Crystal Structures of the Liganded and Unliganded Nickel-binding Protein NikA from Escherichia coli*

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Bacteria have evolved a number of tightly controlled import and export systems to maintain intracellular levels of the essential but potentially toxic metal nickel. Nickel homeostasis systems include the dedicated nickel uptake system nik found in Escherichia coli, a member of the ABC family of transporters, that involves a periplasmic nickel-binding protein, NikA. This is the initial nickel receptor and mediator of the chemotactic response away from nickel. We have solved the crystal structure of NikA protein in the presence and absence of nickel, showing that it behaves as a “classical” periplasmic binding protein. In contrast to other binding proteins, however, the ligand remains accessible to the solvent and is not completely enclosed. No direct bonds are formed between the metal cation and the protein. The nickel binding site is apolar, quite unlike any previously characterized protein nickel binding site. Despite relatively weak binding, NikA is specific for nickel. Using isothermal titration calorimetry, the dissociation constant for nickel was found to be ~10 μM and that for cobalt was approximately 20 times higher.

Nickel is a transition metal with appreciable affinity for oxygen, nitrogen, and sulfur. Its biological role in animals remains obscure, although it has been implicated in both metabolism and reproduction of mammals and birds (1, 2). The biology of nickel is much better understood in bacteria. Its properties allow it to bind both DNA and proteins and disrupt many cellular functions when present in excess (3). Several bacterial nickel efflux systems have been described that confer resistance to high levels of the metal (4, 5). At the same time, bacterial nickel efflux systems have been described that confer resistance to high levels of the metal (4, 5). At the same time, bacteria must be able both to import and export nickel to maintain an appropriate non-toxic concentration in the cytoplasm. Seven enzymes are known that depend on nickel (6) and is essential for anaerobic metabolism (7), so several enzymes and signaling peptides. An analysis of the genome locus is dependent on the nickel-containing enzyme urease (EC 3.5.1.5). A number of accessory proteins are required for the production of active urease including the nickel metallochaperone UreE (9, 10). Nickel incorporation in Escherichia coli hydrogenases also requires a GTP-binding accessory protein, HypB (11). The protein sequences of a number of microbial nickel uptake systems have been described (12–14), but to date no structural information has been obtained. Nickel uptake by bacteria is of interest for bioremediation and water purification as well as its biological function and involvement in pathogenesis.

The nickel transporter of E. coli encoded by the nik operon was originally discovered by the loss of hydrogenase activity in mutants generated using the MudI transposon (15, 16). The addition of 0.5 mM nickel led to the full recovery of hydrogenase activity, suggesting that the mutants were defective in nickel transport. This was later demonstrated directly in nickel transport experiments using 63Ni2⁺ (17). Sequencing the nik locus showed five open reading frames, nikA-E-encoding proteins with significant similarity to the ABC-type dipeptide and oligopeptide import systems (18). ABC transporters in Gram-negative bacteria such as E. coli consist of three components: integral membrane proteins that create a pore through the inner membrane, membrane-associated ATP-hydrolyzing proteins, and a periplasmic binding protein (PBP).1 These transporters allow the cell to import selectively the available nutrients and signaling peptides. An analysis of the E. coli genome suggests that it encodes 44 import systems and 13 export systems in the ABC family (19). The crystal structures of more than a dozen different periplasmic binding proteins have been determined and show a number of common features (20). These proteins serve as the initial receptor for their respective ligands, which diffuse freely through the outer membrane of Gram-negative bacteria. In Gram-positive bacteria, these proteins are tethered to the cell by lipophilic tails (21). Periplasmic binding proteins vary in size from 25 to 59 kDa, the largest being the oligopeptide-binding protein OppA, which shows considerable sequence similarity to NikA. NikA is expressed as a pre-protein 524 amino acid residues long. The N-terminal 22 residue leader sequence directs translocation to the periplasm where it is removed to leave a 502 residue mature protein with a molecular mass of 56.3 kDa (17).

Overall, solute-binding proteins show little sequence similarity but do show a number of conserved features. The two relatively rigid halves are connected by a hinge, which closes on substrate binding, completely enclosing the substrate in a manner often compared with a Venus fly-trap. The large conformational change has been confirmed for several solute-binding

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1 The abbreviations used are: PBP, periplasmic binding protein; DTT, dithiothreitol; PDB, Protein Data Bank.
proteins by x-ray scattering in solution (22). Until 1998, all of the known PBP structures could be classified into two groups depending on the topology of the connection between the two lobes (23). The crystal structures of two metal binding proteins in the solute-binding protein family, however, show a very different connection between the two domains, forming a new family of metal binding receptors (24). TraA is a zinc-binding protein from Treponema pallidum (25, 26), and PsaA is a manganese/zinc-binding protein from Streptococcus pneumoniae (27). In these proteins, the polypeptide chain passes from one domain to the other only once, this connection being a long helix running most of the length of the protein. Unlike the β strands, which connect the lobes of most solute-binding proteins, the helix is rigid and permits only a very small conformational change on ligand binding. It has been suggested that this is necessary for tight metal binding given the small ligand size (26). The crystal structure of a molybdate-binding protein from Azotobacter vinelandii, however, shows a more usual topology with a flexible hinge (28).

OppA and DppA (the E. coli dipeptide-binding protein) are nearly twice the size of the smallest members of the PBP family, the extra residues forming a highly conserved domain, which is shared with NikA (29, 30). OppA is the most unspecific PBP, being able to bind short peptides with relatively little side-chain preference (31, 32). In contrast, most solute-binding proteins are highly selective and show dissociation constants of around 1–0.1 μM for their ligands. Two previously published reports (33, 34) suggest very different values for the Ka for Ni²⁺ of NikA. We have solved the crystal structure of the protein in both liganded and unliganded forms and determined the binding constants for Ni²⁺ and Co²⁺ using titration calorimetry.

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification**—The region of the nikA gene encoding mature NikA protein was cloned by PCR from E. coli JM109 chromosomal DNA and inserted into the isopropyl-1-thio-galactopyranoside-inducible expression vector pET28b (Novagen) using Ncol and Xhol restriction sites. The nucleotide sequence of the gene was further purified was necessary, NikA was passed down a Hiload 26/60 Superdex 200 gel filtration column (Pharmacia) at a flow rate of 1 ml/min. The protein was eluted with a descending ammonium sulfate gradient and 100 mM sodium chloride. Thin plates appeared within 1 week but took several months to thicken sufficiently for data collection. Apo-NikA crystallized in space-group P2₁ (two molecules per asymmetric unit) as triangular prisms, which diffracted to 1.85 Å. Multiwavelength data were collected to 2.5 Å from a selenomethionine-containing crystal at beamline BL44B2 at SPring8 (Harima, Japan). The anomalous signal from the selenium atoms was used to derive phases using SOLVE and RESOLVE programs (36, 37).

**Refinement**—The apo-NikA protein model was built using ARPwARP (38) and TURBO (39) programs. REFMAC (40) was used for refinement, and the CCP4 package (41) was used for general data manipulation. The nickel-bound structure was solved by molecular replacement using AMoRE (42) with two separate halves of the nickel-free model cut at the hinge region. This provided clear solutions at both the rotation and translation steps in space group P2₁2₁2₁. A complete molecule built from the two halves was then used as a search model in MOLREP (43) to find both complete molecules in the asymmetric unit. Data collection and refinement statistics are shown in Table I. Overall, the quality of the maps is excellent. An analysis of the structures was carried out with XTALVIEW (44). Fitting regions of the refined structures to each other was carried out with the program FIT written by Dr. Guoguang Lu (Purdue University). The center of gravity and principal rotation axes of models were found using AMoRE (42). Coordinates and x-ray data have been deposited in PDB with entry codes 1uiu (unliganded) and 1uiv (liganded).

**Analytical Ultracentrifugation**—Experiments were carried out using a Beckman XL-I instrument using an AnTi 60 rotor. All of the experiments were carried out at 20 °C. Sedimentation velocity data were collected at 40,000 rpm and analyzed with SEDFIT (45) to yield plots of concentration versus sedimentation coefficient. Buffer density and protein partial molar volume were estimated using SEDNTERP (46). Sedimentation equilibrium data were collected at 9, 12, and 16 k rpm using six-channel centerpieces and three different concentrations of protein between A₂₈₀nm of 0.25 and 1.0. The buffer used was 0.1 M Tris-Cl, pH 7.5, and 0.1 M sodium chloride. Nickel chloride (where present) was used at a final concentration of 1 mM. Absorption and interference data were analyzed separately using the manufacturer’s software.

**Isothermal Titration Calorimetry**—Experiments were performed using a CSC 4200 instrument (Calorimetry Sciences Corporation). NikA was purified using a final gel filtration step with EDTA- and DTT-containing buffer to remove all of the traces of heavy metal ions. The protein was then extensively dialyzed against 50 mM HEPES, pH 7.0, and 100 mM sodium chloride. The NikA concentration used was typically 90 μM, and all of the experiments were carried out at 25 °C. 25 injections were made of 10 μl of the same buffer containing 1 mM nickel chloride, cobalt chloride, or calcium chloride. Blank runs with no protein present were used to measure the background dilution heats. Each experiment was repeated three times, and the results were analyzed with the manufacturer’s software.

**RESULTS**

**Structure Determination**—Crystallization of the protein was carried out using the hanging drop method in both the presence and absence of nickel. Nickel-free NikA crystallized in space group P2₁2₁2₁ as triangular prisms, which diffracted to 1.85 Å. Molecular replacement using the OppA model proved impossible, not surprisingly, given the variable extent to which the hinge may open. Phases were therefore derived by multi-wavelength anomalous dispersion using selenomethionine-substituted protein. A nearly complete model could be built automatically from the experimental phases after density modification. Excellent electron density was derived from the trigonal
Table I

| Data collection and phasing statistics | Native | Nickel complex |
|---------------------------------------|--------|----------------|
| Space group/unit cell (Å)             | P3₃/a = b = 126.9, c = 60.5 | P2₁2₁2₁ = 69.7, b = 192.8, c = 75.1 |
| Reflections (measured/unique)         | 20.0±1.85  | 20.0±1.85 |
| Completeness (overall) (%)            | 91.1/78.6  | 94.6/72.1 |
| Redundancy (overall)                  | 4.5/38.4  | 4.1/11.4 |
| Mean (I/Σ(I)) (overall)               | 2.5  | 4.2 |
| σ Cut-off reflections used             | 17.5  | 18.7 |
| R-factor/free R-factor (%)            | 0.1/24.9  | 20.4/25.5 |
| Root mean square deviation bond       | 0.023/2.0  | 0.023/2.1 |
| Water atoms                           | 553  | 504 |
| Average B-factor (protein/water/Ni) (Å²) | 34/29  | 26/30/35 |
| Ramachandran plot                     | 89.4  | 91.7 |
| Residues in most favorable regions (%)| 10.0  | 7.8 |
| Residues in additional allowed regions (%) | 0.6  | 0.5 |
| Root mean square deviation bond angles (Å) | 0.023/2.0 = 0.023/2.1 |

a Completeness and R_mean are given for overall data and for the highest resolution shell. The highest resolution shells for the native, nickel complex, and multi-wavelength anomalous dispersion datasets are 1.92–1.85, 2.02–1.95, and 2.59–2.50 Å, respectively.
b R_mean = Σ Iᵢ / (Iᵢ + 2Σ₁, where Iᵢ is intensity of an observation and 〈I〉 in the mean value for that reflection. Summations are over all equivalents. R-factor = Σ [||Fᵢo|| - ||Fᵢc||] / Σ ||Fᵢo||, where Fᵢo and Fᵢc are the observed and calculated structure factor amplitudes, respectively. The free R-factor was calculated with 5% of the data excluded from the refinement.

apoNikA crystals using the anomalous signal from 10 selenium atoms/monomer. Residues 1–3 at the N terminus and 500–502 at the C terminus are not visible in the final 1.85-Å electron density map. All of the residues number refers to the mature protein.

In the presence of nickel, orthorhombic crystals were grown, which diffracted to 1.95 Å. Clear systematic absences were observed along the h00 and 0k0 axes, but the possibility of space group P2₁2₁2₁ could not be ruled out completely from the inspection of the diffraction pattern alone. The open apoform of the protein showed clearly that the protein consists of two lobes, residues 4–245 and 471–499 forming lobe I and residues 246–470 forming a contiguous domain, lobe II. Molecular replacement using these separate lobes allowed the nickel-bound orthogonal crystal form to be solved very rapidly in space group P2₁2₁2₁. On structure refinement, it became clear that a mutation had occurred at the surface residue Glu-361, which has been replaced by an arginine. Resequencing all of the clones from the original PCR showed that one of these clones has a single base mutation in this codon. Although the mutation is 30 Å from the nickel binding site, fresh wild-type protein was prepared for nickel binding studies. Isothermal titration calorimetry was carried out with both wild-type and mutant NikA. The crystal structures are of the mutant protein.

Overall Shape—The closed nickel-bound form of NikA has a flattened tear shape highly similar to DppA and OppA. A DALI (47) search of PDB with apoNikA revealed high similarity to DppA (PDB code 1dpe; Z-score 34.3) and OppA (PDB code 1jev; Z-score 29.0) but much poorer scores for other structures. The overall structure of the protein is shown in Fig. 1. Of the 27 PDB structures found by DALI, only two others were PBPs, the lysine-arginine-ornithine-binding protein and molybdate-binding protein, with Z-scores of 3.2 and 2.2, respectively. Because there are two copies per symmetric unit for both crystal forms of NikA, there are four ways of matching a liganded and unliganded monomer. Least-squares fitting the 271 Ca atoms of lobe I in the apoform and bound form gave root mean square deviations between 0.56 and 0.99 Å. The largest shift is found for a small loop around Leu-172, which appears to be flexible. Subsequently, overlapping the 225 lobe II Ca atoms gave root mean square deviations between 0.60 and 0.75 Å. The rotation between lobes was between 12.2 and 17.5°, and the translation in each case was <0.3 Å. The conformational change is therefore very close to a pure rotation of one rigid lobe relative to another. A stereoview of the superimposed apoprotein and nickel-bound structure is shown in Fig. 2. This rotation is quite modest compared with other PBPs but much larger than the 4° observed in the case of the zinc-binding protein TroA (25, 26). The hinge motion in NikA is similar to those in OppA and DppA but smaller (30, 48). OppA for example shows a hinge opening of 26° in the crystal structure of the open unliganded form (48). The apoNikA crystal structure suggests no reason why, in solution, the hinge motion could not be much larger. Although to a good approximation, the two lobes behave as rigid bodies, some residues do show significant side-chain movements. The largest changes in side-chain conformation involve Asn-220, Glu-221, Glu-247, and Arg-396. Hinge closure on nickel binding brings Arg-396 close to Glu-221, pushing this residue toward the nickel binding site. Asn-220 forms an Asn-lysine-arginine-ornithine-binding protein and molybdate-binding protein, with Z-scores of 3.2 and 2.2, respectively. Because there are two copies per symmetric unit for both crystal forms of NikA, there are four ways of matching a liganded and unliganded monomer. Least-squares fitting the 271 Ca atoms of
The nature of the binding site (shown schematically in Fig. 4) is quite unlike previously described nickel (or metal ion) binding sites in proteins. NikA forms no direct coordination bonds with the nickel ion but hydrogen-bonds to a coordinating water while bound to NikA was unexpected because it must be protonated to form a hydrogen bond with a nearby buried carbonyl group. Despite the nearby Arg-137 side chain, the binding pocket overall is calculated to have a negative electrostatic potential (Fig. 5). The binding site residues show little side-chain movement on nickel binding with two exceptions being Arg-97 and Glu-221, which both point toward the nickel ion (Fig. 6). Two tryptophan residues (Trp-100 and Trp-398) line the binding site, which explains the intrinsic fluorescence change on nickel binding, although this is small (data not shown).

Analytical Ultracentrifugation—Analytical centrifugation was used to check the oligomeric state of the protein for crystallization studies. Sedimentation coefficient distributions from velocity experiments showed clearly that the protein is almost entirely a single species in solution but with a small amount (roughly 2%) moving with a sedimentation coefficient approximately twice that of the main peak. The molecular masses derived from fitting a single ideal species model to the sedimentation equilibrium data were 55,160 and 55,908 Da for the apoprotein and nickel-bound protein, respectively. These values are within 3 and 1% of the expected value, 56,302 Da. Preliminary solution x-ray scattering data, collected to very low resolution using a rotating anode x-ray generator, suggest a similar shape for the liganded and unliganded protein, but higher resolution data are needed to observe the small shape change found between the two crystal forms of NikA.

Isothermal Titration Calorimetry (ITC)—Two previous reports in the literature suggest very different values for the affinity of nickel binding to NikA. de Pina et al. (33) used the intrinsic fluorescence to measure \( K_d \) and found a value of 0.1 \( \mu \)M. Salins et al. (34) used a fluorophore to enhance the signal and found that NikA could detect nickel down to 10 nM concentration. Although they did not explicitly give a \( K_d \) value, the binding curve they present shows a \( K_d \) of approximately 10 \( \mu \)M. We have used ITC to measure the binding of Ni\(^{2+}\) and Co\(^{2+}\) ions to NikA (Fig. 4). In both cases, the observed enthalpy of binding was small, but the enthalpy and free energy of binding could be determined reproducibly. Consistent with previous findings, the ITC results show NikA to bind nickel selectively over cobalt. The \( K_d \) for Ni\(^{2+}\) binding was found to be 11 \( \mu \)M \( \pm \) 1.7 \( \mu \)M, and the \( K_d \) for Co\(^{2+}\) was found to be 246 \( \pm \) 29 \( \mu \)M. Ca\(^{2+}\) showed no detectable binding. The \( K_d \) value derived from ITC agrees well with the result of Salins et al. (34) but not that of de Pina et al. (33). We also attempted to determine the \( K_d \)
using intrinsic fluorescence, but in our hands the signal was too small to estimate $K_d$ reliably (data not shown). ITC results for both the wild-type and Gln-361/Arg NikA were in close agreement. Fitting the data with no restraints suggested a stoichiometry of binding of 0.73 mol of Ni$^2+$ to 1 mol of NikA. The error is probably due to the calculated concentration of NikA using a calculated extinction coefficient of 75,530 M$^{-1}$ cm$^{-1}$ (Fig. 7).

**DISCUSSION**

From a chemical viewpoint, nickel has both hard and soft properties. From a biological viewpoint, it is both useful and dangerous. *E. coli* requires nickel for glyoxalase I activity (50) as well as hydrogenases expressed when oxygen is scarce (16, 18). Transcription of the *nik* operon is increased under anaerobic conditions by the fumarate and nitrate regulatory protein FNR and decreased by the DNA-binding protein NikR in the presence of excess nickel (51). NikR has been the subject of several studies showing both high affinity nickel binding and a change in coordination geometry on nickel-NikR binding to operator DNA (52–54). Interest in bacterial nickel-binding proteins has been fueled by the realization that nickel deprivation may be a useful method of inhibiting bacterial growth or pathogenicity (55). Periplasmic binding proteins also have potential as biosensors for their respective ligands (56), and NikA has been touted a possible biosensor for nickel (34). Genetically engineered bacteria with en-
hanced nickel uptake properties also show promise for bioremediation (57).

The nature of the NikA binding site was completely unexpected, even though the solute-binding proteins have an established history of surprising interactions. The phosphate-binding protein was found to have a binding site with marked negative charge (58), and the molybdate-binding protein was found to be specific through size selectivity (28). Given the absence of cysteines from the mature protein, it was expected that at least one of the ten histidines in the sequence would coordinate to the nickel ligand. In fact, uniquely among all of the nickel-binding proteins described to date, NikA forms only indirect bonds with the metal ion. NikA achieves selective binding using a hydrophobic pocket shaped to fit a penta-coordinate-hydrated nickel ion. In general, the first row metals prefer tetrahedral geometry over square planar due to size considerations but ions with incomplete d shells (such as Ni^{2+}, a d^8 species) may prefer a planar conformation with small, strong field ligands due to the stronger d orbital splitting. Higher coordination is more stable (where sterically permissible) because more bonds are formed but with decreasing return since each metal-ligand bond is weakened by increasing coordination. The nickel repressor protein NikR has been shown to have square planar or octahedral coordination depending on the binding to operator DNA (52). For nickel, the transition from blue, paramagnetic octahedral complexes to yellow, diamagnetic, square planar complexes is a temperature-, pH-, and ionic strength-dependent equilibrium. The structure of urease
shows that the two active site nickel ions are penta- and hexa-coordinate (59). Co³⁺ ions (and low spin d₆ ions in general) greatly prefer octahedral geometry. The arginine side chain at the binding site will also repel metal ions, which do not fit snugly, and prefer divalent to trivalent cations. Explaining the selectivity for Ni²⁺ over Co³⁺ is more difficult. Ligand binding appears to be driven by cation–π interactions with Trp-100 and Trp-398. These interactions are estimated to provide 1–4 kcal/mol (60) so that the two tryptophans could provide the entire binding energy. Rather than use imidazole ligands to which cobalt will bind strongly, NikA appears to use the coordination geometry of nickel to achieve selective binding.

The crystal structures of NikA described here show the protein to be a classical periplasmic binding protein rather than a member of the recently described metal binding receptors (24). Two other members of the NikA subfamily of PBPs have been solved, OppA and DppA (29, 30). DppA mediates the chemo- tactic response of E. coli toward dipeptides, but OppA has no such function. OppA is the most thoroughly characterized PBP (31, 32). The protein binds peptide ligands with affinities spanning at least three orders of magnitude (Kₐ = 10⁻³–10⁻¹⁰ M⁻¹). The absence of nickel coordinating side chains on NikA is consistent with the rather low ligand affinity determined by ITC. Further evidence for weak nickel binding was provided by the appearance of apoNikA crystals from hanging drops containing only a 2-fold molar excess of nickel over the protein at 7.5 mg/ml. A dissociation constant of 10⁻¹⁰ M would lead to a 13-fold reduction in Nickel NikA. Drinking water in the USA contains only a 2-fold molar excess of nickel over the protein at 7.5 mg/ml. A concentration factor of 13 is therefore sufficient to exert a retentive effect, reduces diffusion back out of the periplasm by a factor of (1–10⁻¹⁰)⁻¹. This value would be raised significantly by smaller estimates of the periplasmic volume. Silhavy et al. (61) showed by simple mathematical modeling that when a PBP is present in large excess of its ligand, it exerts a retentive effect, reduces diffusion back out of the periplasm by a factor of (1–P/Kₐ). Thus, a Kₐ for NikA binding to nickel of 10⁻¹⁰ M would lead to a 13-fold reduction in nickel efflux given 120 μM NikA. Drinking water in Europe and the United States has a nickel content of roughly 10⁻¹⁰ M. A concentration factor of 13 is therefore sufficient to present the inner membrane with roughly 0.1 μM nickel for uptake, even from purified water. It should be remembered too that the molecular crowding within the periplasm may be significant. Much has been made of the entropy loss to the PBPs on closing the hinge and adopting a single conformation. Where water activity is reduced by high concentrations of proteins and other macromolecules, the free energy cost of binding water on hinge opening may also be significant. Overall, a 10⁻¹⁰ M Kₐ for NikA is not inconsistent with nickel import and gives the inner membrane components of the system an easier task in pulling nickel into the cytoplasm than a 0.1 μM Kₐ. Weak binding by NikA also helps maintain ligand specificity of the nik permease for nickel over cobalt. Given 20-fold weaker binding of cobalt ions by NikA, the periplasmic level of cobalt is not expected to rise appreciably over that in the medium because of NikA binding. Further work is envisaged using a variety of biophysical methods.

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