Protein Labeling

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Generation of Pseudocontact Shifts in Protein NMR Spectra with a Genetically Encoded Cobalt(II)-Binding Amino Acid**

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There is increasing interest in the paramagnetic labeling of proteins for structural studies by NMR spectroscopy. The resulting paramagnetic effects, particularly pseudocontact shifts (PCSs) and paramagnetic relaxation enhancement (PRE), provide powerful long-range structural information for the rapid structure analysis of proteins, protein–protein complexes, and protein–ligand complexes.[1] Different strategies have been applied for the site-specific labeling of proteins with paramagnetic metal ions. Most rely on single cysteine residues in the protein or peptide fusions.[2] A more widely applicable method would make use of a non-natural metal-binding amino acid that could be incorporated anywhere in the protein without restriction to the N or C terminus of the protein and without consideration of the presence of cysteine residues or disulfide bonds. Herein we show that the site-specific incorporation of the genetically encoded non-natural amino acid bipyridylalanine (BpyAla, Figure 1 a)[3,4] endows the target protein with a site-specific binding site for Co^{II} that generates significant long-range PCSs.

We selected the West Nile virus NS2B-NS3 protease (WNVpro) as the model system. WNVpro is an established drug target which consists of segments from the NS2 and NS3 proteins of the viral polyprotein. In our 28 kDa construct of WNVpro, the NS2B domain was covalently linked to the protease domain NS3 through a Gly_4-Ser-Gly_4 linker,[5] Lys96 was mutated to alanine to prevent self-cleavage,[6] and a His_6 tag was present at the C terminus. The protease was inhibited with 4-nitrophenyl 4-guanidinobenzoate.[7]

BpyAla was incorporated into the protein by cell-free synthesis by using an *Escherichia coli* S30 extract. Cell-free synthesis was chosen because it enabled 1) facile mutation of strategically selected codons to amber stop codons as part of the PCR-amplification protocol without the need for cloning,[8] 2) optimization of the concentrations of suppressor tRNA and the BpyAla aminoacyl-tRNA synthetase (BpyAlaRS), 3) sparing use of the non-natural amino acid, and 4) selective ^{15}N labeling without isotope scrambling.[9,10]

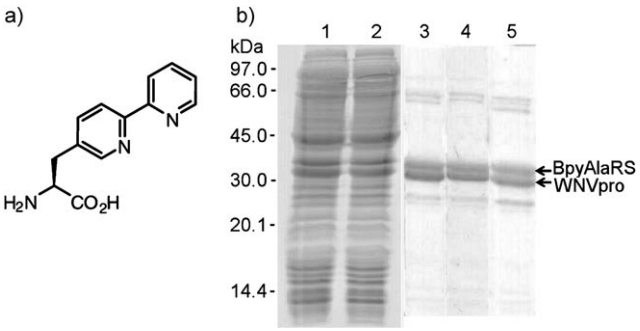

Figure 1. Cell-free expression of BpyAla mutants of WNVpro. a) Structure of BpyAla. b) SDS-PAGE (15%) of BpyAla mutants of WNVpro and analysis with Coomassive Blue staining. Lanes 1 and 2: complete cell-free reaction mixture and soluble fraction of mutant H87BpyAla. Lanes 3–5: purified WNVpro mutants with BpyAla at positions 86, 87, and 88, respectively. The band of BpyAlaRS presents an internal standard of protein yield as BpyAlaRS had an N-terminal His_6 tag and was therefore purified together with the WNVpro mutants to provide an internal standard of protein yield. BpyAlaRS did not appear in the NMR spectra, as it was unlabeled.

Residues Gln86, His87, and Lys88 of NS3, which are located far from the substrate-binding site and the interaction sites between NS2B and NS3, were targeted for mutation to BpyAla. Expression yields were about 1 mg of protein per milliliter of cell-free reaction mixture (Figure 1 b). ^{15}N HSQC spectra of selectively ^{15}N-labeled samples were very similar to that of the wild-type protein, a result indicating structural conservation (see Figures S2–S5 in the Supporting Information). The addition of Co^{II} produced significant PCSs for the H87BpyAla mutant (Figure 2).

The PCSs observed for the His87BpyAla mutant were used to fit the magnetic-susceptibility-anisotropy ($\Delta\chi$) tensor according to Equation (1):

$$\Delta\delta^{PCS} = \frac{1}{12\pi r^3}\left[\Delta\chi_{ax}\left(3\cos^2\theta - 1\right) + \frac{3}{2}\Delta\chi_{rh}\sin^2\theta\cos 2\varphi\right] \quad (1)$$

in which $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ denote the axial and rhombic components, respectively, of the $\Delta\chi$ tensor, r, θ, and ϕ are the polar coordinates of the nuclear spin with respect to the

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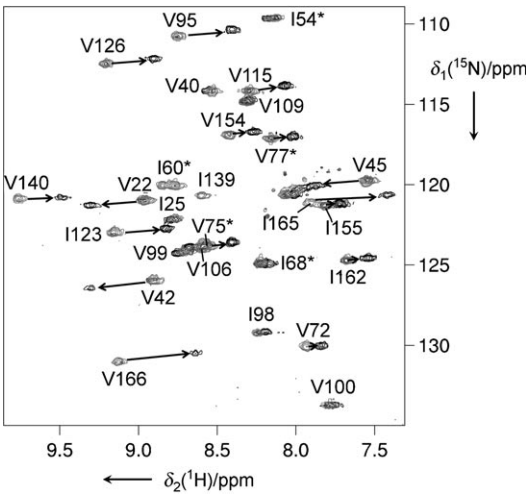

Figure 2. Superimposition of ^{15}N HSQC spectra of an 80 μM solution of ^{15}N-Ile- and ^{15}N-Val-labeled WNVpro H87BpyAla without (gray) and with (black) Co^{II} at 25 °C, pH 6.9. Arrows indicate the pseudocontact shifts induced by Co^{II}. The cross-peaks are assigned as previously reported.[24] NS2B resonances are marked with a star.

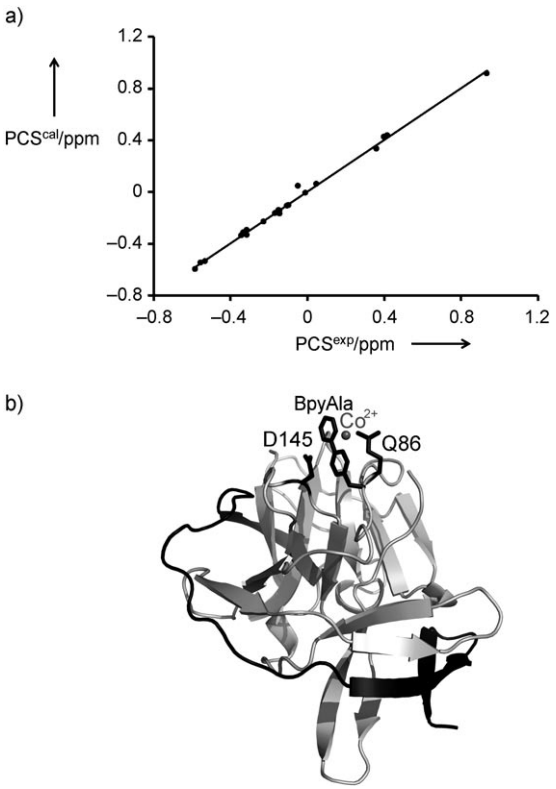

Figure 3. Metal-binding site and $\Delta\chi$ tensor fit of the Co^{II} complex of WNVpro H87BpyAla. a) Plot of back-calculated PCSs (PCS^{cal}) versus experimental PCSs (PCS^{exp}). b) Crystal structure of WNVpro (PDB ID: 2FP7)[11] showing the location of Co^{II} as determined from the PCSs. The side chains of BpyAla87, Asp145, and Gln86 are shown in black. The figure was created with PyMOL (see the Supporting Information for a color version).[17]

principal axes of the $\Delta\chi$ tensor, and $\Delta\delta^{PCS}$ is the pseudocontact shift. We measured ^1H PCSs as the difference between the chemical shifts of paramagnetic and diamagnetic cross-peaks observed in the ^{15}N HSQC spectrum of the protein (see Figure S2 in the Supporting Information). Fitting of the $\Delta\chi$ tensor showed that the Co^{II} ion was located near the side chain of residue 87, as expected.

In a second calculation, the bipyridyl side chain was crafted onto the crystal structure of the protease (PDB ID: 2FP7),[11] and the metal ion was positioned in the plane of the bipyridyl moiety at a distance 1.9 Å from the nitrogen atoms. The χ^1 and χ^2 angles of the bipyridyl side chain were systematically varied, and $\Delta\chi$ tensors were fitted by using the experimental PCSs. The agreement between experimental and back-calculated PCSs for the best fit was excellent (Figure 3a). The metal was within 0.3 Å of the position identified by the fit obtained by using no restraints besides PCSs. The metal position suggests additional coordination by the carboxy group of Asp145 and the side-chain amide group of Gln86 (Figure 3b). The fitted tensor parameters ($\Delta\chi_{ax} = -6.9 \pm 0.4 \times 10^{-32}\ m^3$ and $\Delta\chi_{rh} = -3.5 \pm 0.6 \times 10^{-32}\ m^3$) are characteristic for high-spin Co^{II}.[12]

The PCSs enabled assessment of the 3D structure of WNVpro, as significant paramagnetic shifts were observed for most of the isoleucine and valine residues of the protein, including PCSs of 0.15 ppm as far as 28 Å from the metal ion. The PCSs were perfectly compatible with the cocrystal structures containing tightly binding peptide inhibitors[11,13] and thus indicated that our low-molecular-weight inhibitor induced a very similar conformation. This result is remarkable as, in the absence of an inhibitor, the crystal structure of WNVpro shows NS2B largely dissociated from NS3.[14] Previous NMR spectroscopic data obtained by the use of spin labels had indicated that, in solution, NS2B populates different conformations but tends to be predominantly associated with NS3.[15]

In contrast to the H87BpyAla mutation, the mutants Q86BpyAla and K88BpyAla yielded significant PREs with Co^{II} but no or only very small PCSs (see Figures S3 and S4 in the Supporting Information). The observation of PREs without PCSs is a hallmark of motion of the metal with respect to the protein. Clearly, immobilization of the bipyridyl–cobalt(II) complex is greatly assisted by coordination to additional protein side chains.

In the H87BpyAla mutant, cobalt(II)-bound and free protein exchanged only slowly, as evidenced by the coexistence of diamagnetic and paramagnetic peaks. This behavior is expected in view of the dissociation constant of 1.9 μM reported for the bipyridine–cobalt(II) complex.[16] The slow metal exchange permits accurate PCS measurements from protein spectra when paramagnetic Co^{II} and diamagnetic metal ions are present simultaneously (see Figure S2 in the Supporting Information).

In conclusion, the site-specific incorporation of BpyAla endows proteins with a cobalt(II)-binding site near the protein backbone. Site-specific paramagnetic labeling of proteins with BpyAla–Co^{II} complexes thus opens a window for greatly facilitated structure analysis of proteins and their complexes with binding partners, as PCSs provide detailed structural information even when NMR resonances can only partially be assigned.

Experimental Section

Materials: BpyAla was synthesized as described[4] with a minor modification (see the Supporting Information). The inhibitor 4-nitrophenyl 4-guanidinobenzoate was purchased from Sigma–Aldrich. ^{15}N-labeled isoleucine and valine were from Cambridge Isotope Laboratories. BpyAlaRS was constructed from *Mj*TyrRS (see the Supporting Information). Total tRNA, including suppressor tRNA,[18] was prepared by a previously described procedure (see the Supporting Information).[19] S30 cell extracts were prepared from *E. coli* BL21 Star:λDE3 and concentrated with poly(ethylene glycol) 8000 as described.[20]

Cell-free protein synthesis: Cell-free coupled transcription/translation reactions were carried out in dialysis mode at 30 °C for 10–14 h as described.[8–10] The final concentrations used were 0.525 mg mL^{-1} total tRNA, 40 μM BpyAlaRS (inner buffer), and 1 mM BpyAla (both inner (2 mL) and outer (20 mL) buffer). BpyAla mutants of WNVpro were produced from PCR-amplified DNA templates by the procedure described by Wu et al.,[8] with WNVpro inserted between the *Nde*I and *Eco*RI sites of the vector pRSET5b[21] as the template. The protein samples were purified by using IMAC (immobilized-metal-ion-affinity chromatography) nickel nitrilotriacetic acid spin columns. Treatment with ethylenediaminetetraacetic acid removed any metal ion bound to BpyAla. The purified protein was washed with NMR buffer (2-(*N*-morpholino)ethanesulfonic acid (20 mM) and tris(2-carboxyethyl)phosphane (1 mM) in H_2O/D_2O (9:1) at pH 6.9) and concentrated to 500 μL in a protein concentrator with a molecular-weight cutoff of 10 kDa.

NMR spectroscopy: ^{15}N HSQC spectra were recorded in NMR buffer in the presence of a fivefold excess of the inhibitor at 25 °C on a Bruker Avance 600 MHz NMR spectrometer equipped with a cryoprobe. Each spectrum was recorded for about 4 h with $t_{1max} =$ 45 ms and $t_{2max} = 142$ ms.

$\Delta\chi$ tensor fit: The $\Delta\chi$ tensor and position of the Co^{II} ion were fitted by using the programs Numbat[22] and PyParaTools.[23]

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