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Mapping of the Binding Site on Pseudoazurin in the Transient 152 kDa Complex with Nitrite Reductase

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Electron transfer (ET) reactions between redox proteins are essential in many metabolic processes. A specific protein:protein complex with a short metal-to-metal distance is required to achieve rapid ET.[1] However, the binding constant for complex formation is limited by the high dissociation rate constant, necessary for a high turnover rate. Thus, such transient complexes of redox proteins represent a compromise between specificity and affinity.[2] To understand the molecular details of transient complex formation, the nature of the interfaces needs to be characterized, as we have done using NMR for a number of relatively small complexes (<50 kDa).[3−11]

Here we report the binding interface of pseudoazurin (PAZ) with its partner nitrite reductase (NiR) from the denitrifying bacterium *Alcaligenes faecalis* S-6.[12,13] NiR is a copper-containing enzyme that catalyzes the conversion of nitrite to nitric oxide as part of the denitrification process.[14] It is a trimer (110 kDa) with a type 1 and a type 2 copper site[15] in each subunit.[14] PAZ is a 14 kDa blue copper protein with a type 1 copper site that acts as electron donor of NiR. The interaction site of PAZ and NiR has been investigated via site-directed mutagenesis and kinetic analysis.[16,17] It was shown that the replacement of PAZ lysine residues 10, 38, 57, and 77 by alanines decreases the affinity of PAZ for NiR, and, similarly, NiR mutants E118A, E197A, D201A, and E204A demonstrated reduced affinity for PAZ. Therefore, these charged residues have been suggested as key residues for the electrostatic interactions in the complex.

To map the binding site for NiR on PAZ by NMR, the effects of complex formation on ^{15}N-labeled PAZ were analyzed. Surprisingly, addition of NiR resulted in a proportional decrease of PAZ resonances and only minor line broadening. Apparently, complex formation is slow on the relevant NMR time scale, contrary to other redox protein complexes. A few residues reproducibly demonstrated small chemical-shift perturbations with increasing amounts of NiR, which are ascribed to a weak and fast secondary binding. A titration of ^{15}N-PAZ into NiR demonstrated that PAZ binds to trimeric NiR in a 3:1 ratio with a dissociation constant in the low μM range.

To map the binding site for NiR, the recently described cross-saturation method was employed.[18,19] For this purpose PAZ was enriched with ^{15}N (>98%) and ^{2}H (>93%) and dissolved in a buffer solution of 90% D_2O/10% H_2O. The high percentage of D_2O in the buffer solution was necessary to isolate the amide protons within PAZ to suppress spin diffusion, which would interfere with the cross saturation. Upon complex formation with NiR, magnetization saturation of NiR protons can be transferred to PAZ. This results in selective intensity decrease of the resonances of amide nuclei close to the interface. NiR protons were saturated using selective excitation of the aliphatic region of the spectrum. Saturation will rapidly spread across all NiR protons via spin diffusion. Due to the lack of aliphatic protons in the deuterated PAZ, the amide protons in PAZ only experience magnetization saturation if located close to NiR protons. During the 3-s saturation period, PAZ can dissociate from NiR. The saturation effect on the resonances of the bound PAZ can be observed on the resonances of the free protein, provided the exchange rate is at least on the order of the longitudinal relaxation rate of the amide resonances (~0.3 s^{-1}). To determine the degree of saturation, one spectrum is acquired with saturation of the aliphatic region and one without. It was critical to record these spectra in an interleaved fashion; i.e., for each t_1 increment in the 2D experiment the two FIDs were recorded consecutively. Afterward, the combined data set was deconvoluted into the spectra with and without saturation, and the intensity ratios of the amide resonances were determined. In these experiments, type 2 copper depleted NiR (T2D-NiR)[20] was used to avoid oxidation during the experiment. Both T2D-NiR and PAZ were in the Cu (I) state.

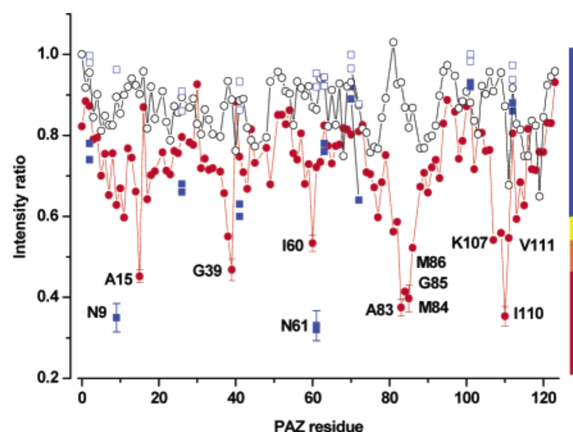

Figure 1. Intensity ratios. The intensity ratio of the resonances of the amide protons in the cross-saturation experiments with and without irradiation of the aliphatic region in the spectrum are plotted against the PAZ residue number. The open and solid symbols represent PAZ in the absence and presence of NiR, respectively. Circles, backbone amide protons; squares, side chain amide protons. Errors are <0.05 and are indicated for most affected residues only. The vertical bar on the right indicates the classification of the intensity ratios used in Figure 2. Conditions: 1 mM ^{2}H−^{15}N-PAZ (CuI), 0.2 mM (trimeric) T2D-NiR(CuI) in 20 mM potassium phosphate pH 6.5 with 8 mM sodium ascorbate in 90% D_2O. NMR spectra were acquired at 293 K on a Bruker AV 750 MHz wide-bore system with 5 mm BBI-2 Grad probe. The saturation-transfer experiment[21] consisted of a TROSY[22] experiment preceded by a 3-s selective saturation period using WURST-20 saturation scheme[23] and ^{1}H decoupling at 0.13 kHz with the offset at −1.2 ppm. The total duration of each scan was 4.2 s. The number of scans and increments was 80 and 410, respectively. The total time for the two interleaved experiments was 77 h.

The results are plotted for all residues in Figure 1. The open symbols indicate the intensity ratios of amide resonances in the experiments with and without irradiation for PAZ in the absence of T2D-NiR. The black circles and blue squares represent backbone and side-chain amide resonances, respectively. A small uniform decrease of the intensities of the amide protons is observed, probably

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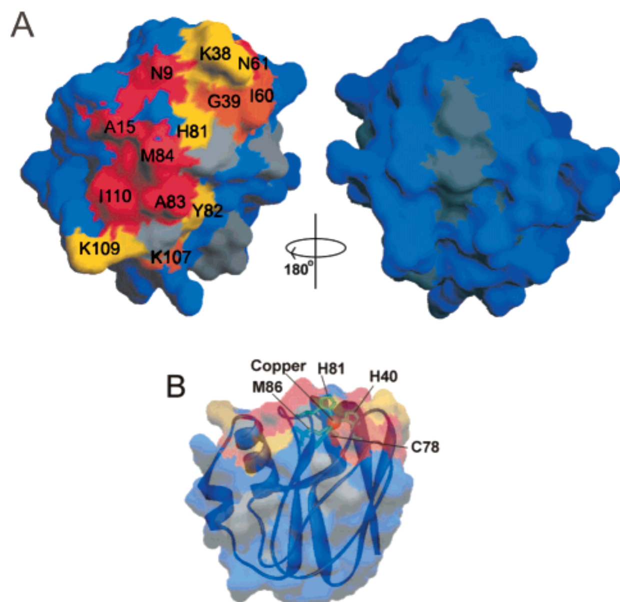

Figure 2. Binding map. (A) Surface representation of the PAZ structure using the color code according to the classification shown in Figure 1: with intensity ratios <0.41 in red, 0.41−0.51 in orange, 0.52−0.55 in yellow and >0.54 in blue. Residues for which no data are available (prolines, K46, D47) are shown in gray. Relevant residues are indicated. (B) Semi-transparent representation, tilted relative to (A) to show the location of the copper site. The figure was produced using GRASP.[28]

due to the saturation transfer from the residual aliphatic protons in the deuterated PAZ. The solid symbols in Figure 1 give the intensity ratios for the mixture of PAZ and T2D-NiR, with red circles and blue squares representing backbone and side-chain amide protons, respectively. A larger general decrease is observed, and a number of residues show substantially smaller ratios due to saturation transfer from T2D-NiR. The intensity ratios have been mapped onto a surface representation of PAZ crystal structure[24] in Figure 2, using the color code according to the classification shown in Figure 1 (color bar). Remarkably, the affected residues all map near to the exposed copper ligand His81. The equivalent His residue has been shown to be involved in ET in other blue copper proteins.[25−27] The results strongly suggest that also in the PAZ:NiR complex ET proceeds through the exposed His ligand. It should be noted that saturation effects can only be observed for amide protons located close to NiR. Interface residues with exposed side chains but buried amide protons may remain unaffected. The residues indicated in Figure 2A thus represent the minimal binding interface. It is interesting to observe that most of the affected residues are apolar. This demonstrates that the interface is at least partially of a hydrophobic nature. Given the effects of charge mutations on the affinity observed in the kinetic studies, it can be concluded that both electrostatic and hydrophobic interactions contribute to complex formation. Clearly, the kinetic studies and NMR results provide complementary information on the process of complex formation. Finally, it is surprising that complex formation is slow on the NMR time scale, indicating that the dissociation rate constant is on the order of a few s^{-1} or less. The steady-state turnover rate of the enzyme is much higher (several hundreds s^{-1}).[16] This apparent contradiction is currently under study.

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Supporting Information Available: Protocol for the production of deuterium-labeled PAZ in *Escherichia coli* and TROSY spectra of the $^{15}N-^{2}H$ PAZ free and in the complex without and with irradiation. This material is available free of charge via the Internet at http://pubs.acs.org.

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