Metal coordination of azurin in the unfolded state

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Abstract **^{1}H NMR data applied to the paramagnetic cobalt(II) derivative of azurin from *Pseudomonas aeruginosa* have made it possible to show that the metal ion is bound to the protein in the unfolded state. The relaxation data as well as the low magnetic anisotropy of the metal ion indicate that the cobalt ion is tetrahedral in the unfolded form. The cobalt ligands have been identified as the residues Gly45, His46, Cys112 and His117. Met121 is not coordinated in the unfolded state. In this state, the metal ion is not constrained to adopt a bipyramidal geometry, as imposed by the protein when it is folded. This is clear confirmation of the rack-induced bonding mechanism previously proposed for the metal ion in azurin.**

Key words: Protein folding; Rack mechanism; Azurin; Blue copper protein; Nuclear magnetic resonance

1. Introduction

How proteins fold and which are the mechanisms that induce them to adopt a determined tertiary structure are debated questions in protein chemistry [1–4]. In the 1960s, Malmström proposed the rack-induced bonding mechanism in order to explain the singular spectroscopic properties of the copper ion in the blue copper proteins (BCPs) [5–7]. According to this concept, a local maximum of energy close to the metal ion can be stabilised if the overall folding of the protein reaches a minimum. This allows the protein to force an atypical coordination of the metal ion, which is not the optimum coordination of the copper in any of its two redox states. In this strained situation, the metal ion can accept or donate an electron without substantial modifications of the overall protein folding and without a large energetic cost and, as a consequence, the electron transfer is favoured. In the folded state, the copper ion of azurin is strongly bonded to three equatorial donor atoms (Cys112Sγ, His46Nδ1 and His117Nδ1, the numbering refers to azurin from *Pseudomonas aeruginosa*) and weakly bonded to two axial ligands (Met121Sδ and Gly45CO, Fig. 1). This distorted trigonal bipyramidal geometry is not favourable for copper(I), and hence, this is a very nice example of the rack-induced metal bonding formalism [8,9].

Folding in azurin has been extensively studied [1,10–15]. According to the free energy diagrams of azurin as a function of the guanidine hydrochloride (GuHCl) concentration, four different forms have been proposed for azurin in the unfolded state [12]. In this unfolded form, the strained coordination of the copper does not exist and thus, metal coordination should be governed by the metal preferences. According to this model, when the protein is unfolded, the copper ion can present two different forms in the oxidised state and two others in the reduced state. Since copper coordination chemistry is largely known, the most stable unfolded form for the copper(II) is supposed to be tetragonal, whereas for copper(I) it is supposed to be trigonal. The other two forms (copper(II) trigonal and copper(I) tetragonal) would not be populated to any significant degree. This logical proposal is based on the coordination chemistry of the copper, although no direct evidence of this coordination has been obtained. On the other hand, from unfolding and refolding experiments the existence of coordination of the metal ion to the protein in the unfolded state has also been suggested. However, again, neither direct (spectroscopic) confirmation of the coordination of the metal ion nor the way of binding of it in this unfolded state has been obtained.

Paramagnetic nuclear magnetic resonance (NMR) is a suitable technique for studying metal ion coordination in proteins. In fact, in a paramagnetic system, the interaction between the unpaired electron(s) and the nuclear spin produces the so-called hyperfine shift [16,17]. Due to this effect, protons belonging to ligands of a paramagnetic metal ion appear shifted far away from the diamagnetic region of the ^{1}H NMR spectrum and thus, they can be easily detected. This has been extensively exploited in the study of a very large number of paramagnetic proteins in solution (for reviews see [16,18]). Recently, this approach has also been used to determine the stability and the conformation of the Fe_4S_4 cluster in the high potential iron-sulphur protein from *Chromatium vinosum* in the unfolded state [19]. However, copper(II) shows very large electronic relaxation times and, hence, this ion is not suitable for use with this methodology. Instead, metal substitution of copper by cobalt(II) and nickel(II) ions, with shorter electronic relaxation times, has made it possible to explore exhaustively the surroundings of the metal ion in BCPs [20,21]. Crystal structures of cobalt(II) and nickel(II) metallo-substituted azurins have been solved [22,23]. In both derivatives the metal ion geometry is a distorted trigonal bipyramid, although the metal ion is displaced toward the carbonyl oxygen of Gly^{45} and, consequently, the M(II)-Met121Sγ bond is weaker than in the native protein. In order to address questions such as whether the metal ion is bonded or not in the unfolded state in azurin, as well as which ligands are coordinated to the metal ion in this state, we have performed a study of the cobalt(II) derivative of azurin by ^{1}H NMR.

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Abbreviations: BCPs, blue copper proteins; GuHCl, guanidine hydrochloride

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2. Materials and methods

2.1. Samples

Azurin from *Pseudomonas aeruginosa* was obtained and purified as previously described [24]. The cobalt(II) derivative was prepared according to a previously published procedure [25]. Samples used in NMR experiments were typically 2–5 mM, phosphate buffer 10 mM, pH 8.0. The NMR experiments were performed at 25°C either in D_2O or in H_2O samples containing 5% D_2O for the lock signal. Samples containing unfolded protein were used for less than 4 h because at these high protein concentrations, progressive loss of the metal ion and irreversible denaturation are produced. GuHCl (99.5%) was obtained from Fluka. UV-visible spectra were recorded in a Cary 1 UV-visible spectrophotometer.

2.2. NMR experiments

One-dimensional ^{1}H NMR spectra were performed either in slow (diamagnetic) conditions to observe slow relaxing signals with complete intensity, or with the superWEFT pulse sequence (RD-P180-τ-P90-AQ) [26] in order to observe fast relaxing signals. In the experiments performed in slow conditions, the acquisition time was 364 ms, the spectral window 8000 Hz and the repetition rate 0.33 s^{-1}. In the superWEFT experiments, the spectral window was 68 000 Hz, the acquisition plus the relaxation delays were 31 ms and the inter-pulse delay, τ, was typically around 29 ms. In the experiment performed to detect very downfield shifted signals (>150 ppm), the spectral window was extended up to 100 000 Hz and the carrier was displaced 100 ppm downfield from the water signal. Relaxation times measurements were performed using the inversion-recovery pulse sequence [27]. Steady-state 1D NOE experiments were performed with the superWEFT pulse sequence applying similar parameters as in the ^{1}H NMR spectrum but collecting alternating spectra after saturating and without saturating the desired signal in the inter-pulse delay, τ.

All NMR experiments were performed in a Varian Unity 400 spectrometer running at 400 MHz. Chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) through the solvent signal.

3. Results

The diamagnetic region (−2, 10 ppm) of the ^{1}H NMR spectrum of a 3.5 mM CoAz sample (phosphate 10 mM, pH 8.0, 25°C) recorded in slow conditions (0.33 s^{-1} repetition rate) is shown at the bottom of Fig. 2A. When GuHCl is added to this sample the shape of this spectrum changes. In fact, for concentrations in GuHCl higher than 2.7 M the characteristic dispersion of signals found in a folded protein starts to disappear whereas most of the protons resonate at chemical shifts very similar to those observed for the free amino acids [28]. This indicates that random structures are being generated, i.e. at these GuHCl concentrations protein denaturation starts to occur. Fig. 2B shows the same titration but recording the paramagnetic region (−40, 80 ppm) of the spectrum and using the superWEFT pulse sequence with a repetition rate of 17 s^{-1}. As observed, the characteristic signals of CoAz in the native state disappear in the slow regime and, at the same time, another set of signals corresponding to the unfolded state (labelled with stars in Fig. 2B) starts to appear. For a GuHCl concentration of 3.7 M, the original signals of CoAz in the folded state have completely vanished.

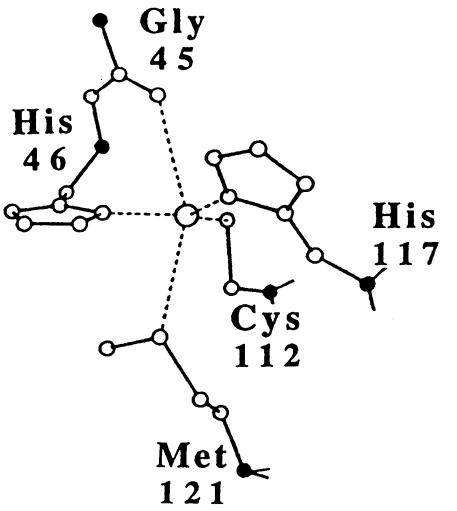

Fig. 1. Active site of azurin, showing the ligands coordinated to the metal ion in the folded form.

The UV-visible spectrum of CoAz in presence of GuHCl 4.0 M is shown in Fig. 3. The spectrum displays basically the same *d-d* bands in the visible region as the protein in the folded state, at 630 and 530 nm, although with lower molar extinction coefficients (240 and 220 M^{-1} cm^{-1}, respectively). The band at 375 nm (assigned to a ligand-metal charge transfer band from Cys112Sγ to the cobalt(II) ion) is not observed in the unfolded state probably because it is overlapped with the characteristic UV band of proteins (at around 280 nm), which becomes broader in presence of GuHCl 4 M.

Table 1
Chemical shifts, longitudinal relaxation times, line widths and tentative assignment of the hyperfine shifted signals of the CoAz in the unfolded state (for comparison, signals corresponding to the same protons in the folded state of CoAz (obtained from [25]) are also given)

Unfolded state					Folded state			
Signal	δ (ppm)	T_1 (ms)	$\Delta\nu_{1/2}$ (Hz)	Tentative assignment	Signal	δ (ppm)	T_1 (ms)	$\Delta\nu_{1/2}$ (Hz)
A	170.4	0.3–0.6[a]	2300	Cys112CHβ	a	285	0.6	2100
					b	232	0.5	2100
B	47.2	2.2	480	His46Hδ2	f	50.6	10.1	195
C	18.0	2.9	340	His117Hδ2	e	56.4	13.3	160
D	13.5	–[a]	300–700[b]					
E	12.97	11.5	200					
F	10.96	10.1	200					
H	9.97	10.1	200					
W	−0.52	57.8	–					
X	−1.07	28.9	–					
Y	−2.10	23.1	140	Met44CH_3ε[c]	–[d]			
Z	−19.6	0.6–0.8	1150	Gly45hα2	z	−29.4	4.7	210

[a] T_1 values could not be obtained accurately due to their short value.
[b] Not possible to determine because of partial overlap with signal E.
[c] Assigned according to its T_1 value and on the basis of the Solomon equation [51].
[d] Not assigned in the folded state.

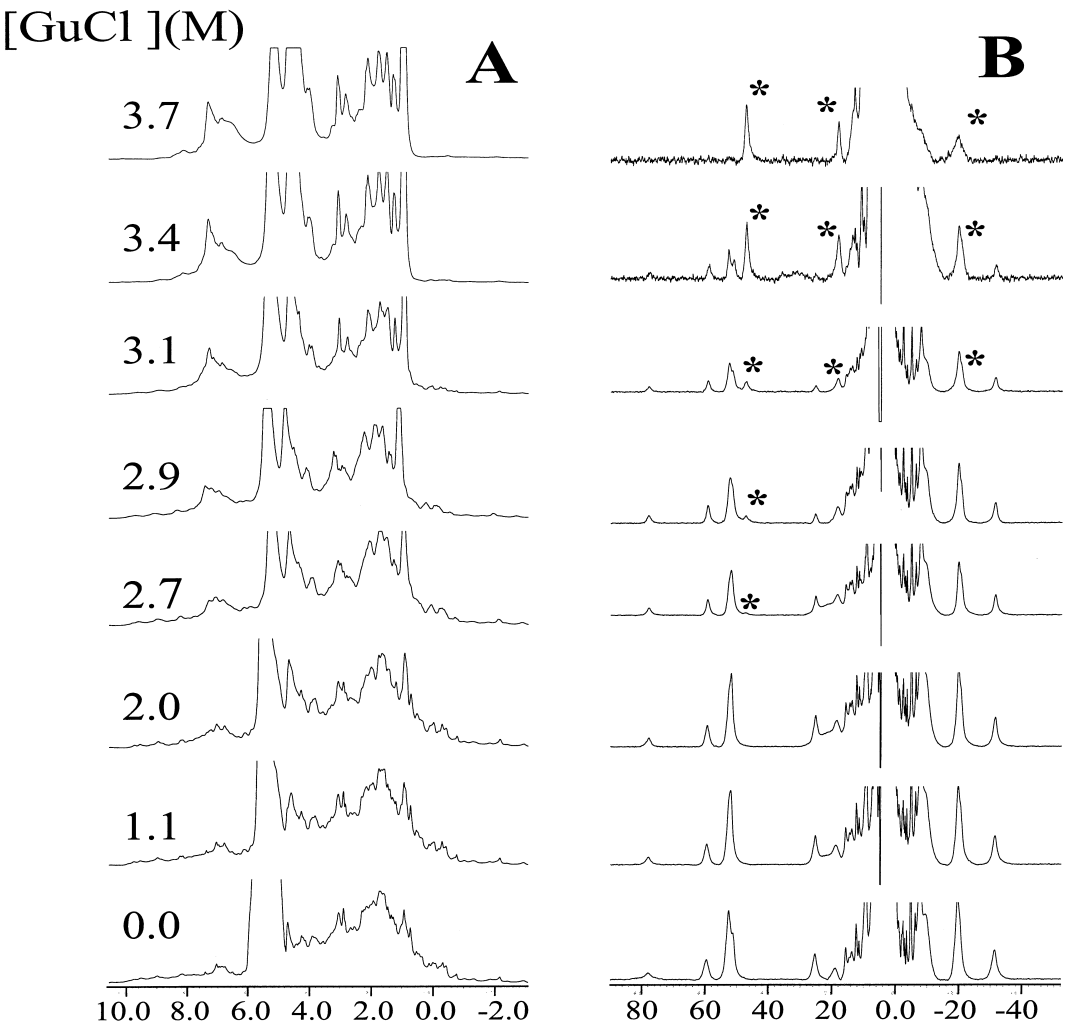

Fig. 2. ^{1}H NMR spectra corresponding to the titration of CoAz with GuHCl. A: Diamagnetic region (spectral window 12 ppm, repetition rate 0.3 s^{-1}). B: SuperWEFT spectrum showing the paramagnetic region (spectral window 120 ppm, repetition rate 17 s^{-1}).

The complete (−40, 200 ppm) ^{1}H NMR spectra recorded with the superWEFT sequence of a CoAz sample in the native state as well as in the presence of GuHCl 3.7 M are shown in Fig. 4. In the folded state, all the hyperfine shifted signals of CoAz have been assigned to protons belonging to residues coordinated to the metal ion [25,29]. By the simple inspection of both spectra several similarities and differences can be observed. First, in the native spectrum of the folded CoAz two very broad signals (labelled *a* and *b*, Fig. 4A) at very low field (285 and 232 ppm) are observed. They have been assigned to the metal ligand Cys112Hβ protons. Analogously, a very broad signal at 170.4 ppm (signal *A* in Fig. 4B), integrating two protons, is found in the unfolded state spectrum. Second, the number of signals with contact contribution, i.e. signals with hyperfine shifts typically larger than ±20 ppm, is clearly lower in the unfolded state (only four signals, labelled *A*, *B*, *C* and *Z* in Fig. 4B) than in the folded state (up to 14 signals can be observed, Fig. 4A). This strongly suggests that the number of ligands of the cobalt ion in the unfolded state is lower than in the folded protein. Third, the spread of the signals close to the diamagnetic region (called the pseudo-diamagnetic region), where protons with only dipolar contribution to their hyperfine shift typically resonate, is larger for the native protein (Fig. 4a) than for the unfolded CoAz (Fig. 4b). In Table 1, the experimental chemical shifts, the longitudinal relaxation times of the corresponding protons and the line widths of the signals in both forms are given. Considering Table 1, another difference can be established. All protons with contact contribution in the unfolded state (signals *A*, *B*, *C* and *Z*) show shorter longitudinal relaxation times and broader line widths

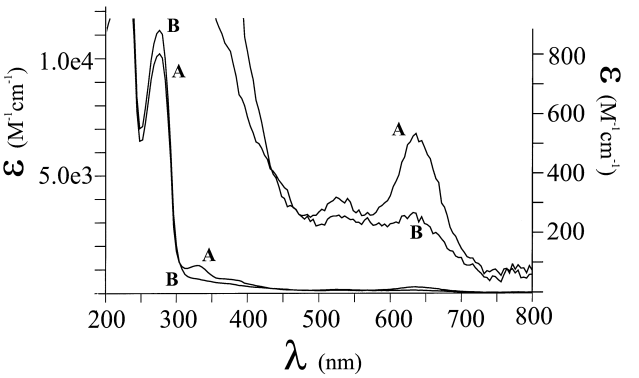

Fig. 3. UV-visible spectrum (200–800 nm) of Co(II)Az (A) in the folded state, and (B) in the unfolded state (at GuHCl 4.0 M). The visible region is also displayed in an enlarged mode (right vertical scale).

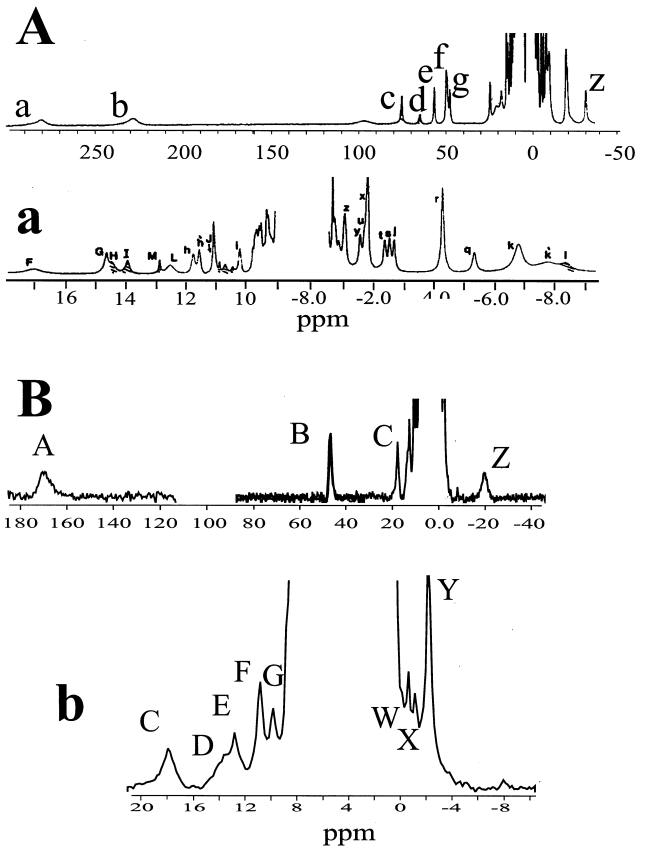

Fig. 4. SuperWEFT ^{1}H NMR spectrum of CoAz in both folded (A) and unfolded (B) states. Figures a and b (lower case) are expanded regions of the pseudo-diamagnetic region (16,−8 ppm) of the same spectra.

than the equivalent protons in the folded state (for assignment see below). This is a consequence of a change either in the geometry of the coordination of the metal ion or in the proton-unpaired electron distances or a combination of both.

Attempts to perform 1D NOE experiments on the hyperfine shifted signals were not successful. The reason resides in metal binding stability. In fact, the protein in the unfolded state progressively loses the metal ion and, at the concentrations necessary to perform 1D NOE experiments in a paramagnetic system (>2 mM), the metalloprotein is not stable for more than 4 h.

Similar experiments were performed on the nickel(II) derivative. However, in this case, as soon as the protein becomes unfolded, the hyperfine shifted signals characteristic of NiAz in the folded state were lost without the appearance of any new signal. In the UV-visible spectrum, the absorption bands of NiAz also disappear without the appearance of any other band. Both facts indicate the loss of the nickel ion in the unfolded state, probably due to the low affinity of the nickel(II) for the donor atoms of the protein ligands. This low affinity has already been observed in the native (folded) azurin at pH values lower than 5.5, where loss of the nickel ion is also found [30].

4. Discussion

4.1. Metal geometry in the unfolded state

Three features, indicated in Section 3, differentiate the ^{1}H NMR spectra of CoAz in the folded and unfolded states: a decrease in the number of signals with contact contribution, a lower dispersion of the pseudo-diamagnetic part of the spectrum and shorter relaxation times of the coordinated protons. The first feature suggests that at least one ligand coordinated in the folded state is not bonded in the unfolded state. In fact, only four hyperfine shifted signals with contact contribution (labelled *A*, *B*, *C* and *Z* in Fig. 4B) are observed, indicating that four ligands are coordinated to the cobalt ion when the protein unfolds. The dispersion of the pseudo-diamagnetic region of the spectrum, i.e. the number of signals with only dipolar contribution, is a direct consequence of the magnetic anisotropy of the considered system. The magnetic anisotropy diminishes with the coordination number for high spin ($S=3/2$) cobalt(II) [20,21], the tetrahedral complexes having the lowest degree of magnetic anisotropy. Cobalt(II) in folded azurin is five-coordinated and thus, there is a large number of signals with noticeable dipolar contribution to their chemical shifts (Fig. 4a). In contrast, very few signals can be observed in the ^{1}H NMR spectrum of CoAz in the unfolded state (Fig. 4b). This clearly indicates that cobalt(II) changes to more a tetrahedral coordination when the protein unfolds.

On the other hand, the electronic relaxation in $S=3/2$ cobalt(II) complexes follows the same rule as the magnetic anisotropy, i.e. six-coordinated Co(II) > five-coordinated Co(II) > four-coordinated Co(II). The shorter relaxation times of the protons in the unfolded state (see Table 1) are also indicative of a tetrahedral coordination for the cobalt(II) ion.

The UV-visible spectrum of cobalt azurin in the unfolded state (Fig. 3B) is also consistent with four-coordination for the cobalt(II). The two bands (at 530 and 630 nm) that appear in the folded protein (Fig. 3A) have been assigned to *d-d* transitions. The molar extinction coefficients decrease when the protein unfolds (220 and 240 M^{-1} cm^{-1}). These values are still in the range of tetrahedral cobalt(II) complexes [31]. According to the crystal structure [22] cobalt(II) presents a highly distorted geometry in the folded state. The lower extinction coefficients of the unfolded protein would indicate a less distorted, more symmetrical (towards tetrahedral) coordination for cobalt(II).

4.2. Assignments of the hyperfine shifted signals

The ^{1}H NMR spectrum of CoAz in the unfolded state (Fig. 4B) shows four hyperfine shifted signals at more than ± 20 ppm, three at low field (signals *A*, *B* and *C*) and another one upfield (signal *Z*). These large shifts in a system with a relatively low magnetic anisotropy (see above) indicate that the corresponding protons experience contact contributions to their hyperfine shifts, i.e. they belong to residues coordinated to the metal ion.

Signal *A* integrates as two protons and shows a chemical shift of 174 ppm. This value is typical for CHβ protons belonging to cysteine ligands not only in cobalt(II) [25,32–34] but also in nickel(II)-substituted proteins [29,35,36], as well as in iron-sulphur proteins [37–39]. As in the native protein (Fig. 4A), this signal can be assigned to both CHβ protons of the cysteine coordinated to the metal ion (Cys112, Fig. 1). Since the hyperfine shift for this signal in the unfolded state is clearly lower than the average for the same signals in the folded state, we can conclude that the Cys112 bond is slightly weaker in the unfolded state than in the folded state. The

similar relaxation times (see Table 1) for the same protons confirm this statement, since, at a same distance, the proton relaxation times should be shorter in a tetrahedral cobalt(II) complex (unfolded state) than in a five-coordinated cobalt(II) species (folded state).

Signals *B* and *C* are hyperfine and downfield shifted (Fig. 4B). In cobalt(II)-imidazole complexes the *meta*-like protons appear upfield shifted [40]; moreover, in all cobalt(II)-substituted proteins studied by ^{1}H NMR [25,29,30,32,33,41–47] *meta*-like protons of imidazole rings of the coordinated histidines (46 and 117, Fig. 1) always appear downfield shifted. Cobalt(II) typically has more affinity for nitrogen donors than for sulphur atoms. As shown above this ion is still coordinated to the Cys112 in the unfolded state, hence it is likely that this ion is coordinated to the imidazole rings of His46 and His117 in the unfolded state. The presence of signals *B* and *C* supports this hypothesis. Thus, these signals can be assigned to the *meta*-like protons (Hδ2 protons) of these two histidines.

Signal *Z* is upfield shifted. In principle, both Met121CHγ or Gly45CHα protons are possible candidates for these two signals. In fact, in the native form of CoAz, as in all cobalt(II) and nickel(II) metallo-substituted BCPs studied by ^{1}H NMR, two protons corresponding to both axial ligands (Met121CHγ2 and Gly45CHα2, or equivalent protons) display upfield hyperfine shifts. At this point, it is interesting to remark that, whatever the possible assignment of these protons is, its short T_1 value (around 0.7 ms, see Table 1) cannot be solely due to a change in the geometry of the metal ion (from trigonal bipyramid to tetrahedral). Such a short T_1 value (shorter than those of signals *B* and *C*) requires a movement of the cobalt(II) ion toward the corresponding ligand. Then, the opposite ligand in the trigonal bipyramid of the metal ion in the folded protein (see Fig. 1) probably is not coordinated. In other words, in the unfolded state cobalt(II) binds stronger to one of the axial donor atoms of the folded protein (Gly45CO or Met121Sδ) and is not coordinated to the other one (Met121Sδ or Gly45CO, respectively).

There are several observations that suggest that signal *Z* does not belong to Met121 and that this residue is not coordinated in the unfolded state. First, if Met121 were coordinated, the methyl group Met121CH$_3$ε should be observed (downfield shifted, according to the low magnetic anisotropy of the system [48]). Second, two signals corresponding to the Met121CHγ protons should appear hyperfine shifted with a similar pattern as Cys112CHβ protons due to the analogous disposition of these protons with respect to the metal ion. Third, the T_1 values of the signals corresponding to the Met121CHγ protons should be as short as that observed for signal *A* (Cys112CHβ protons). Finally, the line widths of these signals should also be as broad as signal *A*. As neither of these situations occurs, we can conclude that Met121 is not coordinated to the cobalt ion in the unfolded form.

On the other hand, there are other findings that point to the coordination of Gly45 and assignment of signal *Z* to one of the Hα protons of this residue. The contact and dipolar contributions for the Met121CHγ2 and Gly45CHα2 protons in the unfolded state have been evaluated [48] and, whereas the GlyHα2 proton shows negative contact contribution to its chemical shift, the Met121Hγ2 proton shows almost negligible contact shift, its upfield shift arising from dipolar effects. In the unfolded state, tetrahedral cobalt(II) displays a low magnetic anisotropy and, consequently, the dipolar contribution is not enough to explain this high negative value (−19.6 ppm) for the hyperfine shift. Although the change in the coordination geometry can modify the mechanism operative in transmitting unpaired spin density on the nuclei through the bonds, it is unlikely that such a drastic change can produce a negative shift in the Met121Hγ2 proton, if this residue were coordinated to the metal ion in the unfolded state. In contrast, if the operative mechanism is maintained in both (folded and unfolded) cobalt(II) proteins, a negative hyperfine shift would be observed for the Gly45Hα2 proton. The observation of a signal with such a negative hyperfine shift supports the coordination of Gly45 (through its carbonyl group) in the unfolded form.

It is quite reasonable to suppose that, if the cobalt ion is bound to His46 in the unfolded state, it has to be close to the Gly45 carbonyl group. In this case, as Gly45 and His46 are consecutive in the sequence, this more rigid segment would be less modified by the unfolding. On the other hand, if His117 varies its relative position with respect to the cobalt then signal *C* (the HisHδ2 proton with the largest hyperfine shift variation when the protein unfolds) should correspond to the His117Hδ2 proton, whereas signal *B* (with a small change in its contact contribution) would correspond to His46Hδ2. In the different azurin crystal structures that have been solved [22,23,49,50], the Met44–His46 segment is in a loop of the protein between two strands of the β-barrel structure, hidden to the solvent. In contrast, His117 as well as Met121 are more exposed to the solvent and very close in the sequence to the carboxy-terminal group (Lys128). This segment (His117–Lys128) forms a sheet of the β-barrel that only interacts with one strand in the structure. Then, this interaction is easier to disrupt, facilitating the unzipping of the C-terminal chain.

Hence, this whole picture is nicely consistent with a displacement of the cobalt(II) ion toward the Gly45 carbonyl group when the protein unfolds, resulting in tetrahedral coordination for the metal ion where the donors are the Gly45CO, the His46Nδ1, the Cys112Sγ and the His117Nδ1 atoms.

4.3. Rack-induced coordination

From our present results, it is clear that the metal ion adopts a coordination that is of minimum energy according to its preferences (tetrahedral for cobalt(II) ion) in the unfolded state. This tetrahedral coordination is reflected in the low magnetic anisotropy of the system as well as in the short T_1 values of the hyperfine shifted signals. In contrast, in the folded state the metal ion coordination is governed by the overall folding of the protein, resulting in a trigonal bipyramidal geometry. It is interesting to remark that, in the C112D azurin mutant [29], the copper is not type 1 any more. Its cobalt(II) derivative (CoC112D) has been studied by ^{1}H NMR and the magnetic anisotropy as well as the proton relaxation times are closer to those of CoAz in the unfolded state than in the folded state. Thus, when the type 1 copper characteristics are lost, the metal adopts a similar coordination (according to its preferences) in the folded protein as in the unfolded state. In contrast, in stellacyanin and in the azurin mutant H46DAz the copper is type 1 and the cobalt(II) derivatives are tetracoordinated. The magnetic anisotropy and the relaxation properties of the observed signals in the ^{1}H NMR spectra of CoSt [33] and CoH46DAz [44] are similar

to those of the CoAz in the folded state. This strongly suggests that in these two derivatives the protein forces the cobalt to adopt strained tetracoordination. This is completely consistent with the rack-induced mechanism proposed 30 years ago [6]. Even though this mechanism has been amply demonstrated, to the knowledge of the authors there has been no direct (spectroscopic) determination of the metal ion coordination of a blue copper protein in the unfolded state.

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