Detection of Scalar Couplings Across NH···OP and OH···OP Hydrogen
Bonds in a Flavoprotein

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Supporting Information

Figure 1. Experimental scheme for the quantitative determination of ^{15}N-^{31}P scalar couplings. The pulse sequence is derived from the [$^{15}N,^1H$]-TROSY experiment (Pervushin, K.; Riek, R.; Wider, G.; Wüthrich, K. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 12366-12371.) and uses gradient ^{15}N coherence selection combined with sensitivity enhancement as described by Yang and Kay (Yang, D.; Kay, L. E. *J. Biomol. NMR* **1999**, *13*, 3-10.). Narrow and wide bars denote rectangular 90° and 180° pulses, respectively, applied with phase x unless specified. The hatched 180° pulses and gradients were only applied in a control experiment to assess the influence of ^{15}N chemical shift anisotropy/^{31}P-^{15}N dipole-dipole relaxation interference. RF field strengths (carrier positions) were 18.5 (4.7 ppm), 7.1 (124.7 ppm), and 12.5 kHz (4.96 ppm) for pulses on ^1H, ^{15}N, and ^{31}P, respectively. ^{31}P decoupling during acquisition was achieved via a 167 Hz GARP-1 modulation (Shaka, A. J.; Barker, P. B.; Freeman, R. *J. Magn. Reson.* **1985**, *64*, 547-552.). The initial 90° Gaussian shaped pulse of 2.5 ms duration aligns the water magnetization along the positive z axis at the end of the sequence and avoids the saturation of fast exchanging amide protons by pulsed field gradients (Grzesiek, S.; Bax, A. *J. Am. Chem. Soc.* **1993**, *115*, 12593-12594; Matsuo, H.; Kupče, E.; Li, H.; Wagner, G. *J. Magn. Reson.*

Ser. B **1996**, *111*, 194-198.). Pulse phases were cycled according to $\phi_1 = 4(x), 4(-x)$; $\phi_2 = x, -x$;

$\phi_3 = 4(x, x, y, y), 4(-x, -x, -y, -y)$; $\phi_4 = 8(x), 8(-x)$; $\phi_5 = x$; $\phi_{receiver} = R, 2(-R), R$ with $R = x$,

$2(-x), x$. All gradients were sine-bell shaped and had durations of 1 ms ($G_{1,2,3,4}$) or 0.5 ms

($G_{5,6,7}$) with the following directions and approximate strengths at their center G_1: x, 5 G/cm;

G_2: y, 7.5 G/cm; G_3: y, 5 G/cm; G_4: xyz, ± 39.4 G/cm; G_5: x, 4 G/cm, y, 5.5 G/cm; G_6: x, 5.5

G/cm, y, 4 G/cm; G_7: xyz, 8 G/cm. N- and P-type signals are collected alternately by inverting

the direction of G_4 along with pulse phase ϕ_5. Axial peaks are shifted to the edge of the

spectrum by incrementing ϕ_1 and the receiver phase by 180° for each value of t_1. The delays τ

and ζ had durations of 2.3 and 0.7 ms, respectively.

The experiment relies on the quantitative *J* correlation concept (Bax, A.; Vuister, G. W.;

Grzesiek, S.; Delaglio, F.; Wang, A. C; Tschudin, R.; Zhu, G. *Meth. Enzymol.* **1994**, *239*,

79-105.) in order to measure the size of scalar ^{15}N-^{31}P interactions. Evolution of these

heteronuclear couplings during the period Δ leads to a build up of ^{15}N antiphase magnetization

with respect to ^{31}P ($N_x (1 - 2H_z) P_z$) which is selected by phase cycling of the 90° pulses on

^{31}P. Since no discrimination of ^{31}P chemical shifts is required the experiment can be carried out

in a two-dimensional version without ^{31}P evolution period, thus providing ^1H-^{15}N correlation

spectra. Cross peak intensities (I_{cross}) are proportional to $\sin^2(\pi J_{NP}\Delta)$. For a quantitative

determination of the desired coupling constants it is necessary to acquire a reference spectrum

in which the signal intensity depends in the same way on ^1H and ^{15}N relaxation rates and

instrumental sensitivity but not on the ^{15}N-^{31}P coupling. This was achieved using an identical

pulse sequence but omitting the phase-cycling of pulses on ^{31}P (i.e. $\phi_1 = 2(x), 2(-x)$; $\phi_2 = x$; $\phi_3 =$

$2(x, y), 2(-x, -y)$; $\phi_4 = x$; $\phi_5 = x$; $\phi_{receiver} = x, 2(-x), x$), such that ^{15}N magnetization is maintained

irrespective of whether it is coupled to ^{31}P or not. Because for small couplings the reference intensities (I_{ref}) are considerably higher than those in the ^{31}P selected experiment a lower number of scans (NS) can be employed here and the coupling constants can be calculated from the relation $I_{cross}/I_{ref} = (NS_{cross}/NS_{ref}) \times \sin^2(\pi J_{NP}\Delta)$.

If 180° pulses on nitrogens are applied in the center of the Δ periods, as indicated by the hatched rectangles, heteronuclear scalar couplings are refocused whereas ^{15}N chemical shift anisotropy/^{31}P-^{15}N dipole-dipole cross correlated relaxation would still be active, giving rise to $N_y (1 - 2H_z) P_z$ antiphase magnetization at the time when the first 90° pulse on phosphorus is applied. The absence of detectable cross peaks in a spectrum recorded with the latter version (see Figure 4C) of the pulse sequence suggests that this effect is negligible in the case of flavodoxin.

Supporting Information Figure 1