Solution Secondary Structure of a Bacterially Expressed Peptide from the Receptor Binding Domain of *Pseudomonas aeruginosa* Pili Strain PAK: A Heteronuclear Multidimensional NMR Study†

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ABSTRACT: The C-terminal receptor binding region of *Pseudomonas aeruginosa* pilin protein strain PAK (residues 128−144) has recently been the target for the design of a synthetic peptide vaccine effective against multiple strains of *P. aeruginosa* infection. We have successfully cloned and bacterially expressed a ^{15}N-labeled PAK pilin peptide spanning residues 128−144 of the intact PAK pilin protein, PAK 128−144(Hs145), and have determined the solution secondary structure of this peptide using heteronuclear multidimensional NMR spectroscopy. The oxidized recombinant peptide exists as a major (*trans*) and minor (*cis*) species in solution, arising from isomerization around the Ile^{138}−Pro^{139} peptide bond. The pattern of NOEs, temperature coefficients, and coupling constants observed for the *trans* isomer demonstrate the presence of a type I β-turn and a type II β-turn spanning Asp^{134}-Glu-Gln-Phe^{137} and Pro^{139}-Lys-Gly-Cys^{142}, respectively. This is in agreement with the NMR solution structure of the *trans* isomer of a synthetic PAK 128−144 peptide which showed a type I and a type II β-turn in these same regions of the sequence [McInnes, C., Sönnichsen, F. D., Kay, C. M., Hodges, R. S., and Sykes, B. D. (1993) *Biochemistry 32*, 13432−13440; Campbell, A. P., McInnes, C., Hodges, R. S., and Sykes, B. D. (1995) *Biochemistry 34*, 16255−16268]. The pattern of NOEs, temperature coefficients, and coupling constants observed for the *cis* isomer also demonstrate a type II β-turn spanning Pro^{139}-Lys-Gly-Cys^{142}, but suggest a second β-turn spanning Asp^{132}-Gln-Asp-Glu^{135}. Thus, the *cis* isomer may also possess a double-turn motif (like the *trans* isomer), but with different spacing between the turns and a different placement of the first turn in the sequence. The discovery of a double-turn motif in the *trans* (and *cis*) recombinant PAK pilin peptide is an extremely important result since the double turn has been implicated as a structural requirement for the recognition of both receptor and antibody. These results pave the way for future isotope-edited NMR studies of the labeled recombinant PAK pilin peptide bound to antibody and receptor, studies integral to the design of an effective synthetic peptide vaccine.

Pseudomonas aeruginosa is a Gram-negative rod-shaped bacterium which causes infections resulting in opportunistic respiratory disease in cancer, cystic fibrosis, and intensive care patients (*1*−*5*). The initial step in the pathogenicity of *P. aeruginosa* is believed to be adherence to the host cell via polar pili on the bacterial surface (*2, 5, 6*). The pili are proteinaceous filaments composed of a homologous polymer of pilin protein (*5*). The semiconserved C-terminal region of the pilin monomer has been shown to contain the binding domain of *P. aeruginosa* to host epithelial cells (*6*−*12*). The seven different strains of *P. aeruginosa* share a common glycosphingolipid cell surface receptor (*7, 13*) where the minimal structural element is a disaccharide, βGalNAc(1−4)βGal, shown to specifically bind strains PAK and PAO (*14, 15*).

In counteracting *P. aeruginosa* infections, an anti-adhesin vaccine has been proposed. Antibodies specific for the pilus C-terminal region can be raised with synthetic peptides and used to counteract infection by blocking bacterial attachment (*16*). Since *P. aeruginosa* exists as seven different strains, production of a cross-reactive antibody effective against all strains would be most desirable for an antibody therapeutic. It is possible to envisage such a cross-reactive antibody since all strains bind to the same receptor (*7, 13, 17*−*19*).

In an effort to understand the structural basis for this cross-reactivity with antibody and receptor, the nuclear magnetic resonance (NMR)[1] solution structures of four synthetic peptide antigens spanning the C-terminal receptor binding regions of four strains of *P. aeruginosa* pilin (PAK, PAO, KB7, and P1) have been studied, and a common structural motif of two sequential β-turns has been revealed (*20*−*22*). All four pilin peptides bind to a common glycosphingolipid cell surface receptor (*14, 15*; H. B. Sheth, personal communications) as well as to a cross-reactive monoclonal antibody, PAK-13, raised against the PAK pilus (*23*).

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[1] Abbreviations: DSS, 2,2-dimethyl-2-sila-5-pentanesulfonate; ELISA, enzyme-linked immunosorbent assay; Hs, homoserine; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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There is evidence that the conserved motif of two sequential β-turns occurs in the epitope region of the pilus (*24, 25*). Competitive ELISA binding assays and single alanine replacement analyses of the synthetic peptide antigen corresponding to the PAK C-terminal receptor binding region have demonstrated that the cross-reactive antibody, PAK-13, and a monoclonal antibody specific for the PAK strain both bind to the PAK sequence which spans the two sequential β-turns (*24*). Furthermore, ^{1}H NMR studies of the interaction between PAK-13 and the four synthetic peptide antigens, PAK, PAO, KB7, and P1, have spectroscopically mapped the antigenic determinant to the two turns in all four peptides. The two turns are thus predicted to constitute the structural element for strain-specific or cross-reactive antibody recognition (*25*).

The discovery of the immunogenic importance of the β-turn in the pilin system is supported by other research groups in other systems. NMR evidence has been found of β-turns in the free solution structures of antigenic peptides (*26*−*31*), and X-ray crystal structures of antibody−peptide complexes have shown β-turns deeply embedded in the antibody−antigen interface (*32*−*37*). In fact, features incorporated into peptide antigens which stabilize β-turns have been shown to increase antibody affinity (*31, 38*). Thus, the β-turn may constitute one of the most important structural motifs for antibody recognition and binding. The turn motif provides a stable and surface-accessible secondary structure (*39, 40*), criteria which are likely necessary in order for binding of an immunogen to a B cell receptor to elicit an immune response (*41*).

There is also evidence that the β-turns are involved in the recognition of a glycosphingolipid cell surface receptor common to many strains of *P. aeruginosa* (*25, 42*). The binding of the PAK peptide to a disaccharide receptor analog, βGalNAc(1−4)βGal, was studied using ^{1}H NMR in order to map the 'adhesintope' recognized by the receptor (*25*). Spectral changes observed in the peptide spectrum with the binding of receptor were similar to those seen for the binding of antibody, suggesting that the epitope recognized by the antibody is structurally coincident with the adhesintope recognized by the receptor. In addition, Wong *et al.* (*42*) have mapped the adhesintope of PAK and KB7 synthetic peptide antigens binding to A549 cells (i.e., the residues which are essential for affinity) using a series of peptides incorporating single alanine replacements, and have identified essential residues in the turn regions of both peptides.

Transferred NOESY experiments of the pilin peptides in complex with PAK-13 antibody and disaccharide receptor analog have probed for conformational changes in the β-turns and hydrophobic pockets of these peptides induced by binding to antibody or receptor (*25*). The bound peptide conformations differed little from the conformations free in solution, suggesting that the free peptide conformation is similar to the bound conformation and is therefore of structural and biological significance. Although this is an interesting result, these experiments have been hampered by the inherent limitations of homonuclear two-dimensional NMR spectroscopy, which does not allow for isotope-edited experiments.

Recently, a bacterial expression and purification system for recombinant pilin peptides has been developed by Tripet *et al.* (*43*) whereby the DNA coding for the pilin peptides has been fused to the DNA coding for half of a two-stranded α-helical coiled-coil dimerization motif (E-coil) which acts as a C-terminal fusion tag. The other half of the two-stranded α-helical coiled-coil dimerization motif (K-coil) is immobilized on an insoluble silica support for selective dimerization affinity chromatography. In such a way, the pilin peptide−E-coil construct has been expressed and purified to >95% on the K-coil affinity column (*43*). We have used this expression and purification system to express ^{15}N-labeled PAK pilin peptide spanning the C-terminal receptor binding residues 128−144 of the intact PAK pilin protein, and are working on the expression and purification of other pilin peptides. The expression of ^{15}N-labeled peptide allows for more sophisticated isotope-edited, multidimensional NMR experiments of the peptide−antibody or peptide−receptor complexes, in order to probe in detail the structural immunology of these interactions. In addition, ^{15}N relaxation experiments of the labeled pilin peptides should allow studies of changes in the mobilities and dynamics of the peptide epitope regions free in solution versus in complex with antibody or receptor.

We present here a heteronuclear multidimensional NMR analysis of the secondary structure of ^{15}N-labeled recombinant PAK 128−144(Hs145) pilin peptide. These structural analyses are necessary in order to determine if the bacterially expressed peptide retains the structural integrity of the two sequential β-turns, especially with the addition of a non-native homoserine residue at the C-terminal end of the peptide (Hs145), a remnant of cyanogen bromide cleavage of a methionine residue engineered at the end of the pilin sequence in the gene construct. To our knowledge, this is also the first published NMR study of a recombinant peptide which is ^{15}N-labeled via a bacterial expression system, and as such provides an economic alternative to expensive chemical synthesis of ^{15}N-labeled peptides.

EXPERIMENTAL PROCEDURES

Expression, Isotope-Labeling, and Purification of Recombinant PAK Pilin Peptide. Construction of the vector pRLDE and PAK−pilin (met) gene fusion has been previously described in detail by Tripet *et al.* (*43*), but for clarity we point out the general features of the cloning, expression, and purification. The DNA coding for the PAK pilin receptor binding domain peptide (residues 128−148, KCTSDQDEQFIPKGCSK) was fused downstream of the omp A signal sequence (for export to the bacterial periplasm) and upstream of the DNA coding for half of a two-stranded α-helical coiled-coil heterodimerization domain motif (E-coil)[2] which when expressed acts as a C-terminal fusion tag. Directly flanking the PAK sequence (at its 5′ and 3′ ends) was the engineering of two codons coding for methionine residues. Purification of the expressed fusion product was carried out by loading the extract onto a silica column containing immobilized K-coil (the other half of the two-stranded α-helical coiled-coil heterodimerization motif). After purification, the PAK pilin−E-coil fusion was cleaved using cyanogen bromide to produce the free PAK pilin peptide with an additional homoserine residue located at the C-terminus [PAK pilin (Hs)].

[2] E-coil and K-coil denote 35-residue peptides that form a heterodimeric 2-stranded α-helical coiled-coil where positions 'e' and 'g' of the heptad repeat 'abcdefg' are occupied by glutamic acid or lysine residues, respectively (*43*).

Growth, Induction, and Lysis of Bacteria Containing Recombinant Peptide. The expression of the ^{15}N-labeled PAK pilin−E-coil peptide construct in bacterial vector pRLDE was carried out in minimal medium consisting of M9 salts as described by Maniatis *et al.* (*44*) and supplemented with $(^{15}NH_4)_2SO_4$. Each 1 L of medium at pH 7.4 contained 12 g of NaH_2PO_4, 6 g of K_2HPO_4, 2 g of NaCl, and 1 g of $(^{15}NH_4)_2SO_4$ to which was added 2 mL of 1 mM $MgSO_4$ and 0.9 mL of 1 mM $FeSO_4$. Glucose (50 mL of 20% in H_2O) was added with supplements of carbenicillin to final concentrations of 10 mg/mL and 100 μg/mL, respectively. All minerals and additives were sterilized separately by autoclaving or ultrafiltration.

E. coli BL21(DE3) cells (*45*) harboring the PAK pilin−E-coil fusion peptide vector were grown overnight at 37 °C, with shaking, in LB medium containing carbenicillin (100 μg/mL). The following morning, six 1 L cultures of minimal media were innoculated with 1/100 dilution of the overnight culture and were grown at 37 °C to an A_{550} of 0.5−0.6. Production of recombinant peptide was then induced by the addition of IPTG to a final concentration of 1 mM. The bacteria were grown for an additional 6 h at 37 °C with shaking, and the expressed peptide was obtained by a modified osmotic shock treatment (*46*). Cells were centrifuged at 4000*g* for 10 min at 4 °C and resuspended in TES buffer (100 mM Tris-HCl, 5 mM EDTA, 20% sucrose, pH 8.0) to a final volume of 80 mL per gram wet weight. Cells were gently shaken at room temperature for 10 min, and the suspension was centrifuged at 5000*g* for 10 min, 4 °C. The pellet was resuspended in 5 mM ice-cold $MgSO_4$ (80 mL per gram wet weight), and the cells were shaken again for 1 h on ice. The suspension was then centrifuged at 7000*g* for 10 min at 4 °C, and the supernatant constituting the periplasmic fraction was lyophilized for the subsequent purification step using K-coil affinity column chromatography (*43*).

K-Coil Affinity Column Chromatography. The lyophilized periplasmic fraction containing the recombinant ^{15}N-labeled PAK pilin−E-coil peptide was resuspended in 30 mL of dH_2O/1 L of culture, and the pH was adjusted to 6.5. Material which was unable to be resolubilized was removed by centrifugation and filtration through a 0.22 μm filter. The periplasmic extract was then loaded onto the K-coil affinity column at a flow rate of 0.5 mL/min. After loading, the column was washed sequentially with a high-salt wash, an acetonitrile wash, and finally with 50% aqueous acetonitrile with 1% trifluoroacetic acid (TFA), as described by Tripet *et al.* (*43*). The final eluent was lyophilized. The affinity-purified product was characterized by RP-HPLC and mass spectroscopy (see below) to confirm the identity and the integrity of the intrachain disulfide bridge. Overall recovery of ^{15}N-labeled PAK pilin−E-coil peptide was found to be ∼10 mg/L of growth culture.

CNBr Digest of PAK Pilin(met)−E-Coil. CNBr cleavage of the PAK pilin(met)−E-coil peptide was carried out by incubating 0.6 μmol of the peptide in 1.0 mL of 0.1 N HCl and 60 μmol of CNBr for 24 h at room temperature in the dark (*47*). The reaction mixture was then diluted 5-fold with dH_2O and lyophilized. Final recovery of the purified CNBr-cleaved PAK pilin(Hs) peptide was obtained using RP-HPLC.

Base-Catalyzed Conversion of the C-Terminal Homoserine Lactone to Homoserine. The PAK pilin peptide C-terminal homoserine residue liberated by the CNBr cleavage process was present as an roughly equimolar mixture of interconverting homoserine and homoserine lactone groups. In order to convert all cleaved PAK pilin peptide into the homoserine form, the peptide was dissolved in 100 mM NH_4HCO_3 and left overnight at room temperature. The base-catalyzed ring cleavage of the homoserine lactone ring to the homoserine (*48*) was monitored by RP-HPLC and verified by mass spectrometry using a Fisons VG Quattro triple quadrupole mass spectrometer (Manchester, England) fitted with an electrospray ionization source operating in the positive ion mode. Following complete interconversion to the homoserine form, the peptide was relyophilized to remove the NH_4HCO_3, and the powder was used directly for preparation of the NMR sample. A schematic drawing showing the expression, ^{15}N-labeling, and purification of the ^{15}N-labeled recombinant peptide, PAK 128−144(Hs145), is presented in Figure 1.

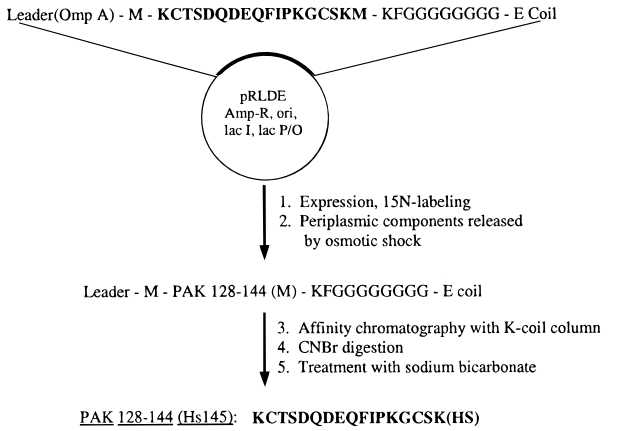

FIGURE 1: Schematic drawing showing the expression, ^{15}N-labeling, and purification of the recombinant ^{15}N-labeled PAK 128−144-(Hs145). The terminal residue (145) is a homoserine (Hs) left from the CNBr cleavage of the pili peptide from the C-terminal E-coil fusion tag in the gene construct.

Competitive ELISA Binding Assays of Recombinant PAK Pilin Peptide Binding to Monoclonal Cross-Reactive Antibody PAK-13. Competitive ELISA was carried out according to the protocol described by Wong *et al.* (*24*) in order to measure the apparent association constant (K_a) for the binding of the recombinant PAK pilin peptide to the monoclonal cross-reactive antibody PAK-13. The binding of the synthetic PAK pilin peptide to PAK-13 was also measured for comparative purposes. Briefly described here, microtiter wells were coated with intact PAK pili and incubated with a solution mixture of PAK-13 antibody and recombinant peptide (or synthetic peptide) at various concentrations of peptide. The K_a for the association of peptide to PAK-13 was then calculated by the formula $K_a = 1/I_{50}$ as described by Nieto *et al.* (*49*). The PAK-13 monoclonal antibody IgG was prepared in mouse ascites as described previously (*10, 23*).

NMR Spectroscopy. Samples were prepared by dissolving recombinant ^{15}N-labeled PAK 128−144(Hs145) in 500 μL of 90% H_2O/10% D_2O PBS buffer to a concentration of 1 mM. DSS was added as an internal chemical shift reference and the pH subsequently adjusted to 5.0 using NaOH and HCl solutions.

All 2D and 3D heteronuclear NMR experiments were carried out using the enhanced sensitivity pulsed field

gradient method (*50, 51*) on a Varian Inova-500 or Unity-600 NMR spectrometer equipped with three channels, a pulsed-field gradient triple resonance probe with an actively shielded z gradient, and a gradient amplifier unit. 2D ^{15}N−^{1}H HSQC spectra (*52*) were acquired at 600 MHz with the following number of complex points, acquisition times, and spectral widths: F1 (^{15}N) 256, 85 ms, 1500 Hz; F2 (^{1}H) 1024, 78 ms, 6600 Hz; 8 transients. 3D ^{15}N-edited NOESY HSQC (*52, 53*) and 3D ^{15}N-edited TOCSY HSQC (*52, 54*) experiments were performed at 600 MHz and at mixing times of 150, 300, and 400 ms for the NOESY and 60 ms for the TOCSY. Both experiments employed the following number of complex points, acquisition times, and spectral widths: F1 (^{1}HN) 240, 24 ms, 5000 Hz; F2 (^{1}H) 1024, 78 ms, 6600 Hz; F3 (^{15}N) 52, 21 ms, 1217 Hz; 12 transients. An HNHA experiment (*55*) was performed at 500 MHz and employed the following number of complex points, acquisition times, and spectral widths: F1 (^{1}HN) 112, 16 ms, 3500 Hz; F2 (^{1}H) 768, 64 ms, 6000 Hz; F3 (^{15}N) 64, 32 ms, 1000 Hz; 16 transients.

All 2D and 3D data sets were processed on SUN Sparc5 and Silicon Graphics Indigo2 workstations using NMRPipe and NMRDraw software provided by F. Delaglio (NIDDK, NIH, Bethesda, MD, unpublished). Spectra assignment was achieved using the interactive graphic-based program PIPP (*56*). Postacquisition solvent subtraction was employed in the spectra where backbone amide protons were detected in the acquisition dimension (*57*). Typically, spectra were processed in the acquisition and indirect dimension with 90° shifted sine-bell squared apodization. For constant time ^{15}N evolution periods, mirror image linear prediction was used to double the time domain signals (*58*). A time domain deconvolution procedure (*51*) was used to minimize the signal from residue water for ^{15}N-edited 3D experiments.

Temperature coefficients ($-\Delta\delta/\Delta T$ ppb) were calculated for the amide protons of recombinant PAK 128−144(Hs145) from linear plots of chemical shift versus temperature measured from ^{15}N−^{1}H HSQC spectra acquired at 5.0, 10.0, 15.0, 20.0, and 25.0 °C. $^{3}J_{\mathrm{NH}\alpha\mathrm{CH}}$ coupling constants were obtained for each residue from the ratio of the intensities of the diagonal peak of residue *i* to its αH−NH (*i,i*) cross-peak in the HNHA spectra (*55*).

RESULTS

Binding of the Recombinant PAK Pilin Peptide to Monoclonal Cross-Reactive Antibody PAK-13. Since the eventual goal of expressing an ^{15}N-labeled PAK pilin peptide is to allow for more sophisticated isotope-edited, multidimensional NMR experiments of the peptide−antibody (or receptor) complexes, it is important to validate the recombinant PAK pilin peptide as being equivalent to the native peptide, not only structurally but also with respect to its affinity to the relevant antibodies and receptors. Competitive ELISA binding experiments were thus perfomed in order to measure the apparent association constant (K_a) for the binding of the recombinant PAK pilin peptide to the monoclonal cross-reactive antibody PAK-13.[3] This is the antibody for which all previous NMR studies of pilin peptide/antibody interactions were studied (25), and for which all future isotope-edited NMR studies of the recombinant peptide−antibody complex will be performed. These competitive ELISA binding experiments produced a K_a of $(6.3 \pm 0.5) \times 10^6$ M^{-1} for the binding of the recombinant PAK pilin peptide to antibody PAK-13, as opposed to a K_a of $(10.3 \pm 1.0) \times 10^6$ M^{-1} for the synthetic PAK pilin peptide. Given the similarity of these binding constants, it would appear that the recombinant PAK pilin peptide is as immunologically active as the native peptide, and therefore is immunologically equivalent. The structural basis for this immunological equivalence will now be examined.

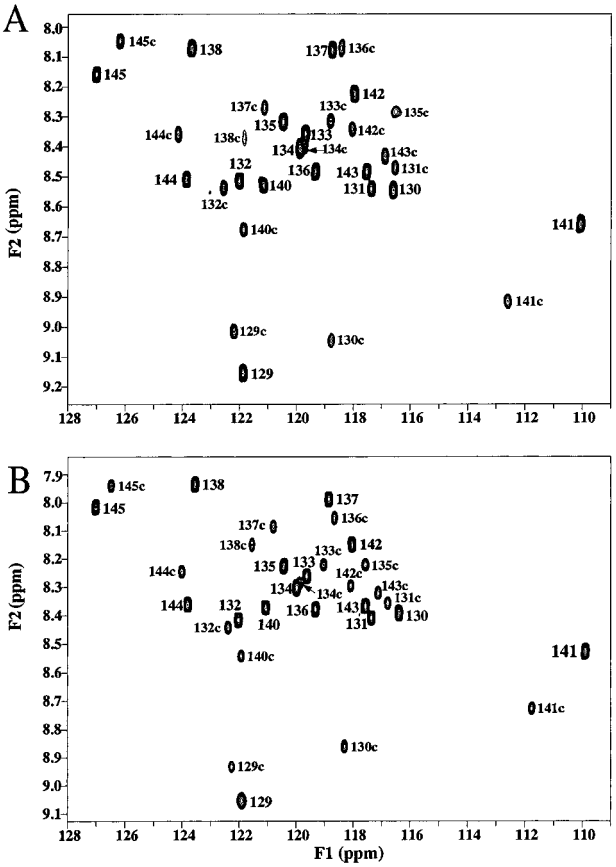

FIGURE 2: 2D ^{15}N−^{1}H HSQC spectra of recombinant ^{15}N-labeled PAK 128−144(Hs145) at (A) 5.0 °C and (B) 25.0 °C showing upfield shifting of resonances with temperature. Peaks arising from the minor *cis* conformer of the PAK peptide are denoted by the letter 'c' after the residue, i.e., 144c. The peptide was in 90% H_2O/10% D_2O, pH 5.0.

cis versus trans Isomers. The oxidized synthetic PAK 128−144 peptide has been found to exist as two distinct conformations in solution which have been demonstrated to arise as a result of isomerization of the Ile^{138}−Pro^{139} peptide bond (*59*). Figure 2 shows the ^{15}N−^{1}H HSQC spectra of the ^{15}N-labeled PAK 128−144(Hs145) at 5.0 °C (2A) and at 25.0 °C (2B), where a doubling of all peaks can be clearly seen arising from *cis/trans* isomerization around the Ile^{138}−Pro^{139} peptide bond in the recombinant peptide. The approximate proportion of *trans* to *cis* isomer can be calculated from the ratio of the intensity of the major to minor HSQC peaks in these spectra as 88:12 at 25.0 °C and 80:20

[3] The sensitivity of the competitive ELISA binding experiment is too low to accurately measure the apparent association constant (K_a) for the binding of the recombinant PAK pilin peptide to the receptor analog βGalNAc(1−4)βGal, since these values are at least 20−50 times weaker than the affinities measured for the recombinant PAK pilin peptide binding to antibody PAK-13. This is in agreement with the affinity measured for the biotinylated synthetic PAK pilin peptide binding to a βGalNAc(1−4)βGal−BSA conjugate from solid phase ELISA binding assays (*15*).

at 5.0 °C, showing that the population of the *cis* isomer increases as the temperature decreases. This is in agreement with the proportions observed for the synthetic PAK peptide where the *cis* isomer was shown to exist in the oxidized form of the peptide to a significant degree (20%) in water at 5.0 °C (*59*).

The conformation of the *trans* isomer is confirmed by the presence of $d_{N\delta}(i,i+1)$ connectivities defining the *trans* Ile^{138}−Pro^{139} peptide bond in the 3D ^{15}N-edited NOESY HSQC at 5.0 °C. However, since the the $d_{\alpha\alpha}(i,i+1)$ connectivity which defines a *cis* Ile^{138}−Pro^{139} peptide bond is absent in the ^{15}N-edited NOESY HSQC, the conformation of the *cis* isomer must be inferred from a comparison of the chemical shifts of this minor isomer to previous assignments of the *cis* isomer of the synthetic PAK pilin peptide (*59*).

Resonance Assignments and Chemical Shifts. The ^{1}H and ^{15}N NMR resonance assignments for the *trans* and *cis* isomers of the recombinant ^{15}N-labeled PAK 128−144-(Hs145) at 5.0 and 25.0 °C are presented in Table 1. These were obtained from the analysis of 3D ^{15}N-edited HSQC NOESY experiments performed at 5.0 °C and TOCSY HMQC experiments performed at 25.0 °C, together with temperature coefficient information (discussed below) and assignments for the ^{1}H chemical shifts of the synthetic PAK 128−144 peptide (*21, 59*). A quick perusal of the dissimilar ^{15}N and ^{1}H chemical shifts for the *trans* versus the *cis* isomer suggests that these peptides adopt different conformations, even in regions of the sequence far removed from the Ile^{138}−Pro^{139} peptide bond.

The conformational dependence of the chemical shifts of the α proton, the amide proton, and the ^{15}N amide (*60*) is well documented. For all three nuclei, upfield shifts are generally found for residues in helices, and downfield shifts are generally found in *β*-strands (*60*). Figure 3 shows plots of the difference in observed versus random coil chemical shift ($\Delta\delta_{obs-rc}$) as a function of residue position for the *trans* (3A) and *cis* (3B) isomers of the recombinant ^{15}N-labeled PAK 128−144(Hs145). Mostly positive values are observed for the N-terminal (129−132) and C-terminal (139−144) regions of both the *trans* and the *cis* isomers, suggesting that these regions are largely extended in solution. However, the central residues (133−138) of both isomers display negative values, especially for the ^{15}N nucleus, suggesting that this region of the sequence may be adopting a more 'folded' structure, helical or otherwise. Significantly, the ^{15}N amide values observed for residues Glu^{135} and Gln^{136} are much more negative for the *cis* isomer than for the *trans* isomer, suggesting that these two residues adopt a different conformation in the *cis* isomer than in the *trans* isomer.

In order to determine to a first approximation if the solution secondary structure of the recombinant PAK pilin peptide is similar to the solution secondary structure of the synthetic PAK pilin peptide, the chemical shifts of the resonances of the *trans* and *cis* isomers of the two peptides were compared. Figure 4 presents a plot of the differences in chemical shift (versus residue position) for the α and backbone amide protons of the *trans* (4A) and *cis* (4B) isomers of recombinant ^{15}N-labeled PAK 128−144(Hs145) versus the synthetic PAK 128−144 peptide (*21, 59*). The largest differences in chemical shifts occur at the ends of the peptide, where differences of greater than ± 0.1 ppm are seen for the α protons of Lys^{128} and Lys^{144} in both the *trans* and *cis* isomers, and for the backbone amide protons of Cys^{129}, Thr^{130}, and Lys^{144} in the *trans* isomer, and Cys^{129} and Lys^{144} in the *cis* isomer. These chemical shift differences are expected since the chemistry of the recombinant peptide differs from that of the synthetic peptide at the N- and C-termini, leading to different local environments. In the recombinant peptide, the N-terminal residue (Lys^{128}) has a free amino group, and the C-terminal residue is a homoserine (Hs145) with a free carboxyl group; whereas in the synthetic peptide Lys^{128} is N-acetylated and the C-terminal residue (Lys^{144}) is amidated. Since the backbone amide of Lys^{128} is unprotected in the recombinant peptide, it exchanges too rapidly with solvent to be detected in any of the ^{15}N-edited spectra (see Figure 2), and therefore cannot be assigned.

The differences in chemical shift observed for the α proton are conformationally more significant than those observed for the amide proton because the α proton chemical shift is uniquely sensitive to changes in secondary structure (*60*), whereas the amide proton chemical shift is sensitive to many factors including solvent environment. Therefore, the absence of significant *trans* or *cis* α proton chemical shift differences between the recombinant versus the synthetic peptide in the region spanned by residues 129−143 strongly indicates that the secondary conformation of this region is the same in the recombinant peptide as it is in the synthetic peptide for both isomers.

Temperature Coefficients. The temperature dependence of the amide proton chemical shift is often used to detect possible sites for intramolecular hydrogen bonding. For a random-coil peptide in water, the temperature coefficients of the amide proton resonances are expected to be $6 \leq -\Delta\delta/\Delta T \leq 10$ ppb, indicating that the backbone is freely solvated by water and that no hydrogen bonds are present which would protect the backbone amides from solvent exchange. However, for amides protected from solvent exchange, these values are expected to decrease to $-\Delta\delta/\Delta T < 5$ ppb (*61*), indicating either the presence of a hydrogen bond or a high degree of solvent shielding arising from other secondary or tertiary elements (*62*). Figure 2 shows the upfield shifting in the F2 (^{1}H) dimension of the amide protons of the recombinant ^{15}N-labeled PAK 128−144(Hs145) from 5.0 °C (2A) to 25.0 °C (2B). The temperature coefficients for each amide backbone proton in the *trans* and *cis* isomers of recombinant PAK peptide are shown in Table 2 and Figure 6.

Small temperature coefficients (≤5 ppb) are observed for the backbone amides of Gln^{133} (4.6 ppb), Asp^{134} (4.6 ppb), Glu^{135} (4.0 ppb), Gln^{136} (5.0 ppb), Phe^{137} (4.2 ppb), and Cys^{142} (3.4 ppb) in the *trans* isomer, and for the backbone amides of Cys^{129} (4.0 ppb), Asp^{132} (4.4 ppb), Gln^{133} (4.4 ppb), Glu^{135} (2.8 ppb), Gln^{136} (<1.0 ppb), and Cys^{142} (2.0 ppb) in the *cis* isomer. Extremely large temperature coefficients are also observed for the backbone amides of Thr^{130} (9.1 ppb), Phe^{137} (9.6 ppb), and Ile^{138} (11.8 ppb) in the *cis* isomer. The temperature coefficient values measured for the *trans* and *cis* isomers of the synthetic PAK pilin peptide (*21, 59*) are very similar (within ± 0.5 ppb) to the values obtained for the recombinant peptide,[4] suggesting again that the secondary conformations of the isomers of the recombinant peptide are similar to those adopted by the isomers of the synthetic peptide.

Table 1: ^{1}H and ^{15}N NMR Resonance Assignments for Recombinant ^{15}N-Labeled PAK 128−144(Hs145)

residue	^{15}N	NH	αH	βH	γH	δH and others
			(A) *trans* Isoform (pH 5.0, 5 °C)			
K_{128}			4.12	1.95		
C_{129}	121.84	9.15	4.88	3.08, 3.29		
T_{130}	116.57	8.55	4.51	4.31	1.22	
S_{131}	117.34	8.54	4.52	3.83, 3.91		
D_{132}	122.00	8.51	4.62	2.73		
Q_{133}	119.66	8.36	4.27	2.00, 2.15	2.36	
D_{134}	119.86	8.41	4.61	2.74, 2.83		
E_{135}	120.46	8.32	4.23	1.99, 2.10	2.36	
Q_{136}	119.31	8.48	4.14	1.89		
F_{137}	118.72	8.07	4.64	3.03, 3.18		δ 7.23, ϵ 7.34, ξ 7.29
I_{138}	123.65	8.07	4.39	1.85	1.11, 1.48 0.84 (γCH3)	0.89 (δCH3)
P_{139}	*	*	4.37	1.95, 2.35	1.98, 2.10	3.63, 3.87
K_{140}	121.14	8.52	4.23	1.85		ϵ 3.00
G_{141}	110.05	8.65	3.92, 4.06			
C_{142}	117.95	8.22	4.78	3.03, 3.24		
S_{143}	117.51	8.48	4.49	3.89		
K_{144}	123.82	8.51	4.36	1.87		1.76, ϵ 3.00
Hs_{145}	127.00	8.15	4.23			
			(B) *trans* Isoform (pH 5.0, 25 °C)			
K_{128}						
C_{129}	121.79	9.04	4.85	3.09, 3.29		
T_{130}	116.34	8.37	4.51	4.31	1.21	
S_{131}	117.26	8.32	4.52	3.82, 3.93		
D_{132}	121.94	8.41	4.62	2.72		
Q_{133}	119.53	8.25	4.28	2.00, 2.14	2.35	
D_{134}	119.27	8.30	4.61	2.73, 2.82		
E_{135}	120.42	8.22	4.24	1.99, 2.10	2.36	
Q_{136}	119.27	8.39	4.15	1.89, 2.15		
F_{137}	118.75	7.98	4.64	3.03, 3.17		δ 7.23, ϵ 7.34, ξ 7.29
I_{138}	123.70	7.93	4.39	1.82	1.11, 1.48 0.84(γCH3)	0.89(δCH3)
P_{139}	*	*				
K_{140}	120.99	8.36	4.23	1.84	1.48	1.70
G_{141}	109.87	8.52	3.92, 4.05			
C_{142}	118.02	8.14	4.78	3.03, 3.25		
S_{143}	117.45	8.37	4.49	3.89		
K_{144}	122.91	8.39	4.35	1.88	1.47	1.78
Hs_{145}	126.95	8.01	4.23	1.87, 2.05	3.63	
			(C) *cis* Isoform (pH 5.0, 5 °C)			
K_{128}			4.15	1.94		
C_{129}	122.17	9.02	5.05	2.96, 3.11		
T_{130}	118.87	9.04	4.53	4.28		
S_{131}	116.56	8.47	4.58	3.60, 3.71		
D_{132}	122.54	8.53	4.66	2.67, 2.82		
Q_{133}	118.78	8.31	4.20	2.04	2.34	
D_{134}	119.65	8.38	4.61			
E_{135}	116.52	8.29	4.05			
Q_{136}	116.40	8.07	4.29	1.95		
F_{137}	121.03	8.27		2.97, 3.17		
I_{138}	121.78	8.39				
P_{139}	*	*	4.72	2.12, 2.41		
K_{140}	121.83	8.67	4.25	1.85		
G_{141}	112.52	8.91	3.87, 4.14			
C_{142}	118.02	8.35	4.56	3.16, 3.27		
S_{143}	116.90	8.43	4.41	3.63, 3.73		
K_{144}	124.14	8.36	4.24	1.86		
Hs_{145}	126.21	8.04				
			(D) *cis* Isoform (pH 5.0, 25 °C)			
K_{128}						
C_{129}	122.30	8.93	5.00	3.01, 3.15		
T_{130}	118.32	8.82	4.52			
S_{131}	116.74	8.36	4.57	3.69, 3.77		
D_{132}	122.30	8.43	4.66	2.67, 2.77		
Q_{133}	118.91	8.21	4.23	2.03	2.34	
D_{134}	119.96	8.25				
E_{135}	117.67	8.22	4.06	2.05	2.32	
Q_{136}	118.64	8.06	4.29	1.93, 2.21		
F_{137}	120.73	8.08		2.97, 3.18		
I_{138}	121.37	8.12	4.35	1.77	0.89(γCH3)	
P_{139}	*	*				
K_{140}	121.88	8.53	4.26	1.85	1.49	

Table 1 (Continued)

residue	^{15}N	NH	αH	βH	γH	δH and others
			(D) *cis* Isoform (pH 5.0, 25 °C)			
G_{141}	111.46	8.69	3.92, 4.11			
C_{142}	118.06	8.33	4.61	3.14, 3.24		
S_{143}	117.06	8.28	4.40	3.62, 3.75		
K_{144}	123.06	8.25	4.29	1.85	1.41	1.74
Hs_{145}		7.94	4.25	1.84		

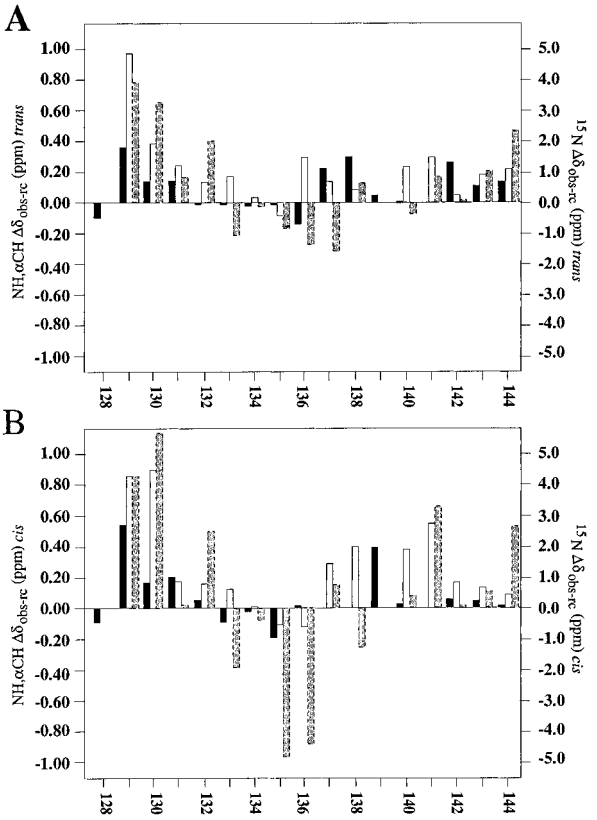

FIGURE 3: Plots of the difference in observed versus random-coil chemical shift ($\Delta\delta_{obs-rc}$) as a function of residue position for (A) the *trans* isomer and (B) the *cis* isomer of recombinant ^{15}N-labeled PAK 128−144(Hs145). The black boxes correspond to the α proton, the white boxes correspond to the amide proton, and the stippled gray boxes correspond to the ^{15}N amide. Data are measured from NMR spectra taken at pH 5.0, 5.0 °C.

The small temperature coefficients of the Phe[137] and Cys[142] backbone amides observed in the *trans* isomers of both the recombinant and synthetic PAK pilin peptides are a likely consequence of their involvement in the (1,4) hydrogen bond between the carbonyl oxygen of the first residue and the amide hydrogen of the last residue of the *β*-turns (Asp[134] CO−Phe[137] NH and Pro[139] CO−C[142] NH) found in the NMR solution structure of the synthetic peptide (*20, 21*). Conversely, the high temperature coefficient of the backbone amide of Phe[137] observed in the *cis* isomers of both the recombinant and synthetic peptides suggests no involvement of the Phe[137] NH in a hydrogen bond, and by inference that the first turn observed in the *trans* isomer is absent in the *cis* isomer. In fact, the high temperature coefficients

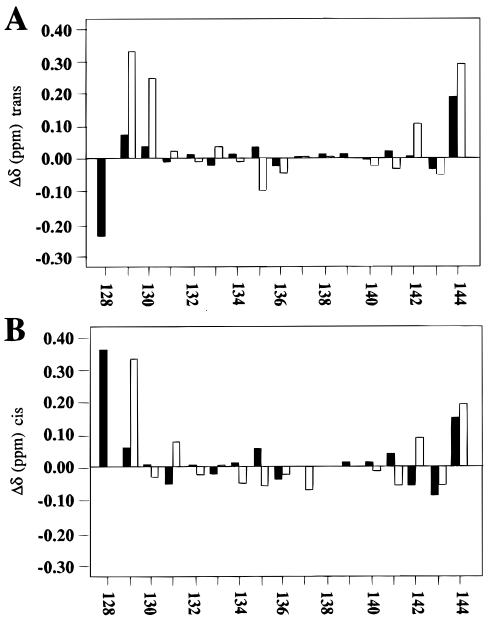

FIGURE 4: Plots of the difference in chemical shift (Δδ) versus residue position for the (A) *trans* and (B) *cis* isomers of recombinant ^{15}N-labeled PAK 128−144(Hs145) versus unlabeled synthetic PAK 128−144. The black boxes correspond to the α proton, and the white boxes correspond to the amide proton. Data are measured from NMR spectra taken at pH 5.0, 5.0 °C.

Table 2: Temperature Coefficient and $^3J_{NH\alpha CH}$ Values for Recombinant ^{15}N-Labeled PAK 128−144(Hs145)

	$-\Delta\delta/\Delta T \times 1000$[a]		$^3J_{NH\alpha CH}$	
residue	*trans*	*cis*	*trans*	*cis*
K_{128}	–	–	–	–
C_{129}	5.6	4.0	7.1 ± 1.0	7.0 ± 1.0
T_{130}	7.6	9.1	8.2 ± 1.1	10.2 ± 1.1
S_{131}	6.6	5.8	7.6 ± 1.0	7.3 ± 1.0
D_{132}	5.2	4.4	7.1 ± 1.0	9.1 ± 1.1
Q_{133}	4.6	4.4	6.2 ± 0.9	4.3 ± 0.7
D_{134}	4.6	6.5	8.0 ± 1.1	–
E_{135}	4.0	2.8	6.2 ± 0.9	–
Q_{136}	5.0	<1.0	6.8 ± 1.0	–
F_{137}	4.2	9.6	7.8 ± 1.0	9.0 ± 1.1
I_{138}	6.6	11.8	8.0 ± 1.1	–
P_{139}	–	–	–	–
K_{140}	8.0	6.6	5.5 ± 0.9	2.8 ± 0.5
G_{141}	6.6	9.2	–	–
C_{142}	3.4	2.0	7.5 ± 1.0	6.3 ± 0.9
S_{143}	5.8	5.2	7.3 ± 1.0	6.9 ± 1.0
K_{144}	7.6	5.2	7.3 ± 1.0	7.5 ± 1.0
Hs_{145}	6.6	6.6	7.3 ± 1.0	7.6 ± 1.0

[a] Uncertainty in the measured temperature coefficient values is ±0.5 ppb/K.

observed for Phe[137] and Ile[138] in the *cis* isomer suggest that this region of the peptide is quite solvent-exposed and may be extended, whereas the very low temperature coefficient observed for Gln[136] in the *cis* isomer (<1.0 ppb) suggests a high degree of solvent shielding arising either from a hydrogen bond or from other tertiary elements. Future NMR

[4] The discrepancy between the temperature values obtained for Asp[132] in the *cis* isomers of the recombinant ^{15}N-labeled PAK 128−144(Hs145) versus the synthetic PAK 128−144 may be a function of an overlap problem of the backbone amides of Asp[132] with Ser[131] and Ser[143] which could not be adequately resolved in the homonuclear spectra of the unlabeled peptide, and may therefore have led to an erroneously measured temperature coefficient for this residue (*59*).

structure calculations of the *cis* isomer of the recombinant peptide should illuminate potential interactions for the amide proton of Gln[136].

The depressed temperature coefficients (<5.0 ppb) of residues Gln[133], Asp[134], and Glu[135] in the *trans* isomer may be partly explained on the basis of the NMR solution structure of the *trans* isomer of the synthetic peptide (*20*, *21*). This solution structure shows that the peptide backbone is folded around a core of hydrophobic side chains (Phe[137], Ile[138], and Pro[139]), placing the side chains of Thr[130] and Glu[135] into close proximity with the backbone of residues 133−135. In the absence of hydrogen bonds involving the amide protons of either Gln[133], Asp[134], or Glu[135], it is likely that the proximity of the Thr[130] and Glu[135] side chains leads to partial solvent sequestering away from the backbone protons of Gln[133], Asp[134], and Glu[135], leading to depressed temperature coefficients for these residues. Future NMR structure calculations of the *trans* isomer of the recombinant peptide should provide further insight into the solvent exposure of this region of the backbone.

Coupling Constants. The $^3J_{NH\alpha CH}$ coupling constants for each residue in the *trans* and *cis* isomers of recombinant pilin peptide are shown in Table 2 and Figure 6. Almost all $^3J_{NH\alpha CH}$ coupling constant values are greater than 6 Hz and less than 8 Hz, indicating that both isomers are quite flexible, undergoing a conformational equilibrium between helical and extended dihedral space. $^3J_{NH\alpha CH}$ values of ≥8 Hz, consistent with a ϕ angle in extended or β-strand dihedral space, are observed for Thr[130] (8.2 Hz), Asp[134] (8.0 Hz), and Ile[138] (8.0 Hz) in the *trans* isomer, and for Thr[130] (10.2 Hz), Asp[132] (9.1 Hz), and Phe[137] (9.0 Hz) in the *cis* isomer. $^3J_{NH\alpha CH}$ values of ≤6 Hz, consistent with a ϕ angle in helical space or position 2 of a type I or type II β-turn, are observed for Lys[140] (5.5 Hz) in the *trans* isomer, and Gln[133] (4.3 Hz) and Lys[140] (2.8 Hz) in the *cis* isomer. Of these, Lys[140] is found in position 2 of the putative type II β-turn found in both the *trans* and the *cis* isomers. The $^3J_{NH\alpha CH}$ coupling constant for Glu[135] at position 2 in the other putative type I β-turn found only in the *trans* isomer is 6.2 Hz. These coupling constants of values less than 6.2 Hz are consistent with a ϕ angle greater than −70°, and therefore consistent with $\phi 2 = -60°$ for most turns (*63*).

The $^3J_{NH\alpha CH}$ coupling constant values measured for the *trans* and *cis* isomers of the recombinant peptide are very similar to those measured for the *trans* and *cis* isomers of the synthetic peptide (*21*, *59*), suggesting that the recombinant and synthetic peptides explore the same conformational space with respect to the dihedral angle, ϕ. In particular, the $^3J_{NH\alpha CH}$ values measured for Glu[135] and Lys[140] in the *trans* isomer of the synthetic peptide were 6.4 and 5.5 Hz, respectively (*21*), identical to those values measured for the *trans* isomer of the recombinant peptide within an error of ±0.2 Hz.

It is interesting to note that the low $^3J_{NH\alpha CH}$ value of 4.3 Hz for Gln[133] in the *cis* isomer coupled with the low temperature coefficient of 4.0 ppb for Glu[135] suggests a second β-turn spanning Asp[132]-Gln-Asp-Glu[135] in the *cis* isomer. The presence of this turn would be confirmed by the presence of a strong $d_{NN}(3,4)$ cross-peak between Asp[134] and Glu[135] and a weak $d_{\alpha N}(2,4)$ cross-peak between Gln[133] and Glu[135] (see below). Unfortunately, the overlap of *trans* and *cis* Asp[134] in both the ^{1}HN and ^{15}N dimensions does not allow resolution of these definitive NOEs. Resolution in a third dimension with the incorporation of ^{13}C labels to the recombinant PAK pilin is necessary in order to confirm the presence of this second turn in the *cis* isomer.

NOE Connectivities. The NOEs diagnostic of β-turns include $d_{\alpha N}(2,3)$, $d_{\alpha N}(3,4)$, $d_{\alpha N}(2,4)$, $d_{NN}(2,3)$, and $d_{NN}(3,4)$ cross-peaks (*64*, *65*) where the numbering indicates the position in the turn. The NOEs required for positive identification of a β-turn are a weak $d_{\alpha N}(2,4)$ cross-peak, corresponding to a distance of 3.6 Å in the type I turn and 3.3 Å in the type II turn, and a strong $d_{NN}(3,4)$ cross-peak, corresponding to a distance of 2.4 Å in both types of turns (*65*). Type I and type II turns may be distinguished by the relative strengths of the $d_{\alpha N}(2,3)$ and the $d_{NN}(2,3)$ cross-peaks. The type I turn displays a medium $d_{\alpha N}(2,3)$ cross-peak and a strong $d_{NN}(2,3)$ cross-peak, corresponding to distances of 3.4 and 2.6 Å, respectively, whereas the type II turn displays a strong $d_{\alpha N}(2,3)$ cross-peak and a weak $d_{NN}(2,3)$ cross-peak, where the corresponding distances are 2.2 and 4.5 Å.

Figure 5 shows strip plots extracted from the 150 ms mixing time ^{15}N-edited NOESY HSQC spectrum of ^{15}N-labeled PAK 128−144(Hs145). The left panel (5A) shows connectivities observed for the *trans* isomer of the peptide, whereas the right panel (5B) shows connectivities observed for the *cis* isomer. Schematic diagrams showing the magnitude of the various NOE connectivities observed for the *trans* and *cis* isomers of PAK 128−144(Hs145) are shown in Figure 6. The *trans* isomer displays strong $d_{NN}(3,4)$ cross-peaks between Gln[136] and Phe[137], and Gly[141] and Cys[142], and weak $d_{\alpha N}(2,4)$ cross-peaks between Glu[135] and Phe[137], and Lys[140] and Cys[142]. These NOEs are diagnostic of two β-turns in the regions Asp[134]-Glu-Gln-Phe[137] and Pro[139]-Lys-Gly-Cys[142]. The *cis* isomer displays moderately weak $d_{NN}(3,4)$ cross-peaks between Gln[136] and Phe[137], and Gly[141] and Cys[142], and a weak $d_{\alpha N}(2,4)$ cross-peak between Lys[140] and Cys[142], but no $d_{\alpha N}(2,4)$ cross-peak between between Glu[135] and Phe[137]. This suggests that only the second β-turn spanning Pro[139]-Lys-Gly-Cys[142] is present in the *cis* isomer.

The configurations of the two β-turns in the *trans* isomer can be approximated from the relative intensities of the $d_{\alpha N}(2,3)$, $d_{NN}(2,3)$, and $d_{\beta N}(2,4)$ cross-peaks (Figure 5A). The presence of a strong $d_{\alpha N}(2,3)$ and a weaker $d_{NN}(2,3)$ cross-peak between Lys[140] and Gly[141] suggests a predominant type II configuration for the turn spanning Pro[139]-Lys-Gly-Cys[142] (*29*). The configuration of the turn spanning Asp[134]-Glu-Gln-Phe[137] is somewhat less obvious based on the relative intensities of the $d_{\alpha N}(2,3)$ and $d_{NN}(2,3)$ cross-peaks. The $d_{\alpha N}(2,3)$ cross-peak between Glu[135] and Gln[136] is about the same magnitude as the $d_{NN}(2,3)$ between these two residues, but comparatively weaker than the $d_{\alpha N}(2,3)$ observed between Lys[140] and Gly[141]. However, a configuration of type I is suggested by moderately strong $d_{\beta N}(2,4)$ connectivities between Glu[135] and Phe[137] since this distance can approach as closely as 2.9 Å in a type I turn, but only as close as 3.6 Å in a type II turn. By contrast, the $d_{\beta N}(2,4)$ connectivity between Lys[140] and Cys[142] is vanishingly weak, consistent with a configuration of type II for the second turn. With respect to the *cis* isomer, the presence of a strong $d_{\alpha N}(2,3)$ and vanishingly weak $d_{NN}(2,3)$ cross-peak between Lys[140] and Gly[141], and the absence of any $d_{\beta N}(2,4)$ connectivity between Lys[140] and Cys[142] (Figure 5B), suggests a type II conformation for the single β-turn spanning Pro[139]-Lys-Gly-Cys[142].

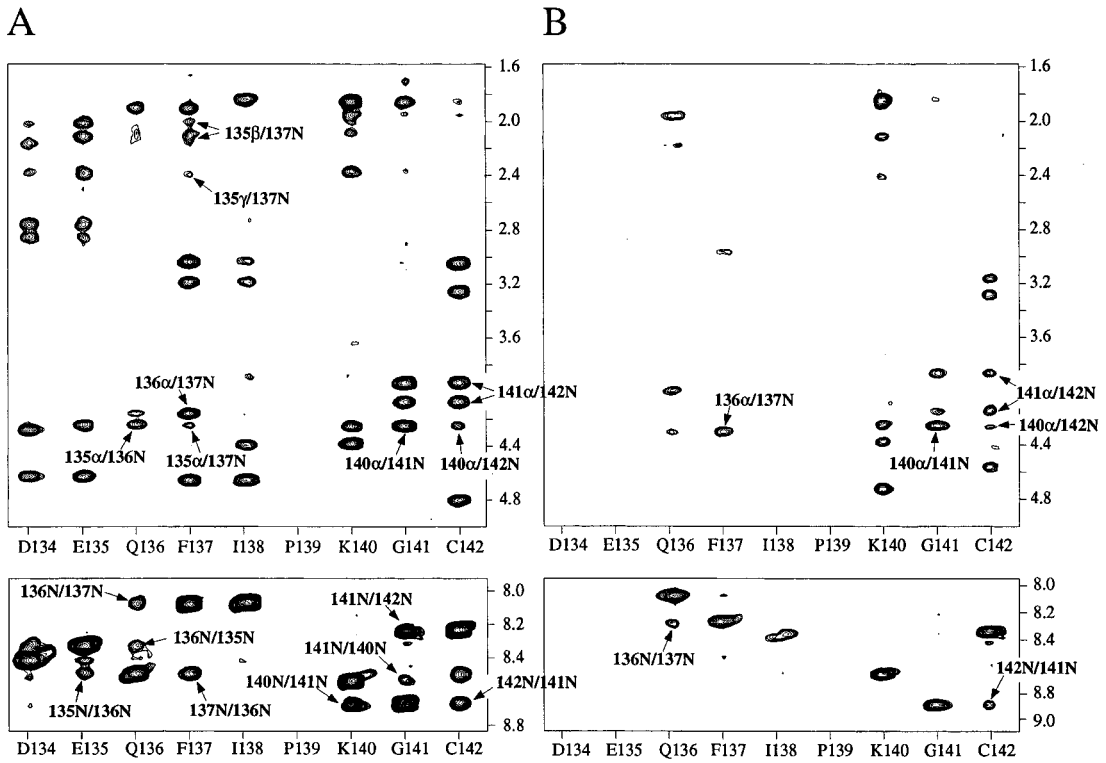

FIGURE 5: Strip plots from the 3D ^{15}N-edited HSQC NOESY spectrum of recombinant ^{15}N-labeled PAK 128−144(Hs145) showing the NOEs identifying β-turns in the antibody epitope region (134−142) for (A) the *trans* isomer and (B) the *cis* isomer.

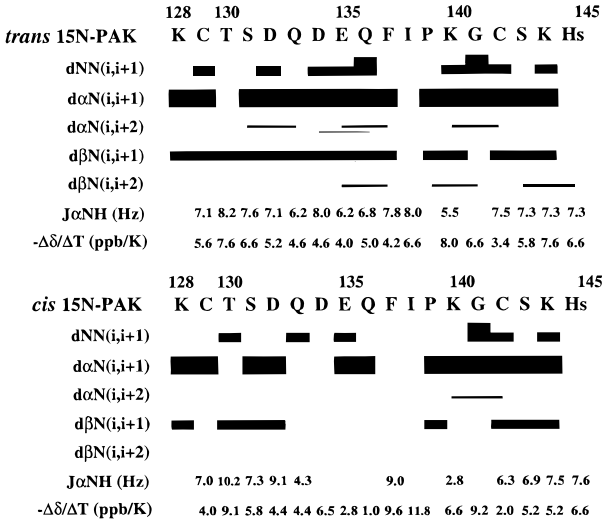

FIGURE 6: Schematic diagram showing the magnitude of various NOE connectivities observed in the 3D ^{15}N-edited HSQC NOESY spectrum of recombinant ^{15}N-labeled PAK 128−144(Hs145), and including coupling constants ($^3J_{NH\alpha CH}$) measured at 5.0 °C and temperature coefficients ($-\Delta\delta/\Delta T$ ppb/K).

The 'turn-diagnostic' NOEs [$d_{NN}(3,4)$, $d_{\alpha N}(2,3)$, $d_{\alpha N}(2,4)$, and $d_{\beta N}(2,4)$] observed for the *trans* and *cis* isomers of the recombinant peptide are found in the same relative intensities in the *trans* and *cis* isomers of the synthetic PAK pilin peptide (*20, 21, 59*), and are consistent with the presence of the type I and type II β-turns seen for the NMR solution structure of the *trans* isomer (*20, 21*).

DISCUSSION

The pattern of NOEs, temperature coefficients, and coupling constants observed for the *trans* isomer of recombinant ^{15}N-labeled PAK 128−144(Hs145) demonstrates the presence of a type I β-turn and a type II β-turn spanning Asp^{134}-Glu-Gln-Phe^{137} and Pro^{139}-Lys-Gly-Cys^{142}, respectively. This is in agreement with the NMR solution structure of the *trans* isomer of the synthetic PAK 128−144 peptide which shows type I and type II β-turns in these same regions of the sequence (*20, 21*). The pattern of NOEs, temperature coefficients, and coupling constants observed for the *cis* isomer of the recombinant peptide also demonstrate a type II β-turn spanning Pro^{139}-Lys-Gly-Cys^{142}; however, the type I β-turn seen in the *trans* isomer appears to be absent in the *cis* isomer. Instead, the low coupling constant measured for Gln^{133} and low temperature coefficient measured for Glu^{135} in the *cis* isomer suggest a second β-turn spanning Asp^{132}-Gln-Asp-Glu^{135}. Thus, the *cis* isomer may also possess a double-turn motif (like the *trans* isomer), but with different spacing between the turns and a different placement of the first turn in the sequence.

Recently, we have used ^{1}H NMR spectroscopy to study the binding of the synthetic PAK pilin peptide to antibody PAK-13 and to the disaccharide receptor analog, βGalNAc-(1−4)βGal (*25*). Both *trans* and *cis* isomers were observed to bind to antibody and receptor primarily at the site of the second type II β-turn spanning Pro^{139}-Lys-Gly-Cys^{142}. Since only this turn is preserved in both the *trans* and the *cis* isomers, this begs the question as to whether the presence of the second turn alone is sufficient for the recognition and binding of the PAK peptide to antibody and/or receptor. From the adhesintope mapping studies of the PAK peptide binding to A549 cells (*42*), two out of the four essential residues for binding, Pro^{139} and Gly^{141}, are located in the i and i+2 positions of the second type II β-turn. Only one of the essential residues (Gln^{136} in position i+2) is located in the first type I β-turn. Therefore, although it is possible that both isomers of the PAK peptide bind antibody and/or receptor in a double-turn motif, the second turn may be more

intimately associated with receptor and therefore may be sufficient alone for binding.

The discovery that the double-turn motif seen in the NMR solution structure of the *trans* synthetic PAK pilin peptide is preserved in the solution structure of the *trans* (and *cis*) recombinant PAK pilin peptide is an extremely important and *necessary* result if the recombinant peptide is to be used in future isotope-edited NMR studies of the labeled recombinant PAK pilin peptide free in solution versus bound to antibody and receptor. The conserved structural identity of the β-turns in the recombinant and synthetic PAK pilin peptides also explains the similarity in the association constants measured for the interaction of these two peptides with antibody PAK-13, and suggests that similar structure leads to similar immune recognition. The immunological equivalence of the recombinant peptide to the synthetic peptide is essential since our eventual goal is to provide high-resolution structures of the recombinant peptide in complex with antibody and receptor, and thereby provide a detailed picture of the complex structural immunology involved in these interactions. In addition, ^{15}N relaxation measurements will allow a study of the dynamics involved in the *trans/cis* isomerization process within the recombinant PAK peptide itself, and should determine whether this has a kinetic effect on the binding of the peptide to antibody or receptor. These studies will be integral to the design of a synthetic peptide vaccine effective against multiple strains of *P. aeruginosa* infection. Finally, the bacterial expression and purification system described in this study provides an economically viable method to produce isotopic-labeled synthetic peptide antigens, and as such allows a new level of sophistication with the use of multidimensional heteronuclear NMR spectroscopic techniques for the study of immune protein−peptide complexes.

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