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Redox-State-Dependent Complex Formation between Pseudoazurin and Nitrite Reductase

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Abstract: Bacterial copper-containing nitrite reductase catalyzes the reduction of nitrite to nitric oxide as part of the denitrification process. Pseudoazurin interacts with nitrite reductase in a transient fashion to supply the necessary electrons. The redox-state dependence of complex formation between pseudoazurin and nitrite reductase was studied by nuclear magnetic resonance spectroscopy and isothermal titration calorimetry. Binding of pseudoazurin in the reduced state is characterized by the presence of two binding modes, a slow and a fast exchange mode, with a K_d^{app} of 100 μM. In the oxidized state of pseudoazurin, binding occurs in a single fast exchange mode with a similar affinity. Metal-substituted proteins have been used to show that the mode of binding of pseudoazurin is independent of the metal charge of nitrite reductase. Contrary to what was found for other cupredoxins, protonation of the exposed His ligand to the copper of pseudoazurin, His81, does not appear to be involved directly in the dual binding mode of the reduced form. A model assuming the presence of a minor form of pseudoazurin is proposed to explain the behavior of the complex in the reduced state.

Introduction

Many enzymes involved in the catalysis of redox reactions interact with small electron-transfer proteins to receive or donate electrons. Turnover rates of such enzymes are commonly on the order of several 100's s^{-1}, which puts a lower limit on the dissociation rate constant for the protein−protein complex. Thus, complexes of redox enzymes and their electron-transfer partners are inherently transient and generally exhibit weak binding. In some well-studied cases, it has been shown that the enzyme binds the oxidized and the reduced states of the electron-transfer protein with different affinities, thus distinguishing the substrate and product states of the partner protein. This is remarkable, because the structures of electron-transfer proteins change little upon oxidation/reduction, thereby limiting the reorganization energy of the electron-transfer reaction.

Plastocyanin, the small copper-containing protein involved in many photosynthetic organisms in electron transfer between cytochrome b_6f and photosystem I, has been reported to bind photosystem I with an affinity that is 6-fold higher in the reduced form, thus enhancing the dissociation of the oxidized, product state of plastocyanin at the cost of a decrease in the driving force.[1,2] Amicyanin receives electrons from the enzyme methylamine dehydrogenase, leading to the conversion of methylamine into formaldehyde. The oxidized form of amicyanin binds more strongly, resulting in a reduction of its midpoint potential upon complex formation.[3,4] It has been argued that this enables electron transfer to another electron-transfer protein, cytochrome *c*-551i, in a ternary complex,[3] although the role of this cytochrome in the electron-transfer chain in vivo has been questioned.[5] Similarly, in the complex of rusticyanin and cytochrome c_4, the former exhibits a drop in the midpoint potential by about 100 mV, compared to that of the free protein.[6,7]

Thus, it appears that redox enzymes are able to tune interactions with electron-transfer proteins according to the specific requirements of the reaction.

Nitrite reductase (NiR) from *Alcaligenes faecalis* S-6[8] catalyzes the conversion of nitrite into nitric oxide as part of the denitrification pathway.[9,10] The enzyme is trimeric and

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(1) Drepper, F.; Hippler, M.; Nitschke, W.; Haehnel, W. *Biochemistry* **1996**, *35*, 1282−1295.
(2) Finazzi, G.; Sommer, F.; Hippler, M. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 7031−7036.
(3) Gray, K. A.; Davidson, V. L.; Knaff, D. B. *J. Biol. Chem.* **1988**, *263*, 13987−13990.
(4) Zhu, Z.; Cunane, L. M.; Chen, Z.; Durley, R. C. E.; Mathews, F. S.; Davidson, V. L. *Biochemistry* **1998**, *37*, 17128−17136.
(5) Van Spanning, R. J. M.; Wansell, C. W.; Reijnders, W. N. M.; Harms, N.; Ras, J.; Oltmann, L. F.; Stouthamer, A. H. *J. Bacteriol.* **1991**, *173*, 6962−6970.
(6) Giudici-Orticoni, M. T.; Guerlesquin, F.; Bruschi, M.; Nitschke, W. *J. Biol. Chem.* **1999**, *274*, 30365−30369.
(7) Abergel, C.; Nitschke, W.; Malarte, G.; Bruschi, M.; Claverie, J. M.; Giudici-Orticoni, M. T. *Structure* **2003**, *11*, 547−555.
(8) Kakutani, T.; Watanabe, H.; Arima, K.; Beppu, T. *J. Biochem.* **1981**, *89*, 453−461.
(9) Zumft, W. G. *Microbiol. Mol. Biol. Rev.* **1997**, *61*, 533−616.
(10) Einsle, O.; Kroneck, P. M. H. *Biol. Chem.* **2004**, *385*, 875−883.

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contains two coppers per subunit. The catalytic sites,[11] harboring a type II[12] copper, are located at the interfaces of two subunits, buried in the protein interior.[13,14] The single electron required for the reaction is donated by the electron-transfer protein pseudoazurin (PAZ).[15−18] The electron enters NiR at the second copper site, which is in a type I configuration. From there, the electron is transferred to the active site via a short covalent electron-transfer pathway.[14,19−22] While the catalytic process has been studied in detail (refs 23−27 and references therein), less is known about the structural and dynamic aspects of the interaction with PAZ. Kinetic studies have demonstrated that electrostatic interactions between the positive patch on PAZ and negative residues on NiR contribute to the affinity of binding.[28,29] Recently, we characterized the binding interface for NiR on PAZ using nuclear magnetic resonance (NMR) spectroscopy, for both the reduced and the Zn-substituted forms of PAZ.[30,31] The latter was used as a substitute for the cupric state, which exhibits large line broadening in the NMR spectra. It was found that, in both cases, the interaction surface is well-defined and located around the exposed copper ligand His-81, involving both polar and hydrophobic residues. On the basis of docking simulations, a model of the complex of NiR and PAZ was proposed.

Here, we report on the dynamic aspects of the complex. It is found that the binding of PAZ to NiR depends in a surprising way on the redox state of the former. The reduced form binds NiR in two modes, one exhibiting slow and the other fast exchange behavior. For the oxidized form, only fast-exchange complex formation is observed.

Experimental Section

PAZ Production and Purification. Unlabeled PAZ was produced in *Escherichia coli* strain BL21 (DE3), transformed with the plasmid pEPsaz.[32] A 10-mL 2xYT/kanamycin (100 mg/L) preculture, incubated at 30 °C and 250 rpm for 6 h, was used to inoculate 1 L of 2xYT. Cultures were incubated under the same conditions to $OD_{600} = 1.0$, at which point expression was induced by addition of 0.5 mM isopropyl β-D-galactopyranoside and the temperature was reduced to 25 °C. After 10 h, cultures were harvested by centrifugation. ^{15}N-labeled PAZ was produced as described.[33] PAZ was purified according to the published procedure.[33] The final 277/595 absorbance ratio of PAZ was 1.9, indicating a purity >95%,[29,34] with a yield of ∼1000 and 30 mg/L of culture for unlabeled and ^{15}N-labeled PAZ, respectively.

The mutation H81A was introduced by following a procedure based on Stratagene's ExSite PCR-based site-directed mutagenesis kit using pEPsaz as the template. Medium-level expression yielding soluble protein was observed in both rich and minimal medium using the same procedure described for the wild type (wt). The protein was produced like wt PAZ. After the bacterial cells were opened using a French press cell, followed by ultracentrifugation, the protein was found in the pellet. The pellet was dissolved in a solution of 6 M guanidium·HCl, 1 mM dithiothreitol, and 20 mM sodium phosphate, pH 7, and after ultracentrifugation, the supernatant was dialyzed under anaerobic conditions against first 1 M urea in the same buffer and then only buffer. The refolded apoprotein was purified like wt PAZ. Upon addition of Cu(II) ions, the [^{15}N,^{1}H] HSQC spectrum of ^{15}N-labeled apo-PAZ H81A showed clear changes, indicating the uptake of Cu. No color change was observed, suggesting that the copper is reduced upon incorporation, similar to what has been found for other blue copper proteins in which the exposed His ligand has been mutated (Prof. Gerard Canters, personal communication).

NiR Production and Purification. The expression vector for the *A. faecalis* NiR gene[35] was kindly provided by Dr. M. J. Boulanger and Prof. M. E. P. Murphy. It contains a kanamycin resistance gene and allows for expression of recombinant genes under the control of the T7 promoter. The construct codes for a C-terminal His tag, introduced to facilitate purification. For the protein interaction studies, this His tag was considered as a possible source of interference. Therefore, the His tag was removed by introducing a stop codon before the His tag sequence by site-directed mutagenesis, yielding pET28b-NiRwt. NiR Cu−Cu was produced in *E. coli* strain BL21 (DE3), transformed with the plasmid pET28b-NiRwt following the procedure for unlabeled PAZ described above. The purification protocol has been described elsewhere.[31] The 280/468 absorbance ratio of purified NiR was 16, in line with the literature value,[8] and the yield was 150 mg/L of culture.

Depletion of the Type 2 Copper and Metal Substitution. NiR Cu−T2D was obtained following the published procedure.[19] Copper depletion was checked by EPR. Substitution of copper by cobalt for both the type 1 and the type 2 sites in NiR was achieved as described.[36] The preparation of PAZ Zn II has been reported elsewhere.[31]

NMR Samples. NMR samples contained 20 mM potassium phosphate buffer, pH 6.5, unless stated otherwise. Samples for direct titrations (NiR titrated into PAZ) contained 0.2 mM ^{15}N-PAZ to which aliquots of a 1.3 mM NiR (trimer concentration) solution were added.

(11) Libby, E.; Averill, B. A. *Biochem. Biophys. Res. Commun.* **1992**, *187*, 1529−1535.
(12) Adman, E. T. *Adv. Protein Chem.* **1991**, *42*, 145−197.
(13) Godden, J. W.; Turley, S.; Teller, D. C.; Adman, E. T.; Liu, M. Y.; Payne, W. J.; LeGall, J. *Science* **1991**, *253*, 438−442.
(14) Kukimoto, M.; Nishiyama, M.; Murphy, M. E. P.; Turley, S.; Adman, E. T.; Horinouchi, S.; Beppu, T. *Biochemistry* **1994**, *33*, 5246−5252.
(15) Ambler, R. P.; Tobari, J. *Biochem. J.* **1985**, *232*, 451−457.
(16) Petratos, K.; Banner, D. W.; Beppu, T.; Wilson, K. S.; Tsernoglou, D. *FEBS Lett.* **1987**, *218*, 209−214.
(17) Thompson, G. S.; Leung, Y. C.; Ferguson, S. J.; Radford, S. E.; Redfield, C. *Protein Sci.* **2000**, *9*, 846−858.
(18) Murphy, L. M.; Dodd, F. E.; Yousafzai, F. K.; Eady, R. R.; Hasnain, S. S. *J. Mol. Biol.* **2002**, *315*, 859−871.
(19) Suzuki, S.; Deligeer; Yamaguchi, K.; Kataoka, K.; Kobayashi, K.; Tagawa, S.; Kohzuma, T.; Shidara, S.; Iwasaki, H. *J. Biol. Inorg. Chem.* **1997**, *2*, 265−274.
(20) Suzuki, S.; Kohzuma, T.; Deligeer; Yamaguchi, K.; Nakamura, N.; Shidara, S.; Kobayashi, K.; Tagawa, S. *J. Am. Chem. Soc.* **1994**, *116*, 11145−11146.
(21) Suzuki, S.; Furusawa, H.; Kataoka, K.; Yamaguchi, K.; Kobayashi, K.; Tagawa, S. *Inorg. React. Mech. (Amsterdam)* **2000**, *2*, 129−135.
(22) Wherland, S.; Farver, O.; Pecht, I. *Chemphyschem* **2005**, *6*, 805−812.
(23) Wijma, H. J.; Canters, G. W.; de Vries, S.; Verbeet, M. P. *Biochemistry* **2004**, *43*, 10467−10474.
(24) Wijma, H. J.; Jeuken, L. J. C.; Verbeet, M. P.; Armstrong, F. A.; Canters, G. W. *J. Biol. Chem.* **2006**, *281*, 16340−16346.
(25) Tocheva, E. I.; Rosell, F. I.; Mauk, A. G.; Murphy, M. E. P. *Science* **2004**, *304*, 867−870.
(26) Pinho, D.; Besson, S.; Brondino, C. D.; de Castro, B.; Moura, I. *Eur. J. Biochem.* **2004**, *271*, 2361−2369.
(27) Antonyuk, S. V.; Strange, R. W.; Sawers, G.; Eady, R. R.; Hasnain, S. S. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 12041−12046.
(28) Kukimoto, M.; Nishiyama, M.; Tanokura, M.; Adman, E. T.; Horinouchi, S. *J. Biol. Chem.* **1996**, *271*, 13680−13683.
(29) Kukimoto, M.; Nishiyama, M.; Ohnuki, T.; Turley, S.; Adman, E. T.; Horinouchi, S.; Beppu, T. *Protein Eng.* **1995**, *8*, 153−158.
(30) Impagliazzo, A.; Ubbink, M. *J. Am. Chem. Soc.* **2004**, *126*, 5658−5659.
(31) Impagliazzo, A.; Krippahl, L.; Ubbink, M. *ChemBioChem* **2005**, *6*, 1648−1653.
(32) Prudêncio, M.; Rohovec, J.; Peters, J. A.; Tocheva, E.; Boulanger, M. J.; Murphy, M. E. P.; Hupkes, H. J.; Kosters, W.; Impagliazzo, A.; Ubbink, M. *Chem. Eur. J.* **2004**, *10*, 3252−3260.
(33) Impagliazzo, A.; Ubbink, M. *J. Biomol. NMR* **2004**, *29*, 541−542.
(34) Kakutani, T.; Watanabe, H.; Arima, K.; Beppu, T. *J. Biochem.* **1981**, *89*, 463−472.
(35) Boulanger, M. J.; Kukimoto, M.; Nishiyama, M.; Horinouchi, S.; Murphy, M. E. P. *J. Biol. Chem.* **2000**, *275*, 23957−23964.
(36) Suzuki, S.; Deligeer; Yamaguchi, K.; Kataoka, K.; Shidara, S.; Iwasaki, H.; Sakurai, T. *Inorg. Chim. Acta* **1998**, *276*, 289−294.

For the inverse titration (PAZ titrated into NiR), samples contained 0.13 mM NiR, to which aliquots of a 3.9 mM ^{15}N-PAZ solution were added. Protein concentrations were determined optically using $\epsilon_{593} = 2.9\ mM^{-1}\ cm^{-1}$ for PAZ Cu II,[34] $\epsilon_{277} = 5.7\ mM^{-1}\ cm^{-1}$ for PAZ Zn II, $\epsilon_{589} = 2.9\ mM^{-1}\ cm^{-1}$ per subunit for NiR Cu II−Cu II,[8] and $\epsilon_{280} = 44.5\ mM^{-1}\ cm^{-1}$ per subunit for NiR Co II−Co II, assuming that, at that wavelength, NiR Cu II−Cu II and NiR Co II−Co II have the same extinction coefficient. NMR samples in the reduced form contained 1.0 mM sodium ascorbate and were prepared in an anaerobic vial. All NMR samples contained 6−10% D_2O for lock, and the solutions were degassed by blowing argon over the surface. For each titration, samples of PAZ and NiR were set at the same pH, and the pH was checked again at the end of the titration.

For the pH titration of free PAZ Cu I, a 1 mM solution of ^{15}N-labeled protein was used. The pH was adjusted by adding microliter aliquots of 1 M NaOH and 1 M HCl, and spectra were recorded over the range of pH 4.0−8.1. Inverse titrations of PAZ and NiR at pH 5.5 and 8.0 were performed as described above. The chemical shift perturbations arising from complex formation between PAZ and NiR were determined with respect to free PAZ at the same pH.

NMR Experiments. All NMR experiments were performed at 14.1 T on a Bruker DMX600 spectrometer operating at 293 K and equipped with a TXI-Z-GRAD (^{1}H, ^{13}C, and ^{15}N) probe. All spectra were processed in AZARA (available from http://www.bio.cam.ac.uk/azara/) and analyzed with Ansig-for-Windows.[37] Assignments of the ^{15}N-PAZ Cu I[33] and ^{15}N-PAZ Zn II HSQC spectra[32] were taken from the literature.

Binding Curves. Binding curves were obtained by plotting the signal intensities of PAZ resonances in a reverse titration against the molar ratio of PAZ and NiR (subunit concentration). Data were fitted to a 1:1 binding model using Origin 6.0 (OriginLab Corp., Northampton, MA), assuming that only free PAZ contributes to the intensity. The equation used for the nonlinear fitting was

$$\mathrm{I} = aP_\mathrm{f} + b \tag{1}$$

where I is the intensity of the PAZ signal, a (in units of M^{-1}) represents the intensity coefficient of the observed nucleus, b is a dimensionless correction term for baseline offset, and P_f is the free PAZ concentration, with

$$P_\mathrm{f} = (P_0{}^i - x_i) \tag{2}$$

where $P_0{}^i$, the total PAZ concentration at titration step i, is related to the concentration of the stock solution of PAZ (P_0), the initial concentration of NiR (N_0), and the molar ratio R_i of the total PAZ and NiR ($N_0{}^i$) concentrations at step i ($R_i = P_0{}^i/N_0{}^i$):

$$P_0{}^i = \frac{R_i N_0 P_0}{N_0 R_i + P_0} \tag{3}$$

Similarly, $N_0{}^i$ is given by

$$N_0{}^i = \frac{N_0 P_0}{N_0 R_i + P_0} \tag{4}$$

In eq 2, x_i is the concentration of the complex of pseudoazurin and NiR, which can be obtained by

$$\frac{1}{K_\mathrm{d}^{\mathrm{app}}} = \frac{x_i}{(P_0{}^i - x_i)(N_0{}^i - x_i)} \tag{5}$$

Substitution yields

$$I = \frac{1}{2} a \left(\frac{N_0 P_0}{N_0 R_i + P_0}(R_i - 1) - K_\mathrm{d}^{\mathrm{app}} + \sqrt{\left(\frac{N_0 P_0}{N_0 R_i + P_0}(R_i + 1) + K_\mathrm{d}^{\mathrm{app}} \right)^2 - 4 \frac{P_0{}^2 R_i N_0{}^2}{(N_0 R_i + P_0)^2}} \right) + b \tag{6}$$

The experimental data were fitted with eq 6, using I and R_i as dependent and independent variables, respectively, and a, b, and K_d^{app} as fitted parameters.

pH Titration Curves. Titration curves of the chemical shift (δ) as a function of the pH were fitted to eq 7,

$$\delta = \frac{(K_\mathrm{a}\delta_\mathrm{H} + [\mathrm{H}^+]\delta_\mathrm{L})}{K_\mathrm{a} + [\mathrm{H}^+]} \tag{7}$$

where δ_H and δ_L are the chemical shifts at high and low pH, respectively.

For resonances that were sensitive to more than one titrating group, the titration curves were fitted to a two-pK_a model,

$$\delta = \frac{([\mathrm{H}^+]^2\delta_\mathrm{L} + [\mathrm{H}^+]K_{\mathrm{a}1}\delta_\mathrm{M} + K_{\mathrm{a}1}K_{\mathrm{a}2}\delta_\mathrm{H})}{(K_{\mathrm{a}1}K_{\mathrm{a}2} + [\mathrm{H}^+]K_{\mathrm{a}1} + [\mathrm{H}^+]^2)} \tag{8}$$

where δ_M is the chemical shift at intermediate pH and K_{a1} and K_{a2} are the protonation constants at low and high pH, respectively. Fits were obtained by nonlinear curve-fitting with $[H^+]$ and δ as the independent and dependent variables, respectively, and δ_H, δ_M, δ_L, K_a, K_{a1}, and K_{a2} as the fitted parameters.

Isothermal Titration Calorimetry. All calorimetric experiments were performed with an VP titration calorimeter (MicroCal Inc., Milton Keynes, U.K.) at 25 °C. Solutions of PAZ Cu II and NiR Cu II−Cu II were dialyzed against 20 mM potassium phosphate buffer at pH 6.5 overnight in the same vessel to minimize differences in buffer composition. All solutions were degassed by stirring under vacuum before use. The reaction cell contained 0.17 mM NiR Cu II−Cu II (trimer concentration). The injection syringe was filled with 3.2 mM PAZ Cu II and was rotated at 300 rpm during equilibration and experiment. Injections were started after equilibration to baseline stability. The experiment consisted of 17 injections of 15-μL volumes, and the time between injections was 460 s. Titration data were corrected for the heat changes observed in control experiments of buffer into buffer and PAZ into buffer under the same experimental conditions. Baseline correction and integration of the peaks was performed manually, and the data were fitted using Origin 6.0 with a user-defined equation for a one-binding-site model.

Results

The Complex of Reduced PAZ and NiR. To characterize the binding between PAZ and NiR in the reduced form, two types experiments were performed: direct and inverse titrations. The direct titration has been performed by recording [^{15}N,^{1}H] HSQC spectra of ^{15}N-PAZ Cu I after addition of microliter aliquots of reduced NiR from which the type II copper had been removed (NiR Cu I−T2D). Removal of the type II copper makes the protein much less prone to reoxidation. Changes in signal intensities, line widths, and chemical shifts were followed in order to determine the binding properties between PAZ Cu I and NiR Cu I−T2D. Upon addition of NiR Cu I−T2D up to 0.9 mol equiv of subunit, the ^{15}N-PAZ Cu I resonances decreased in intensity (Figure 1) and exhibited a small increase in line width in the proton dimension (~5 Hz). It can be assumed that the resonances of PAZ bound to the 111 kDa NiR trimer are broadened beyond detection due to transverse relaxation.

(37) Helgstrand, M.; Kraulis, P.; Allard, P.; Hard, T. *J. Biomol. NMR* **2000**, *18*, 329−336.

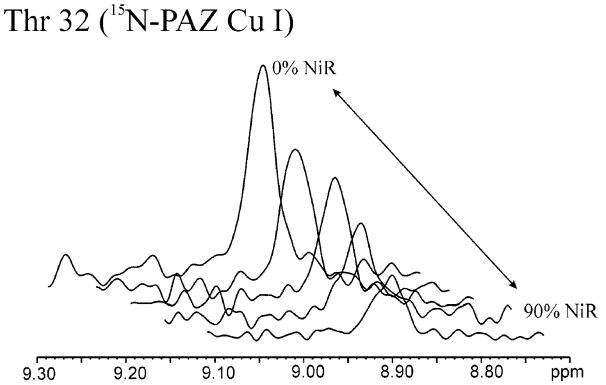


Figure 1. Cross sections through the $^{1}H^{N}$ resonance of Thr32 of PAZ Cu I upon titration with NiR Cu I−T2D. Each spectrum is shifted by −0.035 ppm for clarity.

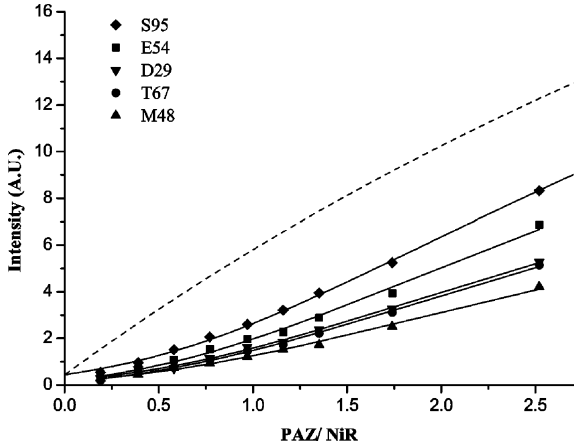


Figure 2. Binding curves for the interaction of ^{15}N-PAZ Cu I with NiR Cu I−T2D in an inverse titration. The intensities of several PAZ amide resonances (arbitrary units, A.U.) are plotted against the PAZ/NiR (subunit) ratio. Solid lines represent a global fit to a model describing one PAZ binding site per NiR Cu I−T2D subunit (eq 6), yielding $K_d^{app} = (1.0 \pm 0.1) \times 10^{-4}$ M. The dashed line represents a simulation of the intensity for Ser95 in the absence of binding, using the same *a* and *b* values as obtained by fitting.

Thus, the observations suggest that binding occurs with modest affinity and that binding and dissociation are slow on the NMR time scale. On the other hand, small chemical shift perturbations were observed, suggesting a fast-exchange regime for binding. In several independent experiments, the same shifts were observed, and these could be shown not to be caused by pH effects or temperature changes. Furthermore, they all map in an area around the proposed electron-transfer port, His81.[31] The largest shift was observed for Lys109 in the ^{1}H dimension, 0.078 ppm, and for Met84 in the ^{15}N dimension, 0.27 ppm at 0.9 mol equiv of NiR subunits. For a further confirmation of these observations, an inverse titration was performed: 0.13 mM NiR Cu I−T2D (trimer concentration) was titrated with ^{15}N-PAZ Cu I. Resonances of many PAZ residues became just observable at 0.4 mol equiv of ^{15}N-PAZ Cu I to NiR subunits, corresponding to a concentration of 0.16 mM PAZ. Free PAZ is easily observable at a concentration of 0.1 mM under the same conditions. It is concluded that PAZ binds to NiR, but with modest affinity. In the case of tight binding, free PAZ would be observable only at molar ratios >1.

The curves of signal intensity versus molar ratio of PAZ/NiR have been plotted for several residues in Figure 2. The deviation from the line predicted for the absence of binding

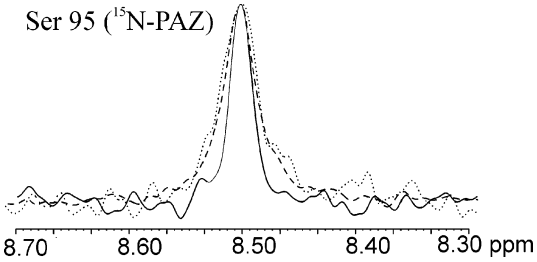


Figure 3. Cross section through the $^{1}H^{N}$ resonance of Ser95 of ^{15}N-PAZ Cu I, in the absence of NiR (solid line, 22 Hz), and at PAZ/NiR (subunit) ratios of 0.4 (dotted line, 41 Hz) and 1.75 (dashed line, 37 Hz). Peak heights have been normalized.

(dashed line) represents intensity loss due to binding to NiR. Global fitting to eq 6 yields a K_d^{app} of $(1.0 \pm 0.1) \times 10^{-4}$ M. In the inverse titration, the decrease in line width between the signals at PAZ/NiR ratios of 0.4 and 1.75 is ∼5 Hz, like in the direct titration, but when the same signal is compared with the signal of the free form of PAZ, the increase is more significant, ∼20 Hz (Figure 3). Moreover, the peaks that were shifting during direct titration showed the same chemical shift perturbations in the inverse titration. The direct titration was repeated under rigorously anaerobic conditions with NiR containing both type I and type II coppers (NiR Cu I−Cu I). The spectra showed the same behavior as with NiR Cu I−T2D, indicating that the depletion of Cu in the type II site does not affect the binding between the two proteins. To establish the effect of the NiR metal charge state on the binding, a direct titration of ^{15}N-PAZ Cu I into a solution of NiR with its coppers replaced by Co II (mimicking NiR Cu II−Cu II) was performed. The effects on the [^{15}N,^{1}H] HSQC spectra of PAZ Cu I upon addition of NiR Co II−Co II were the same as described for the complex between PAZ Cu I and NiR Cu I−T2D, indicating that the charge state on the NiR metals does not influence the binding modes of PAZ.

It can be concluded that reduced PAZ in the complex with NiR experiences two binding modes: a slow-exchange mode, characterized by intensity loss without large line broadening, and a fast-exchange mode, characterized by chemical shift perturbations. The fact that the shifts are small and accompanied by a limited line-width increase suggests that the dissociation constant of this process is larger than the protein concentration used in the NMR experiment (K_d > 0.3 mM). The presence of only one mode cannot explain the complexity of the phenomena observed. A slow-exchange mode cannot account for the presence of the chemical shift perturbations. On the other hand, a fast-exchange mode with K_d > 0.3 mM cannot be the cause of the combination of weak signals (significant binding) and only limited line-width increase. The fact that the chemical shift perturbations are confined to a specific region on the PAZ surface surrounding the exposed copper ligand His81 supports the view that these perturbations represent the binding process.

The Complex of Oxidized PAZ and NiR. ^{15}N-PAZ Cu II was titrated with NiR Cu II−Cu II and NiR Cu II−T2D. Although the presence of the paramagnetic Cu II in ^{15}N-PAZ precludes the observation of resonances from residues close to the metal site of ^{15}N-PAZ, it was possible to detect general changes in the spectral features of the other resonances: increases of ∼20 Hz in the line width in the proton dimension of the amide signals of ^{15}N-PAZ Cu II were observed upon interaction with 0.23 mol equiv of NiR Cu−Cu subunits. Upon

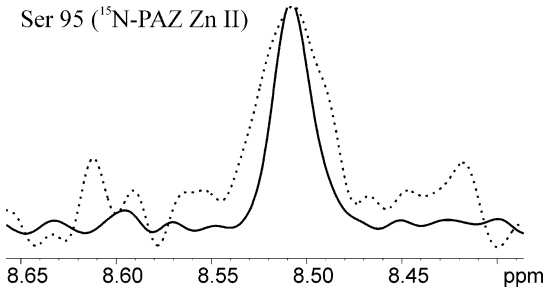


Figure 4. Cross sections through the $^{1}H^{N}$ resonance of Ser95 of ^{15}N-PAZ Zn II of free protein (solid) and with 0.23 mol equiv of NiR Cu I−T2D subunits (dotted). The line width at half-height is 22 and 41 Hz, respectively. Peak heights have been normalized.

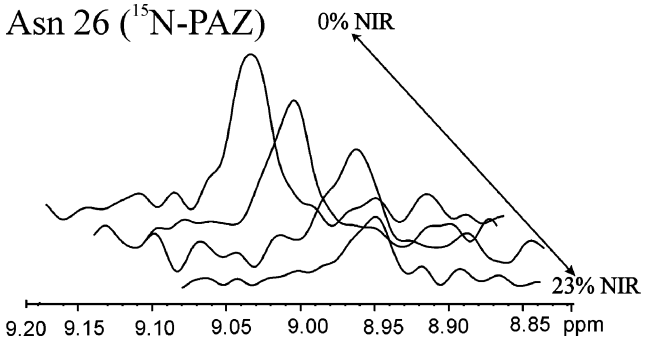


Figure 5. Cross sections through the $^{1}H^{N}$ resonance of Asn26 of ^{15}N-PAZ Zn II upon titration with NiR Cu I−T2D. Each spectrum is shifted by −0.030 ppm for clarity.

addition of 0.3 mol equiv of NiR Cu−Cu subunits, PAZ resonances were no longer detectable due to line broadening. The same was observed in a titration of ^{15}N-PAZ Cu II with cobalt-substituted NiR (NiR Co II−Co II). When the copper atom of ^{15}N-PAZ was replaced by Zn II to create a redox-inactive and diamagnetic protein with the same charge as the copper in the oxidized form, the behavior of the residues around the metal center could be followed. ^{15}N-PAZ Zn II was titrated with NiR Cu−Cu and NiR Cu−T2D in either the reduced or the oxidized state. Similar increases in the line width were observed upon interaction with NiR Cu−T2D (Figure 4). At a ratio of 0.3, PAZ resonances were not longer detectable due to line broadening (Figure 5), with both reduced and oxidized NiR Cu−T2D.

During direct titration of ^{15}N-PAZ Zn II with NiR Cu−Cu and NiR Cu−T2D, a number of peaks showed chemical shift perturbations. At 0.23 mol equiv of NiR subunits, the largest shift was observed in the ^{1}H dimension for Lys109, 0.11 ppm, and in the ^{15}N dimension for Met 84, 0.24 ppm, similar to the titration with PAZ Cu I at much higher NiR/PAZ ratios.

Both the rapid line-width increase and the chemical shift perturbations observed for PAZ Cu II and PAZ Zn II are characteristics of a fast-exchange regime on the NMR time scale ($k_{off} > 300\ s^{-1}$).

Isothermal Titration Calorimetry. Binding affinities between PAZ and NiR, both in the oxidized state, were determined using isothermal titration calorimetry. The results were typical for weak complex formation and could be fitted using a single-site binding model (Figure 6). Titration of PAZ Cu II into NiR Cu II−Cu II yielded $K_d = (1.6 \pm 0.2) \times 10^{-4}$ M with a stoichiometry of PAZ and NiR (subunit) of 0.8 ± 0.1, while the titration of PAZ Cu II into NiR Cu II−T2D produced $K_d =$

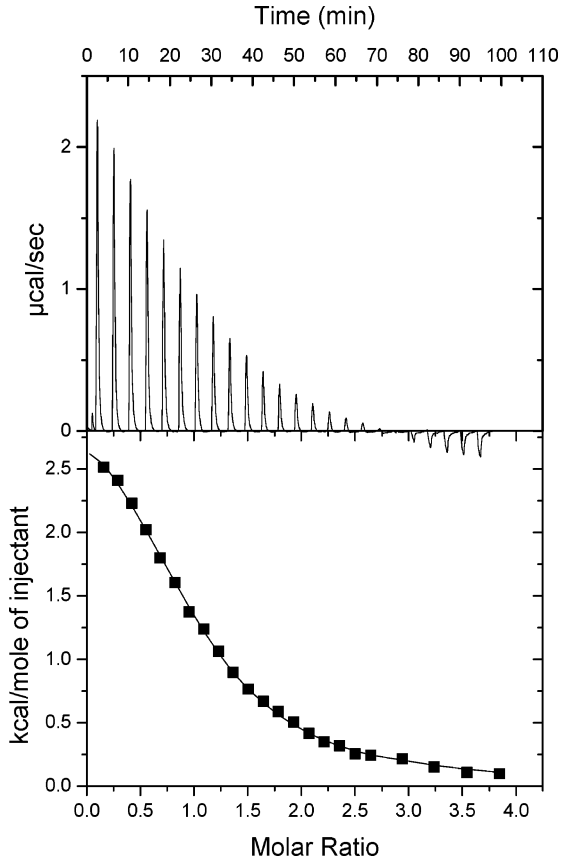


Figure 6. Isothermal titration calorimetric profile of the association of PAZ Cu II with NiR Cu II−Cu II at pH 6.5 and 25 °C in 20 mM sodium phosphate buffer. The top panel shows the uncorrected incremental heat liberation upon titration of 3.2 mM PAZ into 0.17 mM NIR (trimer concentration). The bottom panel shows the integrated areas plotted against the molar ratio of PAZ/NiR subunits. The solid line represents a fit to a single-site binding model, yielding $K_d = (1.6 \pm 0.2) \times 10^{-4}$ M and a stoichiometry of 0.8 ± 0.1.

$(1.1 \pm 0.1) \times 10^{-4}$ M with a stoichiometry of 1.1 ± 0.1. Titration of the reduced proteins (PAZ Cu I and NiR Cu I−T2D) required the presence of ascorbate in all solutions. Reactions of ascorbate with small quantities of dioxygen upon mixing resulted in irreproducible results, and reliable binding data could not be obtained.

pH Dependence of PAZ Binding to NiR. In various protein complexes involving cupredoxin proteins, it has been shown that protonation of His residues can affect the reactivity. To study a possible role of His protonation in the redox-state-dependent binding of PAZ to NiR binding, the pH behavior of free PAZ Cu I and the PAZ-NiR complex was analyzed. Titrations of reduced PAZ were monitored using [^{15}N,^{1}H] HSQC spectra. By fitting the chemical shift perturbations of several residues (Figure 7A), the pK_a of the surface-exposed His6 was determined to be 7.2 ± 0.1, similar to that reported for another pseudoazurin.[38] Figure 7B shows that a large number of residues surrounding His6 are sensitive to its protonation state. The pK_a of His81, the exposed Cu ligand, was established to be 4.8 ± 0.1 (Figure 7C), similar to that of pseudoazurin from *Achromobacter cycloclastes*.[39] Again, many residues around His81

(38) Sato, K.; Dennison, C. *Biochemistry* **2002**, *41*, 120−130.
(39) Dennison, C.; Kohzuma, T.; McFarlane, W.; Suzuki, S.; Sykes, A. G. *Inorg. Chem.* **1994**, *33*, 3299−3305.

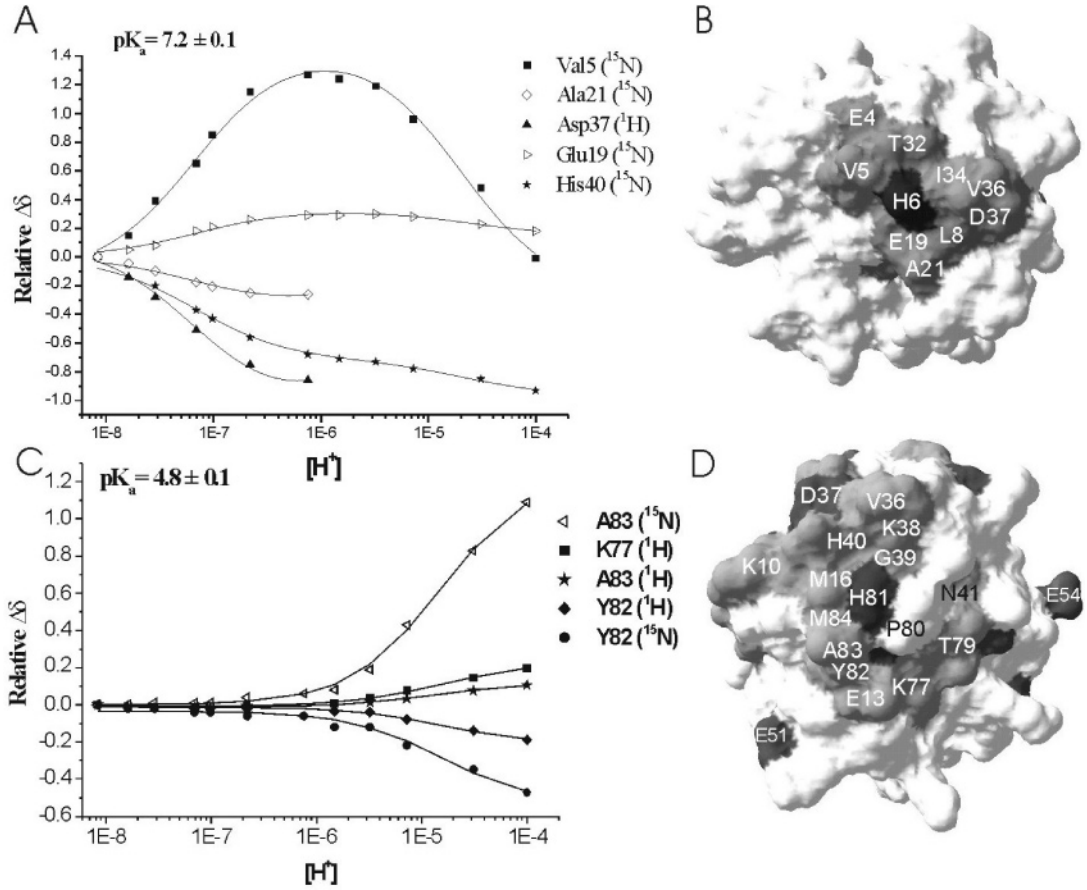


Figure 7. pH titration of free PAZ Cu I. Chemical shift changes ($\Delta\delta$) relative to the δ at pH 8.1 are plotted as a function of the proton concentration for representative residues titration with the pK_a of His6 (A) and His81 (C). Solid lines represent global fits to eq 7 or 8. Surface representation of PAZ facing His6 (B) and His81 (D). In panel B, residues affected by His6 protonation are depicted in gray while His6 is in black. In panel D, residues affected by low pH are depicted in gray while His81 and acidic residues are in black.

experience resonance perturbations due to the protonation (Figure 7D). Table S1 (Supporting Information) lists pK_a's for all perturbed residues.

Inverse titrations of ^{15}N-PAZ Cu I with NiR Cu I−T2D were performed at pH 5.5 and 8.0. At the lower pH, NiR was unstable, leading to precipitation, which excluded a quantitative analysis. Still, it was evident that both chemical shift perturbations and intensity decrease without large line broadening occurred. This indicates that there is no qualitative change in the binding behavior of reduced PAZ. At pH 8, the intensity effects due to complex formation were very small, while chemical shift perturbations of residues Lys109, His81, His40, Met84, Val17, Arg114, and Lys10 were observed (results not shown). Those residues also exhibited perturbations in the experiment performed at pH 6.5. It was estimated that the binding affinity of PAZ Cu I for NiR at pH 8 was at least 6-fold weaker than that at pH 6.5. At pH 8, it is not possible to distinguish the two binding modes observed at pH 6.5 from a single, weak, fast-exchange mode.

Finally, site-directed mutagenesis was performed to produce the PAZ variant H81A. A direct titration of the reduced form of this variant with NiR Cu I−Cu I indicated a 3-fold decrease in the affinity, but no qualitative change in the dual binding mode behavior of reduced PAZ was observed (results not shown).

Discussion

Different Binding Modes for PAZ Cu I and PAZ Cu II/Zn II. The binding of PAZ to NiR with both partners in different oxidation states has been studied by NMR spectroscopy. Direct and inverse titration experiments of PAZ Cu I with NiR Cu I−Cu I or NiR Cu I−T2D reveal two exchange modes occurring simultaneously: a slow-exchange mode evidenced by the decrease of signal intensities without extensive line broadening, and a fast-exchange mode represented by chemical shift perturbations of amide resonances located in a specific area on the PAZ surface. The pH titration of free PAZ eliminates the possibility that the perturbations can be attributed to small changes in pH, because the shift patterns with pH are entirely different. Furthermore, the perturbations are consistently found for the same residues, mapping all in the hydrophobic patch of PAZ.[31] The perturbations were largest at the higher ratios of NiR/PAZ but decreased only little with decreasing ratios. Similarly, line broadening was observed for PAZ Cu I due to the presence of NiR, which decreased only marginally with increasing PAZ concentrations in the inverse titration. These observations suggest that PAZ interacts with NiR in a fast-exchange mode, with very weak affinity. For a K_d that is similar to or larger than the highest PAZ and NiR concentrations, only small changes in chemical shift perturbations and line broadening are expected in the inverse and direct titrations. Similar behavior is observed when PAZ Cu I is titrated with NiR Co

II−Co II, indicating that the metal charge on NiR does not affect the mode of binding to PAZ.

A different binding behavior is observed when the direct titration is performed with ^{15}N-PAZ Cu II /Zn II and NiR Cu−Cu or NiR Cu−T2D, in either the oxidized or the reduced form. Already at an NiR/PAZ ratio of 0.3, ^{15}N-PAZ Zn II amide signals are no longer detectable due to line broadening. The difference in line widths of the signal between free PAZ and its bound form at a ratio of 0.23 was ~20 Hz, which is similar to that of the reduced form at a ratio of 1.7. Chemical shift perturbations are observed for resonances of residues located on the same surface area around the metal center as in the reduced form. These results suggest that PAZ Zn II/Cu II binds to NiR in a fast-exchange mode with a K_d on the order of 10^{-4} M or less. This is confirmed by the K_d values obtained via ITC. There is no evidence for a dual binding mode in the case of PAZ Cu II/ Zn II.

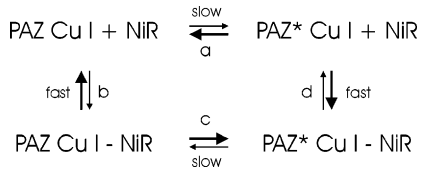
Redox-state-dependent binding, as described here for PAZ and NiR, has been observed before for other transient complexes involving blue copper proteins through different techniques. In the work of Zhu et al., the molecular basis for the effect of complex formation of amicyanin with its partner methylamine dehydrogenase (MADH) is described.[4] Protonation of the His95 copper ligand in reduced amicyanin[40] has been shown to be responsible for the pH dependence of its midpoint potential. However, when amicyanin is in complex with MADH, its potential is not pH dependent. By comparing the crystal structures of free and bound amicyanin, it was observed that the conformational change of His95, responsible for the described phenomena, would be sterically hindered in the complex. Consequently, His95 is prevented from dissociating from the copper upon reduction, maintaining the potential suitable for the electron transfer to occur.

The binding of plastocyanin to photosystem I has been analyzed by the technique of perturbed angular correlation of γ rays to establish the importance of the charge on the metal in the copper site.[41] In line with previous work,[1] the dissociation constant was shown to be dependent on the charge of the metal ion of plastocyanin. By comparing the structures of Cd and Ag plastocyanin and their complex with photosystem I, it was suggested that the metal site structure, involving the His87 metal ligand, is involved in regulating the dissociation constant of the complex and, more specifically, that binding of Ag plastocyanin to photosystem I stabilizes the Ag metal center structure. It is also important to note that the structural change of the metal center was not attributed to a protonation of the $N^{\delta 1}$ of His87.

From these studies, the conclusion arises that conformational changes in the metal center due to the metal oxidation state can change the binding characteristics and, inversely, that complex formation can affect the metal center structure.

Modeling of PAZ Cu I Binding Mode. The redox-state dependence of the binding modes suggests that the reduced form of PAZ exists in two forms, PAZ Cu I and PAZ* Cu I, while the oxidized form, PAZ Cu II, exists only in one. In the HSQC spectrum of PAZ, single resonances are observed for each amide, indicating that, in the case of two forms, either they exist in fast exchange or one is dominant. To explain all the observations, the model in Scheme 1 is proposed. PAZ and PAZ* are in equilibrium (a), PAZ is assumed to be the dominant conformation and the conversion is slow on the NMR time scale ($k_{ex} \leq 100\ s^{-1}$). Furthermore, PAZ* binds NiR with high affinity (d), while PAZ does so with low affinity (b). Equilibrium (c) is also slow and shifted to the right relative to (a), to match the equilibrium ratio of (d) and (b). In this scheme, slow-exchange binding of PAZ (the observed form of PAZ) occurs via (a) and (d) or (b) and (c), because conversion from PAZ to PAZ* (free or bound) is slow. Fast-exchange binding occurs via (b). The K_d^{app} determined from the inverse titration is $(1.0 \pm 0.1) \times 10^{-4}$ M. This value is based on the decrease of the intensity due to the binding, assuming that bound PAZ does not contribute to the intensity of the HSQC signals. Modeling with PAZ/PAZ* $\geq$ 10, PAZ-NiR/PAZ*-NiR $\approx$ 0.25, $K_d^{(d)} \approx 10\ \mu$M, and $K_d^{(b)} \geq 0.4$ mM yields fractions of PAZ and PAZ-NiR (equilibrium (b)) that match those expected from the chemical shift perturbations of the fast-exchange mode observed at several different PAZ/NiR ratios. These values also yield $K_d^{app} = 100\ \mu$M. Thus, it is possible to reproduce all experimental observations with the model in Scheme 1 assuming a high PAZ/PAZ* ratio.

Scheme 1. Model for the Interaction between PAZ Cu I and NiR Involving Two Binding Modes



The binding of the oxidized PAZ can be described with a single binding mode in fast exchange, similar to (b), with a K_d of about 100 μM. Interestingly, this K_d and the K_d^{app} for the reduced state are similar, suggesting that both redox states bind with similar effective affinities.

Nature of the Conversion between PAZ and PAZ*. The data suggest that reduced pseudoazurin exists in two slowly exchanging forms, while the oxidized form does not. This implies that the difference between the two forms in PAZ Cu I is related to differences between reduced and oxidized pseudoazurin. Several of such differences have been reported. In pseudoazurin from *Ac. cycloclastes*, His6 exhibits pK_a values of 7.2 and 6.4 for the reduced and oxidized forms, respectively.[38,39] This difference is too small to explain the presence of different binding modes for the oxidized and reduced pseudoazurin at the experimental pH (6.5). Also, from our pH titration, it is clear that His6 protonation is fast on the NMR time scale, ruling it out as the explanation for the PAZ/PAZ* conversion.

Protonation of the exposed copper ligand is specific for the reduced form of blue copper proteins. His81 of *A. faecalis* pseudoazurin exhibits a pK_a of 4.8, which means that, at pH 6.5, the deprotonated form is dominant (98%). The protonated form could represent PAZ*. This would be in line with the observation that binding is much weaker at pH 8, because at higher pH, equilibrium (a) in Scheme 1 shifts to the left, leading to a decrease in the apparent binding constant. However, like for His6, protonation of His81 is fast on the NMR time scale. Furthermore, the replacement of His81 with Ala leads to a general weakening of the binding but, contrary to expectation,

(40) Lommen, A.; Canters, G. W.; Van Beeumen, J. *Eur. J. Biochem.* **1988**, *176*, 213−223.
(41) Danielsen, E.; Scheller, H. V.; Bauer, R.; Hemmingsen, L.; Bjerrum, M. J.; Hansson, O. *Biochemistry* **1999**, *38*, 11531−11540.

does not abolish the two binding modes. Therefore, not all observations support His81 protonation as the cause of the PAZ/PAZ* equilibrium.

Other potential causes for the occurrence of two species of PAZ are the possibility to form dimers, as found for *Paracoccus pantotrophus* PAZ,[17,42,43] and a transition at alkaline pH.[44,45] However, in those cases, it has been shown that the oxidized form is involved in these equilibria. Dimer formation did not occur in the reduced state, while it is not known whether the PAZ Cu I exhibits an alkaline transition. Thus, these phenomena could not explain our observations. Finally, a comparison of the crystal structures of oxidized and reduced PAZ shows subtle structural differences. Met7 and Pro35 move closer to His40 upon reduction.[46] It could be possible that residues in this region are responsible for the two forms in the reduced state.

Conclusions

The binding of reduced PAZ to NiR exhibits a slow mode with high affinity as well as a weak, fast mode. Binding of the oxidized form can be described by a single, fast binding process. The physiological relevance of this phenomenon remains to be established, but the strong pH dependence of the binding of the reduced PAZ can result in different K_d values of the oxidized and reduced PAZ for NiR, shifting the midpoint potential of PAZ upon binding to NiR. The turnover number of 700 s^{-1} for NiR at pH 6.5[24] puts a stringent lower limit on the association and dissociation rates of PAZ, which seems difficult to reconcile with a slow binding mode of $\leq$100 s^{-1}. However, as discussed above, this could be related to a slow conversion the PAZ rather than to the binding process itself. Further structural and kinetic studies on PAZ variants may help to establish the molecular basis for the double binding modes.

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Supporting Information Available: Table with pK_a values for all titrating amides in PAZ. This information is available free of charge via the Internet at http://pubs.acs.org.

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(42) Williams, P. A.; Fulop, V.; Leung, Y. C.; Chan, C.; Moir, J. W. B.; Howlett, G.; Ferguson, S. J.; Radford, S. E.; Hajdu, J. *Nature Struct. Biol.* **1995**, *2*, 975−982.
(43) Pauleta, S. R.; Guerlesquin, F.; Goodhew, C. F.; Devreese, B.; Van, Beeumen, J.; Pereira, A. S.; Moura, I.; Pettigrew, G. W. *Biochemistry* **2004**, *43*, 11214−11225.
(44) Kohzuma, T.; Dennison, C.; McFarlane, W.; Nakashima, S.; Kitagawa, T.; Inoue, T.; Kai, Y.; Nishio, N.; Shidara, S.; Suzuki, S.; Sykes, A. G. *J. Biol. Chem.* **1995**, *270*, 25733−25738.
(45) Dennison, C.; Kohzuma, T. *Inorg. Chem.* **1999**, *38*, 1491−1497.
(46) Libeu, C. A. P.; Kukimoto, M.; Nishiyama, M.; Horinouchi, S.; Adman, E. T. *Biochemistry* **1997**, *36*, 13160−13179.