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Forum

Insights into Partially Folded or Unfolded States of Metalloproteins from Nuclear Magnetic Resonance

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Nuclear magnetic resonance (NMR) provides detailed insights into the conformational features of unfolded and partially folded proteins. In the case of metalloproteins, special attention should be devoted to the characterization of the properties of the metal binding sites, and specific approaches need to be developed depending on the nature of the metal ion and its coordination environment. At the same time, metal-based NMR parameters may help in getting a better picture of the average structural properties of the metalloprotein. A critical evaluation of the limits of applicability of paramagnetic effects for solution structure determination in partially folded or unfolded proteins is presented. The coupling between NMR characterization of structure and dynamic of the polypeptide chain and of the metal environment provides insights into the stabilizing role of metal ions in metalloproteins. The overall approach is illustrated for some case examples of increasing flexibility obtained far from native conditions for cytochrome *c* and superoxide dismutase, two metalloproteins that have been extensively studied in our lab and whose misfolded forms may be relevant for important biological processes.

Unfolded or Partially Folded Metalloproteins

The characterization of the structure of non-native states of proteins which occur in the presence of denaturants, at high temperatures, or far from physiological pH values is believed to be of great interest to understanding the protein folding mechanisms; the partially unfolded forms, detected by equilibrium studies, are representative of folding intermediates while the fully unfolded forms provide a picture of the conformational ensemble at the starting point of the folding process. NMR is the technique of choice for such studies given its ability to provide information at the level of individual residues on the dynamic and conformational features in the intrinsically heterogeneous ensemble that characterizes each of these states. In a recent view, NMR of partially folded or unfolded states is gaining increasing importance for determining the role of such conformationally heterogeneous states in crucial biological processes.

It is emerging that many proteins or protein domains are only partially structured under physiological conditions and fold toward stable structures only upon binding to their biological partners.[1−3] Metal binding can be seen as a special case of the protein−biological partner recognition. This is particularly true for all those proteins that are involved in specific metal trafficking pathways as well as for those proteins that determine metal-mediated signaling.[4−11]

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Unfolded states of proteins also play a role in the transport of proteins across membranes.[12] Protein translocation across the membrane of subcellular organelles is of general importance to understand the basic mechanism of noncytosolic metalloprotein reconstitution, which occurs after the polypeptide chain has been synthesized on ribosomes. It is more and more clear that proteins have to be largely unfolded in order to cross the membrane and then reach their final destination where metal reconstitution and consequent structuring occurs. In the particular case of eukaryotic cytochrome *c*, the reverse process is also relevant, as release of cytochrome *c* from mitochondria to the cytosol is at the basis of caspase-dependent apoptosis.[13]

Finally, misfolded states of proteins are thought to be relevant for amyloid diseases or, more generally, for those diseases that are related to the formation of protein aggregates.[14] This is the case of copper,zinc superoxide dismutase (Cu,ZnSOD) mutants that are associated with familial amyotrophic lateral sclerosis (FALS). One of the dominant hypotheses to explain mutant toxicity relies on the idea that mutant proteins may become misfolded and therefore oligomerize into high-molecular weight species, of increasing size, that ultimately lead to the death of motor neurons.[15] The ability of the technique to detect high molecular weight aggregates is limited to solid-state NMR, which is out of the scope of this review; however, attempts are in progress to detect the first steps of protein oligomerization by solution NMR, as well as to characterize the structural and dynamic properties of the isolated mutants and understand the molecular determinants for the increased aggregation tendency.

A major challenge for NMR is that of studying molten globule states or, in general, protein states that are highly fluxional, with exchange among the many conformational states being on a time scale from milliseconds to fractions of nanoseconds. The three systems described in this paper represent examples of increasing fluxionality, from the molten globule state represented by the alkaline form of yeast cytochrome *c*,[16,17] to the globular random coil monomeric Cu,ZnSOD[18] through the intermediate situation of cytochrome *c* in the presence of sodium dodecylsulfate (SDS) micelles.[19] The three examples will be discussed as a function of the differences in the relevant NMR parameters.

What Can We Learn About the Polypeptide Chain

Diamagnetic NMR. The approach to study unfolded and partially folded proteins through NMR has been extensively reviewed in recent times[20−22] and will be quickly summarized here. The potential of the NMR technique relies on the availability of isotopically enriched samples. ^{13}C and ^{15}N nuclei chemical shifts are indeed more sensitive to local amino acid sequence rather than to the overall structural environment and, therefore, maintain a significant chemical shift dispersion even in fully unfolded proteins. The ^{1}H chemical shift, instead, is characterized by extensive signal overlap for largely unfolded states, which makes the spectral analysis difficult. The situation is depicted in Figure 1, where the progressive loss of structure in Cu,ZnSOD upon addition of increasing amounts of guanidinium chloride (GdmCl) can be easily monitored by the decreased spreading in ^{1}H chemical shifts for backbone amides.[18]

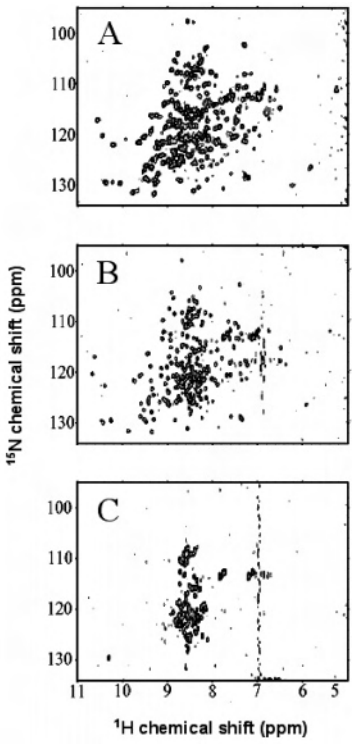

Figure 1. Progressive loss of structure upon addition of GdmCl to monomeric Cu,Zn superoxide dismutase as monitored by $^{15}N-^{1}H$ HSQC experiments. The ^{1}H chemical shift in the absence of denaturant covers the range 6−11 ppm (A); with 3 M GdmCl all the backbone signals are concentrated between 8 and 9 ppm, those falling outside this region being due to side chains (C). Spectrum in panel B has been acquired in the presence of 2 M GdmCl, and the signals of the folded and unfolded species are both detectable, the two protein forms being in slow exchange on the NMR time scale. (Adapted from ref 18.)

Three-dimensional triple resonance experiments to establish sequential connectivities via ^{15}N and ^{13}C resonances constitute the most general approach to obtain unambiguous resonance assignments. Triple resonance experiments developed specifically for the assignment of unfolded states of proteins are reviewed elsewhere.[21,23] It is worth mentioning here that, in recent years, our lab has been heavily committed

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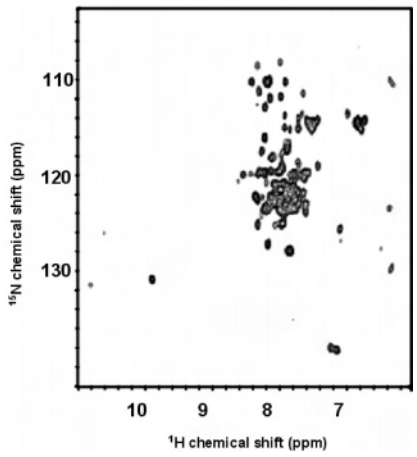

Figure 2. ^{15}N−^{1}H HSQC spectrum recorded at 500 MHz for horse heart cytochrome *c* in the presence of SDS micelles. (Adapted from ref 19.)

to the development of "protonless" pulse sequences based on ^{13}C direct detection that have the advantage of allowing sequential assignment without making use of proton resonances. An approach of this type can be used also in the presence of largely paramagnetic metal ions that make the ^{1}H signals undetectable in a wide sphere around the metal ion.[24−27]

Once the backbone assignment is obtained, information can be gained directly on the conformational preferences of the ensemble, keeping in mind that when a single set of resonances is observed NMR parameters simply reflect a population weighted average over all the accessible conformations of the system that are in fast equilibrium on the NMR time scale. The most sensitive probe of the secondary structure features is the chemical shift index, a parameter that measures the sequence-corrected variations in chemical shift with respect to random coil values.[28,29] Some insight into the secondary structure propensity can also be obtained from the analysis of coupling constants that are directly related to backbone dihedral angles. For example, $^{3}J_{\mathrm{HNHA}}$ values are correlated to the backbone ϕ angles.[30] In the case of unfolded monomeric Cu,Zn SOD, the $^{3}J_{\mathrm{HNHA}}$ values are indicative of a protein that has an essentially random coil structure for the backbone, in agreement with other spectroscopic data.[18] In the case of yeast iso-1 cytochrome *c* in the presence of SDS micelles,[19] the $^{3}J_{\mathrm{HNHA}}$ values are the result of a fast equilibrium on the NMR time scale between a protein form with α helical structure and extended random coil conformations. The presence of residual secondary structure is consistent with a nonfully collapsed ^{1}H chemical shift spreading for backbone amides, as shown in Figure 2.

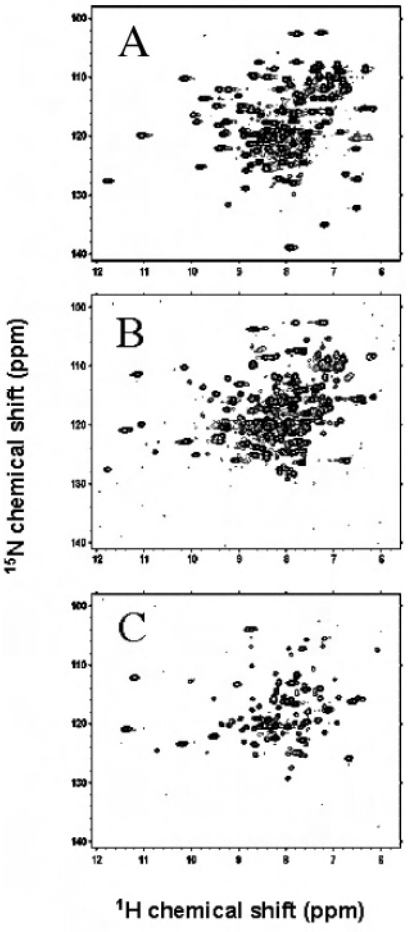

Figure 3. ^{15}N−^{1}H HSQC spectra recorded at 800 MHz for the alkaline form of K79A yeast cytochrome *c* at pH 7.1 (A), 9.7 (B), 11.1 (C). (Adapted from ref 16.)

The alkaline form of cytochrome *c*[16] has well resolved resonances, covering a chemical shift range comparable to that of the protein under native conditions (Figure 3). The $^{3}J_{\mathrm{HNHA}}$ values for the residues that in the native structure give rise to four out of the five helices are still typical for α helical conformation.

Solution structures of folded proteins are obtained primarily thanks to the use of medium and long-range distance information available from the nuclear Overhauser effect (NOE). NOEs are detectable for each pair of nuclei separated by less than 5.5−6 Å in space. Normally, about 15−20 NOEs are detectable for each residue possessing a well defined conformation. The intensity of the NOE cross-peaks depends on the inverse sixth power of the internuclear distance; thus, a solution structure is usually based on the availability of several thousands of upper distance limits derived from the intensity to distance conversion of the observed NOEs. The conversion of NOE intensities into distances, however, holds as long as the dipolar interaction between two nuclei close in space is modulated by the molecular tumbling. When motions faster than tumbling occur, the NOE effect is quenched and could even become undetectable. This is the case of unfolded SOD and of cytochrome *c* in SDS.[18,19]

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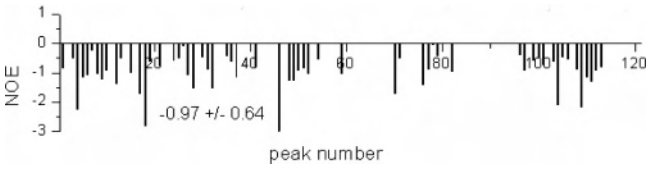

Figure 4. In the unfolded form of monomeric Cu,ZnSOD, the $\{^1H\}-^{15}N$ NOE values measured at 400 MHz are zero or negative, consistent with the large reduction in the effective correlation time that drops from an overall 9.3 ns in the folded protein to a distribution of values in the range 0.8−2 ns.

Moreover, the conversion of the NOE into distance is often difficult as the correlation time for the dipolar interaction is unknown.

The above considerations introduce the issue of the peculiar dynamic features of nonfully folded protein states. The common description of the dynamics of a folded protein is based on the presence of a single tumbling time (of the order of nanoseconds) which roughly corresponds to the correlation time from the Stokes−Einstein equation, and a series of local motions that are much faster than the tumbling time. The model-free approach,[31,32] that is extensively used for the analysis of the backbone relaxation data of uniformly ^{15}N-labeled proteins, i.e., ^{15}N R_1 and R_2 and $\{^1H\}^{15}N$ NOE, is based on this type of description. Unfolded or partially folded states of proteins are more correctly described as experiencing a mobility governed by a distribution of correlation times on the nanosecond time scale, as it results clearly from the analysis of the $\{^1H\}^{15}N$ NOEs, where the internuclear distance is substantially constant and the main determinant of the observed effect comes from the correlation times that modulate the dipolar interaction. In fully folded proteins, $\{^1H\}^{15}N$ NOE values are usually quite uniform and high (0.8) along the protein sequence, while in unstructured polypeptide sequences they can display a large variability and can even be negative for the most flexible areas, as shown in Figure 4 for the unfolded Cu,ZnSOD. The presence of a distribution of correlation times makes the model-free approach useless, and therefore, new approaches have been developed that are reviewed by Dyson and Wright.[21] In practice, effective correlation times for the dipolar interaction shorter than the tumbling time of the protein under native conditions are often estimated from the field dependence of $\{^1H\}^{15}N$ NOE intensity.[17−19]

Paramagnetic NMR. In the case of paramagnetic metalloproteins, a further structural constraint that has been extensively used for protein structure determination is the pseudocontact shift. This contribution to the shift originates from the anisotropic part of the dipolar interaction between unpaired electron(s) and the nucleus, which averages to a value different from zero upon rotation in solution when the metal center possesses magnetic anisotropy. The pseudocontact shift δ^{pc} is given by the following equation,[33,34] which holds within the metal-centered point−dipole/point−dipole approximation:

$$\delta^{pc} = \frac{1}{12\pi r^3}\left[\Delta\chi_{ax}^{para}(3\cos^2\theta - 1) + \frac{3}{2}\Delta\chi_{rh}^{para}(\sin^2\theta\cos 2\phi)\right] \quad (1)$$

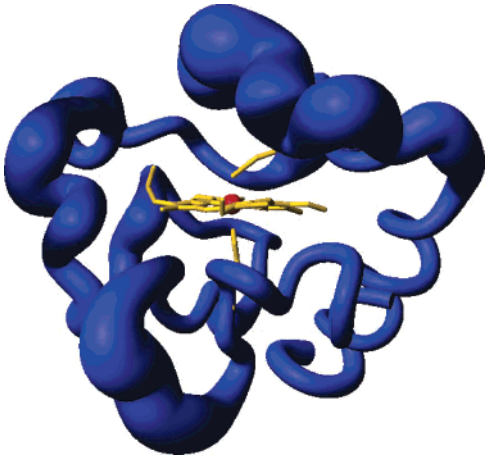

Figure 5. Family of solution structures for the alkaline form of K79A cytochrome *c*. The protein is essentially a molten globule with conserved secondary structure elements and important conformational rearrangement of the loop bearing Met80. The native Met80 ligand is replaced by Lys73, which constitutes the only anchoring point of the distal loop to the rest of the structure. The large RMSD values, indicated by the large radius of the tube representing the family of structures, do reflect an intrinsic mobility of the protein.

where $\Delta\chi_{ax}^{para}$ and $\Delta\chi_{rh}^{para}$ are axial and rhombic anisotropies of the magnetic susceptibility tensor, θ and ϕ are cylindrical coordinates of the position vector of a proton, and r is the distance between the paramagnetic center and the proton in question.

The pseudocontact shift is the only paramagnetic contribution to the paramagnetic shift for nuclei that do not belong to metal ligands and can be evaluated by subtracting from the observed shift of the paramagnetic species the chemical shift of the corresponding diamagnetic analogue. Availability of these nonconventional NMR constraints for solution structure determination of proteins allows us to frame the metal center within the protein[35−39] and also to obtain a better resolution in the proximity of the paramagnetic metal ion, where often NOE intensity is strongly reduced (at the limit of detectability) owing to the reduction in nuclear T_1 caused by the paramagnetic-induced relaxation. Pseudocontact shifts become even more important in the case of partially unfolded states of metalloproteins, where the total number of NOEs is reduced by mobility.

A nice example of pseudocontact shift applicability is represented by the characterization of the unfolded form of the alkaline form of cytochrome *c*. Its solution structure has been determined by NMR at pH 11.1 (Figure 5).[16] The

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overall result is that of a protein characterized by a structure, on average, of the same size of that of the native protein, with the most important conformational differences concentrated in the distal site of the heme where the native Met80 ligand is replaced by a Lys (see below). However, the whole protein is by far more flexible with respect to the native one, as exemplified by the fact that the correlation time for heteronuclear NOEs drops from 5.2 ns of the native form, typical for a protein of this size, to a distribution of effective correlation times with values in the loop region as low as 3 ns. As a consequence, the number of ^{1}H−^{1}H NOE constraints meaningful for structure calculation is reduced: 1361 in the native and 838 in the alkaline protein. Nevertheless, 180 pseudocontact shift values provided very useful constraints for solution structure determination.

Other useful constraints to obtain structural information in solution are the residual dipolar couplings. In diamagnetic proteins, partial alignment of the molecule at high magnetic fields originates from intrinsic magnetic anisotropy of secondary structure elements and aromatic rings.[40−44] Partial alignments give rise to effects that are detectable through NMR spectroscopy[45,46] such as the noncompletely averaged dipolar couplings or residual dipolar couplings[47,48] that represent a source of structural information.[49−52] The effect can be enhanced by the presence of orienting media.[53−55] In the case of diamagnetic heme proteins, a further contribution to molecular magnetic anisotropy is provided by the heme.[55−57] The use of "diamagnetic" residual dipolar couplings in nonfolded states can give rise to useful information on the overall backbone topology and has been proposed as a new method to obtain structural constraints,[22] provided the necessary orienting media do not interact with the exposed hydrophobic residues.

In low spin iron(III) and high spin iron(II), the molecular magnetic anisotropy arises from the diamagnetic contributions (χ^{dia}) and the dominant paramagnetic contribution of the iron(III) ion (χ^{para}),[56−59] χ^{para} being the same as in the equation of the ^{1}H pseudocontact shifts.[17,33,34,36,37,58,60−64] More generally, a sensible paramagnetic contribution to residual dipolar coupling exists for any metal center possessing magnetic susceptibility anisotropy. The resulting equation for the paramagnetic contribution to the residual dipolar coupling (rdc) is[17]

$$\Delta\mathrm{rdc}^{para}(B_0) = -\frac{1}{4\pi}\frac{{B_0}^2}{15kT}\frac{\gamma_H\gamma_N h}{4\pi^2 {r_{HN}}^3}S\left[\Delta\chi_{ax}^{para}(3\cos^2\theta - 1) + \frac{3}{2}\Delta\chi_{rh}^{para}(\sin^2\theta\cos 2\phi)\right] \quad (2)$$

where $\Delta\chi_{ax}^{para}$ and $\Delta\chi_{rh}^{para}$ are the same as in eq 1, θ and ϕ are polar coordinates describing the orientation of the N−H bond vector in the (axis) frame of the χ^{para} tensor, and S is the generalized order parameter, whose meaning will be discussed below. By analogy with the pseudocontact shift, the $\Delta\mathrm{rdc}^{para}$ can be obtained from the difference between the residual dipolar coupling of the paramagnetic form and that of the corresponding diamagnetic analogue, measured at the same magnetic field. $\Delta\mathrm{rdc}^{para}$ values are generally significantly larger than the diamagnetic ones, and they can be easily measured in the absence of orienting media.

Unfortunately, we have found the $\Delta\mathrm{rdc}^{para}$ para to be not as useful as pseudocontact shifts as structural constraints. Indeed, it is now established that mobility on a time scale shorter than milliseconds affects rdc values by reducing them.[53,65] That is the origin of the generalized order parameter in eq 2, which represents a scaling factor (with values ≤ 1) for the rdc value expected for a well defined orientation of the NH vector. In proteins, under native conditions, the extent of this effect is often negligible, but it can become very important in partially folded states. This effect was first observed in the alkaline form of cytochrome *c*,[17] where the measured $\Delta\mathrm{rdc}^{para}$ values are sensibly smaller, in absolute value, than those expected on the basis of eq 2 and the anisotropies of the magnetic susceptibility tensor derived from pseudocontact shifts. The fact that the latter adequately represent the electronic situation of the metal center is demonstrated by the full compatibility with the other constraints used for solution structure calculations (NOE and

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Table 1. The $\Delta rdc^{700-400}$ Values of Cytochrome *c* in SDS Micelles[a]

$\Delta rdc^{700-400}$ (Hz)		
−1.28	−0.18	−0.02
−0.86	−0.18	0.00
−0.81	−0.17	0.00
−0.80	−0.16	0.02
−0.68	−0.15	0.06
−0.62	−0.14	0.09
−0.48	−0.14	0.15
−0.47	−0.13	0.22
−0.47	−0.13	0.30
−0.44	−0.11	0.31
−0.41	−0.09	0.33
−0.29	−0.07	0.37
−0.27	−0.07	0.39
−0.26	−0.05	0.39
−0.23	−0.05	0.49
−0.23	−0.05	1.03
−0.23	−0.04	1.04
−0.20	−0.03	1.09
−0.18	−0.03	

[a] The values are arranged in order of increasing $\Delta rdc^{700-400}$.

dihedral angle constrains). Introduction of pseudocontact shifts as structural restraints improves the definition of the family of structures leaving unaltered the target function that measures the agreement between calculated and experimental constraints. On the other hand, the presence of submillisecond mobility is in agreement with the above-discussed reduction in effective correlation times for the NOEs.

We report here, for the first time, a similar behavior for the unfolded form of cytochrome *c* in concentrated solutions of SDS. The backbone $^{15}N-^{1}H$ ^{1}J values could be safely measured for 56 well resolved amides of cytochrome *c* in SDS micelles at two fields (700 and 400 MHz). As discussed in the Experimental Section, lack of the corresponding diamagnetic protein form does not allow the estimate of the Δrdc^{para}. Nevertheless, the total contribution (diamagnetic and paramagnetic) to molecule self-orientation can be inferred from the resulting $\Delta rdc^{700-400}$ values, that are found to span a very narrow range (Table 1). Most of them are smaller than 0.5 Hz (in absolute value), while only four of them are greater than 1 Hz (in absolute value), i.e., much smaller than those expected on the basis of magnetic susceptibility tensor considerations. Indeed, the values of magnetic susceptibility tensor anisotropies expected for high spin iron(III) form of cytochrome *c* in SDS are comparable to those of the low spin iron(III) heme proteins[59] and $\Delta rdc^{700-400}$ values between −2 and +2 Hz were expected in SDS. Consistent with the reduction in $\Delta rdc^{700-400}$ measured values, an extensive subnanosecond backbone mobility characterizes this protein form as found by ^{15}N mobility measurements.[19]

Figure 6 shows, in a pictorial way, the origin of the different sensitivity of the two NMR parameters to the backbone mobility. The fact that pseudocontact shifts are not quenched by the mobility that quenches residual dipolar couplings indicates that, even in the presence of backbone mobility, on average the same metal-to-proton distances are maintained, and motions do not sensibly affect the angular part of eq 1. Therefore, pseudocontact shifts maintain their relevance in protein forms characterized by large mobility of the backbone. On the contrary, residual dipolar couplings lose their meaning as structural constraints. This is due to

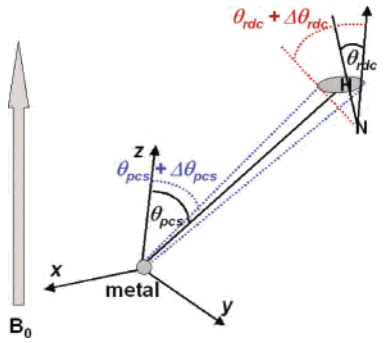

Figure 6. Effect of a change in the orientation of the NH vector on the θ angles, which are relevant for the angular dependence of pseudocontact shifts (blue) and paramagnetic residual dipolar couplings (red). The longer metal−proton distance makes the effect on pseudocontact shifts substantially negligible.

the different effect of the reorientation of the NH vector on the angular part of eqs 1 and 2: a change in the orientation of an NH bond determines a sensible change in the angle between the NH vector and the magnetic axis, while the angle between the same axis and the metal−H vector is only slightly affected. The orientational average of an NH vector may reduce residual dipolar couplings, whereas the average polar coordinates of an amide proton, which determine the pseudocontact shift, may vary only a little.

The most important conclusion of this section, therefore, resides in the consideration that long range constraints are able to maintain their relevance in proteins that are largely fluxional, while those based on more local effects are by far more sensitive. At the same time, the use of paramagnetic residual coupling can constitute a further way to characterize the dynamics of the conformational ensemble.

What Can We Learn about the Metal Site

Histidine Ligands. Histidines are often found as metal ligands in metalloproteins. Therefore, detecting the binding mode of His residues to the metal ion is important to establish the structural features of the metal center under non-native conditions. From the NMR point of view, this goal can be achieved through $^{15}N-^{1}H$ HSQC experiments where the INEPT delay is optimized for the detection of the small ^{2}J coupling between the imidazole nitrogens and the protons bound to the adjacent carbon nuclei. The two nitrogens can be discriminated because two cross-peaks are expected for the Nϵ and only one for the Nδ. Metal bound His species have a characteristic shift pattern, with the binding nitrogen falling in the range 200−230 ppm, while the noncoordinated imidazole nitrogen has shift in the range 170−185.[66,67] The situation is depicted in panel A of Figure 7 for monomeric Cu,ZnSOD containing copper in the +1 oxidation state under native conditions: in this protein the copper ion is bound to the Nδ of His46 and to the Nϵ of His48 and His120, while the zinc ion is bound to the Nδ of His63, His71, and His80. Upon unfolding, metals are removed from their native

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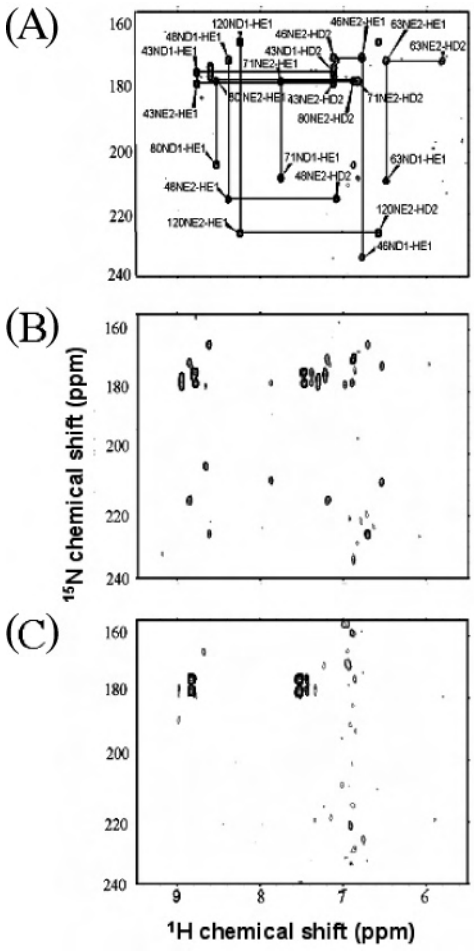

Figure 7. ^{2}J ^{15}N−^{1}H HSQC spectra aquired at 500 MHz on copper(I) monomeric Cu,ZnSOD to monitor His binding to the metal ions: in the absence of denaturant (A), with 2 M GdmCl (B), with 3 M GdmCl (C). (Adapted from ref 18.)

binding sites,[18] and the histidine residues become free to rotate and experience fast equilibria between the two tautomeric forms, as indicated in panel C of Figure 7, where all the resonances of His side chains are collapsed in the region typical for unstructured polypeptides. The method fails when the exchange regime between the tautomeric species is quasislow on the NMR time scale, where signals can become undetectable. This has been observed in the case of some flexible parts of metal chaperones in their apo form (work in progress). Owing to the severe line broadening induced on the ^{1}H resonances of protons belonging to metal ligands, the method has no applicability in the case of His coordinating paramagnetic metal ions.

Heme Metal Cofactors. Heme proteins possess particular features that can be exploited to monitor the changes in the axial ligand properties that often are coupled to loss of the native structure. The change from hexacoordination (with two strong axial donor atoms usually encountered when the axial ligands are protein amino acids side chains) to pentacoordination is obviously detected due to the change in the spin state of the heme iron: the heme methyl resonances resonate in the 100−60 ppm ^{1}H chemical shift range for high spin species, and in the 40−10 ppm range for the low spin heme iron.[68] This is the case for cytochrome *c* in SDS, where, upon unfolding, the native bond between Met80 and iron is broken.[69] Water binding to the sixth coordination position is almost indistinguishable from pentacoordination. From the NMR point of view, the two forms can be distinguished only by the presence of broad meso resonances in the upfield part of the spectrum for pentacoordinate heme iron(III).[68] However, these signals are usually far too broad to be detected in partially unfolded states of heme proteins, where they are further broadened due to conformational exchange. If a native axial ligand is substituted by another strong protein ligand (good donor atoms are potentially the nitrogens of His, Lys, or the N-term of the protein, provided they are deprotonated at the pH of the experiment) the protein remains low spin and the resonances of the new ligands can be detected, as was the case of alkaline cytochrome *c*, where broad signals attributable to Lys73 binding the iron were detected. Once the nature of the new axial ligand is identified, semiempirical equations can be used for protein containing c-type or b-type hemes in order to define the orientation of the axial ligand.[70−72] These equations simply rely on the pattern of ^{1}H chemical shifts of the heme methyl resonances. Two different equations have been developed and extensively tested for a series of well behaving systems: the one for bis-His axial coordination provides the relative orientation between the two imidazole planes and the direction of the projection of the bisector of the angle between these two planes onto the heme plane. When one axial ligand is a His and the other is a ligand that has π orbitals with cylindrical symmetry, the equation allows us to define the orientation of the proximal His ligand with respect to the heme plane. This is the case of alkaline cytochrome *c*, where this approach has allowed us to establish that the orientation of His18 does not change in the molten globule state.[16]

Other Metal Cofactors. The behavior, upon protein unfolding, of metal centers in copper and iron sulfur proteins has been monitored by NMR.[73−76] At the moment, however, well established methods that allow us to easily interpret the spectral changes in the NMR spectrum in terms of precise variations in metal binding environment upon loss of the native structure have not been developed. Detection of the protons of ligands is a challenge for many paramagnetic centers. Nevertheless, the possibility of direct detection of the ^{13}C resonances of protein amino acids binding to paramagnetic metal ions that we are pioneering in our lab

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should open new chances to better understand the details of the coordination environment of the metal ions in largely unstructured proteins.

Experimental Section

Twenty $^1J_{NH}$-modulated 2D $^1H-^{15}N$ HSQC spectra were recorded at 400 and 700 MHz for yeast cytochrome *c* in a solution of 100 mM SDS, following the standard procedure.[17,77] The difference between the $^1J_{HN}$ values measured at the two different fields in the case of a paramagnetic metalloprotein depends on the total magnetic susceptibility of the molecule which results from a tensorial combination of the diamagnetic tensor arising from the backbone, aromatic ring, and porphyrin contribution and the paramagnetic tensor due to the presence of the paramagnetic metal center.[56] In this case, it was not possible to subtract the diamagnetic residual dipolar couplings from the observed ones, because a diamagnetic protein form is lacking. Nevertheless, the difference between the values measured at the two fields allows an evaluation of the extent of the expected range values by considering that in heme proteins the diamagnetic contribution is essentially due to the heme moiety and is very similar also for proteins with largely different folds.[17,56,57] The observed values are too small for any possible orientation of the NH vector with respect to the metal center.

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