Lrp is a global regulator that controls the expression of a large \textit{Escherichia coli} operons. The family is named after two proteins from Bacteria and Archaea but do not appear to be present in eukaryotic genomes. From the \textit{Neisseria meningitidis}, a causative agent of bacterial meningitis, has a relatively small repertoire of transcription factors, including NMB0573 (annotated AsnC), a member of the Lrp/AsnC family of regulators that are widely expressed in both Bacteria and Archaea. In the present study we show that NMB0573 binds to L-leucine and L-methionine and have solved the structure of the protein with and without bound amino acids. This has shown, for the first time that amino acid binding does not induce significant conformational changes in the structure of an AsnC/Lrp regulator although it does appear to stabilize the octameric assembly of the protein. Transcriptional profiling of wild-type and NMB0573 knock-out strains of \textit{N. meningitidis} has shown that NMB0573 is associated with an adaptive response to nutrient poor conditions reflected in a reduction in major surface protein expression. On the basis of its structure and the transcriptional response, we propose that NMB0573 is a global regulator in \textit{Neisseria} controlling responses to nutrient availability through indicators of general amino acid abundance: leucine and methionine.

\textit{Neisseria meningitidis}, the \textit{L-leucine-responsive regulatory protein (Lrp)}\textsuperscript{2}/AsnC family of transcription factors are widely distributed in both Bacteria and Archaea but do not appear to be present in eukaryotic genomes. The family is named after two proteins from \textit{Escherichia coli} implicated in the control of amino acid metabolism; the Lrp and AsnC, which share \textasciitilde 25\% sequence identity. Lrp is a global regulator that controls the expression of a large number of operons in \textit{E. coli}, including those involved in the synthesis and degradation of amino acids (1), whereas AsnC is a specific regulator of the \textit{asnA} gene, which codes for asparagine synthetase, responsible for converting aspartate to asparagine in an ATP-dependent reaction, and an autorepressor of its own expression (2). As the name implies, many Lrp-responsive operons are co-regulated by L-leucine that can either antagonize or potentiate the effects of Lrp (3). L-Leucine is one of the most common amino acids in proteins and the reciprocal effect of Lrp on amino acid metabolism suggests that intracellular Lrp abundance mediates transitions between “feast and famine” (3, 4). AsnC, on the other hand, appears to be responsible for controlling asparagine levels via negative feedback regulation of asparagine synthetase expression (2, 5).

Insight into the relationship between the structure of Lrp/AsnC family proteins and their function has come from x-ray crystallography. To date, four x-ray crystal structures from the family have been reported, two from Archaeal sources; \textit{Pyrococcus furiosus} LrpA (Protein Data Bank code 1I1G (6) and the close homologue, FL11, from \textit{Pyrococcus sp. OT3} (PDB code 1R17 (7) and two from bacterial sources: \textit{E. coli} AsnC in complex with asparagine (PDB code 2CG4) and \textit{Bacillus subtilis} LrpC (PDB code 2CFX) (8). The Lrp/AsnC family fold revealed by analysis of these structures consists of a N-terminal DNA-binding domain containing a helix-turn-helix motif connected to a C-terminal ligand binding domain (9), which has a \(\beta\alpha\beta\alpha\beta\) fold topology (6). The basic structural unit of Lrp/AsnC biological assemblies is dimeric, with the C-terminal \(\beta\)-sheet regions of each monomer interacting to generate a tightly bound \(\beta\)-cage structure. The association of the dimers into an octameric array is consistent with a model for DNA-protein interaction involving DNA being wrapped around the perimeter of the disc (7, 8). The ligand-bound structure of AsnC confirms an earlier prediction based on sequence alignments (9) and shows that the asparagine-binding site is formed at the interface between adjacent dimers. This results in eight L-asparagines bound in the AsnC octameric array (8).

In view of their potential role as both global and specific transcriptional regulators, we have targeted the Lrp/AsnC family proteins as part of a structural proteomics and transcriptional study of pathogenic \textit{Neisseria} spp. The genomes of \textit{Neisseria meningitidis} serogroups A (strain Z2491) and B (strain MC58) and \textit{Neisseria gonorrhoeae} (strain FA 1090) have been sequenced and each shown to contain two Lrp/AsnC family regulators (10). The two regulators exemplified by the gene

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\textsuperscript{2}The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

The atomic coordinates and structure factors (code 2PSV, 2P6S, and 2P6T) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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2 The abbreviations used are: Lrp, \textit{L-leucine-responsive regulatory protein}; r.m.s., root mean square.
products of NMB0573 (annotated asnC) and NMB1650 (annotated lrp) in N. meningitidis serotype B are each highly conserved between the two species of pathogen (≥99% sequence identity) and are 27% identical to each other. In this report we describe the cloning and expression of the NMB0573 gene, which we have designated as NMB0573. We have identified the amino acid ligands of the protein as methionine and leucine and have determined its structure by x-ray crystallography with and without bound amino acids. The regulatory function(s) of NMB0573 have been investigated by comparing the growth phenotype and transcriptomes of wild-type and knock-out strains of N. meningitidis. Analysis of microarray data indicates that NMB0573 is predominantly a global activator of gene expression. In light of these results, the annotation of NMB0573 as AsnC, implying that it is a specific regulator of asparagine synthetase, will need to be revised.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions—**N. meningitidis strain MC58 was used as the template for structural studies and for the phenotypic/transcriptional analysis. All experiments and the NMB0573 mutant were prepared using a strain in which the terminal sialotransferase (siaD; NMB0067) had been deleted, through insertion of an erythromycin cassette, to reduce its virulence and increase the safety of handling the strain in the laboratory. The mutant was prepared by replacing the whole of the coding region of NMB0573 with a kanamycin resistance cassette comprising the kanamycin cassette from puc4kan and the neisserial recA promoter. This was inserted into the EcoRI site within a cloning linker created between a 5′-flanking region amplified using primers: GTTCAAGTCCGGCCGGCG and ggggtacacattgatcgggATAT ATAAGTATTACCCTACTC- CGGGGAC and a 3′-flanking region amplified with primers ggggtacacattgatcgggATAT ATTATACCGTCATC- CCGGC and CAGCAGGTTGGTTCGTCAGCCGC. In each case the uppercase bases indicate the native sequence, and the lowercase bases represent the engineered insertion sites. Deletion mutants were obtained using natural transformation of linear DNA using standard techniques and selection on 50 mg/liter of kanamycin, followed by confirmation of the mutants by PCR and sequencing. Bacteria were grown on GC Agar (Difco Laboratories) containing the Kellogg supplements and 5 mg/liter ferric nitrate (11) at 37 °C, or 40 °C in air supplemented with 5% CO₂, overnight, or RPMI 1640 with 1-glutamine media (Invitrogen) supplemented with 1% bacterial agar and 5 mg/liter of ferric nitrate. Cells were harvested after 16 h of incubation at 37 °C in air supplemented with 5% CO₂. Liquid cultures used similar media without agar and with 0.0042% bicarbonate as a source of CO₂. Pre-cultures for growth curves were grown on the equivalent solid media, so that the bacteria would not need to adjust to a differing nutrient composition during the lag phase of liquid growth. Cultures for transcription profiling were prepared from solid media, grown overnight, and harvested from plates grown typically for 16 h (while still in an active and rapid phase of growth). The meningococcal expression profiling was performed using RNA prepared from 8 independently grown biological replicate cultures, on 5 separate occasions/batches of media. Two data sets were excluded due to a large global transcriptional change in one comparison and poor data quality and number of reported genes in another, leaving six independent biological replicates for analysis.

**cDNA Generation, Labeling, and Microarray Hybridization—**

Total RNA was prepared as described in Ref. 12. Reagents and enzymes for the preparation of materials for microarray hybridizations were sourced from the 3DNA Array 900 MPX kit (Genisphere, PA) unless otherwise stated. Three to 5 μg of RNA was reverse transcribed into unlabeled cDNA using SuperScript III reverse transcriptase (Invitrogen) at 42 °C for 2 h. The cDNA was cleaned using a Clean & Concentrate-5 column (Zymol Research) and poly-T tailed with terminal deoxynucleotidyl transferase. Dye-specific capture sequences were ligated to the poly-T tails, and the tagged cDNAs were cleaned using a Clean & Concentrate-5 column. The pan-Neisseria microarray version 2 (13) containing probes to N. gonorrhoeae and N. meningitidis genes was used for these experiments. Microarray slides were pre-hybridized in 3.5× SSC, 0.1% SDS, and 10 mg/ml bovine serum albumin at 65 °C for 20 min, washed with water and isopropyl alcohol, dried with an airbrush, and pre-scanned to check for array defects. The capture sequence-tagged cDNAs were hybridized onto the microarray slide for 16 h at 60 °C in a SlideBooster with the power setting at 25 and a pulse/pause ratio of 3:7. Following the first hybridization, the slides were washed in 2× SSC, 0.2% SDS for 10 min at 60 °C, followed by washes at 2× SSC and 0.2× SSC for 10 min, each at room temperature. The slides were dried with an airbrush and hybridized with the Cy3 and Cy5 capture reagents at 55 °C for 4 h in a SlideBooster. The slides were again washed in 2× SSC, 0.2% SDS (10 min at 55 °C) followed by 10 min room temperature washes in 2× SSC and 0.2× SSC and dried with an airbrush. Dried slides were scanned using a ScanArray ExpressHT (PerkinElmer Life Sciences) using autocalibration.

**Microarray Data Analysis—**Images were analyzed using BlueFuse for Microarrays (BlueGnome). Spot data were extracted from images and manually flagged to remove artifacts before fusion. Fused data were filtered according to pON value (BlueGnome). The pON values associated with an absence of visible spot signals was determined for each slide, and used to eliminate the bias generated by the inclusion of un-hybridized spots in the statistical interpretation of the data. The results were then analyzed using BASE (14). Data were globally normalized to the median fold ratio of the central 60% of the data with an intensity of greater than 200 in both channels. The mean overall fold ratio for the combined normalized data from the biological replicates was determined using the MGH fold change algorithm. Statistical analyses were performed using both the Student’s t test and a local implementation of Cyber-T within BASE (using a sliding window size of 75, and a Bayes confidence estimate value of 15), and the confidence intervals were determined within a separate local BASE tool. Summary data of the changes discussed in the article are given in supplementary Table S1. The complete data are available in an interactive GBrowse data base online (www.combio.ox.ac.uk/data). The complete microarray details and experimental data have been deposited in the ArrayExpress data base (www.ebi.ac.uk/arrayexpress) with the accession number E-MEXP-925.
**Protein Production**—NMB0573 gene sequences were amplified from genomic DNA (N. meningitidis strain MC58) using the following pairs of primers (gene-specific sequences are in capital letters). NMB0573 forward primer 1: 5’-aagtctgtttcagggcccgATGTT- and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG- and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG-GTGTGGTAGGCG-3’ and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG-GTGTGGTAGGCG-3’ and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG-GTGTGGTAGGCG-3’ and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG-GTGTGGTAGGCG-3’ and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG-GTGTGGTAGGCG-3’ and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG-GTGTGGTAGGCG-3’ and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG-GTGTGGTAGGCG-3’ and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG-GTGTGGTAGGCG-3’ and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG-GTGTGGTAGGCG-3’ and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG-GTGTGGTAGGCG-3’ and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG-GTGTGGTAGGCG-3’ and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG-GTGTGGTAGGCG-3’ and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG-GTGTGGTAGGCG-3’ and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG-GTGTGGTAGGCG-3’ and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG-GTGTGGTAGGCG-3’ and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG-GTGTGGTAGGCG-3’ and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG-GTGTGGTAGGCG-3’ and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG-GTGTGGTAGGCG-3’ and the common reverse primer 5’-atgtctagaagcttCTATTCCTTCAGCAG-GTGTGGTAGGCG-3’. The resulting PCR products were inserted into the vector pOPINF (15) using In-Fusion® enzyme (Clontech). The expression constructs contained a N-terminal His tag and 3C protease cleavage site (16). Soluble expression was evaluated at small scale as described in Refs. 15 and 17 and large scale purification carried out as described in Ref. 18. Selenomethionine-labeled proteins were produced in the B834 (DE3) using SelenoMet media (Molecular Dimensions). Full incorporation of selenomethionine was confirmed by amino acids, a thermal shift assay using Sypro Orange dye (Molecular Probes) was performed (19, 20). Briefly, 20–μl reactions containing 0.2–0.5 mg/ml protein, Sypro Orange dye, and either buffer alone or amino acid solutions (0–30 mM) were heated from 20 to 90 °C in 0.5 °C steps in a real time PCR machine (iCycler iQ Real-Time Detection System, Bio-Rad). Each step was held for 15 s and fluorescent intensity was measured at excitation/emission wavelengths of 490/575 nm. The midpoint temperature of transition between folded and un-folded states (Tm) was obtained from the first derivative of the plot of fluorescence intensities at different temperatures. The ΔTm was calculated as the difference between the Tm of the protein in the presence of an amino acid compared with the protein alone. Assays were performed in triplicate.

**Crystallization and Data Collection**—NMB0573 protein (8.6 mg/ml) was crystallized following removal of the N-terminal His tag. An initial screen of 672 crystallization conditions was carried out using the sitting drop vapor diffusion method in 200-nl drop size (1:1 protein/precipitant ratio) prepared using a Cartesian Technologies pipetting system (21, 22). Crystals of NMB0573 were grown from 95 mM HEPES-Na, pH 7.5, 190 mM calcium chloride polyethylene glycol 400 (26.6%, v/v) and glyc- erol (5%, v/v). Crystals were optimized using the standard nanoliter optimization procedure as described (22). For soaking experiments, crystals were incubated in reservoir solution with either L-methionine or L-leucine (10 mM) for 5–30 min before mounting. Multiple wavelength anomalous dispersion data for selenomethionine-labeled NMB0573 crystals were collected to 2.0-Å resolution at beamline BM14, the ESRF (Grenoble, France). Following a fluorescence scan, 1060 images of 0.5° oscillation at the peak wavelength were collected. 180° of data were also collected at a remote wavelength. Subsequent data collections from crystals of NMB0573 incubated with amino acids were carried out at the ESRF beamline ID14.1. Crystals were flash frozen and maintained at 100 K under a stream of nitrogen gas during data collections. Indexing and integration of data images of NMB0573 and amino acid complexes were carried out with DENOVO and data were merged using SCALEPACK (23). The images of the complexes showed two diffraction lattices. In the case of the NMB0573-methionine complex, the two lattices were indexed and integrated separately and then merged together. The selenomethionine data were processed with HKL2000. The statistics for x-ray data are given in Table 1.

**Structure and Function of NMB0573**

| Data collection details | Unliganded NMB0573 (selenomethionine) | NMB0573/L-Met | NMB0573/L-Leu |
|-------------------------|--------------------------------------|---------------|---------------|
| ESRF beamline           | BM14                                 | ID14-1        | ID14-1        |
| Wavelength (Å)          | 0.979 (peak)                         | 0.935         | 0.934         |
| Space group             | P2                                    | P2_1          | P2_1          |
| Unit cell (a, b, c (Å); β (°)) | 65.07, 149.48, 77.72, β = 106.41  | 64.83, 149.68, 77.67, β = 106.01  | 65.39, 149.77, 77.85, β = 105.68  |
| Resolution range (Å)    | 30.0–2.0 (2.1–2.0)                    | 30.0–2.0 (2.1–2.0) | 30.0–2.0 (2.1–2.0) |
| Unique reflections       | 74,733 (2,860)                        | 35,471 (3,499) | 28,887 (2,007) |
| Completeness (%)         | 77.7 (29.9)                          | 99.9 (99.4)   | 91.2 (63.2)   |
| Redundancy               | 7.4 (3.4)                            | 11.5 (8.1)    | 2.7 (2.1)     |
| Average I/σ              | 20.2 (2.4)                           | 13.7 (1.2)    | 14.6 (1.8)    |
| Rmerge                   | 0.082 (0.424)                        | 0.061 (0.331) | 0.183 (0.653) |
| Refinement statistics    |                                      |               |               |
| Resolution range (Å)     | 30.0–2.0                             | 30.0–2.0      | 30.0–2.9      |
| No. of reflections       | 70,865/3,804                         | 33,226/1,751  | 28,166/1,454  |
| R-factor (Rwork/Rfree)   | 0.176/0.238                         | 0.232/0.305   | 0.234/0.300   |
| No. of atoms             | 9,780/761/138                        | 9,852/218/153 | 9,852/133/119 |
| R.m.s. bond length (Å)   | 0.008                                | 0.007         | 0.007         |
| R.m.s. bond angle (°)    | 1.1                                  | 1.0           | 1.1           |
| Mean B-factor (Å²)       | 47/53/54/63                          | 73/74/35/77   | 57/59/32/64   |

*Data are essentially complete to 2.3 Å.
*Rwork and Rfree are defined by r = Σ|Fobs|−|Fcalc|/Σ|Fobs|, where h, k, l are the indices of the reflections (used in refinement for Rwork; 5% not used in refinement, for Rfree).
*Mean B-factor for main chain, side chain, water, and other atoms.
The side chains identifiable. The partially built model by RESOLVE was rebuilt manually using the program O (26) and then refined with CNS (27) using simulated annealing and positional refinement with main chain NCS restraint followed by individual isotropic B factor refinement. There are apparent structural differences in some segments (residues 1–13, 22–50, and 78–82) among the 8 monomers, and these segments are not included in the NCS restraint. The NMB0573-L-methionine and NMB0573-L-leucine complexes were orientated and positioned in the unit cell using rigid-body refinement and the structures were then refined with CNS and REFMAC (28). The electron density maps for the bound L-methionine and L-leucine are shown in Fig. 4, e and f, respectively. The crystallographic refinement statistics for all three structures are given in Table 1. The atomic coordinates and structure factors have been deposited in the Protein Data Bank under the accession codes 2P5V, 2P6S, and 2P6T.

RESULTS
Protein Expression and Purification—From the sequence of the NMB0573 gene there are two potential start sites and although neither is preceded by a consensus Shine-Delgarno motif, i.e. AGG-AGG (29), multiple sequence alignment suggested that the second ATG is the authentic start site for the gene (Fig. 1). Sequences corresponding to both potential initiation codons were amplified from N. meningitidis genomic DNA and cloned into an expression plasmid containing a resident N-terminal His6 tag for detection and purification. Expression screening at small scale showed that whereas the
short-form version was produced as a soluble protein, expression of the longer version was not detected (data not shown). We conclude that the authentic start site is the second in-frame ATG and this version of the protein is presumed to be full-length. Production of the NMB0573 gene product as a Histagged protein was scaled up for structural studies. The protein was biosynthetically labeled with selenomethionine, purified, and characterized. The oligomeric state of the protein in solution was assessed by size exclusion chromatography and determined to be an oligomeric mixture including some octamers (calculated molecular mass of 145.6 kDa) (Fig. 2).

Effect of Amino Acids on Protein Thermal Unfolding—Lrp/AsnC family regulators appear to function as either nonspecific DNA scaffolding proteins, as specific or global regulators of amino acid metabolism, or both (e.g. B. subtilis LrpC). Their function appears to be modulated by amino acids and direct binding to the proteins has been demonstrated (8, 30). To investigate the binding of amino acids to the NMB0573 protein, a thermal shift assay was performed using Sypro Orange dye. The assay used a real time PCR machine to detect changes in fluorescence during thermal unfolding of the protein. The fluorescent dye Sypro Orange shows increased emission intensity in regions of low dielectric constant such as the hydrophobic regions exposed in unfolded proteins. Thus, as the reaction mixture is heated, the protein unfolds and there is an increase in fluorescence. Using this parallelized thermal shift assay (19), 11 potential amino acid ligands were screened for binding to NMB0573 at a concentration of 10 mM (Fig. 3), including alanine, asparagine, leucine, and valine each of which have been previously identified as Lrp/AsnC family effectors (1). Of the amino acids tested, L-methionine showed the largest effect on the unfolding transition temperature of NMB0573, with an increase in the midpoint temperature of 2.5 °C compared with the protein alone. L-Leucine also caused a measurable shift in the transition temperature, whereas the effect of other amino acids was generally less than ±1 °C (Fig. 3a). The stabilizing effect of L-methionine and L-leucine on NMB0573 was concentration dependent with both amino acids showing similar efficacies (Fig. 3, b and c). These data provide strong evidence for an interaction between NMB0573 and these amino acids. Interestingly, gel filtration of the protein in the presence of either L-methionine or L-leucine (10 mM) resulted in most of the protein migrating as octamers (Fig. 2). Together with the thermal shift data, these results suggest that binding of the amino acid ligands favors the assembly of the protein in an octameric state. On the basis of these results, the interaction between NMB0573 and L-methionine and L-leucine was investigated at the structural level (see below).

Overall Structure—The structure of the apo form of NMB0573 was determined using selenomethionine-substituted protein by multiple wavelength anomalous dispersion to 2.0-Å resolution. The subunits of the protein are made up of two domains connected by a single β-strand (β1). The N-terminal domain consists of three α-helices (α1, α2, α3) with a helix turn helix motif between residues 23 and 48 (Fig. 4, c and d). The C-terminal domain comprises a four-stranded anti-parallel β-sheet (β2–5) sandwiched between two α-helices (α4 and α5) plus a C-terminal β-stranded tail (Fig. 4, c and d). Examination of the molecular packing within the crystal lattice clearly shows that NMB0573 is assembled into an octameric disc, similar to those previously reported for PfLrpA (6), E. coli AsnC, and B. subtilis LrpC (8). N-terminal DNA-binding domains are located around the periphery of the ring, with the octamer formed as a tetramer of dimers (Fig. 4, a and b). The interac-
The structure of NMB0573. In (a), the overall structure, the polypeptide chains are drawn as coils and ribbons, and each monomer is individually colored. The 8 calcium ions in the un-liganded structure are shown as purple spheres. L-Methionines from the NMB0573-L-methionine complex are overlapped onto the un-liganded structure and shown as black spheres to mark the amino acid binding sites. The two amino acid binding sites between adjacent dimers are related by a 2-fold axis of symmetry such that four sites are presented on one face of the octameric array and four on the opposite face. b, is an overlay of the overall structure of NMB0573 with (red trace) or without bound methionine (gray trace). 100% of the two molecules can be overlapped with an r.m.s. deviation of 0.5 Å. Bound methionines are shown as ball and sticks (colored by atom) to mark the amino acid binding sites. In (c) and (d) the structure of NMB0573 is compared with E. coli AsnC. c, shows the Cα backbones of NMB0573 (red) and AsnC (gray) monomers overlaid with every 20 residues of NMB0573 marked by small green spheres and secondary structure features labeled; 75% Cαs of the two molecules can be overlapped with an r.m.s. deviation of 1.1 Å. Larger differences appear at the N-terminal domain (DNA binding domain) between residues 12 and 32 encompassing the α2 helices and in the C-terminal domain (effector binding domain) in the position of the α4 helices relative to the β2 strands. d, a ribbon diagram showing the superimposed dimers of NMB0573 (red and green) and AsnC (gray) with the secondary structure features labeled on one pair of dimers. e and f show the amino acid binding site of NMB0573, which comprises residues from three peptide chains, of which the backbones are shown as ribbons and coils and colored individually. The side chains of residues either lining the pocket or having any atom interact with the ligand are drawn as sticks and colored by atoms. Hydrogen bonds are shown as cyan dashed lines. Simulated annealing omit electron density maps contoured at 3.5σ for the bound L-methionine (e) and L-leucine (f) are shown as semi-transparent green surfaces.
tions that form the dimer are similar to the other Lrp/AsnC family proteins, in that a hydrophobic core is formed by residues in the four-stranded β-sheet (β2–5) of each subunit. The crystals were grown in the presence of calcium chloride (190 mM) and a single calcium atom was observed at the interface between each of the dimers of the protein coordinated by Asp-107. The biological significance of this is not clear from the structure.

Refinement of the structures of the NMB0573 protein obtained from crystals soaked with either L-leucine or L-methionine showed density corresponding to the amino acids, located in a cleft between adjacent dimers in the octameric array such that 8 amino acid molecules were bound in the overall structure. This region is close to the position occupied by calcium in the apo structure (see below). To investigate the effect of amino acid binding on the overall structure of NMB0753, the apo- and amino acid-bound structures were compared. The octamers of the apo- and L-methionine-bound NMB0573 were overlaid with a r.m.s. deviation for the Cα of 0.5 Å indicating that the structure of the assembly is largely unchanged following amino acid binding (Fig. 4b). A similar result was obtained for the L-leucine-bound structure (data not shown). Furthermore, overlay of the subunit dimer of the NMB0573 apo structure with that of AsnC from E. coli (8) showed a conservation of overall structure with a r.m.s. deviation for the Cα comparison of 1.1 Å. The major differences observed between the dimers of NMB0573 and AsnC were a shift in the position of the α4 helix relative to the β2 strand and the positions of the α2 helices (Fig. 4, c and d).

Amino Acid Binding—Although all of the eight binding sites in the octamer contained a bound ligand, examination of amino acid binding revealed some differences in the residues involved at each site. In all cases the peptide carbonyl oxygen and amide nitrogen of residues Thr-103 and Gly-104 flipped 180° so that L-methionine, and L-leucine formed H-bonds with the corresponding amide nitrogen and carbonyl oxygen of Gly-104 (Fig. 4, e and f). An additional H-bond was also formed between the amide nitrogen of Ser-139 and the carbonyl oxygen of L-methionine at all sites (Fig. 4e). In the E. coli AsnC structure, the corresponding residues, Gly-103 and Thr-139, were also observed to interact directly with the bound asparagine residue in the structure. The fact that the glycine and serine/threonine residues are conserved in all Lrp/AsnC family sequences confirms the key role of these residues in ligand binding (9). In some of the NMB0573 binding sites, either the carbonyl oxygen of Asp-107, the carbonyl oxygen of Thr-106, or both also contribute to the H-bonding network securing the amino acid in the pocket between the dimer subunits depending upon the exact positioning of the L-methionine residue. It is interesting to note that mutation of the equivalent of Asp-107 in E. coli Lrp (Asp-114) abolishes the response of Lrp to exogenous L-leucine (31) but not high affinity binding (30). Another difference between the binding sites is the presence or absence of a calcium atom, which was observed in 3 of the 8 sites coordinated by Glu-105, presumably displaced from its position in the apo structure by amino acid binding. Comparison of the apo- and amino acid-bound structures of NMB0573 indicates that the residues local to the binding site are largely unchanged. The only exception is the side chain of Arg-83, which points into the binding pocket in the apo structure and rotates to accommodate leucine or methionine binding in the amino acid bound structures (Fig. 5, a and b).

The thermal shift response of NMB0573 was specific for L-methionine and L-leucine with no effect observed for other amino acids, including asparagine, raising the question of what determines amino acid selectivity. Comparison of the structures of NMB0573 and AsnC of E. coli indicates that specificity is due to the polarity and size of the residues in the binding site. In the Neisseria protein a hydrophobic pocket is formed by residues from both chains of adjacent dimers, favoring interaction with amino acids with hydrophobic rather than hydrophilic or polar side groups. For example, the binding pocket formed by the interface of the two dimers chains G-H and chains A-B, is lined by Val-124, Leu-129, and Ala-137 from chain G, Leu-123 from chain B, and Ala-101 from chain A (Fig. 4, e and f). By contrast, in the E. coli AsnC protein Leu-129 is replaced by Gln-128 (Fig. 5), which appears to form a key hydrogen bond with the carboxyl oxygen of the asparagine bound in the structure (8). Another key discriminating position appears to be Ala-101 in NMB0573, which corresponds to Tyr-100 in AsnC, the larger side chain of which, although accommodating the asparagine is likely to clash with more extended amino acids, such as L-methionine (Fig. 5b). Consistent with the above, in E. coli Lrp, which binds to L-leucine, the residues corresponding to Leu-129 and Ala-101 in NMB0573 are also hydrophobic in nature (Fig. 1, Leu-108 and Leu-137).

Growth Characteristics and Expression Profiling of Wild-type and NMB0573 Knock-out Strains—Structural analysis of NMB0573 confirms its assignment as a member of the Lrp/AsnC family of transcriptional regulators and shows that the protein binds to L-methionine and L-leucine. To investigate the physiological significance of these results, a NMB0573 knock-out mutant of N. meningitidis strain MC58 (capsule negative) was constructed by insertion of a kanamycin gene replacing the entire coding region and selecting the transformants with kanamycin. The growth phenotype of the mutant strain was compared with wild-type N. meningitidis grown in both rich (GC media with supplements), and nutrient restricted defined media (RPMI with ferric nitrate and bicarbonate). The growth curves are shown in Fig. 6. The knock-out mutant showed no reduction in the rate of log-phase growth in rich media and only differed from the parent strain in having a shorter lag phase that suggests that it was initially better adapted to these conditions. By contrast, in nutrient poor conditions mutants displayed slower growth, a longer lag phase, and a slightly lower final growth than the parent strains. Overall, it appears that Neisseria requires the presence of NMB0573 for optimum growth in poorer nutrient conditions.

Functional Classes of Genes That Are Differentially Expressed in Wild-type and NMB0573 Knock-out Strains—To avoid the effects of measuring the secondary consequences of reduced growth in the poor nutrient conditions, and thus to focus upon the direct and indirect effects of the absence of the regulator, mRNA expression profiling was performed on early growth phase cultures grown on solid rich GC media. The data pre-
sented are based upon the results from six independent biological replicates (supplemental Table S1). The predominant change in the NMB0573 knock-out compared with the wild-type strain was a reduction of transcript abundance (74 of 91 genes, 81%) suggesting that this protein, if acting directly, is primarily a transcriptional activator. Normally, these regulators bind to DNA as a complex with their cognate ligands and the absence of the amino acid is associated with a reduction in DNA binding. Hence, the mutant would mimic a situation in which there was a lack of the monitored intracellular amino acid. The main groups of genes with annotated functions showing changes are dominated by the most abundant cell surface proteins, and components of aerobic metabolism/the TCA cycle.

Overall the metabolic changes combine to create a picture of a diversion of resources from the use of pyruvate for energy production to the formation of amino acids and central metabolic proteins. Reduction in energy production is not limited to the TCA cycle, and other genes involved in glycolysis, fermentation, and electron transport are also reduced. These observations are broadly consistent with a role of NMB0573 in the adaptation to growth in relatively poor nutrient conditions.

Consistent with the relatively similar rates of growth of the NMB0573 and parent strains, transcription of the genes of the division cell wall cluster are predominately unchanged. However, the exceptions to this are that there are 1.5-fold decreases in the expression of both \( ftsZ \) \( (p < 0.01) \) and \( murD \) \( (p < 0.01) \) in the mutant, which are notable in that each has additional promoters within the \( dcw \) cluster (32, 33) and may represent points of fine control of cell division in response to nutrient conditions.

The surface proteins showing the greatest changes include: \( porB \), \( porA \), pilE, pilO, opc, opa, outer membrane protein NMB1946, adhesin protein NMB2095, serotype 1 antigen, and \( magB \) genes, as well as subunits of two ABC transporters. The simplest explanation for these changes is that under nutrient limiting conditions the cell re-focuses its protein synthesis away from these abundant proteins, toward proteins required for cellular survival and core functions. This diversion of resources may account for the faster growth of the knock-out, compared with the parent, under nutrient-rich conditions. Genes coding proteins responsible for synthesis of cell surface elements peptidoglycan (\( murD \), 1.5-fold, \( p < 0.01 \)), fatty acids (\( accC \) and \( accD \), both 1.6-fold, \( p \leq 0.01 \)), and polysaccharides and lipopolysaccharides (\( amiC \), 1.7-fold, \( p = 0.02 \), \( n = 3 \)) are also decreased in the absence of NMB0573. Moreover \( ftsZ \), which is responsible for septum formation in cell division, follows the same pattern (1.5-fold, \( p < 0.01 \)).

Generally, the tRNA and amino acid synthetases are not abundant transcripts and do not report from all replicates (supplemental Table S1), but the most strongly increased gene in the absence of NMB0573 was arginyl-tRNA synthetase (0.4-fold, \( p < 0.02 \), \( n = 2 \)), in addition to increases in leucyl-tRNA synthetase (0.6-fold, \( p < 0.02 \), \( n = 4 \)), glutamyl-tRNA synthetase (0.6-fold, \( p < 0.01 \), \( n = 5 \)), and tryptophanyl-tRNA synthetase (0.6-fold, N.S., \( n = 2 \)). All of these changes are consistent with an adaptive role related to amino

**FIGURE 5. Comparison of amino acid binding sites.** (a) shows a stereo pair of an overlay of the apo and methionine-bound sites of NMB0573, the Cα backbones and side chains of NMB0573/L-methionine are shown in red and orange and those of apo-NMB0573 in cyan and blue, respectively, with the bound amino acid drawn in thicker bonds. Residue Arg-83 is labeled. (b) shows a stereo pair of an overlay of the binding sites of NMB0573/L-methionine and *E. coli* AsnC/asparagine. The Cα backbones and side chains of NMB0573/L-methionine are shown in red and orange and those of AsnC/asparagine in cyan and blue, respectively, with the bound amino acid drawn in thicker bonds. For clarity only the Ala-101 and Leu-129 in NMB0573 and the corresponding residues Tyr-100 and Gln-128 in *E. coli* AsnC are labeled. The orientation of both figures is the same as in Fig. 4, e and f.
acid metabolism and control, and perhaps they represent a response to increase the efficiency of tRNA loading and utilization of a limited amino acid pool.

**DISCUSSION**

A recent analysis of 150 published bacterial and Archaeal genomes identified 439 putative Lrp/AsnC family transcription factors (34), although relatively few have been functionally or structurally characterized. In this report we describe the structure, amino acid binding specificity, and preliminary functional characterization of the NMB0573 gene product of *N. meningitidis* that has been annotated as an AsnC-like transcriptional regulator. In common with other members of this family, we show that NMB0573 forms an octamer and have identified L-methionine and L-leucine as the amino ligands of the protein. The binding pocket is formed by the interface of adjacent dimers and is therefore a property of the oligomeric state of the protein. Interestingly, we observed minimal differences between the overall conformation of the unbound and amino acid bound forms of the protein showing that amino acid binding has no direct effect on either the DNA-binding domain or its relative orientation with respect to the C-terminal effector domain. In the case of *E. coli* Lrp, the binding of L-leucine appears to modulate the higher order oligomeric state of the protein such that in the presence of L-leucine, association of dimers into octamers is favored over hexadecamers (35). Thus an effect of amino acid binding on the conformation of the protein in the octameric state *per se* might not necessarily be expected. The results of the thermal shift assay and gel filtration reported here are consistent with the stabilization of the octameric state of NMB0573 by the amino acids, L-methionine and L-leucine. Thus the small local changes in the structure at the binding site, notably the movement of the side chain of Arg-83, that occur on amino acid binding appear to be sufficient to stabilize the octameric form of the protein.

The effect of l-leucine on *E. coli* Lrp oligomerization has been linked to the modulation of Lrp-dependent gene regulation by l-leucine (35) and its role as a global regulator of gene expression in *E. coli*. By altering the oligomeric state of the protein, leucine appears to effect the efficiency with which Lrp binds to relatively degenerate sequences in the promoter regions of target genes (36). Therefore, it is conceivable that l-methionine and l-leucine also modulate the activity of the NMB0573 regulator via an effect on its oligomeric state. This raises the question of the physiological role of the NMB0573 regulatory protein in *Neisseria*.

Expression profiling of wild-type and NMB0573 knock-out strains of *N. meningitidis* suggests that NMB0573 is a global regulator of gene expression. The growth properties and transcriptional response of the knock-out mutant indicate that NMB0573 plays a role in adaptation to low nutrient conditions. The interactions with leucine and methionine are consistent with a central role in controlling the response to the intracellular availability of the key resources for the initiation and synthesis of proteins. Investigation of the genes that are significantly changed in the absence of NMB0573 on BioCyc (37) showed that 19 of them are controlled in *E. coli* by the cAMP receptor protein. cAMP receptor protein is a global activator that regulates the response of the cell to carbon and energy shortage. *Neisseria* do not have a cAMP receptor protein homologue and although the results may be coincidental, they may also suggest that NMB0573 directly or indirectly serves a similar role to cAMP receptor protein in *Neisseria*. In common with other global regulators we would expect that the NMB0573 acts both directly on target promoters to recruit RNA polymerase and indirectly via intermediary transcription factors.

In conclusion, we propose that NMB0573 broadly represents a neisserial equivalent of the Lrp global regulator, controlling an adaptive response to low intracellular amino acid availability. This indicates that members of this regulator family can function as global regulators outside of the enteric bacteria. We conclude that the annotation of this gene as *asnC* is incorrect, and that NMB0573 probably controls responses to methionine and leucine. The ligand specificity of the AsnC/Lrp family of regulators is determined by the identifiable differences in size and charge properties of the ligand binding region, and that the determination of their DNA binding is primarily mediated through changes in their oligomeric state, rather than through structural changes in their DNA-binding regions.

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