Detection of Short-Lived Transient Protein−Protein Interactions by Intermolecular Nuclear Paramagnetic Relaxation: Plastocyanin from *Anabaena variabilis*

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The formation of transient peptide−protein and protein−protein complexes is an essential part of many biological processes. Well-established NMR techniques, based on intermolecular ^1H−^1H dipolar interactions (the nuclear Overhauser enhancements (NOEs)), are being used extensively in studies of the contact surfaces and structures of relatively stable complexes.[1] For sufficiently short-lived complexes, where the NOE approach cannot be applied, the much stronger electron−nucleus dipole interaction can be exploited if at least one of the interacting molecules is paramagnetic. This was demonstrated in an elegant study of the interaction between cytochrome *f* and plastocyanin,[2] where the changes in chemical shift of plastocyanin nuclei upon binding to paramagnetic cytochrome *f* were used to determine the structure of the complex. However, even this approach fails for more short-lived complexes where the intermolecular interaction is nearly diffusion controlled, as in the electron self-exchange process of plastocyanins, or in the initial stage of the electron transfer between cyanobacteria plastocyanins and cytochrome *f*.[3,4]

Here an NMR approach is presented that can detect short-lived, transient interactions between two protein molecules, using *Anabaena variabilis* (*A.v.* PCu) plastocyanin and the electron self-exchange between its reduced (diamagnetic) and oxidized (paramagnetic) forms as an example. The interaction is monitored by the *inter*molecular paramagnetic contribution to the longitudinal relaxation of protons at or close to the contact surface. Not only the unpaired electrons of the parent molecule but also those of the interacting molecule may affect the relaxation of these protons. However, unlike the first-mentioned effect the latter is concentration dependent and can, therefore, be detected by the concentration dependence of the nuclear relaxation rates.

The paramagnetic relaxation enhancement, R_{1p}, of protons more than about five bonds from the paramagnetic ion is caused only by the modulation of the dipolar spin−spin interaction between the nuclei and the unpaired electrons, e.g. the unpaired Cu^{2+} electron, and is to a good approximation given by[5,6]

$$R_{1p} = \frac{2}{5}\left(\frac{\mu_0}{4\pi}\right)^2 S(S+1)g_{eff}^2 \mu_B^2 \gamma_I^2 \Delta^2 \frac{\tau_{c,1}}{1 + \omega_I^2 \tau_{c,1}^2} \quad (1)$$

Here $\Delta^2 = r^{-6}$ if the point dipole approximation applies,[5] *r* being the geometric distance between the nucleus and the unpaired electron, while $\tau_{c,1}$ is the correlation time for the modulation of the electron−nucleus dipolar interaction.[7] The parameters μ_0, S, g_{eff}, μ_B, γ_I, and ω_I are defined in ref 7.

The observed longitudinal relaxation of the nuclei can be affected by chemical exchange processes,[8] e.g. the electron self-exchange
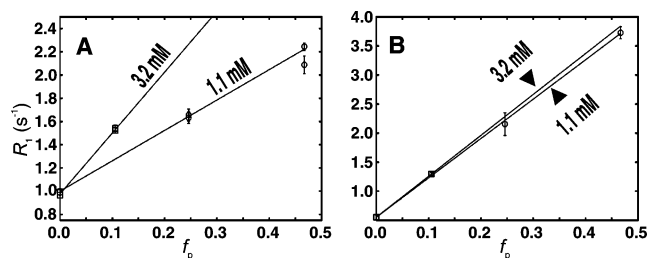
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Figure 1. Observed R_1 relaxation rates of the α-protons of residues (A) D44 and (B) F43 in *A.v.* PCu at (○) 1.1 mM and (□) 3.2 mM *A.v.* PCu, for different fractions of the oxidized species. The straight curves correspond to a least-squares fit of eq 2 to the relaxation rates, using $k_{ese} = 186 \pm 13$ mM^{-1} s^{-1} as determined previously.[11] Both α-protons are close to the fast exchange regime, i.e. eq 2 reduces to $R_1 \approx R_{1d} + f_p R_{1p}$.[8] For residue D44 (A) the paramagnetic relaxation enhancement, R_{1p}, clearly depends on the concentration of the protein. For residue F43 (B) no concentration dependence of R_{1p} is observed.

(ESE). If the process is sufficiently fast, as in the case of the electron self-exchange of *A.v.* PCu, the longitudinal relaxation of the protons is single exponential,[9] and the rates obtained from the exchange-averaged signals are given by[9−11]

$$R_1 = R_{1d} + \frac{1}{2}(R_{1p} + k_{ese}c) - \sqrt{\left(\frac{R_{1p} + k_{ese}c}{2}\right)^2 - R_{1p}k_{ese}cf_p} \quad (2)$$

Here R_{1d} is the longitudinal relaxation rate in the diamagnetic species, R_{1p} is the longitudinal paramagnetic relaxation enhancement, k_{ese} is the second-order rate constant for the ESE process, f_p is the molar fraction of the oxidized species, and *c* is the total concentration of the protein.

The R_{1d} and R_{1p} rates of 40 α-protons in *A.v.* PCu[12,13] were obtained from IR-TOCSY experiments[14,15] by a least-squares fit of eq 2 to the signal recoveries, as shown in Figure 1. The rates of the remaining α-protons could not be obtained because the protons are too close to the paramagnetic center or because of severe signal overlap. To ensure a high precision, each rate was determined from more than one sample, using samples with different f_p as shown in Figure 1.

The concentration-dependent relaxation enhancements, ΔR_{1p}, observed in *A.v.* PCu are shown in Figure 2. Significant enhancements are found for about one-third of the observed α-protons, clearly showing that interactions take place between individual *A.v.* PCu molecules. Moreover, as shown in Figure 3 the concentration-dependent enhancements are confined to certain regions on the surface of the molecule, indicating that the interactions are specific. Thus, the residues 95 and 34, which are part of the hydrophobic patch on the "north side" of the molecule, have significant ΔR_{1p}
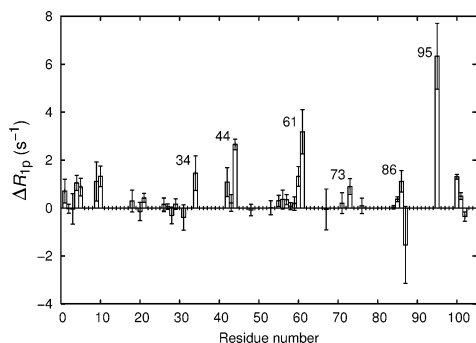
Figure 2. Observed concentration-dependent paramagnetic relaxation enhancements, $\Delta R_{1p} = R_{1p}$ (3.2 mM) $- R_{1p}$ (1.1 mM), of α-protons in *A.v.* PCu. The relaxation rates were not obtained for residues without error bars (see text). Residues 42–44 are located in a β-sheet, where the α-protons of 42 and 44 point toward the surface, while the α-proton of 43 points toward the core of the protein. Therefore, only the paramagnetic relaxation of 42 and 44 is enhanced.
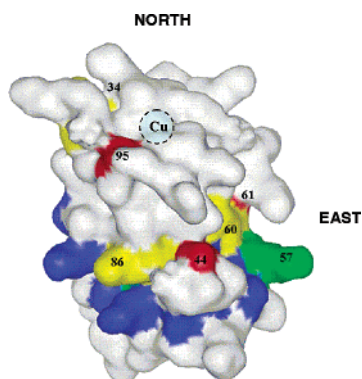

Figure 3. NMR solution structure of *A.v.* PCu (PDB entry 1FA4). Regions with concentration dependent paramagnetic relaxation enhancements, $\Delta R_{1p} = R_{1p}$ (3.2 mM) $- R_{1p}$ (1.1 mM) of the α-protons are colored; red, $\Delta R_{1p} > 2$ s^{-1}; yellow, 1 s$^{-1} < \Delta R_{1p} \leq 2$ s^{-1}; green, 0 s$^{-1} < \Delta R_{1p} \leq 1$ s^{-1}; blue $\sigma(\Delta R_{1p}) > \Delta R_{1p}$, $\sigma(\Delta R_{1p})$ being the experimental error.

values. Similar observations are made for the residues 42, 44, 60, 61, and 73 that are located on the "eastern face" of the molecule. These results indicate that the two regions are engaged in intermolecular interactions, which may be important for the electron self-exchange taking place in the sample. Interestingly, recent ^{15}N NMR relaxation studies of the backbone dynamics of *A.v.* PCu showed an increased mobility on the microsecond−millisecond time scale for both of these regions,[16] in accordance with the general observation that active regions of proteins often have enhanced mobility. Moreover, recent site-directed mutagenesis studies indicate that both regions are involved in the interaction of *Phormidium laminosum* (*P. laminosum*) plastocyanin[4] with cytochrome *f*. Further support of an eastern interaction surface is provided by the crystal structure of this plastocyanin[17] where both H61 and D44 are located at the crystal packing surfaces of the crystal trimer. Finally, it is worth noticing that none of the interior protons observed here shows concentration-dependent relaxation enhancements.

In conclusion it is shown that paramagnetic longitudinal proton relaxation can provide detailed information about short-lived, transient protein complexes where the intermolecular interactions are close to the diffusion limit. For the specific case of *A.v.* PCu two regions of interaction were identified. These regions may serve as the contact surfaces in the electron exchange process. This is supported by an increased mobility on the microsecond−millisecond time scale of these regions,[16] by the functional importance of the corresponding regions in *P. laminosum* plastocyanin,[4] and by the crystal structure of *P. laminosum* plastocyanin.[17]

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(14) The inversion recovery TOCSY (IR-TOCSY) experiments[15] consisted of 11 partly relaxed ^{1}H spectra recorded at 298.2 K on a Varian Inova 500 spectrometer. The time domain signals included 512 t_1 slices each with 4096 data points. The sweep width was 10000 Hz, and the mixing time was 50 ms. Signal intensities were determined by a least-squares fitting procedure.[9] The relaxation rates were derived from the signal recoveries by three-parameter single exponential fits. Several of the relaxation rates were determined independently from more than one cross-peak in the F_2 dimension (see Figure 1).
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