Purification and characterization of *Klebsiella aerogenes* UreE protein: A nickel-binding protein that functions in urease metallocenter assembly

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Abstract

The *Klebsiella aerogenes ureE* gene product was previously shown to facilitate assembly of the urease metallocenter (Lee, M.H., et al., 1992, *J. Bacteriol.* 174, 4324–4330). UreE protein has now been purified and characterized. Although it behaves as a soluble protein, UreE is predicted to possess an amphipathic β -strand and exhibits unusually tight binding to phenyl-Sepharose resin. Immunogold electron microscopic studies confirm that UreE is a cytoplasmic protein. Each dimeric UreE molecule ($M_r = 35,000$) binds 6.05 ± 0.25 nickel ions (K_d of $9.6 \pm 1.3 \mu\text{M}$) with high specificity according to equilibrium dialysis measurements. The nickel site in UreE was probed by X-ray absorption and variable-temperature magnetic circular dichroism spectroscopies. The data are most consistent with the presence of Ni(II) in pseudo-octahedral geometry with 3–5 histidyl imidazole ligands. The remaining ligands are nitrogen or oxygen donors. UreE apoprotein has been crystallized and analyzed by X-ray diffraction methods. Addition of nickel ion to apoprotein crystals leads to the development of fractures, consistent with a conformational change upon binding nickel ion. We hypothesize that UreE binds intracellular nickel ion and functions as a nickel donor during metallocenter assembly into the urease apoprotein.

Keywords: *Klebsiella aerogenes*; metallocenter assembly; nickel; urease; UreE

Urease (EC 3.5.1.5), a nickel-containing enzyme found in certain plants and many microorganisms, hydrolyzes urea to yield ammonia and carbamate; the carbamate spontaneously decomposes to form a second molecule of ammonia and carbonic acid (Andrews et al., 1988;

Mobley & Hausinger, 1989). In addition to being important in nitrogen metabolism, the enzyme has been implicated as a bacterial virulence factor in various human and animal diseases (reviewed by Mobley & Hausinger, 1989). The most extensively characterized microbial urease is that from the gram-negative enteric bacterium, *Klebsiella aerogenes* (a non-nitrogen-fixing *Klebsiella pneumoniae*). The urease enzyme possesses three different subunits ($M_r = 60,304$ [α], 11,695 [β], and 11,086 [γ] [Mulrooney & Hausinger, 1990]) where each catalytic unit is comprised of subunits in an apparent $\alpha_1\beta_2\gamma_2$ stoichiometry and contains a bi-nickel active site (Todd & Hausinger, 1987, 1989). When *K. aerogenes* cells are grown in the absence of nickel, urease apoenzyme is produced. Although the purified apoenzyme cannot be reactivated by the simple addition of nickel ions, an energy-dependent recon-

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Abbreviations: CHESS, Cornell High Energy Synchrotron Source; EXAFS, extended X-ray absorption fine structure; FT, Fourier transform; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N'*-(2-ethanesulfonic acid); MOPS, 3-(*N*-morpholino)propanesulfonic acid; PBS, phosphate-buffered saline; PEB, buffer composed of 20 mM potassium phosphate, 0.5 mM EDTA, 1 mM 2-mercaptoethanol (pH 7.2); SDS, sodium dodecyl sulfate; SPEAR, Stanford Positron-Electron Accelerating Ring; SSRL, Stanford Synchrotron Radiation Laboratory; TBST, Tris-buffered saline, pH 7.4, with 0.05% (v/v) Tween 20; VTMD, variable-temperature magnetic circular dichroism; XAS, X-ray absorption spectroscopy.

stitution of the protein was observed in intact cells (Lee et al., 1990).

DNA sequence analysis revealed the presence of several additional genes that are part of the *K. aerogenes* urease gene cluster. The three urease structural genes (*ureA*, *ureB*, and *ureC*) are immediately preceded by the *ureD* gene and followed by the *ureE*, *ureF*, and *ureG* genes (Mulrooney & Hausinger, 1990; Lee et al., 1992). The auxiliary genes have been shown to be required for urease metallocenter assembly by using recombinant *Escherichia coli* cells containing plasmids with derivatives of the *K. aerogenes* urease gene cluster (Lee et al., 1992). In cells containing deletion derivatives affecting the *ureD*, *ureF*, and *ureG* genes, the urease protein was shown to be synthesized in an inactive form that was devoid of nickel, whereas cells containing deletion derivatives of *ureE* possess a reduced urease activity and the nickel content of the purified urease is correspondingly reduced. Each of the four genes encode accessory proteins that are required for nickel incorporation into urease. Although specific functions have not been identified for these proteins, UreE is a strong candidate for a nickel-binding protein because the translated *ureE* gene sequence reveals the presence of several potential metal-binding sites including a carboxyl-terminus where 10 of 15 residues are histidine (HGHH HAHHDHHAHSH; Mulrooney & Hausinger, 1990).

Here we describe the purification and characterization of UreE protein. In particular, we demonstrate that UreE is indeed a nickel-binding protein and detail the properties of its novel nickel metallocenter.

Results

Purification of UreE

UreE was highly purified by using a combination of DEAE-Sepharose and carboxymethyl-Sepharose column chromatographies. Samples at this stage of purification were estimated to be over 95% homogeneous (Fig. 1) and were deemed suitable for most of the experiments reported below. However, an additional step involving Mono S chromatography was included prior to generation of polyclonal monospecific antibodies. In spite of the presence of multiple histidine residues at its carboxyl-terminus, UreE did not exhibit any affinity to a chelating-Superose column that was charged with nickel. In contrast, UreE does bind tightly to phenyl-Sepharose resin. Indeed, elution of protein from the hydrophobic resin requires the inclusion of 40% dimethylsulfoxide in the PEB buffer.

Cellular localization of UreE

The tight interaction observed between UreE and phenyl-Sepharose resin raises the possibility of a membrane localization for the peptide in vivo. Such an interaction is not predicted from calculations of UreE hydrophobicity that show a lack of significant hydrophobic regions. In con-

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified UreE. Cell extracts from *E. coli* DH5 (pKAU17) (lane 1) and purified UreE (lane 3) were subjected to SDS-polyacrylamide gel electrophoresis by using a 10–15% gradient gel, followed by Coomassie blue staining. Molecular weight markers (lane 2) were phosphorylase b, M_r 92,500; bovine serum albumin, M_r 66,200; ovalbumin, M_r 45,000; carbonic anhydrase, M_r 31,000; soybean trypsin inhibitor, M_r 21,500; and lysozyme, M_r 14,400.

trast, however, an unusually strong feature (maximum of 0.85 with approximately 180° rotation per residue) can be found in the region from residue 18 to 34 in calculations of the amphipathic moment for UreE. The use of such predictive algorithms can lead to intriguing speculation (vide infra); however, it does not directly assess the in vivo interaction between the UreE peptide and the membrane. For this purpose, immunogold electron microscopic localization studies were carried out (Fig. 2). The gold particle labeling patterns clearly show that UreE in *K. aerogenes* (pKAU19) (Fig. 2A) and *E. coli* (pKAU17) (Fig. 2B) is a cytoplasmic protein. Wild-type *K. aerogenes* cells were insufficiently labeled by the immunogold technique to allow localization (not shown), probably because of insufficient levels of UreE protein (Kellenberger et al., 1987). *E. coli* (pKAU17ΔureE-1) that does not possess UreE did not bind significant levels of anti-UreE antibody (Fig. 2C).

Characterization of UreE: A nickel-binding protein

UreE protein was shown to exist as an apparent dimer (M_r = 35,000) when subjected to gel filtration analysis.

Fig. 2. Immunogold localization of UreE in recombinant *K. aerogenes* and *E. coli* cells. Thin sections of (A) *K. aerogenes* (pKAU19), (B) *E. coli* (pKAU17), and (C) *E. coli* (pKAU17ΔureE-1) cells were reacted with anti-UreE antibodies and labeled with anti-rabbit IgG-gold particles. UreE was localized to the cytoplasmic portion of the cell.

When purified in the presence of buffer containing 0.5 mM EDTA, UreE was found to be free of metal ions; however, equilibrium dialysis experiments were used to demonstrate that dimeric UreE binds 6.05 ± 0.25 nickel ions with a K_d of $9.6 \pm 1.3 \mu\text{M}$ (Fig. 3). This value for K_d is an average of the individual values for each of the six nickel sites. Bovine serum albumin and lysozyme were used as controls in this experiment. Lysozyme has no reported affinity for nickel ion, whereas, bovine serum

Fig. 3. Equilibrium dialysis of UreE with NiCl_2 . UreE ($2 \mu\text{M}$) (●) in $50 \mu\text{M}$ sodium phosphate (pH 7.2) buffer containing 0.5% NaCl was equilibrated with the indicated concentrations of NiCl_2 solutions containing $^{63}\text{NiCl}_2$ ($1.2 \mu\text{Ci/mL}$). Bovine serum albumin (○) and lysozyme (■) were used as controls.

albumin is analogous to the human serum albumin, which is known to bind 1 mole of nickel per mole of protein (Glennon & Sarkar, 1982).

The specificity of nickel binding was assessed by examining the effects of various concentrations of other divalent cations on the binding of ^{63}Ni that was present at a concentration of $20 \mu\text{M}$ (Fig. 4). The presence of excess magnesium, manganese, and calcium ions had negligible effects on nickel binding even to concentrations of $400 \mu\text{M}$. Cobalt ion appeared to compete reasonably well at approximately one of the four sites that bind nickel at this concentration and only weakly competed with the remaining nickel-binding sites. In a separate experiment, $200 \mu\text{M}$ cobalt was found to have little effect on the total number of nickel ions that UreE could bind and only slightly shifted the value for K_d (data not shown). Neglecting different affinities for cobalt among the six metal ion sites, the overall apparent K_i for cobalt ion interference of nickel binding was found to be $\sim 640 \mu\text{M}$. Copper ion also interferes differentially with the nickel-binding sites, but in this case copper ion competed well at approximately three of the four sites that bound nickel at $20 \mu\text{M}$ and competed weakly for the remaining site. Zinc ion exhibited the greatest ability to compete with nickel ion at the concentrations shown, but again this metal preferentially interfered with binding at a subset (approximately half) of the sites. At concentrations higher than $100 \mu\text{M}$, zinc ion exhibited anomalous behavior as if it prevented equilibration of nickel ion across the dialysis membrane even in the control sample containing no protein.

Characterization of the UreE metallocenter

The Ni X-ray absorption edge spectrum of UreE (Fig. 5) is qualitatively similar to the edge spectrum of (Ni(imid-

Fig. 4. Effect of divalent metal ions on nickel binding to UreE. Equilibrium dialysis of 2 μM UreE with nickel ion at a concentration of 20 μM was performed in the presence of various concentrations of metal ions. **A:** Experiments with MgSO₄ (◇), MnCl₂ (◆), and CaCl₂ (▲) were carried out in 50 mM sodium phosphate buffer (pH 7.2), CoCl₂ (○) in 50 mM HEPES buffer (pH 7.2), and CuSO₄ (●) in 50 mM Tris-HCl buffer (pH 7.6), each containing 0.5% NaCl. **B:** Experiments with ZnCl₂ were carried out in buffer containing 50 mM sodium phosphate (pH 7.2)–0.5% NaCl. Data at higher concentrations of zinc ion could not be obtained due to anomalous interference with distribution of Ni ions across the dialysis membrane (see text).

azole)₆(BF₄)₂ (Colpas et al., 1991) and the 8,333-eV 1s → 3d peak area is 0.028 eV, within the range expected for octahedral geometry (0.006–0.040 eV) (Colpas et al., 1991). The appearance of the outer-shell FT peaks in the Ni EXAFS of UreE (Fig. 6) suggests the presence of histidine imidazole ligands at the Ni(II) sites. The EXCURVE curved-wave multiple-scattering formalism (Strange et al., 1987) was used to simulate this imidazole outer-shell scattering, and the best average number of imidazoles per nickel was determined to be four (fit 2, Table 1). However, we cannot rule out average imidazole coordination numbers of three or five (fits 1 and 3, Table 1). Addition of the extra (N,O)-containing first-shell ligands simply completes the six-coordinate (pseudo)octahedral geometry suggested by the edge spectrum.

The wavelengths and intensities of the ligand field transitions can also provide information on the coordination

Fig. 5. Ni X-ray absorption edge spectrum of UreE. The arrow indicates the position of the 1s → 3d transition, the integrated area of which suggests a nearly octahedral symmetry for the Ni(II) site (see text for details).

geometry and the nature of the ligands. However, our attempts to identify the Ni(II) *d-d* transitions by UV/visible/near-IR absorption spectroscopy were unsuccessful. No clearly discernible bands were observed above the

Fig. 6. Ni K-edge EXAFS data (**A**) and Fourier transform (**B**) for UreE. The solid lines represent experimental data, and the dashed lines are the best-fit simulation (fit 2, Table 1). The FTs were performed with EXCURVE using k^3 weighting over the $k = 3.4$ – 13.4 Å⁻¹ range and are phase-corrected using Ni-N scattering phases.

Table 1. EXCURVE curve-fitting results for Ni EXAFS of UreE^a

Sample	Fit	Group	Shell	N_s	R_{as} (Å)	σ_{as}^2 (Å)	f'^b
UreE	1	Imid	Ni-N ₁	(3) ^c	2.08	0.0030	0.033
		Imid	Ni-C ₂		3.09	0.0045	
		Imid	Ni-N ₃		4.23	0.0060	
		Imid	Ni-C ₄		4.29	0.0060	
		Imid	Ni-C ₅		3.03	0.0045	
UreE	2	Imid	Ni-N	(3)	2.11	0.0055	0.029
		Imid	Ni-N ₁	(4)	2.08	0.0040	
		Imid	Ni-C ₂		3.09	0.0065	
		Imid	Ni-N ₃		4.23	0.0085	
		Imid	Ni-C ₄		4.29	0.0085	
UreE	3	Imid	Ni-C ₅	(2)	3.03	0.0065	0.034
		Imid	Ni-N	(5)	2.08	0.0045	
		Imid	Ni-N ₁		3.09	0.0070	
		Imid	Ni-N ₃		4.23	0.0090	
		Imid	Ni-C ₄		4.29	0.0095	
UreE	4	Imid	Ni-C ₅	(1)	3.03	0.0075	0.042
		Imid	Ni-N	(6)	2.11	0.0025	
		Imid	Ni-N ₁		2.08	0.0045	
		Imid	Ni-C ₂		3.09	0.0090	
		Imid	Ni-N ₃		4.23	0.0120	
UreE		Imid	Ni-C ₄		4.29	0.0120	
		Imid	Ni-C ₅		3.03	0.0090	
		Imid	Ni-C ₅		3.03	0.0090	

^a Group is the chemical unit defined for the multiple scattering calculation. N_s is the number of scatterers (or groups) per metal; R_{as} is the metal-scatterer distance; σ_{as}^2 is a mean square deviation in R_{as} .

^b f' is a goodness-of-fit statistic normalized to the overall magnitude of the $k^3\chi(k)$ data:

$$f' = \frac{[\sum |k^3(\chi_{\text{obsd}}(i) - \chi_{\text{calc}}(i))|^2/N]^{1/2}}{(k^3\chi)_{\text{max}} - (k^3\chi)_{\text{min}}}$$

^c Numbers in parentheses were not varied during optimization.

scattering background for the samples of UreE used in the VTMCD studies. Hence only an upper limit can be placed on the molar extinction coefficients of the Ni(II) $d-d$ bands. Based on the Ni(II) concentration, we conclude that the $d-d$ bands have $\epsilon < 50 \text{ M}^{-1} \text{ cm}^{-1}$, which is consistent with pseudo-octahedral coordination geometry.

VTMCD at liquid helium temperatures and high magnetic fields provides an alternative and more sensitive method for identifying the ligand field transitions of paramagnetic Ni(II) centers in biological samples (Kowal et al., 1988; Finnegan et al., 1991; Kiick, 1991). Figure 7 shows VTMCD UV/visible/near-IR spectra for UreE recorded at temperatures between 2 K and 50 K with a magnetic field of 4.5 T. At low temperatures, octahedral Ni(II) complexes are dominated by negative temperature-dependent MCD bands with maxima close to the absorption maxima of the spin allowed $d-d$ transitions (Kiick, 1991). Hence the negative bands centered at 360 nm, 615 nm, and 1,000 nm that increase in intensity with decreasing temperature are assigned to the three spin-

Fig. 7. Variable temperature MCD spectra of UreE. Spectra were recorded with a magnetic field of 4.5 T at 2.0 K, 4.2 K, 9.4 K, 23 K, and 49 K. All MCD bands increase in intensity with decreasing temperature.

allowed $d-d$ transitions of an octahedral Ni(II) center, i.e., ${}^3A_{2g} \rightarrow {}^3T_{1g}(P)$, ${}^3A_{2g} \rightarrow {}^3T_{1g}(F)$, and ${}^3A_{2g} \rightarrow {}^3T_{2g}$, respectively. The MCD intensities under comparable conditions are very similar to those of the spin-allowed $d-d$ bands of other octahedral Ni(II) complexes, i.e., $\Delta\epsilon$ values $< -0.5 \text{ M}^{-1} \text{ cm}^{-1}$ and $> -3.0 \text{ M}^{-1} \text{ cm}^{-1}$, at approximately 2 K and 4.5 T (Kiick, 1991). The temperature-dependent derivative feature centered at 420 nm is attributed to a minor low spin Fe(III) cytochrome impurity. Ligand field analysis of the Ni(II) $d-d$ transition leads to $Dq = 1,020 \pm 20 \text{ cm}^{-1}$ and $B = 900 \pm 20 \text{ cm}^{-1}$. Based on the ligand field parameters of a wide range of octahedral Ni(II) complexes with N and O donor atoms (Rosenberg et al., 1975), these values are consistent with three to six N donor atoms in the first coordination sphere. Because the ligand field-splitting parameter for $(\text{Ni}(\text{imidazole})_6)^{2+}$ ($Dq = 1,131 \text{ cm}^{-1}$ and $B = 849 \text{ cm}^{-1}$; Reimann [1968]) is significantly larger than for the Ni(II) center in UreE, coordination by six histidine ligands seems unlikely. Rather the ligand field parameters, taken together with the Ni EXAFS results, are indicative of pseudo-octahedral Ni(II) with three, four, or five histidine ligands.

Crystallography

Typical crystals of UreE apoprotein are shown in Figure 8. Preliminary diffraction analysis of these crystals revealed measurable intensities to a limiting resolution of 5 Å and indicated hexagonal unit cell parameters of $a = b = 190 \text{ Å}$, $c = 175 \text{ Å}$, with $\alpha = \beta = 90^\circ$, and $\gamma = 120^\circ$. Precession photographs confirm these unit cell parameters and also reveal the presence of $p6mm$ symmetry in the $hk0$ zone. Additionally, systematic absences along $00l$ for $l \neq 6n$ are observed in precession photographs of the $h0l$ zone. Therefore, the space group $P6_122$ (or its enantiomorph,

Fig. 8. Hexagonal bipyramid crystals of UreE apoprotein.

P6₅22) is assigned to hexagonal bipyramid crystals of UreE. The unit cell volume of these crystals is $5.47 \times 10^6 \text{ \AA}^3$; in order to exhibit the average packing volume V_m of $2.4 \text{ \AA}^3/\text{Da}$ found in a survey of crystalline proteins (Matthews, 1968), a total of six UreE apoprotein dimers must reside in the asymmetric unit of the unit cell (this corresponds to a solvent content of 49%). Given that we measure a density of $1.25 \pm 0.05 \text{ g mL}^{-1}$ for crystals of UreE apoprotein, it is unlikely that these crystals have a higher solvent content. Confirmation of the contents of the asymmetric unit must await further diffraction experiments.

At the CHESS, 30° of diffraction data were collected from one crystal of UreE apoprotein, of which the limiting resolution was 2.9 Å. Crystal lifetime in the X-ray beam was excellent, with an average 10% decay measured for a total exposure time of 10 min per crystal. A total of 28,228 intensity measurements with $I \geq 3\sigma$ were collected on 30 image plate frames, and these intensities were merged together with $R_{\text{merge}} = 0.13$; a sample diffraction plate image is found in Figure 9. Clearly, the prospects for a successful three-dimensional structure determination of UreE apoprotein are good. Because these crystals crack when equilibrated with 5 mM NiSO₄, we hypothesize that a conformational change accompanies the saturation of protein metal-binding sites with nickel. Accordingly, we are attempting to crystallize the nickel-bound UreE holoprotein so that we may ultimately compare the structures of metal-free and metal-bound forms.

Discussion

Isolation of UreE

UreE, a cytoplasmic nickel-binding protein that assists in the functional incorporation of nickel ion into urease, has been purified and characterized. Mulrooney and

Fig. 9. Typical UreE diffraction recorded on a Fuji image plate at the CHESS. Data were measured with a 1° crystal oscillation for 20 s; the wavelength of the incident beam was 0.91 Å. The crystal-to-film distance was 231 mm, and the limiting resolution of diffraction data was 2.9 Å.

Hausinger (1990) speculated that UreE could function in binding nickel based on sequence analysis of the *ureE* gene. The protein sequence was shown to possess numerous potential metal ion-binding sites including a carboxyl-terminus that contained 10 histidines among the last 15 residues. Other laboratories genetically attach polyhistidine tails to various proteins in order to facilitate their purification by metal ion affinity chromatography (e.g., Hochuli et al., 1988; Smith et al., 1988; Ljungquist et al., 1989; Lilius et al., 1991). UreE thus represents a naturally occurring example of such a protein with a poly-His tail. In contrast to other poly-His proteins, however, we found that UreE does not bind to an iminodiacetic acid metal ion affinity resin. Although metal ion affinity chromatography was not useful for peptide isolation, UreE readily can be purified by a simple two-column procedure.

Structural features of UreE

Immunogold electron microscopic studies were used to localize UreE to the cytoplasm of the cell, consistent with the lack of hydrophobic regions in the peptide based on computer calculations. Yet, UreE tightly binds to phenyl-Sepharose resin. The observed interaction with this hydrophobic resin may be related to the high amphipathicity in the region from residues 18 to 34. The sequence of this region (Leu-Pro-Ile-Asp⁽⁻⁾-Val-Arg⁽⁺⁾-Val-Lys⁽⁺⁾-Ser-Arg⁽⁺⁾-Val-Lys⁽⁺⁾-Val-Thr-Leu) contains alternating

hydrophobic (underlined) and hydrophilic residues. We speculate that this region may form an amphipathic β -strand in which one face of the peptide is hydrophilic and the other face is hydrophobic and could, perhaps, contribute to the tight binding to the phenyl-Sepharose resin. The hydrophobic face does not appear to bind to cellular membranes *in vivo*, but rather it may participate in protein-protein interactions. For example, this motif may contribute to the observed dimerization of the UreE peptide.

Nickel-binding properties of UreE

Dimeric UreE binds ~6 nickel ions in a highly specific manner. The average coordination sphere of UreE-bound nickel ions appears to be comprised of three to five histidine imidazole groups with additional nitrogen or oxygen donors. If one assumes that any one imidazole can ligate a single metal, then dimeric UreE would be able to bind 4–5 nickel ions at the carboxyl-termini. The fact that additional nickel ions were bound demonstrates that other parts of the UreE structure are essential for ligating a portion of the nickel. The peptide possesses five additional histidine residues at positions 91, 96, 109, 110, and 112. The presence of distinct nickel-binding sites within UreE is consistent with the cobalt, copper, and zinc competition studies that indicate preferential competition with subsets of the total nickel. The *in vivo* effects of cobalt, copper, and zinc ions on urease metallocenter assembly are unknown; however, Lee et al. (1990) demonstrated that these cations cannot substitute for nickel during urease biosynthesis in the absence of nickel ion.

Possible role of UreE in urease metallocenter assembly

Although our results clearly demonstrate that the carboxyl terminus of *K. aerogenes* UreE can bind nickel ions, we have not established that this equilibrium process is important for incorporation of nickel into urease. Indeed, the carboxyl-termini of UreE peptides from *Proteus mirabilis* (Jones & Mobley, 1989) and *Helicobacter pylori* (Cussac et al., 1992) possess eight and only one histidine, respectively, and only one of the five non-carboxyl-terminal histidines is conserved in the *H. pylori* peptide. It is possible, however, that some other protein could functionally compensate for a UreE protein that was deficient in nickel-binding ability. Consistent with this view, deletion of most of the *ureE* gene from a plasmid containing the urease genes does not abolish all urease activity in recombinant *E. coli*, but only reduces the activity levels and correspondingly reduces the nickel content (Lee et al., 1992). Potential candidates for nickel-binding proteins that could substitute for UreE include auxiliary peptides that are essential for hydrogenase biosynthesis (hydrogenase is another nickel-containing enzyme [Haus-

inger, 1987]). For example, ORF5 in the *Azotobacter vinelandii* *hox* gene cluster possesses a 13-residue region containing 10 histidines (Chen & Mortenson, 1992) and ORF4 in the *Rhodobacter capsulatus* *hup* gene cluster possesses a region that contains 23 histidines in 62 residues (Xu & Wall, 1991). The roles of these hydrogenase-related genes have not been elucidated but they are related in sequence to *hypB*, an *E. coli* gene that is known to function in hydrogenase activation involving nickel incorporation (Lutz et al., 1991).

UreE is only one of the four accessory proteins involved in urease metallocenter assembly. Nothing is known about the functions of the *ureD* and *ureF* gene products in this process. Similarly the role of UreG has not been demonstrated; however, sequence analysis reveals a P-loop motif (Saraste et al., 1990) that is found in a variety of ATP- and GTP-binding proteins. Furthermore, an energy dependence for *in vivo* nickel ion incorporation was observed (Lee et al., 1990). These features, together with the above UreE-related results, lead us to hypothesize that UreE may bind nickel and act as a nickel donor to the urease apoprotein, whereas UreG binds ATP or GTP and couples its hydrolysis to the nickel incorporation process. Further experiments to test and expand this hypothesis are currently under investigation.

Materials and methods

Bacterial strains and growth conditions

Klebsiella aerogenes CG253 was transformed with plasmid pKAU19 (Mulrooney et al., 1989), and *E. coli* DH5 was transformed with pKAU17 (Mulrooney et al., 1989) or pKAU17 Δ ureE-1, a *ureE* deletion mutant (Lee et al., 1992). Recombinant *K. aerogenes* cells were grown at 37 °C in MOPS-glutamine medium (Mulrooney et al., 1989) containing 100 μ M nickel chloride and chloramphenicol (30 μ g/mL). Recombinant *E. coli* cells were grown at 37 °C in LB medium containing 1 mM nickel chloride and ampicillin (50 μ g/mL) as previously described (Lee et al., 1992).

Purification of UreE protein

Cultures (3 L) of *K. aerogenes* CG253(pKAU19) or *E. coli* DH5 (pKAU17) were grown to late exponential phase (optical density at 600 nm = 3.5) and harvested by centrifugation. The cells were washed twice with PEB buffer, resuspended in an equal volume of PEB buffer containing 0.5 mM phenylmethylsulfonyl fluoride, disrupted by three passages through a French pressure cell (American Instrument Co.) at 18,000 lb/in.², and centrifuged at 100,000 $\times g$ for 90 min at 4 °C. The cell extracts were chromatographed on a DEAE-Sepharose column (2.5 $\times$ 15 cm) at 4 °C, in the same buffer and eluted with a 400-mL linear salt gradient to 1 M KCl. UreE eluted from

the column at approximately 0.3 M KCl. The pooled sample was desalted and concentrated by using an Amicon pressure filtration stirred-cell with a YM 10 ultrafiltration membrane in PEB buffer (pH 6.9), applied to a carboxymethyl-Sepharose column (2.5×17 cm), and eluted with a linear salt gradient to 0.5 M KCl. Nearly homogeneous UreE that was suitable for most experiments eluted approximately at 0.2 M KCl. For antibody preparation, however, this sample was further purified by using a Mono-S column (0.5×5 cm) and a linear gradient to 1 M KCl at room temperature. All resins and columns were purchased from Pharmacia-LKB. The presence of UreE protein in column fractions was assessed by polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out by using the buffers of Laemmli (1970) and included either a 12 or 15% polyacrylamide running gel or a 10–15% polyacrylamide gradient running gel with a 4.5% polyacrylamide stacking gel. Gels were stained with Coomassie brilliant blue.

Peptide structural calculations

Prediction of the UreE structural properties made use of a set of sequence analysis programs described by Devereux et al. (1984). In particular, the amphipathic moment was calculated by the MOMENT program, whereas the hydrophobicity and the potentials for forming secondary structures were calculated by the PEPTIDESTRUCTURE program.

Cellular localization by immunogold electron microscopy

Antibodies directed against UreE protein were generated in a white, female, New Zealand rabbit by injecting 200 μ L (2.5 mg/mL) of homogeneous protein in PBS emulsified with the same volume TiterMax adjuvant (CytRx corporation). The rabbit was boosted after 28 days, and after an additional 22 days, the IgG fraction was purified from the serum (McKinney & Parkinson, 1987). Antibodies were titrated by using standard dot blot (Cleveland et al., 1981) and ELISA methods (Engvall & Perlmann, 1972). For immunogold detection, wild-type *K. aerogenes*, *K. aerogenes* (pKAU19), *E. coli* DH5 (pKAU17), and *E. coli* DH5 (pKAU17 Δ ureE-1) were grown to stationary phase in LB medium supplemented with 1 mM nickel chloride. After centrifugation, the cells were washed once in 10 mM potassium phosphate, 1 mM EDTA (pH 7.0), and fixed in 0.1 M potassium phosphate (pH 7.2) containing 1% (v/v) glutaraldehyde for 30–60 min at room temperature. The fixed cells were resuspended in 1% (w/v) Noble agar, dehydrated in ethanol, and embedded in Lowicryl K4M

(Armbruster et al., 1982). Polymerization was carried out for 2 days at 6 °C under UV irradiation. Thin sections were cut by using an LKB Ultratome III microtome and placed on Butvar B-98-coated nickel grids. Sections were floated first on a drop of TBST for 5 min and transferred to 1 or 3% (w/v) bovine serum albumin in TBST for 15 min in order to block nonspecific binding. The samples were transferred to solutions containing anti-UreE IgG (200 μ g/mL) in TBST for 1 h, washed three times for 15 min each in TBST, and floated on gold particles that were attached to goat anti-rabbit IgG (15 nm, Jansen) for 1 h (Bendayan, 1984). After washing in TBST and H₂O, the samples were stained with uranyl acetate and lead citrate. Sections were observed with a Philips CM-10 electron microscope.

Native size and nickel content of UreE protein

The molecular weight for native *K. aerogenes* UreE protein was estimated by using a Superose 12 column (1.0×30 cm) in 20 mM potassium phosphate, 0.5 mM EDTA, 1 mM 2-mercaptoethanol (pH 7.4) containing 0.2 M KCl. The column was standardized with thyroglobulin, gamma-globulin, ovalbumin, myoglobin, and vitamin B₁₂ (M_r = 670,000, 158,000, 44,000, 17,000, and 1,350). Metal contents of ashed samples were determined by using a Varian Spectra AA-400Z atomic absorption spectrometer as previously described (Lee et al., 1992).

Protein determination

Protein concentrations were routinely assessed by the spectrophotometric assay of Lowry et al. (1951) using bovine serum albumin as a standard.

Equilibrium dialysis

Equilibrium dialysis of UreE with ⁶³NiCl₂ (1,455 mCi/mmol; Du Pont NEN Research Products, Inc.) diluted with various concentrations of unlabeled NiCl₂ was performed either in a Spectrum five-cell equilibrium dialyzer (Spectrum Medical Industries, Inc.) or an equilibrium microvolume dialyzer (Hoeffer Scientific Instruments) with precut dialysis membranes (MW cutoff = 12,000–14,000). Equilibrium dialysis metal ion competition studies could not be carried out in phosphate buffer for cobalt ion because of precipitation problems, so instead, HEPES buffer was used. Similarly, addition of copper to either phosphate or HEPES buffer led to precipitation, hence Tris-HCl buffer was used. Purified UreE (2 μ M) was analyzed for nickel binding in 50 mM sodium phosphate (pH 7.2), 50 mM HEPES (pH 7.2), or 50 mM Tris-HCl (pH 7.6) each containing 0.5% NaCl to reduce the Donnan effect. After a 3-h equilibration period at room temperature, radioactivity was measured in aliquots from each compartment by using a Beckman LS7000 liquid

scintillation system. Nickel ion binding to UreE in a control sample was not affected by either HEPES or Tris-HCl buffer. Calculation of the bound nickel was performed by standard procedures (Bell & Bell, 1988).

Spectroscopic characterization of UreE holoprotein

UreE (35 mg/mL) in the presence of 3.1 mM NiCl₂, 0.1 mM EDTA, 20 mM potassium phosphate buffer (pH 7.6), and 30 % glycerol was examined by X-ray absorption, UV-visible-near IR absorption, and VTMCD spectroscopies.

XAS data collection was performed at the SSRL with the SPEAR ring running at 3.0 GeV with an average current of 40 mA. Data reduction was performed using the XFPACK software package developed by one of us (R.A.S.) as described previously (Scott, 1985). Details of the data collection and reduction are summarized in Table 2. Curve-fitting analyses of the EXAFS data were performed using EXCURV86, which makes use of curved-wave single and multiple scattering (Strange et al., 1987). First-shell scattering functions were checked against model compound-tested FEFF v3.25 (Rehr et al., 1991) scattering function templates as described previously (Scott et al., 1992), yielding $\Delta E_0 = 31.0$ eV, this value being fixed in all subsequent curve fitting. (The amplitude reduction factor, AFAC, was also fixed at 0.9 for all fits.) A constrained group refinement procedure was used in fitting histidinyl imidazoles. The imidazole ring was treated

as a rigid group with an averaged structure taken from crystallographic data on a number of metal-imidazole compounds. The Ni was forced to lie in the plane of the imidazole ring and only the Ni-N₁ distance and the Ni-N₁-C₂ angle were allowed to change. The position of the Ni-(C₂,C₅) FT peak at ~ 3 Å in test simulations was compared to the experimental FT to set the Ni-N₁ distance. Tilted structures with the Ni-N₁-C₂ angle set at ± 5 and $\pm 10^\circ$ from that for a symmetrical imidazole were tested to match both the ~ 3 -Å (Ni-(C₂,C₅)) and ~ 4 -Å (Ni-(N₃,C₄)) experimental FT peaks. Once the positioning of the (average) imidazole ring was set in this way, integral coordination numbers were chosen, and the Debye-Waller factors for each shell were varied to optimize the fit (with single-scattering contributions from any other required ligands included). Possible coordination numbers of histidyl imidazole ligands were selected from fits that generated physically reasonable Debye-Waller factors for these outer shells, as determined by test fits on a number of metal-imidazole compounds. In a number of metal-imidazole model compounds, we find that the following Debye-Waller factors are typical: M-N₁, $\sigma_{as}^2 = 0.003$ – 0.006 Å², M···(C₂,C₅), $\sigma_{as}^2 = 0.006$ – 0.012 Å²; M···(N₃,C₄), $\sigma_{as}^2 = 0.010$ – 0.016 Å².

UV-visible-near IR absorption spectra were recorded using a Shimadzu UV3101PC spectrometer fitted with an integrating sphere detector to decrease background scattering in the UV region. VTMCD measurements over the temperature range 1.5 K to 300 K in the UV-visible-near IR region were carried out at a magnetic field of 4.5 T using an Oxford Instruments SM3 superconducting magnet mated to either a Jasco J500C (200–1,000 nm) or J720 (700–2,000 nm) spectropolarimeter. The experimental protocols used in the VTMCD measurements have been described in detail elsewhere (Johnson, 1988). Samples for VTCMD studies were diluted with an additional 50% (v/v) glycerol to ensure formation of a glass on freezing. $\Delta\epsilon$ Values are based on the Ni(II) concentration.

Table 2. X-ray absorption spectroscopic data collection and reduction for UreE samples

SR facility	SSRL
Beamline	VII-3
Monochromator crystal	Si(220)
Energy resolution, eV	~ 2
Detection method	Fluorescence
Detector type	13-Element solid state array ^a
Scan length, min	21
Scans in average	20
Metal concentration, mM	3.1
Temperature, K	10
Energy standard	Ni foil (1st inflection)
Energy calibration, eV	8,331.6
E ₀ , eV	8,350
Pre-edge background	
Energy range, eV (polynomial order)	8,400–9,040 (2) ^b
Spline background	
Energy range, eV (polynomial order)	8,375–8,500 (2)
	8,500–8,750 (3)
	8,750–9,040 (3)

^a Maintained by NIH Biotechnology Research Resource at SSRL.

^b Background was calculated from fitting this (EXAFS) region, then a constant was subtracted so that the background matched the data just before the edge.

Crystallography

Crystallization of UreE apoprotein was achieved by the sitting-drop method in sealed crystal growth chambers (Charles Supper Company, catalogue no. MVD/24). Ten-microliter sitting drops consisted of: 5 μ L of 8–10 mg/mL protein solution containing 20 mM KH₂PO₄-HCl buffer at pH 7.8, 0.5 mM EDTA, 1 mM dithiothreitol, and 5 μ L of reservoir solution containing 1.0 M Li₂SO₄, 100 mM *N*-(2-acetamido)-iminodiacetic acid-LiOH at pH 6.3, and 10% methanol. Droplets were equilibrated against 1.0–1.5 mL of reservoir solution at room temperature. Hexagonal bipyramid crystals first appeared in these droplets within 2 weeks, and crystals grew to maximum size of $0.5 \times 0.5 \times 0.4$ mm within 4 weeks.

Preliminary diffraction analysis of these crystals was carried out by using a Siemens X-100A multiwire area de-

tector mounted on a Rigaku RU-200 rotating anode X-ray generator (powered at 45 kV, 65 mA) with the software BUDDHA (Durbin et al., 1986).

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