of primers, shown below, were designed to amplify the open reading frames (ORFs) using a cDNA library (41) as a template to construct plasmids to produce EhNifs and EhNifU fusion proteins with a histi
dine tag or glutathione S-transferase at the amino terminus, respectively. The EhNif/S ORF was amplified with (5'-GATGCAAATTGACAAATAACTAGT-3') and an antisense (5'-CCAGGATC- CTTAGAAGCATGTGTGGAAGAATGTTG-3') primer, where BamHI sites are underlined and the translation initiation and termination codons are italicized. The initial step, denaturation at 94 °C for 2 min with platinum px DNA polymerase (Invitrogen), was followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and elongation at 68 °C for 2 min, and a final extension for 10 min at 68 °C.

The ~1.2-kb PCR fragment was digested with BamHI, electrophoresed, purified with GeneClean kit (BIO 101, Vista, CA), and cloned into BamHI-digested PET-15b (Novagen) in the same orientation as the T7 promoter. The Eh/NifU ORF was amplified using a sense (5'-ATGTCACAAATGTTGAGGTCGC-3') and antisense (5'-CTTCTTTTTGATATTTAAGGT-3') primer from a cDNA library (the translation initiation and termination sites are italicized). A PCR fragment containing Eh/NifU was end-blunted and cloned into the EcoRI-digested and end-filled site of pGEX2T (Amersham Biosciences). The final constructs were designated pHisEhNifS and pGSTEhNifU, respectively.

Expression and Purification of Recombinant EhNifS and EhNifU Proteins—To express the recombinant proteins in E. coli, pHisEhNifS or pGSTEhNifU was introduced into BL21 (DE3) or DH5α cells, respectively. Expression of the recombinant EhNifS (rEhNifS) and EhNifU (rEhNifU) fusion proteins was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 5–6 h. The rEhNifS and rEhNifU fusion proteins were purified using a Ni2+-NTA column (Novagen) or glutathione-Sepharose 4B column (Amersham Biosciences), respectively, according to the manufacturer's instructions with a few modifications. The bacterial cells were washed, sonicated in the lysis buffer (50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 5 mM 2-mercaptoethanol, and 10 mM imidazole) containing 0.1% Triton X-100 (v/v), 100 μg/ml of lysozyme, and Complete Mini EDTA free protease inhibitor mixture (Roche, Tokyo, Japan), and centrifuged at 24,000 × g for 15 min at 4 °C. The histidine-tagged rEhNifS protein was eluted from the Ni2+-NTA column with 100 mM imidazole in 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 0.1% Triton X-100 (v/v) and extensively dialyzed in 50 mM NaCl (pH 7.4), 2 mM EDTA, 150 mM NaCl, and 0.1% Triton X-100 (v/v) containing 10% glycerol (v/v) and the protease inhibitors. To obtain the rEhNifU, bacterial cells were lysed in 100 mM sodium phosphate buffer (pH 7.4), 2 mM DTT, 0.1% Triton X-100 (v/v), 1 mM phenylmethylsulfonyl fluoride, and 100 μg/ml of lysozyme. After the GST-EhNifU fusion protein was bound to the glutathione-Sepharose 4B column, the rEhNifU was obtained by digestion of GST-EhNifU fusion proteins with thrombin (Amersham Biosciences) in the column or on the column, the rEhNifU was obtained by digestion of GST-EhNifU fusion protein was bound to the glutathione-Sepharose 4B column (Amersham Biosciences), respectively, according to the manufacturer's instructions with a few modifications. The bacterial cells were washed, sonicated in the lysis buffer (50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 5 mM 2-mercaptoethanol, and 10 mM imidazole) containing 0.1% Triton X-100 (v/v), 100 μg/ml of lysozyme, and Complete Mini EDTA free protease inhibitor mixture (Roche, Tokyo, Japan), and centrifuged at 24,000 × g for 15 min at 4 °C. The histidine-tagged rEhNifS protein was eluted from the Ni2+-NTA column with 100 mM imidazole in 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 0.1% Triton X-100 (v/v) and extensively dialyzed in 50 mM NaCl (pH 7.4), 2 mM EDTA, 150 mM NaCl, and 0.1% Triton X-100 (v/v) containing 10% glycerol (v/v) and the protease inhibitors. To obtain the rEhNifU, bacterial cells were lysed in 100 mM sodium phosphate buffer (pH 7.4), 2 mM DTT, 0.1% Triton X-100 (v/v), 1 mM phenylmethylsulfonyl fluoride, and 100 μg/ml of lysozyme. After the GST-EhNifU fusion protein was bound to the glutathione-Sepharose 4B column, the rEhNifU was obtained by digestion of GST-EhNifU fusion proteins with thrombin (Amersham Biosciences) in the column or on the column, the rEhNifU was obtained by digestion of GST-EhNifU fusion proteins with thrombin (Amersham Biosciences) in the column or on the column.

Iron Assay—The iron content of EhNifU was determined by the O-phenanthroline method as described, except that the volume of the reaction mixture was reduced (33). The EhNifU samples were acidified with 6 M HCl and diluted with buffer or distilled water to 0.2 ml. The mixtures were heated to 80 °C for 10 min and cooled down to room temperature; then 0.6 ml of water, 40 μl of 10% hydroxylamine hydrochloride, and 0.2 ml of 0.1% O-phenanthroline were added. The mixtures were further incubated at room temperature for 30 min, and then absorbance at 512 nm (A512) was measured with 0–100 μl of ferrous ammonium sulfate as the standard.

Anion-Exchange Chromatography of the Native and Recombinant EhNifS and EhNifU—Approximately 2 × 107 E. histolytica trophozoites (250–300 mg) was resuspended in 1.0 ml of 100 mM Tris-HCl (pH 8.0), 1.0 mM EDTA, 2.0 mM DTT, and 15% glycerol containing 10 μg/ml of transferrin and 0.3% (v/v) phenylmethylsulfonyl fluoride. The mixture was incubated for 24 h. However, it was reduced to 25–30% when stored without any additive at room temperature, 4, or 20 °C for 24 h.

Size Exclusion Chromatography of the Native and Recombinant EhNifS and EhNifU—Approximately 500 μg of recombinant proteins or the fractions that were eluted from the anion-exchange chromatography were dialyzed against gel filtration buffer (100 mM Tris-HCl (pH 8.0), 0.1 M NaCl) at 4 °C and concentrated to 1 ml with the Centricon YM-10. A Sephacryl S300 HR Hiptreamed column (7 cm long and 1.6-mm in diameter) (Amersham Biosciences) was preequilibrated, loaded, and eluted with the gel filtration buffer of a flow rate of 0.5–0.6 ml/min. The same column was calibrated with ferritin (200 kDa), albumin (67 kDa), ovalbumin (45 kDa), and chymotrypsinogen A (25 kDa) (Amersham Biosciences).

Immunoblot Analysis—Polyclonal antisera against recombinant EhNifS and EhNifU were raised in rabbits by Kitayama-Rabes (Nagano, Japan). Immunoblot analysis was carried out as described previously (46). Primary antibodies were used at either 1:500 (for anti-EhCS1 antisera) (41) or 1:1000 (for anti-methionine γ-lyase 2 (EhMGL2)) (46) and anti-EhNifS antisera. The blots were visualized using alkaline phosphatase-conjugated anti-rabbit IgG antibody (ICN-Cappel, Cappel, OH) (1:2000) with nitro blue tetrazolium/5-bromo-4-chloro-3-indolylo phosphate solution (Roche) according to the manufacturer’s instructions.

Heterologous Expression of the Amebic NiFs and NiF Genes in the iscA/mutant E. coli Strain—A plasmid to coexpress EhNifS and EhNifU in E. coli was constructed. The EhNifS and EhNifU ORF were PCR-amplified using a sense (5’-CTTCTCTAGAGATGATCAATGAAATACAAAATGCTATTGTCCTAG-3’) and an antisense (5’-GGTCATGTTTAAACACTCAGCATATGATATAAGTGCCTAGAGATGATAGAAATACAAAATGCTATTGTCCTAG-3’) primer (EhNifU), where XbaI, PciI, Nol, and NheI sites are underlined, the translation initiation and termination codons are italicized, and the ribosome binding sequence is shown in lowercase. Because of the engind the translation initiation and termination codons are italicized, and the ribosome binding sequence is shown in lowercase. Because of the engind the translation initiation and termination codons are italicized, and the ribosome binding sequence is shown in lowercase. Because of the engind the translation initiation and termination codons are italicized, and the ribosome binding sequence is shown in lowercase. Because of the engind the translation initiation and termination codons are italicized, and the ribosome binding sequence is shown in lowercase. Because of the engind the translation initiation and termination codons are italicized, and the ribosome binding sequence is shown in lowercase. Because of the engind the translation initiation and termination codons are italicized, and the ribosome binding sequence is shown in lowercase. Because of the engind the translation initiation and termination codons are italicized, and the ribosome binding sequence is shown in lowercase. Because of the engind the translation initiation and termination codons are italicized, and the ribosome binding sequence is shown in lowercase. Because of the engind the translation initiation and termination codons are italicized, and the ribosome binding sequence is shown in lowercase. Because of the engind the translation initiation and termination codons are italicized, and the ribosome binding sequence is shown in lowercase. Because of the engind the translation initiation and termination codons are italicized, and the ribosome binding sequence is shown in lowercase. Because of the engind the translation initiation and termination codons are italicized, and the ribosome binding sequence is shown in lowercase. Because of the engind the translation initiation and termination codons are italicized, and the ribosome binding sequence is shown in lowercase. Because of the eng
FIG. 1. Multiple alignment of deduced amino acid sequences of NifS/IscS from *E. histolytica* and other organisms. Protein sequences were aligned using the CLUSTAL W program (www.ebi.ac.uk/clustalw/). Sequences are as follows: *Escherichia coli* IscS (accession number A65030); *Azotobacter vinelandii* IscS and NifS (AAC24472, P05341); human IscS (AAD01587); Saccharomyces cerevisiae IscS (NS1p, tRNA splicing protein SPL1 M98808); *Trichomonas vaginalis* IscS (TviscS-2, AAK69174); Giardia lamblia IscS (AAK39427); *Plasmodium yoelii* IscS (EAA21518); Campylobacter jejuni NifS (CAB72709); Helicobacter pylori NifS (D64547); *E. histolytica* NifS (AB112427); and *Anabaena sp.* PCC 7120 NifS (P12623). Conserved residues involved in PLP binding are highlighted with a gray background. Invariable cysteine and other residues involved in substrate binding are boxed with thin lines. A carboxyl-terminal extension consisting of 20–21 amino acids including the consensus sequence SPL(W/Y)(E/D)(M/L)X(K/Q)XG(I/V)D(L/I)XX((/V)XWXXX found in proteobacterial and eukaryotic IscS, and a similar extension found in nitrogen-fixing bacteria is also boxed with thin lines. A circle on the alignment (also boxed) indicates the conserved histidine involved in the substrate deprotonation. Asterisks indicate identical amino acids; colons and dots indicate conserved amino acid substitutions; and dashes indicate computer-generated gaps.
contains the E. coli iscUA-hscBA or sufABCDSE operon in pRKNMC, respectively (6, 8), pRKEhNifSU, or pRKNMC was introduced into E. coli strain UT109 [iscUA-hscBA::Kmr, sufABCDSEU::Gmr] harboring pKO3-SUF, a complementation plasmid carrying E. coli sufAB-CDSE and the temperature-sensitive replication origin. The transfor-
mants were plated and grown at 30°C (permissive temperature) and

![Figure 2](http://www.jbc.org/)

**Fig. 2.** Multiple alignment of deduced amino acid sequences of NifU/IscU/Nfu from a variety of organisms including *E. histolytica*. Protein sequences were aligned using the CLUSTAL W program. Sequences are as follows: *S. cerevisiae* Isu2p (NP_014869); *E. coli* IscU (AP002562.1); *G. lamblia* IscU (EAA38480); *A. vinelandii* IscU and NifU (AAC24473, AAA22167); human IscU1 (AAG37427); *P. yoelii* IscU (EAA18972); *Cyanothece sp.* PCC 8801 NifU (AAC33371.1); *H. pylori* NifU (NP_222928.1); *C. jejuni* NifU (NP_281434.1); and *E. histolytica* NifU (AAK85709). Conserved cysteine and aspartate residues are boxed with a gray background. Unique insertions shared by NifU from *E. histolytica* and *E. coli* proteobacteria are boxed. Asterisks indicate identical amino acids; colons and dots indicate conserved amino acid substitutions; and dashes indicate computer-generated gaps.
RESULTS

Identification and Features of EhNiF/S and EhNiF/U Genes and Their Encoded Proteins—We identified putative NiF/S/IscS/SufS and NiF/U/IscU gene homologs in the E. histolytica genome database as described in "Experimental Procedures." Putative EhNiF/S and EhNiF/U genes contained an ORF of 1173 and 1047 bp encoding a protein of 390 and 348 amino acids with predicted molecular masses of 42.8 and 38.9 kDa and a pI of 5.9 and 5.6, respectively. Neither the PSORT II program, which predicts protein localization in cells\(^5\) nor a Kite-Doolittle hydropathy plot suggested any possible cellular localization other than a cytosolic distribution for both EhNiF/S and EhNiF/U. A protein alignment of 21 NifU/IscU/Nfu1p homologs from Archaea, bacteria, fungi, protists, plants, and metazoa revealed: (i) His\(^{106}\) (numbered according to EhNiF/S), which is involved in the initial deprotonation of the substrate; (ii) the PLP-binding site with the Schiff base-forming amino acids Lys\(^{208}\), Asp\(^{182}\), and Gln\(^{185}\) that bind the pyridine nitrogen and the phenolate oxygen of PLP, respectively; (iii) Thr\(^{76}\), His\(^{207}\), SerThr\(^{205}\), and Thr\(^{243}\) involved in the formation of an additional six hydrogen bonds anchoring the phosphate group of PLP (2, 9); and (iv) the substrate-binding site including Cys\(^{330}\), which provides a reactive cysteinyl residue (3), as well as Arg\(^{356}\), Asn\(^{157}\), and Asn\(^{35}\), which anchor the cysteine with a salt bridge and hydrogen bond (9). A carboxyl-terminal extension consisting of 20–21 amino acids including the consensus sequence SPL(W/Y)(E/D)(M/L) was previously proposed to differentiate proteobacterial and eukaryotic IscS from homologs of other organisms (34) but also is present in nitrogen-fixing bacteria and cyanobacteria (Fig. 1). However, this extension is absent in the NiF/S homologs from E. histolytica and \(\varepsilon\)-proteobacteria.

A protein alignment of 21 NiF/U/IscU/IscS homologs from Archaea, bacteria, fungi, protists, plants, and metazoa revealed (an alignment of only representative members is shown in Fig. 1) that EhNiF/S showed greatest homology (52–55% identity) to NiSs from \(\varepsilon\)-proteobacteria Campylobacter jejuni and Helicobacter pylori; 37–42% identity to NiF/U from nitrogen-fixing Euarchaea including A. vinelandii, Klebsiella pneumoniae, and cyanobacteria; and 32–33% identity to IscSs from nitrogen-fixing and non-fixing Euarchaea, fungi, and other protists including G. lamblia, T. vaginalis, P. yoelii, and Cryptosporidium parvum, and metazoa. EhNiF/U also showed limited homology (21–24% identity) to other (group II) NiF/S/IscS/SufS homologs including CsdA (cysteine sulfinate desulfsinate) and SufS (CsdB or selenocysteine lyase) from E. coli, a hypothetical protein from Synechocystis, and a chloroplast homolog from Arabidopsis thaliana (data not shown; see also “phylogenetic analyses” and Fig. 3A). All of the residues of the active site and amino acids implicated in the cysteine desulfurase activity were conserved: (i) His\(^{106}\) (numbered according to EhNiF/S), which is involved in the initial deprotonation of the substrate; (ii) the PLP-binding site with the Schiff base-forming amino acids Lys\(^{208}\), Asp\(^{182}\), and Gln\(^{185}\) that bind the pyridine nitrogen and the phenolate oxygen of PLP, respectively; (iii) Thr\(^{76}\), His\(^{207}\), SerThr\(^{205}\), and Thr\(^{243}\) involved in the formation of an additional six hydrogen bonds anchoring the phosphate group of PLP (2, 9); and (iv) the substrate-binding site including Cys\(^{330}\), which provides a reactive cysteinyl residue (3), as well as Arg\(^{356}\), Asn\(^{157}\), and Asn\(^{35}\), which anchor the cysteine with a salt bridge and hydrogen bond (9). A carboxyl-terminal extension consisting of 20–21 amino acids including the consensus sequence SPL(W/Y)(E/D)(M/L) was previously proposed to differentiate proteobacterial and eukaryotic IscS from homologs of other organisms (34) but also is present in nitrogen-fixing bacteria and cyanobacteria (Fig. 1). However, this extension is absent in the NiF/S homologs from E. histolytica and \(\varepsilon\)-proteobacteria.
Iron-Sulfur Cluster Formation in E. histolytica

Carboxyl-terminal half of EhNifU (178–348) showed limited homology (12–14% identity) to Nfu1p homologs from S. cerevisiae and A. thaliana. Nine of 12 cysteine residues of EhNifU (Cys54, Cys81, and Cys131) in the amino terminus, which constitute a binding site for iron and transient Fe-S cluster (5) and are essential for the function of IscU, Isu1p, and NifU (12, 47). In contrast, Cys56 was conserved only in EhNifU and among NifU proteins from \(-\)proteobacteria. The central portion of EhNifU contains four conserved cysteine residues, Cys103, Cys165, Cys196, and Cys206, which were shown to be responsible for permanent \([2Fe-2S]\)^2 cluster binding as described in a mutational study on A. vinelandii NifU (12). Two additional cysteines (Cys293 and Cys294) at the carboxyl-terminal region that are present in mammalian Nfu and have been shown to function as a scaffold protein for one transient \([4Fe-4S]\)^2 cluster per two Nfu monomers (29) are also conserved in EhNifU. Asp56 was conserved in all species and was implicated in the release of the NifU-bound transient Fe-S cluster (10).

Phylogenetic Analysis of EhNifS and EhNifU---NifS/IscS/SufS homologs were shown previously to form two distinct groups, I and II (48). Distal members of the homologs that belong to group II (SufS and CsdA) were used as the outgroup to examine the phylogenetic relationship of EhNifS. IscS from a wide range of non-diazotrophic Eubacteria other than \(-\)proteobacteria, fungi, metazoa, and parasitic protists (G. lamblia, T. vaginalis, P. yoelii, and C. parvum) and NifS from nitrogen-fixing Eubacteria formed independent clades that were well supported by high bootstrap values (99.8–100%) (Fig. 3A). The amebic NifS formed a separate clade (also well supported by a 100% bootstrap value) with \(-\)proteobacteria H. pylori and C. jejuni. This, together with high amino acid identities among this group, reinforces a close kinship of EhNifS with homologs from \(-\)proteobacteria.

The phylogenetic relationship of EhNifU with 20 other NifU/IscU homologs from Archaea, bacteria, fungi, protists, plants, and metazoa was presented as a radial unrooted tree as an appropriate outgroup was not available (Fig. 3B). IscU from nitrogen-fixing and non-fixing bacteria, fungi, protists except for E. histolytica, plants, and metazoa and NifU from nitrogen-fixing bacteria, cyanobacteria, \(-\)proteobacteria, and E. histolytica formed two independent clades, well supported by high bootstrap values (92–96%). EhNifU showed strong affinity for homologs from \(-\)proteobacteria as shown in the case of EhNifS and represents an independent group well separated from the group of NifU from nitrogen-fixing bacteria. This, together with the high amino acid identities and common insertions, clearly supported a close association of E. histolytica NifU with NifU from \(-\)proteobacteria.

Expression and Purification of rEhNifS and rEhNifU Proteins—The rEhNifS protein was purified at high yield, ~100 mg/l of E. coli culture. Cells, cell extracts, and purified EhNifS were yellowish in color. The rEhNifS protein revealed an apparently homogeneous band of 45 kDa on SDS-PAGE (Fig. 4A), which was consistent with the predicted size of the deduced EhNifS protein with the extra 25 amino acids added at the amino terminus. The purified rEhNifS protein was evaluated ~98% pure by densitometric measurement of the Coomassie-stained SDS-PAGE gel. The cell pellet of the E. coli that overexpressed the GST-EhNifU fusion protein was brown in color. The purified recombinant GST-EhNifU protein also showed a slight brownish color, characteristic of Fe-S proteins. The recombinant GST-EhNifU protein showed an apparently homogeneous band of 65 kDa on SDS-PAGE run under reducing or non-reducing conditions (Fig. 4B, data for under the non-reduc-

![Fig. 4. Expression and purification of recombinant EhNifS and EhNifU proteins. A, EhNifS was expressed as a protein containing the amino-terminal histidine tag and purified with the Ni2+-NTA column as described in “Experimental Procedures.” The total cell lysate and samples at each purification step were electrophoresed on 12% SDS-PAGE gel and stained with Coomassie Brilliant Blue. Lane 1, protein marker; lane 2, control (an E. coli transformant with pET-15b control vector); lane 3, total lysate of cells expressing EhNifS; lane 4, supernatant of lane 3 after 24,000 × g centrifugation; lane 5, unbound fraction of the Ni2+-NTA column of lane 4; lane 6, eluted from the Ni2+-NTA column with 100 mM imidazole; lane 7, elute from the gel filtration column. The right panel shows a Western blot for EhNifS: lanes 8 and 9, 10 ng of rEhNifS protein and 10 μg of soluble E. histolytica lysate protein, respectively. B, EhNifU was expressed as a GST-fusion protein and purified with a glutathione-Sepharose 4B column. Lane 1, protein marker; lane 2, total cell lysate of an E. coli transformant expressing EhNifU; lane 3, supernatant of lane 2 after 24,000 × g centrifugation; lane 4, unbound fraction of the glutathione-Sepharose 4B column; lane 5, fraction eluted with 10 mM glutathione from the glutathione-Sepharose 4B column; lane 6, thrombin-digest of lane 5; lane 7, purified rEhNifU after the removal of the GST portion; lane 8, GST-EhNifU purified by gel filtration chromatography. The right panel shows a Western blot for EhNifU: lanes 9 and 10, 10 ng of rEhNifU protein and 10 μg of soluble E. histolytica lysate protein, respectively.](http://www.jbc.org/fig4)
ical of a PLP-binding protein, a characteristic peak at 370–380 nm (Fig. 5A). Incubation with 0.5 mM l-cysteine shifted the peak to 348 nm, with a broad shoulder at 410 nm (Fig. 5A), similar to NifS from *H. pylori* (13). In contrast, *A. vinelandii* NifS showed major and minor peaks at 416 and 370 nm, respectively, in the presence of L-cysteine (2). The spectral change was observed previously for NifS-like protein from *T. maritima* and proposed to be attributable to deprotonation/protonation of aldimine, leading to the formation of either germinal diamine or enolimine species (49), which occurs in conjunction with persulfide formation (9). The whole reaction required 10–15 min to complete. At 30 min after the addition of cysteine, no further spectral change was observed. The optimum pH for EhNiFS was 10–10.5; the half-maximum activity was reached at about pH 9.0 (Fig. 5B). The rEhNiFS protein showed cysteine desulfurase activity, as measured in sulfide production, of ~9.5 nmol/min/mg pure protein with 0.5 mM cysteine, which is comparable to NiFS from *H. pylori* (9.9 nmol/min/mg with 50 mM cysteine) (13) and lower than other bacterial homologs (*A. vinelandii* NiFS, 89.4 nmol/min/mg with 0.5 mM cysteine (2); *A. vinelandii* IscS, 67.6 nmol/min/mg with 2.5 mM cysteine (4); *E. coli* IscS, 78 nmol/min/mg with 2.5 mM cysteine (16); *E. coli* CSD, 3.4 μmol/min/mg (*V*<sub>max</sub>) (48); and *E. coli* SufS, 0.019 or 0.9 μmol/min/mg (*V*<sub>max</sub>) in the absence or presence of SufE, respectively (25)). Among a variety of possible substrates, EhNiFS was shown to be specific for L-cysteine and L-cystine. No
sulfide production was observed when D-cysteine, N-acetylcysteine, DL-homocysteine, DL-selenocysteine, cysteine sulfinic acid, or L-cysteic acid was used at up to 5 mM. rEhNifS showed 40–50% cysteine desulfurase activity toward L-cystine (4.0 nmol sulfide/min/mg) in the presence of DTT. Both alanine and cyanolysis assays also showed the comparable cysteine desulfurase activity of rEhNifS (9.1 nmol alanine/min/mg and 8.0 nmol sulfur/min/mg, respectively) with 0.5 mM cysteine, whereas rEhNifS showed the specific activity of 3.0 nmol alanine/min/mg against L-cystine by alanine assay.

Biochemical Characterization of Recombinant EhNifU—Spectrophotometric analysis of the purified recombinant EhNifU revealed peaks at 330, 420, and 460 nm and a shoulder at 550 nm, which indicates the presence of a [2Fe-2S]_{2}^{2+} cluster (Fig. 6), as described for NifU of A. vinelandii (11). Extinction coefficients of EhNifU per 38-kDa subunit were also consistent with the premise that EhNifU contains a [2Fe-2S]^{2+} center: the peaks at 330 nm (extinction coefficient, 11700 M^{-1} cm^{-1}), 420 nm (6800 M^{-1} cm^{-1}), and 460 nm (7000 M^{-1} cm^{-1}), a shoulder at ~550 nm (4700 M^{-1} cm^{-1}), and a ratio between 280 and 335 nm (A_{335}/A_{280}) of 0.27. The dithionite-reduced rEhNifU showed, in contrast, a relatively featureless spectrum, which increased in intensity at shorter wavelengths because of dithionite. Effects of Fe chelators on the cluster structure were assessed by treating oxidized and reduced rEhNifU with a 40-fold excess of 2,2'-dipyridyl or EDTA. No change of A_{520} was observed over 2 h (results not shown), indicating that the Fe-S cluster is either stable or inaccessible to solvent, which is also similar to A. vinelandii NifU (11). The iron analysis indicates that EhNifU contains two irons per subunit (2.1 ± 0.084 Fe/subunit from three independent EhNifU preparations).

Determination of Multimeric Structure of EhNifS and EhNifU—The cysteine desulfurase activity of rEhNifS was eluted at the predicted molecular mass of 85–95 kDa (Fig. 7) by gel
filtration chromatography. This is consistent with the notion that rEhNifS exists as a dimer of two identical subunits (42.5 kDa plus 2.6 kDa corresponding to the histidine tag), which is similar to all other organisms reported earlier. EhNiFU was eluted at 150–170 kDa; GST-EhNiFU was also eluted at 260–280 kDa (Fig. 7). These results show clearly that EhNiFU is a tetramer irrespective of the presence or absence of a fusion partner. Elution patterns of EhNiFU remained unchanged when EhNiFU was pretreated with 2 mM DTT and fractionated in the presence of DTT (results not shown).

**Anion-Exchange Chromatographic Separation of Native and Recombinant Cysteine Desulfurase Activity**—To correlate native cysteine desulfurase activity in the *E. histolytica* lysate with the recombinant enzyme, the lysate from the trophozoites and rEhNiFS were subjected to chromatographic separation on a Mono Q anion exchange column and analyzed by cysteine desulfurase assay and also immunoblotting using anti-EhCS, EhMGL, and EhNiFS antibodies. *E. histolytica* trophozoites possess two isoforms of CS (CS1 and CS2) (41) and two isoforms of MGL (MGL1 and MGL2) (46), both of which showed substantial cysteine desulfurase activity.6 Thus, we attempted to separate native NiFS from the CS and MGL isoforms. The *E. histolytica* lysate showed three major peaks of cysteine desulfurase activity (Fig. 8A). Fractions corresponding to the last and largest cysteine desulfurase peak (fractions 20–27) contained the protein that reacted well with the anti-EhNiFS and anti-CS antibodies (Fig. 8B). In contrast, fractions corresponding to the first and second cysteine desulfurase peaks did not react with anti-EhNiFS antibody but reacted with anti-MGL and anti-CS antibodies, respectively. rEhNiFS was fractionated under the same conditions and showed a single peak eluted at a slightly lower ionic strength (0.5 ml earlier elution volume) than the native EhNiFS (Fig. 8C), which is consistent with the fact that recombinant histidine-tagged EhNiFS has a slightly higher pI (6.15) than the native protein (5.9). These results suggest that the third dominant cysteine desulfurase peak of the parasite lysate represents activity mainly attributable to native EhNiFS.

**Heterologous Expression of the Amoebic NiFS and NiFU in the isc/suf-Mutant E. coli Strain**—To assess the in vivo role of EhNiFS and EhNiFU, we attempted heterologous complementation of an *E. coli* mutant UT109 in which the chromosomal isc and suf operons were deleted. This strain was lethal unless we provided the suf or isc operon in a complementing plasmid carrying a temperature-sensitive replicative origin.7 At the restrictive temperature, introduction of the *E. coli* isc or suf operon in a complementing plasmid carrying a temperature-insensitive replicative origin complemented the growth defects of the UT109 strain under both the aerobic and anaerobic conditions (Fig. 9). In contrast, coexpression of EhNiFS and EhNiFU rescued the growth of UT109 only under the anaerobic, not aerobic, conditions at the restrictive temperature. Thus, the amoebic NiFS and NiFU are apparently necessary and sufficient for the Fe-S cluster formation under anaerobic conditions in this heterologous system. Although CS and MGL showed cysteine desulfurase activity in vitro, coexpression of CS1 or MGL2, together with EhNiFU, did not complement the growth defects of UT109 under either aerobic or anaerobic conditions (data not shown).

**DISCUSSION**

We have identified and characterized two necessary and sufficient components, NiFS and NiFU, of the NIF-like system for the assembly of Fe-S clusters in a human intestinal protozoan anaerobe. As far as we are aware, this is the first demonstration of the NIF-like system in eukaryotes. Despite a thorough search of the *E. histolytica* genome database, no other proteins that were shown to be involved in ISC/SUF systems of other organisms were found, suggesting that this parasite possesses the NIF-like system as a sole and non-redundant system for the biosynthesis of all Fe-S proteins. Because *E. histolytica*

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6 V. Ali and T. Nozaki, unpublished data.
7 U. Tokumoto and Y. Takahashi, manuscript in preparation.
does not possess nitrogenase and is incapable of nitrogen fixation, the presence of the NIF-like system and the lack of other systems in this organism reinforce the premise that the NIF system is not specific for the Fe-S cluster formation of nitrogenase but is involved in the Fe-S cluster assembly for nitrogenase and non-nitrogenase proteins, as proposed for the NIF-like system in E. coli (13). Our in vivo complementation of a temperature-dependent growth defect of the E. coli isclsf mutant strain by expression of a heterologous NIF-like system indicates that the NIF-like system plays an interchangeable role in the Fe-S cluster assembly of non-nitrogenase proteins with the ISC or SUF system under anaerobic conditions. Despite common catalytic and scaffold mechanisms shared by the NIF, ISC, and SUF systems, there are a number of differences among these systems (10); the ISC and SUF systems appear to be considerably more complex than the NIF system. For example, heat-shock-cognate (Hsc) proteins have been suggested to have chaperone-like functions in the ISC system for the formation of transient Fe-S clusters or insertion into various target proteins in bacteria and yeast (7, 26, 30, 50, 51). Our in vivo complementation study revealed that the NIF-like system does not require any additional component other than NifS and NifU for Fe-S cluster assembly under anaerobic conditions. However, it is also possible that as yet unidentified proteins that remain in the isclsf-mutant strain of E. coli function together with the exogenous NifS and NifU. It is also conceivable that the NIF-like system is shared by other anaerobic parasitic organisms. However, two other well-characterized anaerobic protozoan parasites G. lamblia and T. vaginalis appear to possess the ISC and lack the NIF system (32, 34). Thus, the presence of the NIF-like system is likely unique to E. histolytica, which might be attributable to a rare horizontal gene transfer from a NIF-like-containing prokaryotic organism (see below).

Several lines of evidence support close kinship between the amebic NifS and NifU and their homologs from e-proteobacteria. First, phylogenetic analyses indicate that E. histolytica and e-proteobacteria represent an independent clade well separated from other NifS and NifU homologs. Second, this close phylogenetic association is also supported by the common insertions and deletions of amino acids shared by NifS and NifU from these organisms. Third, the multimeric structure of the amebic and H. pylori NifU is identical (i.e. tetramer), whereas NifU from A. vinelandii and IscU from E. coli and yeast form a dimer. Fourth, UV/visible absorption spectra and specific activity of EhNifS were comparable to the H. pylori NifS but notably different from A. vinelandii. These data suggest that amebic nif-like genes were likely obtained from an ancestral organism currently represented by e-proteobacteria by lateral gene transfer, as suggested for other metabolic enzymes that are proposed to have been transferred from Archaea and/or bacteria by a similar mechanism (52–54).

Fractionation of the crude extract of E. histolytica by anion-exchange chromatography revealed that at least three groups of enzymes, CS, MGL, and NifS, contributed to cysteine desulfurase activity, i.e. activity to mobilize the sulfur or sulfide from L-cysteine, and thus possibly provide sulfur for Fe-S cluster synthesis. Although both CS and MGL showed cysteine desulfurase activity (see also “Results” and Ref. 16 for E. coli CS) and also in vitro activity to convert an apo form of recombinant amebic [4Fe-4S]2+ ferredoxin (53, 55) into a holo form, we argue that EhNifS is the sole protein that functions in Fe-S cluster biosynthesis in vivo. Expression of CS or MGL (i.e. EhCS1 or EhMGL2), when coexpressed with EhNifU, did not complement the Fe-S cluster formation of the isclsf-mutant strain of E. coli (data not shown). This reinforces the previous observation on E. coli cysteine synthase A, B, and γ-cystathionase (16) and indicates that the in vitro conversion assay of apo Fe-S protein does not reflect in vivo function.

We did not demonstrate in the present study the species of the temporal Fe-S cluster (i.e. [2Fe-2S]2+ or [4Fe-4S]2+) that formed on EhNifU in addition to the stable [2Fe-2S]2+ cluster shown in Fig. 6. However, it is conceivable, by analogy to NifU from A. vinelandii involved in the Fe-S cluster formation of nitrogenase, that amebic NifU functions as an intermediate site for the transient [2Fe-2S]2+ cluster assembly (12). The putative labile [2Fe-2S]2+, if present, was likely lost during purification under aerobic conditions. We also speculate that the transition of Fe-S clusters on EhNifU may occur under anaerobic conditions; one [4Fe-4S]2+ cluster may be formed from two [2Fe-2S]2+ clusters, as shown for Azotobacter IscU (5). Because the amebic NifU forms a tetramer, the mechanism of the [4Fe-4S]2+ cluster formation from [2Fe-2S]2+ on NifU may differ from Azotobacter NifU, which exists as a dimer. Because
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*E. histolytica* is not a nitrogen-fixing organism, the NIF-like system is not necessarily specific for nitrogenase proteins, but they are also involved in Fe-S cluster assembly for other Fe-S proteins, as shown previously for *H. pylori* (13). Although all characterized or putative Fe-S proteins in the genome database of *E. histolytica*, including ferredoxins and pyruvate:ferredoxin oxidoreductase, likely contain 2[4Fe-4S] 2 cluster in *E. coli* indicates that the amebic NIF-like system is involved in the biosynthesis of all forms of Fe-S clusters.

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An Intestinal Parasitic Protist, *Entamoeba histolytica*, Possesses a Non-redundant Nitrogen Fixation-like System for Iron-Sulfur Cluster Assembly under Anaerobic Conditions

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