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 1a,[3, 4] endows the target protein with a site-specific binding site for CoII 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 (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 drug target which consists of segments from the NS2 and NS3 proteins for structural studies by NMR spectroscopy. Support of this research by the Australian Research Council included fellowships to K.O. and T.H. components, respectively, of the nuclear spin with respect to the polar coordinates of the nuclear spin with respect to the nuclear spin with respect to the nuclear spin with respect to the
principal axes of the $\Delta \chi$ tensor, and $\Delta \sigma_{\text{PCS}}$ is the pseudocontact shift. We measured $^1$H 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 $\chi'$ and $\chi''$ 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 ($\Delta \sigma_{\text{ax}} = -6.9 \pm 0.4 \times 10^{-3} \text{ m}^3$ and $\Delta \sigma_{\text{rh}} = -3.5 \pm 0.6 \times 10^{-3} \text{ m}^3$) 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 co-crystal 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\(^6\) 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 M/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 Star3DE3 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.\(^{16–19}\) 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 WNVPpro were produced from PCR-amplified DNA templates by the procedure described by Wu et al.\(^{8}\) with WNVPpro inserted between the Ndel and EcoRI sites of the vector pRSET5b\(^{21}\) as the template. The protein samples were purified by using IMAC (immobilized-metal-affinity chromatography) nickel nitrilotriacetic acid spin columns.

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 4 h with \(t_{\text{max}} = 45 \text{ ms}\) and \(t_{\text{max}} = 142 \text{ ms}\). The A\(^x\) tensor fit: The A\(^x\) tensor and position of the Co\(^{6}\) ion were fitted by using the programs Numbat\(^{22}\) and PyParaTools\(^{23}\).

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