Characterization of the NifU and NifS Fe−S Cluster Formation Proteins Essential for Viability in *Helicobacter pylori*†

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Received July 26, 2000; Revised Manuscript Received October 26, 2000

ABSTRACT: The Fe−S cluster formation proteins NifU and NifS are essential for viability in the ulcer causing human pathogen *Helicobacter pylori*. Obtaining viable *H. pylori* mutants upon mutagenesis of the genes encoding NifU and NifS was unsuccessful even by growing the potential transformants under many different conditions including low O_2 atmosphere and supplementation with both ferric and ferrous iron. When a second copy of *nifU* was introduced into the chromosome at a unrelated site, creating a mero-diploid strain for *nifU*, this second copy of the gene could be disrupted at high frequency. This indicates that the procedures used for transformation were capable of *nifU* mutagenesis, so that the failure to recover mutants is solely due to the requirement of *nifU* for *H. pylori* viability. *H. pylori* NifU and NifS were expressed in *Escherichia coli* and purified to near homogeneity, and the proteins were characterized. Purified NifU is a red protein that contains ∼1.5 atoms of iron per monomer. This iron was determined to be in the form of a redox-active $[2Fe\text{-}2S]^{2+,+}$ cluster by characteristic UV−visible, EPR, and MCD spectra. The primary structure of NifU also contains the three conserved cysteine residues which are involved in providing the scaffold for the assembly of a transient Fe−S cluster for insertion into apoprotein. Purified NifS has a yellow color and UV−visible spectra characteristic of a pyridoxal phosphate containing enzyme. NifS is a cysteine desulfurase, releasing sulfur or sulfide (depending on the reducing environment) from L-cysteine, in agreement with its proposed role as a sulfur donor to Fe−S clusters. The results here indicate that the NifU type of Fe−S cluster formation proteins is not specific for maturation of the nitrogenase proteins and, as *H. pylori* lacks other Fe−S cluster assembly proteins, that the *H. pylori* NifS and NifU are responsible for the assembly of many (non-nitrogenase) Fe−S clusters.

Iron−sulfur clusters are prosthetic groups within proteins that perform many important redox, catalytic, and regulatory roles (*1*, *2*). The steps in which Fe−S clusters are assembled are only now becoming elucidated. It has recently been shown that proper metallocluster formation requires the presence of "chaperone"-like proteins. Two of the most central proteins involved in Fe−S cluster formation are those of the Isc (*i*ron *s*ulfur *c*luster) proteins IscU and IscS and the closely related Nif (*ni*trogen *f*ixation) proteins NifS and NifU. The IscS/NifS proteins provide the sulfur via an L-cysteine desulfurase activity, while the IscU/NifU proteins provide a scaffold onto which the nascent Fe−S cluster is assembled (*3*). Generally, the Isc proteins are considered to serve the "housekeeping" needs of the cell, while the Nif proteins usually serve to alleviate the increased demands put on the cell for Fe−S clusters associated with nitrogen fixation. With the availability of whole genome sequences, it has become clear that most organisms have multiple Fe−S cluster formation proteins. The genome sequence of non-diazotrophic *Helicobacter pylori* predicts only two such proteins, NifU and NifS (*4*). *H. pylori* is a human pathogen which, like almost all organisms, has genes that encode for many proteins which require Fe−S clusters for their proper function (see Table 1). Iron metabolism in pathogenic bacteria is of keen interest as iron acquisition and its associated metabolism are often related to virulence in pathogenic organisms. Indeed, iron restriction by the host is a major strategy used to evade or restrict infection by microorganisms, in part due to the ubiquitous nature and essential functions of Fe−S cluster proteins in nearly all bacteria. Because of the importance of iron to *H. pylori* for basic metabolic cellular processes and as factors likely to influence virulence, it is important to understand the way in which iron is metabolized by *H. pylori*. Here we report the characteristics of the NifU and NifS proteins of *H. pylori* that place these as key proteins in Fe−S cluster metabolism. Indeed, we show they are absolutely required for viability of *H. pylori*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. H. pylori ATCC strain 43504 was the parent strain. *H. pylori* was grown on Brucella agar (Difco) supplemented with 10% defibrinated sheep blood. Kanamycin (15 μg/mL), chloramphenicol (20 μg/mL), $FeCl_2$ (14 μg/mL) and $Fe(NH_4)_2(SO_4)_2$ (5 μg/mL) were added to the media as noted. Cells were incubated in CampyPak Plus (Becton Dickinson) atmosphere in jars or in a CO_2/O_2 incubator with 5% CO_2, 95% relative humidity,

† This work was funded by the Georgia Research Alliance and the National Institutes of Health (Grant GM62524 to M.K.J.).

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10.1021/bi001744s CCC: $19.00
Published on Web 11/23/2000

Table 1: *H. pylori* Proteins Predicted To Contain Iron−Sulfur Clusters

TIGR no.	protein	type
HP 0799	aconitase	4Fe-4S
HP 0126	phosphoglucanate dehydratase	4Fe-4S
HP 0631	hydrogenase (small subunit)	2 (4Fe-4S) 1 (3Fe-3S)
HP 0191	fumarate reductase (iron−sulfur subunit)	1 (2Fe-2S) 1 (3Fe-3S) 1 (4Fe-4S)
HP 0221	NifU	2Fe-2S
HP 1109	pyruvate−ferredoxin oxidoreductase (subunit d)	2 (4Fe-4S)
HP 0588	2-oxoglutarate:acceptor oxidoreductase (subunit d)	2-(4Fe-4S)
HP 1508	ferredoxin	2Fe-2S
HP 0588	ferredoxin	2Fe-2S
HP 0227	ferredoxin	2Fe-2S
HP 1356	quinolinate synthetase	4Fe-4S
HP 1540	ubiquinol cytochrome *c* oxidoreductase	2Fe-2S
HP 1260−HP 1273, HP 0037	NADH−Q oxidoreductase (complex I)	up to 9 clusters (both 2 and 4 Fe)

37 °C, and O_2 concentrations as indicated. DNA manipulations were done in *Escherichia coli* strain DH5α (BRL), and overexpression was done in *E. coli* strain BL21 (DE3) RIL (Stratagene). LB and LB agar were used for growing *E. coli*, supplemented with ampicillin (100 μg/mL), kanamycin (30 μg/mL), or chloramphenicol (20 μg/mL) as noted.

Cloning of nifU and nifS. The genes encoding NifU and NifS were amplified by polymerase chain reaction (PCR) using genomic DNA from strain 43504 as template and PFU polymerase. The primers for *nifU* amplification are as follows: 5′ GGCATATGGCAAAACATGATTTAGTGG 3′ and 5′ CCGAATTCAAATCGGTAACACCCTGATACT 3′. These primers engineer a *Nde*I restriction site into the 5′ end and a *Eco*RI site into the 3′ end of the gene, to facilitate direct cloning into *Nde*I/*Eco*RI-digested pET21A (Novagen), yielding the plasmid pET-NifU vector used for expressing NifU in *E. coli*. *nifS* was PCR amplified using the following primers: 5′ GCGCATATGTTACAACGAATTTATTTAG 3′ and 5′ CGGGATCCATAAGAGCTTGAAATATTTCTC 3′. The PCR product was first cloned into the *Sma*I site of p(bluescript)KS+, yielding pKS-NifS. This step was done because the gene for NifS contains an internal *Nde*I site and a partial digestion had to be performed to obtain the full-length *nifS Nde*I/*Bam*HI fragment. This was then ligated to *Nde*I/*Bam*HI-digested pET21A, yielding pET-NifS.

Construction of the Mero-Diploid nifU Strain. Previously, our laboratory had determined that introduction of foreign DNA into the region of the *H. pylori* genome corresponding to ATCC strain 26695 HP 0405 (*4*) was not deleterious to *H. pylori* growth (data not shown). We used this fact to introduce a second copy of the *nifU* into this open reading frame. The plasmid pEU39 (purchased from The Institute for Genomic Research, Rockville, MD) contains a 2.04 kb fragment of *H. pylori* genomic DNA corresponding to bases 416433−417953 of the *H. pylori* 26695 genome, including all of HP 0405, cloned into the *Sma*I site of pUC18. The chloramphenicol resistance cassette was then cloned into a unique *Sma*I site within the insert, generating pEU39 cm. This plasmid has a unique *Eco*RV site adjacent to the cm^r cassette, into which a 1.54 kb fragment of *H. pylori* genomic DNA, including all of the *nifU* gene, was inserted, generating the plasmid pNUcm. *H. pylori* 43504 was then transformed with pNUcm, creating the *H. pylori* mero-diploid strain 43504:CD84 (see Figure 1).

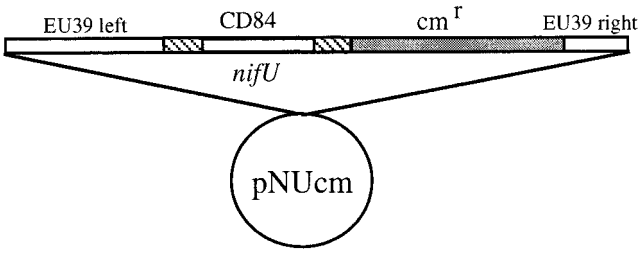

FIGURE 1: Diagram of pNUcm, the plasmid used to create the *nifU* mero-diploid strain. EU39 left contains *H. pylori* genomic DNA 416433−417953; EU39 right contains *H. pylori* genomic DNA 417953−418474. CD84 contains *H. pylori* genomic DNA from 229316 to 230822, including all of *nifU*. Transformation of 43504 with pNUcm yielded strain 43504:CD84, in which the cm^r cassette and a second copy of *nifU* have been inserted into the chromosome at bp 417953. Nucleotide numbers refer to those designated for strain 26695 (*4*).

Construction of Clones Designed for Mutagenesis of H. pylori 43504 and 43504:CD84. Both *nifU* and *nifS* were cloned into p(bluescript)KS+, and the coding sequence was then interrupted with the kanamycin resistance cassette *aph*A1. This cassette was inserted into a unique *Sph*I site in pKS-NifU, yielding pNifU:Kan, and into the *Nde*I site of pKS-NifS, yielding pNifS-Kan. That the cassette was inserted at the correct position within the genes was determined by the correct pattern of restriction fragments for three separate restriction endonucleases (data not shown).

Mutagenesis of H. pylori 43504 and 43504:CD84. Growing cells were harvested by centrifugation and washed five times with ice-cold 9% sucrose and 15% glycerol. These competent cells were used immediately for transformation or flash frozen and stored at −80 °C. DNA (2 μg) was added to 50 μL competent cells and electroporated with a pulse of 2.5 kV in a transporator plus (BTX) apparatus. Cells were resuspended in 50 μL of Mueller Hinton (Difco) broth, spotted onto a cold nonselective blood agar plate, and incubated in Campypac atmosphere. After 48 h on nonselective plates, the cells were resuspended by swab and plated onto selective plates. Resistant colonies (if any) appeared in 72−96 h and were confirmed to contain the correct insert by Southern blotting of genomic DNA.

Purification of NifU. The overexpression plasmid pET-NifU was transformed into *E. coli* strain BL21 (DE3) RIL. Cells were grown at 30 °C in LB ampicillin media supplemented with 14.5 mg/L $FeCl_2$, shaking (350 mL in a 2 L flask) at 200 rpm. When the A_{600} reached 0.6, cells were induced for NifU production for 2 h by addition of 0.5 mM IPTG. Cells were then harvested by centrifugation, washed once with cold TND buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM DTT), and resuspended in TND at approximately 2 mL/g of cell pellet. All subsequent steps were done at 4 °C, all buffers were argon sparged, and procedures (centrifugations, precipitations, and dialysis) were performed in closed vessels under an argon atmosphere. Cells were broken by two passages through a French pressure cell at 138 000 kPa and unbroken cells removed by centrifugation at 15000*g* for 20 min. The crude extract was then precipitated

with 1% streptomycin sulfate with constant stirring for 30 min and cleared by centrifugation at 20000*g* for 20 min. The streptomycin-treated supernatant was then precipitated by slowly adding 35% solid ammonium sulfate with constant stirring over a period of 60 min. The precipitated supernatant fraction was then collected by centrifugation (20000*g*, 20 min) and the pellet resuspended in the original volume of TND and then dialyzed at 4 °C versus 1 L of TND. The protein was then loaded onto a 5 mL Q-Sepharose column (Pharmacia Corp.) preequilibrated with TND, and the column was washed with 5 column volumes of TND and then subjected to a 60 mL 50−600 mM NaCl (in 50 mM Tris-HCl, pH 7.5, 2 mM DTT) gradient. Two milliliter fractions were collected and immediately sparged with argon, and the test tubes were stoppered. NifU-containing fractions (as determined by SDS−PAGE) were then collected and dialyzed against TND and used for assay as described.

Purification of NifS. NifS was overproduced from the plasmid pET-NifS in *E. coli* strain BL21 (DE3) RIL. Cells were grown, induced with IPTG, harvested, broken, and streptomycin sulfate precipitated as described for purification of NifU, except the $FeCl_2$ was omitted from the media, and all of these steps were performed aerobically. The crude extract was fractionated by adding 25% solid ammonium sulfate with constant stirring over a 30 min period and centrifuged at 20000*g* for 20 min. The supernatant was then collected and then precipitated further by addition of solid streptomycin sulfate to a final concentration (w/v) of 45%. The precipitated supernatant fraction was then collected by centrifugation (20000*g*, 20 min), resuspended in the original volume of TND, and dialyzed overnight versus 1 L of TND. The protein was then loaded onto a 5 mL Q-Sepharose column that had been preequilibrated with TND, and the column was washed with 5 column volumes of TND and then subjected to a 60 mL 50−600 mM NaCl (in 50 mM Tris-HCl, pH 7.5, 2 mM DTT) gradient. Two milliliter fractions were collected and assayed for NifS by SDS−PAGE.

Spectroscopic Methods. All samples of NifU were in 50 mM Tris-HCl buffer, pH 7.5, with 2 mM DTT. Concentrations are based on protein determinations and are expressed per NifU monomer. UV−visible absorption spectra were recorded under anaerobic conditions in septum-sealed 1 mm or 1 cm cuvettes, using a Shimadzu 3101PC scanning spectrophotometer. Variable temperature MCD spectra were recorded using samples containing 55% (v/v) glycerol in 1 mm cuvettes using an Oxford Instruments Spectromag 4000 (0−7 T) split-coil superconducting magnet (1.5−300 K) mated to a Jasco J-715 spectropolarimeter. X-band (∼9.6 GHz) EPR spectra were recorded using a Bruker ESP-300E EPR spectrometer equipped with a dual-mode ER-4116 cavity and an Oxford Instruments ESR-9 flow cryostat. Frequencies were measured with a Systron-Donner 6054B frequency counter microwave frequency counter, and the magnetic field was calibrated with a Bruker ER035M gaussmeter. Spin quantitations were carried out under nonsaturating conditions against a 1 mM Cu(II)EDTA standard, and spectral simulations were carried out using Simfonia (Bruker).

NifS Assays. All NifS assays were done at room temperature in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 50 mM L-cysteine. DTT (2 mM) was added when assaying for sulfide. Sulfane sulfur was assayed by the cold cyanolysis method as described (*5*). Sulfide was assayed by the methylene blue binding assay as described (*6*). The UV−visible spectroscopy of purified NifS was done on a Beckman DU 640 B spectrophotometer.

Iron Assay. Iron content in NifU was determined by the *o*-phenanthroline method. The NifU samples were acidified by addition of 10 μL of concentrated HCl and then made to 1 mL in deionized water. The samples were then heated to 80 °C for 10 min and cooled to room temperature, and then the following were added with mixing between each addition: 3 mL of water, 0.2 mL of 10% hydoxylamine hydrochloride, and 1 mL of 0.1% *o*-phenanthroline. Samples were incubated at room temperature for 30 min, and then the absorbance was read at 512 nm and compared to a standard curve made with 0−100 nmol of ferrous ammonium sulfate.

Gel Filtration. Native size determinations were performed by loading 200 μL (∼5 mg/mL) of each protein to a Superose 6 (Pharmacia Biotech) 10 cm × 16 cm column preequilibrated with Tris-HCl, pH 7.5, 50 mM NaCl, or TND buffer as indicated. The column had previously been calibrated with the following gel filtration molecular weight markers (all from Sigma): β-amylase (M_r 200 000), alcohol dehydrogenase (M_r 150 000), bovine serum albumin (M_r 66 000), and carbonic anhydrase (M_r 29 000).

RESULTS

nifU and nifS Are Required for Viability in H. pylori. Neither *nifU* nor *nifS* were able to be insertionally inactivated by electrotransformation using plasmids in which the ORF's encoding *nifU* (pNifU:kan) and *nifS* (pNifS:kan) are interrupted by the *aphA*3 kanamycin-resistant cassette. Transformations were done using standard techniques, as well as transformations in which the recovery phase (nonselective) and growth phase plates contained additional ferric iron or ferrous iron, and the plates were incubated at 2% O_2 (partial pressure) conditions. In all cases, positive control plasmids were transformed into other *H. pylori* genes, and the anticipated gene was inactivated with a yield $>10^3$ CFU/μg of DNA. To show that the constructs would be capable of transforming *H. pylori* should the mutation not be lethal, a mero-diploid strain was constructed for one (*nifU*) of these genes. In this construct, a fragment of *H. pylori* genomic DNA containing the entire *nifU* reading frame and ∼600 bp of flanking DNA was inserted into the *H. pylori* genome at an unrelated site, within the reading frame of HP 0405 (see diagram, Figure 1), creating the strain 43504:CD84. This strain was easily transformed with the plasmid pNifU:kan, yielding $>10^3$ CFU/μg of DNA. Genomic DNA from two of these kan^r colonies (one from each of two separate transformations) was obtained and, when probed with the *nifU* reading frame by Southern blot, indicated that each strain had one intact (990 bp; lanes 1−3, Figure 2) and one insertionally inactivated (∼2300 bp; lanes 2 and 3, Figure 2) copy of *nifU*. It should be noted that further Southern blot analysis revealed that, in both cases, the *aphA*3 cassette was inserted into the second copy of *nifU* (data not shown). This was what was expected as the natural promoter for *nifU* is not contained within the genomic fragment CD84, so that the second copy is probably not correctly expressed.

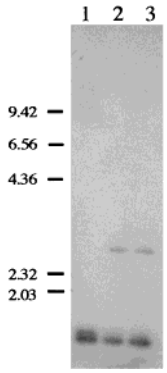

FIGURE 2: Southern blot of NifU:kan. Each lane contains *H. pylori* genomic DNA from strain 43504 (lane 1) or two independent isolates of NifU:kan (lanes 2 and 3) digested with *Hin*dIII probed with a internal fragment of *nifU*. DNA markers (λ DNA digested with *Hin*dIII) are indicated to the left of the figure in kilobases.

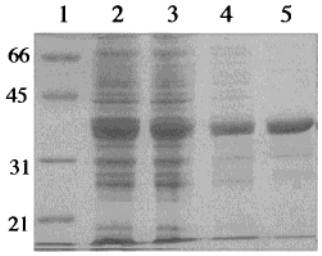

FIGURE 3: Purification of NifU: 12.5% SDS−PAGE NifU fractions. Lane 1 contains molecular mass markers (in kilodaltons); lanes 2−5 contain 5 μg each of the following: crude extract, streptomycin sulfate fraction, ammonium sulfate fraction, and Q-Sepharose pooled fractions.

Purification and Characterization of NifU. NifU was cloned by PCR into the overexpression vector pET21a (Novagen) and expressed at high levels in *E. coli* (Figure 3, lane 2). Although a significant proportion of the expressed protein was originally insoluble, we found we could increase the soluble/insoluble ratio by adding $FeCl_2$ to the media and incubating at lower (30 °C) temperatures. Purification of NifU was achieved using an ammonium sulfate precipitation followed by ion-exchange chromatography using Q-Sepharose. NifU fractions were analyzed by SDS−PAGE and were essentially pure, with only a minor contaminant at about 31 kDa (Figure 3). The protein migrated at essentially the predicted mass of 36 318 Da. The red color of purified NifU indicated the presence of an Fe−S center, and iron analysis yielded 1.43 ± 0.14 iron atoms per monomer. Gel filtration of purified NifU determined it has a native molecular mass of 130 000, nearly twice as large as the *Azotobacter vinelandii* enzyme, suggesting that *H. pylori* NifU may be a homotetramer. Molecular mass determinations performed in both the presence and absence of reducing agent (2 mM DTT) produced identical elution times (data not shown).

Spectroscopic Characterization of the Fe−S Center in NifU. The UV−visible absorption spectra of the as-purified and dithionite-reduced *H. pylori* NifU (Figure 4) are consistent with the presence of a $[2Fe\text{-}2S]^{2+,+}$ cluster. The spectra are qualitatively and quantitatively similar to those previously reported for *A. vinelandii* NifU (*7*), and in accord with the Fe analyses, the extinction coefficients at 320, 440, 460, and 550 nm for the as-purified protein (12.4, 5.8, 5.4, and 2.8 mM^{-1} cm^{-1}, respectively) are indicative of 0.7 ± 0.2 $[2Fe\text{-}2S]^{2+}$ clusters per monomer.

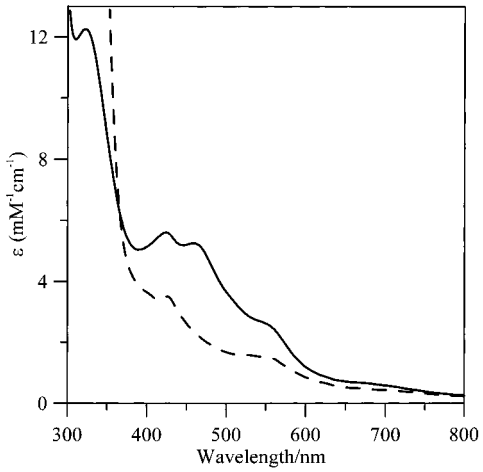

FIGURE 4: Room temperature UV−visible absorption spectra of *H. pylori* NifU as purified (solid line) and dithionite reduced (broken line). The protein concentration was 0.3 mM, and the sample was anaerobically reduced with a 10-fold excess of sodium dithionite. The dominant absorption centered at 314 nm in the reduced spectrum results from excess dithionite.

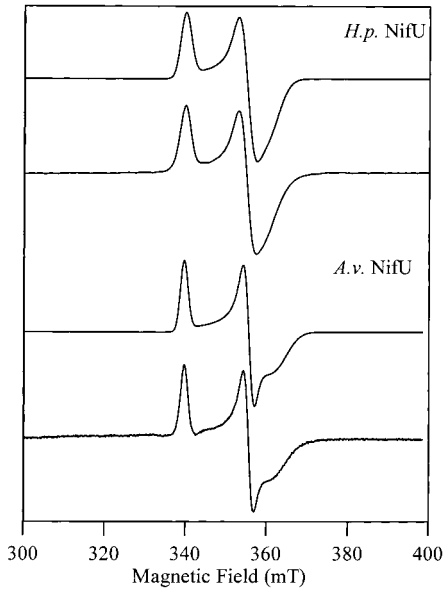

FIGURE 5: Comparison of the X-band EPR spectra of dithionite-reduced NifU from *H. pylori* and *A. vinelandii*. Conditions of measurement for both spectra: microwave frequency, 9.60 GHz; modulation amplitude, 6.43 G; microwave power, 1 mW; temperature, 20 K; protein concentration, 0.3 mM. Simulated spectra are offset directly above each of the experimental spectra. The spectra were simulated with the following parameters: *H. pylori* NifU, $g_{1,2,3}$ = 2.017, 1.930, 1.902 and line widths $l_{1,2,3}$ = 2.6, 3.2, 5.6 mT; *A. vinelandii* NifU, $g_{1,2,3}$ = 2.019, 1.927, 1.892 and line widths $l_{1,2,3}$ = 1.1, 1.1, 3.3 mT.

The ground- and excited-state properties of the paramagnetic $[2Fe\text{-}2S]^{+}$ cluster in reduced *H. pylori* NifU were investigated using EPR (Figure 5) and variable temperature MCD (Figure 6) spectroscopies, and the results are compared to those previously obtained and analyzed for *A. vinelandii* NifU (*7*). Reduced *H. pylori* NifU exhibits an $S = {}^1/_2$ EPR signal, g = 2.017, 1.930, and 1.902 (based on spectral simulation), that is observed without significant broadening up to 70 K and accounts for 0.8 ± 0.1 spins per monomer. These relaxation properties are characteristic of a $[2Fe\text{-}2S]^{+}$ cluster and the g-value anisotropy is very similar to that of

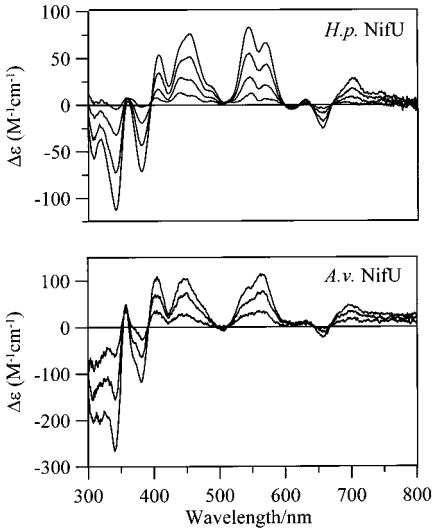

FIGURE 6: Comparison of the variable temperature MCD spectra of dithionite-reduced NifU from *H. pylori* and *A. vinelandii*. Protein concentrations were 0.13 and 0.11 mM for *H. pylori* and *A. vinelandii* NifU, respectively, and both samples contained 55% (v/v) ethylene glycol. MCD spectra were recorded in 1 mm cuvettes with a magnetic field of 6.0 T at 1.70, 4.22, 10.0, and 35 K for *H. pylori* NifU and 1.70, 4.22, and 10.0 K for *A. vinelandii* NifU. All bands increase in intensity with decreasing temperature.

the $[2Fe\text{-}2S]^{+}$ cluster in *A. vinelandii* NifU, $g = 2.019$, 1.927, and 1.892. The only significant difference in the resonances lies in increased line widths for *H. pylori* NifU, which presumably reflect greater heterogeneity in the cluster environment. The variable temperature MCD spectrum of paramagnetic $[2Fe\text{-}2S]^{+}$ clusters provides a much more sensitive monitor of the excited-state properties than the absorption spectrum. Hence, the one to one correspondence in the temperature-dependent MCD bands for the $[2Fe\text{-}2S]^{+}$ centers in *H. pylori* and *A. vinelandii* NifU (Figure 6) attests to very similar excited-state structures for these chromophores. The spectra differ only in the relative intensities of discrete bands, and such differences can arise from minor perturbations in the composition and energies of electronic excited states. Magnetization studies indicate that all of the electronic transitions resolved in the MCD spectra of *H. pylori* NifU arise from the EPR-characterized $S = {}^{1}/_{2}$ ground state. Overall, the UV−visible absorption, EPR, and MCD spectra attest analogous ground- and excited-state electronic structures for the redox-active $[2Fe\text{-}2S]^{2+,+}$ clusters in *H. pylori* and *A. vinelandii* NifU and indicate that *H. pylori* NifU contains approximately one [2Fe-2S] center per monomeric unit.

Purification and Characterization of NifS. NifS was cloned by PCR into the overexpression vector pET21a (Novagen). The first attempts to overexpress NifS with the normal BL21 (DE3) cells were largely unsuccessful, due to very poor production of NifS. When *nifS* was expressed from pET-NifS in the BL21 (DE3) RIL strain (Stratagene), production of NifS was increased dramatically (Figure 7). This expression strain contains a plasmid with extra copies of four rare (for *E. coli*) codons, AGA, AGG, AUA, and CUA. These codons are used in *H. pylori nifS* a total of 13 times. NifS purified from this strain had the characteristic yellow color of a pyridoxal phosphate containing enzyme, as do other examples of NifS (*8*, *9*). NifS was purified by two ammonium sulfate precipitations followed by Q-Sepharose chromatography. Purification of NifS was monitored by SDS−PAGE, and pure NifS migrated slightly larger than the predicted mass of 42 000 Da (Figure 7). Gel filtration of purified NifS gave a native molecular mass of 76 000, consistent with NifS being a homodimer. NifS UV−visible absorption spectra revealed a characteristic peak at 382 nm, consistent with a pyridoxal phosphate containing protein. Addition of 0.5 mM L-cysteine shifted the peak to 348 nm, with a shoulder at 412 nm (Figure 8). This is in contrast to the *A. vinelandii* enzyme, in which the major peak is at 416 nm and the minor peak at 370 nm (*8*). NifS is a cysteine desulfurase, catalyzing the stoichiometric release of sulfane sulfur (nonreducing conditions, data not shown) or sulfide (in the presence of DTT) from L-cysteine. Kinetic analysis monitoring sulfide as product revealed that purified NifS catalyzed the release of 9.9 nmol of sulfide min^{-1} (mg of pure protein)$^{-1}$. This compares with 89.4 nmol min^{-1} mg^{-1} for the *A. vinelandii* NifS (*8*), 67.6 nmol min^{-1} mg^{-1} for *A. vinelandii* IscS (*10*), and 78 nmol min^{-1} mg^{-1} for the *E. coli* NifS (*9*).

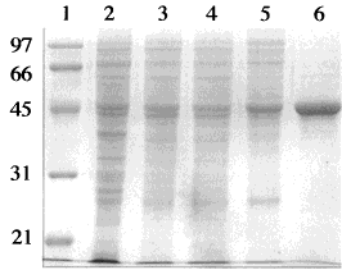

FIGURE 7: Purification of NifS: 12.5% SDS−PAGE of NifS fractions. Lane 1 contains molecular mass markers (in kilodaltons); lanes 2−6 contain 5 μg each of the following: crude extract, streptomycin sulfate treated supernatant, 25% ammonium sulfate supernatant, 45% ammonium sulfate precipitate, and pooled Q-Sepharose fractions.

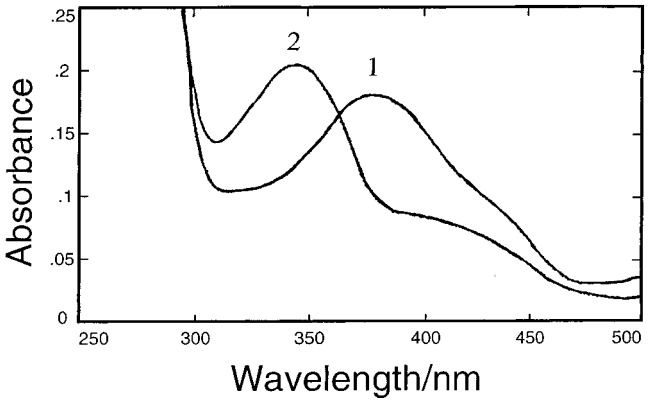

FIGURE 8: UV−visible spectra of purified NifS (2 mg/mL) in the absence (spectra 1) and presence (spectra 2) of 0.5 mM L-cysteine.

DISCUSSION

NifU and NifS are both essential for the viability of *H. pylori*. Numerous attempts to individually disrupt the genes encoding for these proteins were unsuccessful, indicating that the loss of function of either of these proteins is lethal. To show that the transformation procedure would in fact interrupt the gene of interest, a second copy of *nifU* was supplied on a unrelated locus within the *H. pylori* genome.

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Hp NifU    1 MAKHDLVGSALWDAYSKEVQRRMDNPTHLGVITEEQAKAKNAKLIVADYGAEACGDAVRL  60
Av NifU    1            MWDYSEKVKEHFYNPKNAGAVEGAN--------AIGDVGSLSCGDALRL  41
Av IscU    1             MAYSDKVIDHYENPRNVGKLDAQDP-----DVGTGMVGAPACGDVMRL  43
                           **  *  .  ** . * .                *. .*** .**

Hp NifU   61 YWLVDESTDIIIDAKFKSFGCGTAIASSDMMVELCLNKRVQDAVKITNLDVERGLRDDPD 120
Av NifU   42 TLKVDPETDVILDAGFQTFGCGSAIASSSALTEMVKGLTLDEALKISNQDIADYLDG---  98
Av IscU   44 QIKVN-EQGIIEDAKFKTYGCGSAIASSSLATEWMKGRTLEEAETIKNTQIAEELA----  98
                *     .* ** *...***.*****    *   .  . .*  * *  .   *

Hp NifU  121 TPAVPGQKMHCSVMAYDVIKKAAGMYLGKN-AEDFEEEIIVCECARVSLGTIKEVIRLND 179
Av NifU   99 ---LPPEKMHCSVMGREALQAAVANYRGETIEDDHEEGALICKCFAVDEVMVRDTIRANK 155
Av IscU   99 ---LPPVKIHCSVLAEDAIKAAVRDYK-------HKKGLV                     128
                .*  *.****.  . .. *   *           .

Hp NifU  180 LKSVEEITNYTKAGAFCKSCVR------PGGHEKRDYYLVDILKEVREEMEAEKLKATAN 233
Av NifU  156 LSTVEDVTNYTKAGGGCSACHEAIERVLTEELAARGEVFVAAPIKAKKKVKVLAPEPAPA 215
Av IscU  129                                                             128

Hp NifU  234 KSQNGELAFREMTMVQKIKAVDKVIDENIRAMLMMDGGDLEILDIKESDDYIDVYIRYMG 293
Av NifU  216 PVAEAPAAAPKLSNLQRIRRIETVLAA-IRPTLQRDKGDVELIDV----DGKNVYVKLTG 270
Av IscU  129                                                             128

Hp NifU  294 ACDGCMSATTGTLFAIENALQELLDRSIRVLPI           326
Av NifU  271 ACTGCQMASM-TLGGIQQRLIEELGEFVKVIPVSAAAHAQMEV 312
Av IscU  129                                           128
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FIGURE 9: Primary sequence alignment of *H. pylori* and *A. vinelandii* NifU and *A. vinelandii* IscU. Identical residues are indicated by a star (*), similar residues are indicated by a period (.), and gaps in the sequence are indicated by hyphens. The conserved cysteine residues are in bold type. The *H. pylori* and *A. vinelandii* NifU sequences have 33% identity and 52% similarity.

This second copy of *nifU* could be readily genetically disrupted using the same procedures initially used on the parent cells, indicating that the lack of transformants to phenotypically identify *nifU* mutants is clearly due to the adverse effects of disrupting the gene. Using this procedure, only the second copy of *nifU* was found to be mutated in all transformants tested (data not shown). This is probably because *nifS* and *nifU* are likely to be transcribed together as indicated from the sequence data, and the inserted copy of *nifU* lacks a recognizable promoter. This copy therefore only acts as a target for mutagenesis, not as a functional gene. That NifU and NifS are required for growth is not completely unexpected, as the IscS/IscU genes are thought to be required for the viability of other organisms, both bacterial (*10*) and eucaryotic (*11*, *12*).

Although we were unable to study the phenotypes of mutations within these genes, we could overproduce the gene products in *E. coli* and then study the purified components. NifU is a modular protein (*3*, *13*); the primary sequences of *A. vinelandii* and *H. pylori* NifU, as well as *A. vinelandii* IscU, are compared in Figure 9. The N-terminus is shared between the NifU and IscU, and this portion of the protein contains the three conserved cysteine residues that have been shown to be the site of NifS-directed assembly of a "transitory" [2Fe-2S] center within NifU (*3*). More recently, IscS-directed cluster assembly studies on IscU have shown that this domain provides a scaffold for the sequential assembly of [2Fe-2S] and [4Fe-4S] clusters (*14*). The second domain of NifU, absent in the IscU proteins, contains the four conserved cysteine residues that ligate the "permanent" Fe−S cluster. This is the [2Fe-2S] cluster that is always present in NifU and the one that has been characterized here in the *H. pylori* protein. The function of this domain of the protein is still uncertain, but it almost certainly is related to a redox role. IscU-like proteins which lack this second domain are usually encoded by genes in genetic proximity to the ferredoxin encoding gene *fdx* (*10*). Although these ferredoxins have little primary sequence homology to the second domain of NifU, the properties of the [2Fe-2S] centers within these ferredoxins are almost identical to the permanent clusters within *A. vinelandii* NifU (*15*), as well as the *H. pylori* permanent cluster characterized here. These ferredoxins probably work in conjunction with the IscU-type proteins, fulfilling the redox role of the second domain. The third domain of the NifU proteins contains two conserved cysteine residues but has not been assigned a specific function in any of the members of this family (*3*, *13*).

Members of the NifS/IscS class of proteins carry out the desulfurization of L-cysteine in a mechanism involving the donation of the sulfur atoms to form the Fe−S cluster. Consistent with a role in sulfur donation, the *H. pylori* NifS is a L-cysteine desulfurase. The mechanism proposed for the NifS reactions is PLP-dependent (*16*), and the UV−visible absorption spectrum of *H. pylori* NifS is consistent with a PLP-containing enzyme (Figure 8). In addition to its role in Fe−S cluster formation, the IscS protein from *E. coli* has also been proposed to be the source of sulfur donation in the synthesis of thionucleosides (*17*), biotin (*18*), thiamin, and NAD (*19*). Whether or not this sulfur is donated directly from cysteine or through an intermediate Fe−S cluster is not clear.

It is clear that the presence of the second and third domains of NifU (which includes the Fe−S cluster binding ligands) distinguishes the NifU-type proteins from the IscU proteins (Figure 9). A BLAST search of the GenBank protein database using the *H. pylori* NifU retrieves 19 protein sequences sharing significant homology to the second and third domains (BLAST score >40). The top three matches

are proteins from the two sequenced strains of *H. pylori* and the closely related human gut pathogen *Campylobacter jejuni*, all corresponding to NifU. The remaining 16 matches are also NifU but from organisms capable of fixing nitrogen. The available data from the nitrogen-fixing organism *A. vinelandii* suggests that NifU is specifically involved as a scaffold for assembling Fe−S clusters that are destined for insertion into the nitrogenase protein (*3*, *13*). In contrast, IscU appears to function as a scaffold for general Fe−S cluster biosynthesis. Since *H. pylori* is not a nitrogen-fixing organism, the present work clearly demonstrates that the NifU type of Fe−S scaffold protein is not necessarily specific to the nitrogenase proteins.

The characteristics of the purified NifS and NifU proteins, their primary sequence, and their requirement for viability point to the fact that these proteins are almost certainly responsible for Fe−S cluster formation and/or repair in *H. pylori*. The absence of any other potential Fe−S cluster assembly genes in the *H. pylori* genome (*4*) suggests that NifS and NifU are responsible for the assembly of all Fe−S centers. A list of the Fe−S-containing proteins of *H. pylori* is shown in Table 1. Not only would we expect many of these Fe−S containing proteins to play essential roles themselves, deletion of NifS and NifU would almost certainly upset the balance of Fe homeostasis within the cell. *H. pylori* is microaerobic, and the sole known ecological niche of *H. pylori* is the mucosa of the human stomach. This inability to tolerate aerobic conditions (or any oxidative bursts derived from the host response) would certainly be exacerbated by free iron within the cell.

ACKNOWLEDGMENT

The authors thank David McGee for invaluable advice and for use of his *H. pylori* reagents and methods.

REFERENCES

1. Beinert, H., Holm, R. H., and Münck, E. (1997) *Science 277*, 653−659.
2. Johnson, M. K. (1998) *Curr. Opin. Chem. Biol. 2*, 173−181.
3. Yuvaniyama, P., Agar, J. N., Cash, V. L., Johnson, M. K., and Dean, D. R. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 599−604.
4. Tomb, J. F., White, O., Kerlavage, A. R., Clayton, R. A., Sutton, G. G., Fleischmann, R. D., Ketchum, K. A., Klenk, H. P., Gill, S., Dougherty, B. A., Nelson, K., Quackenbush, J., Zhou, L., Kirkness, E. F., Peterson, S., Loftus, B., Richardson, D., Dodson, R., Khalak, H. G., Glodek, A., McKenney, K., Fitzegerald, L. M., Lee, N., Adams, M. D., Hickey, E. K., Berg, D. E., Gocayne, J. D., Utterback, T. R., Peterson, J. D., Kelley, J. M., Cotton, M. D., Weidman, J. M., Fujii, C., Bowman, C., Watthey, L., Wallin, E., Hayes, W. S., Borodovsky, M., Karp, P. D., Smith, H. O., Fraser, C. M., and Venter, J. C. (1997) *Nature 388*, 539−547.
5. Wood, J. L. (1987) *Methods Enzymol. 143*, 25−28.
6. Siegel, L. M. (1965) *Anal. Biochem. 11*, 126−132.
7. Fu, W., Jack, R. F., Morgan, T. V., Dean, D. R., and Johnson, M. K. (1994) *Biochemistry 33*, 13455−13463.
8. Zheng, L., White, R. H., Cash, V. L., Jack, R. F., and Dean, D. R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2754−2758.
9. Flint, D. H. (1996) *J. Biol. Chem. 271*, 16068−16074.
10. Zheng, L., Cash, V. L., Flint, D. H., and Dean, D. R. (1998) *J. Biol. Chem. 273*, 13264−13272.
11. Garland, S. A., Hoff, K., Vickery, L. E., and Culotta, V. C. (1999) *J. Mol. Biol. 294*, 897−907.
12. Schilke, B., Voisine, C., Beinert, H., and Craig, E. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 10206−10211.
13. Agar, J. N., Yuvaniyama, P., Jack, R. F., Cash, V. L., Smith, A. D., Dean, D. R., and Johnson, M. K. (2000) *J. Biol. Inorg. Chem. 5*, 167−177.
14. Agar, J. N., Krebs, C., Frazzon, J., Huynh, B. H., Dean, D. R., and Johnson, M. K. (2000) *Biochemistry 39*, 7856−7862.
15. Jung, Y. S., Gao-Sheridan, H. S., Christiansen, J., Dean, D. R., and Burgess, B. K. (1999) *J. Biol. Chem. 274*, 32402−32410.
16. Zheng, L., White, R. H., Cash, V. L., and Dean, D. R. (1994) *Biochemistry 33*, 4714−4720.
17. Kambampati, R., and Lauhon, C. T. (2000) *J. Biol. Chem. 275*, 10727−10730.
18. Kiyasu, T., Asakura, A., Nagahashi, Y., and Hoshino, T. (2000) *J. Bacteriol. 182*, 2879−2885.
19. Lauhon, C. T., and Kambampati, R. (2000) *J. Biol. Chem. 275*, 20096−20103.

BI001744S