Journal of Biomolecular NMR **29:** 541–542, 2004.
© 2004 *Kluwer Academic Publishers. Printed in the Netherlands.*

Letter to the Editor: ^{1}H, ^{13}C and ^{15}N resonance assignment of Cu(I)-pseudoazurin from *Alcaligenes faecalis S-6*

Antonietta Impagliazzo & Marcellus Ubbink*
Leiden Institute of Chemistry, Leiden University, Gorlaeus Laboratories, P.O. Box 9502, 2300 RA Leiden, The Netherlands

Received 21 January 2004; Accepted 15 March 2004

Key words: alcaligenes, copper, pseudoazurin

Biological context

Pseudoazurin (PAZ) is a copper-containing redox protein of 123 amino acids that is isolated from denitrifying bacteria, like *Alcaligenes faecalis S-6* (Kakutani et al., 1981). It functions as electron donor to a copper-containing nitrite reductase (NiR), which catalyses the reduction of nitrite to nitric oxide as part of the denitrification process. The proteins form a transient complex (Kukimoto et al., 1995, 1996) to enable electron transfer from pseudoazurin to NiR.

The complex requires a high turnover to avoid that electron transfer, rather than the chemical conversion, becomes rate limiting. However, efficient electron transfer also requires the formation of a specific complex, with a short distance between the redox centres (Marcus and Sutin, 1985). We aim to determine the dynamic and structural features of the complex of NiR and PAZ to understand how such proteins can associate and dissociate rapidly, yet with sufficient specificity to allow for electron transfer. NMR spectroscopy is the technique of choice to study the features of the complex under native conditions. Chemical shift perturbation can identify the binding site. Line shape analysis provides information about the dissociation rates and the orientation of the proteins in the complex can be determined using distance information obtained from paramagnetic shifts, as we demonstrated for the complex of cytochrome *f* and plastocyanin (Ubbink et al., 1998; Crowley et al., 2001). A necessary step towards the determination of the complex interface is the assignment of the NMR spectra of ^{1}H, ^{15}N and ^{13}C of the free PAZ, which is reported here.

*To whom correspondence should be addressed. E-mail: m.ubbink@chem.leidenuniv.nl

Methods and results

Part of the PAZ gene, coding for the mature protein, was subcloned in pET-28a(+), creating a plasmid for expression in the cytoplasm of *Escherichia coli*. The subcloning procedure introduced two additional residues at the N-terminus, a Ser and Ala. The protein was produced in *E. coli* strain HMS174, cultured on minimal medium containing $^{15}NH_4Cl$ and U-^{13}C- glucose. Cultures were incubated at 37 °C with 250 rpm shaking to an OD_{600} of 0.7. Expression was induced with 0.5 mM IPTG, and 100 μM copper citrate was added. Ten hours after induction cultures were harvested by centrifugation. Cell pellets were resuspended in 20 mM phosphate buffer pH 7.0 containing 500 mM NaCl, 1 mM PMSF, DNAse and 0.5 mM $CuCl_2$ and lysed using a French pressure cell (15.000 PSIG). After centrifugation for 15 min at 10.000 rpm the supernatant was dialysed against 20 mM phosphate buffer pH 7.0 and loaded onto a CM column equilibrated with the same buffer. PAZ eluted at circa 90 mM using a gradient of 0–250 mM NaCl. The fractions containing PAZ were concentrated and purified further on a Superdex 75 FPLC gel filtration column. The 277/595 absorbance ratio of PAZ was 1.9 indicating a purity $> 95\%$ (Keiichi et al., 1987), with a yield of 30 mg/L.

NMR samples contained 2–3 mM ^{15}N-PAZ or ^{15}N-^{13}C PAZ, 2 mM sodium ascorbate and either 10% or $> 99\%$ D_2O in 20 mM potassium phosphate buffer pH 7.0.

Samples were placed into 5 mm Shigemi micro NMR tubes. All NMR spectra were acquired at 312K on a Bruker DMX 600 MHz NMR spectrometer. Backbone resonances were assigned using ^{15}N-HSQC, HNCA, HNCACB, HNCO and

Figure 1. ^{1}H-^{15}N-HSQC spectrum of PAZ with assignments (20 mM potassium phosphate pH 7.0, 312K). Resonances of Asn and Gln side chains amides are connected by horizontal lines. The asterisk indicates the amide resonances of ^{15}N-acetamide, which is used as internal standard.

HNCACO spectra. Side-chain carbon and proton resonances were assigned using 2D ^{15}N-HSQC-TOCSY, 2D ^{15}N-HSQC-NOESY, ^{13}C-HSQC, H(CCO)NH, and HCCH-TOCSY spectra. Resonances of aromatic side-chains were assigned using ^{13}C-HSQC, HCCH-TOCSY and ^{13}C-HSQC-NOESY spectra optimised for detection of the aromatic region of ^{13}C spectrum. Spectra were processed with AZARA (http://www.bio.cam.ac.uk/azara/) and analysed with ANSIG for WINDOWS 1.0 (Kraulis, 1989; Helgstrand et al., 2000).

Extent of the assignment and data deposition

Resonances have been assigned for all the ^{1}H, ^{15}N and ^{13}Cα nuclei backbone except for residues A-1, K46 and D47, which do not appear in the ^{1}H-^{15}N-HSQC spectrum (Figure 1), probably due to fast exchange with the solvent.

Resonances for all side chain have been assigned except for several nuclei in residues E13, I49, P80, M86, P108 and L115 and the Lys residues 24, 38, 46, 57, 59, 77, 106, 107 and 109. In conclusion, the extent of PAZ assignment is: 98% of the amide resonances, 98% of Cα, 97% of Hα, 96% of CO, 94% of ^{1}H and ^{13}C side-chain and 54% of aromatics ^{1}H and ^{13}C resonances.

Chemical shifts of all assigned nuclei have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) entry BMRB-6043.

Acknowledgements

Dr M. Boulanger and Prof M.E.P. Murphy are acknowledged for providing the PAZ expression vector. We thank Prof G.W. Canters for fruitful discussions. This work was supported by the 'Jonge Chemici' Programme of the Netherlands Organisation for Scientific Research (NWO), grant 99512.

References

Crowley, P.B. and Ubbink, M. (2001) *J. Am. Chem. Soc.*, **123**, 10444–10453.

Helgstrand, M., Kraulis, P., Allard, P. and Härd, T. (2000) *J. Biomol. NMR*, **18**, 329–336.

Kakutani, T., Watanabe, H., Arima, K. and Beppu, T. (1981) *J. Biochem.*, **89**, 463–472.

Kraulis, P.J. (1989) *J. Magn. Reson.*, **84**, 627–633.

Kukimoto, M., Nishiyama, M., Ohnuki, T., Turley, S., Adman, E.T., Horinouchi, S. and Beppu, T. (1995) *Protein Eng.*, **8**, 153–158.

Kukimoto, M., Nishiyama, M., Tanokura, M., Adman, E.T. and Horinouchi, S. (1996) *J. Biol. Chem.*, **271**, 13680–13683.

Marcus, R.A. and Sutin, N. (1985) *Biochim. Biophys. Acta*, **811**, 265–322.

Ubbink, M., Ejdebäck, M., Karlsson, B.G. and Bendall, D.S. (1998) *Structure*, **6**, 323–335.

Yamamoto, K., Uozumi, T. and Beppu, T. (1987) *J. Bacteriol.*, **169**, 5648–5652.