1D- and 2D-NMR Studies of the pH Effects on the Metal-site Geometry in Nickel(II)–Azurin from *Pseudomonas aeruginosa*

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The isotropically shifted proton resonances in nickel(II)–azurin have been assigned on a firm basis by using 1D- and 2D-NMR methods which has allowed the subtle structural changes on the metal binding site associated with the pH induced conformational change in this protein to be revealed.

The electron transfer reactions of redox proteins have been the subject of intense research activity over the last decade, as these processes are of vital importance in biological organisms. However, despite much recent effort, the mechanistic details of biological electron transfer are not at all well understood.[1,2]

Azurins are blue copper proteins which have been found in the respiratory chains of several denitrifying bacteria, where they act as electron carriers.[3,4] The structure of the *Pseudomonas aeruginosa* (*Pae*) azurin has recently been determined by X-ray crystallography at 1.93 Å resolution.[5] The copper ion is strongly bound to two imidazole nitrogens N(δ1) (His-46, His-117) and to a cysteine thiolate sulfur (Cys-112), which form a distorted trigonal planar arrangement around the metal. Two additional weakly coordinated groups in axial positions, a methionine sulfur (Met-121) and a carbonyl oxygen atom (Gly-45), complete a distorted bipyramidal geometry. From recent studies using azurin mutants it has been proposed that the electron transfer reactions proceed through the copper ligand His-117 and that the surrounding hydrophobic patch is most important as a molecular recognition site for the presumed physiological redox partners of azurin, cytochrome c_{551} and nitrite reductase.[6]

There is now convincing evidence that *Pae* azurin undergoes a pH-dependent conformational change associated with deprotonation of His-35. Moreover, the redox potential is also pH dependent decreasing about 60 mV over the pH range 5 to 8 and it has been interpreted as due to deprotonation of His-35.[7] Although it has been assumed that the conformational transition in azurin plays a regulatory role in its redox activity, the scope of this transition is not at all clear.

Ni^{II} and Co^{II} have been widely used to monitor the structure and reactivity of several metalloproteins.[8,9] The studies on the Co^{II} and Ni^{II} azurin have been of considerable utility to analyse some pH dependent properties as well as to interpret the optical spectra of this protein.[4,10,11] However, our understanding of the scope of the structural changes in the metal binding site, associated with the pH dependent conformational transition, remains incomplete. The study of the Ni^{II} azurin, by one- and two-dimensional NMR spectroscopy, has allowed us a more detailed analysis of the effects of the conformational change on the ligand arrangement, and the results of which are reported herein.

The ^1H NMR spectrum of Ni^{II}-substituted azurin at pH 4.7 shows nine main well resolved and paramagnetically shifted signals (**a–i**, Fig. 1). These signals arise from the coordinated groups and only signal **g**, which integrates three protons, has been previously assigned to the methyl group of Met-121.[11] In D_2O, signals **e** and **f** disappear and so they can be assigned to the exchangeable NH(ε2) protons of the two coordinated histidines (His-46, His-117). At pH 8, the ^1H NMR spectrum exhibits the same pattern but only one exchangeable signal **f'** is detected. The labelling of the signals indicates the correspondence of signals between the species at the two pH values. The ^1H NMR titration shows that only signal **c**, assigned to His-117 (see below), remains at the same position. All the

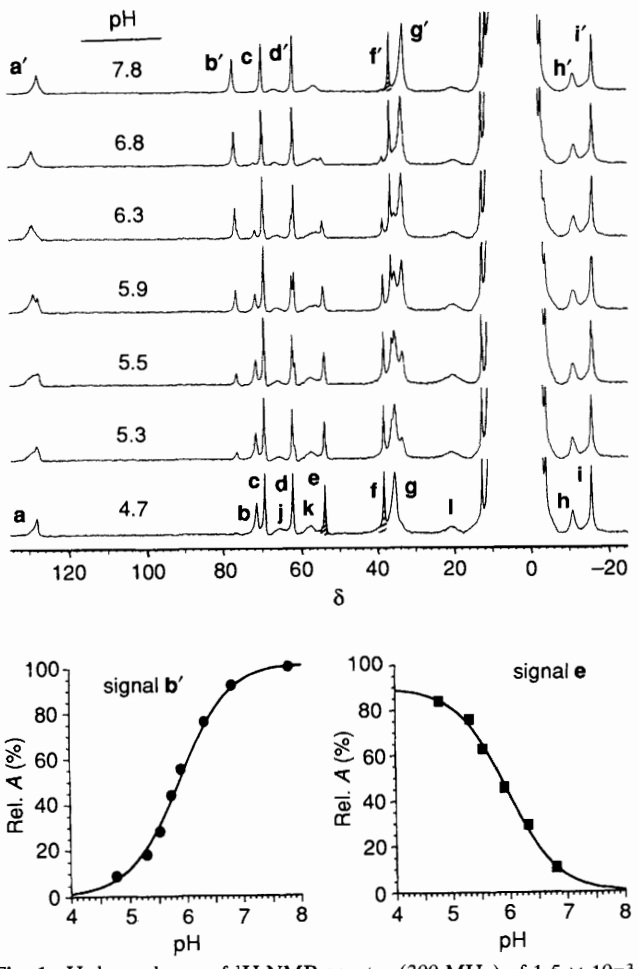

Fig. 1 pH dependence of ^{1}H NMR spectra (300 MHz) of 1.5×10^{-3} mol dm^{-3} Ni^{II}–azurin at 298 K (shaded signals disappear in D_2O). The relative area of signals **b'** and **e** as a function of pH are also shown with the best fitting curve.

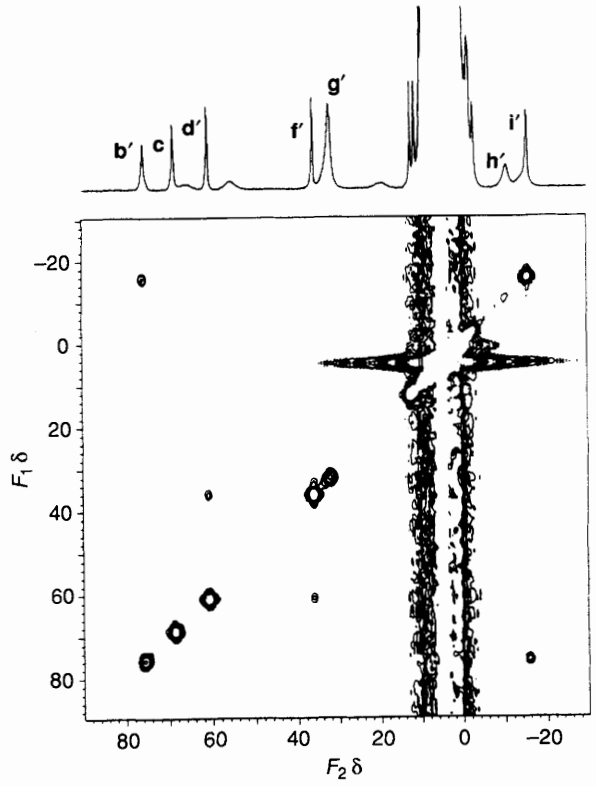

Fig. 2 300 MHz NOESY spectrum of 5.6×10^{-3} mol dm^{-3} Ni^{II}–azurin at pH 8 and 298 K. The data were collected with a mixing time of 6 ms.

other signals change their shifts some ppm in a slow exchange regime on the NMR time-scale and the process is completed at pH ~ 7.5. From the pH dependence of the relative area of signal **b'** a pK_a value of 5.9 ± 0.1 was obtained. This value is completely consistent with that associated with the ionization equilibrium of His-35 in Cu^{II}–azurin.[12] Therefore, the obtained results clearly reveal that the slow conformational change, triggered by the deprotonation of His-35, implies some structural rearrangements in the metal binding site. On the other hand, the exchangeable signal **e** decreases in intensity as the pH is increased and finally becomes undetectable at pH ≥7.5. Curiously, the same pK value is also obtained from the pH dependence of the relative area of this signal. Because this pK value is too low for reflecting the deprotonation of a coordinated histidine, the behaviour of signal **e** can be explained by assuming that, at high pH, the NH(ε) proton of a coordinated histidine exchanges rapidly with bulk water on the NMR time-scale. The temperature dependence of the NMR spectrum of Ni^{II} azurin is also consistent with this interpretation (data not shown). Therefore, as a consequence of the pH induced conformational change, the NH(ε) of a coordinated histidine will be more exposed to solvent and enters in fast exchange and so becomes undetectable. Of the two coordinated histidines, His-46 is situated inside the protein, whereas His-117 is allocated in the centre of the so-called hydrophobic patch and it is somewhat exposed to the solvent, as indicated by X-ray crystallographic studies.[5] Indeed the NH(ε2) proton of His-117 is hydrogen-bonded to a water molecule. Therefore, signal **e** can be assigned to the NH(ε2) proton of this histidine.

Until recently, 1D NOE (nuclear Overhauser effect) has been the only technique successfully used in several paramagnetic metalloproteins to identify spatial correlations among protons and it has provided important structural information about the metal-binding sites in those proteins. On the contrary, considerably much less attention has been devoted to the 2D NMR techniques whose application to the study of paramagnetic metalloproteins has been particularly hindered by the fast nuclear relaxation rates. Despite these drawbacks, several studies directed towards extending the applicability of the 2D NMR techniques to the paramagnetic metalloproteins have been published in the last two years.[13,14] In particular, the 2D NOESY experiments allow the detection of dipolar connectivities among isotropically shifted signals.

The 300 MHz NOESY spectrum of the Ni^{II} azurin at pH 7.9 is shown in Fig. 2. Two cross peaks were observed between hyperfine-shifted resonances, namely, between signals **b'**–**i'** and **d'**–**f'**. Although signals **b'** and **i'** have T_1 values shorter than signals **d'** and **f'**, they generated more intense cross peaks reflecting their origin as geminal protons while the **d'**–**f'** pair of protons being vicinal protons [HC(δ2), HN(ε2)] of a coordinated histidine, His-46. In order to check the **b'**–**i'** and **d'**–**f'** correlations we performed steady-state NOE experiments.[14] Saturation of signals **b'** and **d'** induces NOEs to **i'** and **f'** respectively (Fig. 3). The obtained interproton distances of 1.7 ± 0.2 and 2.4 ± 0.2 Å for the **b'**–**i'** and **d'**–**f'** pairs of protons are consistent with the NOESY results. Furthermore, ^{1}H NOE experiments performed at pH 5 indicate that signals **c** and **e** are also correlated through NOE and the determined interproton distance of 2.4 ± 0.2 Å agrees with **c** and **e** being vicinal protons [HC(δ2), HN(ε2)] of the other coordinated histidine, His-117.

In an attempt to assign the **b'**–**i'** pair of protons to a specific residue we have analysed the proton relaxation rates. As shown in Table 1, the T_1 values are practically pH independent. Indeed only signal **c** shows a significative change beyond the experimental error. By using the Solomon equation,[15]

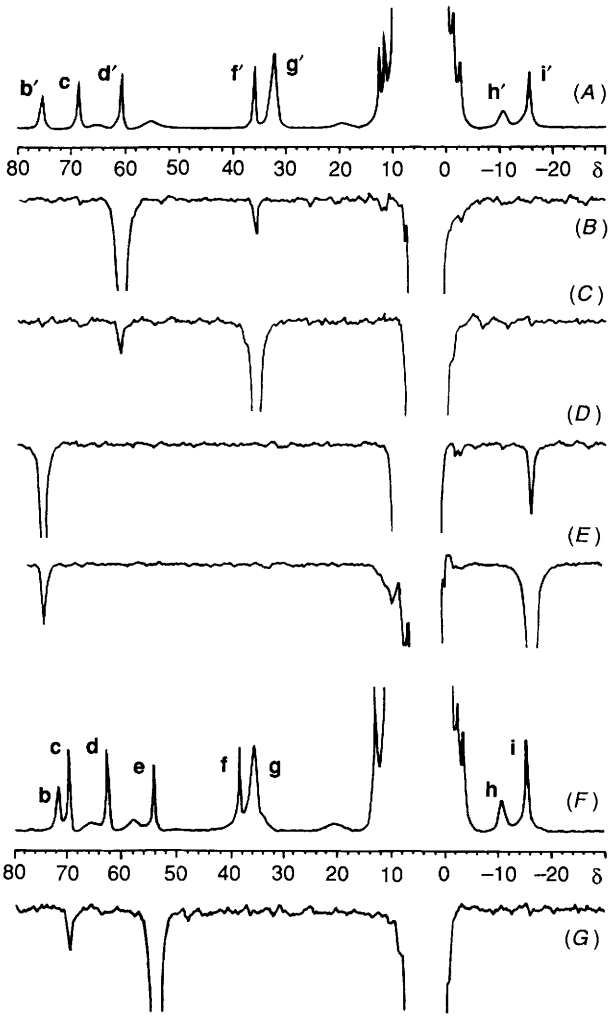

Fig. 3 200 MHz ^{1}H NMR spectra of 3.0×10^{-3} mol dm^{-3} Ni^{II}–azurin in water at 298 K: reference spectra at pH 8 (*A*) and pH 5 (*F*); traces from (*B*)–(*E*) show steady-state NOE difference spectra obtained at pH 8 by saturating signals **d′**, **f′**, **b′** and **i′**; saturation of signal **e** was performed at pH 5 (*G*)

$T_{1M}^{-1} = Kr^6 f(\tau_c)$ where K is a constant, r the Ni···H distance and $f(\tau_c)$ a function of the correlation time, and an average distance of 4.17 Å for the $CH_3(\varepsilon)$ protons of Met-121, as determined by X-ray crystallography,[5] we obtained a τ_c value of 7×10^{-12} s. This value is in agreement with the expected one for Ni^{II} with a trigonal bipyramid coordination. The calculated distances for the other protons together with their known crystallographic distances in native azurin[5] are indicated in Table 1. According to our calculations, the Ni···H distances for the **b′–i′** pair of geminal protons must be in the range 4.7–5.0 Å and only the β-CH_2 protons of Met-121 are acceptable candidates on this criterion.

Therefore, our results indicate that the metal–ligand distances are almost unaffected by pH (< 0.1 Å), and agree with recent X-ray crystallographic and EXAFS studies.[5,16] However, as a consequence of the conformational transition, the NH(ε2) of His-117 enters in a fast exchange regime altering the well-defined hydrogen bond between His-117 and a water molecule. This behaviour is also observed in cobalt(II)–azurin (results not shown). Thus, it could have some mechanistic implications, because this H-bond has been proposed as a pathway for electron transfer reactions.[17]

We conclude that, despite the fast nuclear relaxation rates observed for Ni^{II}–azurin, 1D- and 2D-NMR are valuable techniques for revealing subtle structural changes on the metal binding site in azurin. Work is continuing in an attempt to explore the structural changes on residues close to the metal centre by using 2D-NMR methodology.

Table 1 ^{1}H NMR data (200 MHz) for isotropically shifted signals in Ni^{II}–azurin at 298 K and pH 4.9

Signal	T_1[a]/ms	Assignment	Cu^{II}–H[b]/Å	Ni–H[c]/Å
a	2.6 (2.7)	—	—	—
b	7.3 (7.5)	Met-121 H(β1)	4.63	4.69
c	13.1 (11.6)	His-117 H(δ2)	5.33	5.18
d	8.2 (7.9)	His-46 H(δ2)	5.22	4.79
e	9.5 (—)	His-117 H(ε2)	5.16	4.90
f	11.2 (10.6)	His-46 H(ε2)	5.02	5.04
g	3.6 (3.8)	Met-121 $CH_3(\varepsilon)$	4.17	4.17
h	3.5 (3.5)	—	—	—
i	10.1 (9.6)	Met-121 H(β2)	4.85	4.96

[a] Values at pH 7.8 are indicated in parentheses. [b] Distances from ref. 5. [c] Calculated by the Solomon eqn.

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