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ARTICLE

A strong ^{13}C chemical shift signature provides the coordination mode of histidines in zinc-binding proteins

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Abstract Zinc is the second most abundant metal ion incorporated in proteins, and is in many cases a crucial component of protein three-dimensional structures. Zinc ions are frequently coordinated by cysteine and histidine residues. Whereas cysteines bind to zinc via their unique S^{γ} atom, histidines can coordinate zinc with two different coordination modes, either $N^{\delta 1}$ or $N^{\varepsilon 2}$ is coordinating the zinc ion. The determination of this coordination mode is crucial for the accurate structure determination of a histidine-containing zinc-binding site by NMR. NMR chemical shifts contain a vast amount of information on local electronic and structural environments and surprisingly their utilization for the determination of the coordination mode of zinc-ligated histidines has been limited so far to ^{15}N nuclei. In the present report, we observed that the ^{13}C chemical shifts of aromatic carbons in zinc-ligated histidines represent a reliable signature of their coordination mode. Using a statistical analysis of ^{13}C chemical shifts, we show that $^{13}C^{\delta 2}$ chemical shift is sensitive to the histidine coordination mode and that the chemical shift difference $\delta\{^{13}C^{\varepsilon 1}\} - \delta\{^{13}C^{\delta 2}\}$ provides a reference-independent marker of this coordination mode. The present approach allows the direct determination of the coordination mode of zinc-ligated histidines even with non-isotopically enriched protein samples and without any prior structural information.

Keywords NMR spectroscopy · ^{13}C chemical shifts · Histidine · Zn-ligated histidine · Zinc-binding protein · Coordination mode of histidine · Histidine tautomers · Chemical shift analysis

Introduction

NMR chemical shifts are strongly sensitive to local electronic environment and therefore contain an immeasurable amount of information on protein structures and dynamics (Wishart and Case 2002). This information is largely used in the different steps of the structure determination process by NMR, namely chemical shift resonance assignment (Wuthrich 1986; Oh et al. 1988; Grzesiek and Bax 1993), secondary structure and backbone angle restraints predictions (Wishart and Sykes 1994; Cornilescu et al. 1999; Shen et al. 2009), determination of side-chain rotamer conformation (London et al. 2008; Hansen et al. 2010; Hansen and Kay 2011), detection of cis-peptide bonds (Schubert et al. 2002), prediction of the oxidation state of cysteines (Sharma and Rajarathnam 2000), and even complete determination of three-dimensional structures (Cavalli et al. 2007; Shen et al. 2008; Wishart et al. 2008).

A large number of proteins incorporate metal ions as part of their structure (hence called metalloproteins). According to various estimates, about one-quarter to one-third of all proteins require metal ions to perform their biological function (Rosenzweig 2002; Dupont et al. 2010). Zinc is only the second most abundant metal ion in living

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organisms after iron, but regarding the large variety of zinc-binding protein domains, zinc is the most widespread incorporated metal ion in protein structures. Indeed, in eukaryotes, about 9 % of all proteins are predicted to include at least one zinc ion in their structure (Dupont et al. 2010; Andreini et al. 2006b). Zinc is commonly coordinated by four ligands provided by protein side chains in structural sites and by only three protein ligands in catalytic sites in which the coordination sphere is completed by a water molecule involved in catalysis (Auld 2001). The most common protein side chains involved in zinc-coordination are by far cysteines and histidines, found with similar usage, followed by aspartate and glutamate (Andreini et al. 2006a).

Contrary to cysteine ligands, for which the atom that directly contacts the zinc ion is unique (Cys S^{γ}), histidine ligands bind to zinc ions with two different coordination modes involving either one or the other of the two endocyclic nitrogens $N^{\delta1}$ and $N^{\varepsilon2}$ (Fig. 1a). It is important to notice that these coordination modes are intrinsically associated with the tautomeric form of the histidine ring, where a $N^{\delta1}$ coordination mode is uniquely compatible

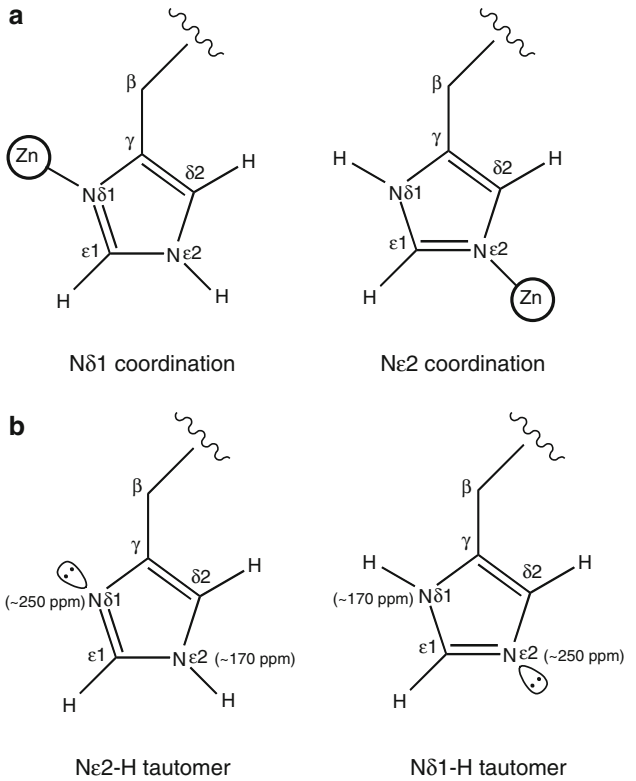

Fig. 1 Chemical structure and atom nomenclature of an histidine side chain. **a** Chemical structure and atom nomenclature for the two coordination modes of zinc-ligated histidines, namely the $N^{\delta1}$ and the $N^{\varepsilon2}$ coordination modes. **b** Chemical structure and characteristic ^{15}N chemical shifts for the two tautomeric form of neutral histidines, namely the $N^{\varepsilon2}$–H and the $N^{\delta1}$–H tautomeric form

with a protonation on $N^{\varepsilon2}$ and vice versa (Fig. 1a, b). Since the two endocyclic nitrogens are 2.2 Å apart, the correct determination of the histidine coordination mode is of primary importance for the accurate structure determination by NMR of a histidine-containing zinc-binding site.

Two different approaches are currently used for this purpose. In the first approach, a preliminary structure is determined without any information on the histidine-coordination mode, and this one is latter deduced from the analysis of the bundle of structures (Lee et al. 1989; Omichinski et al. 1990; Dempsey et al. 2004; Malgieri et al. 2007; Bessière et al. 2008; Cordier et al. 2008; Briknarová et al. 2011). This method is effective when NOE patterns to $H^{\delta2}$ and $H^{\varepsilon1}$ aromatic protons are sufficiently different to unambiguously position a single endocyclic nitrogen in the vicinity of the zinc ion. Note that this approach can sometimes be unreliable, since the differences between corresponding distances present in the two alternative structures, formed via $N^{\delta1}$ or $N^{\varepsilon2}$ coordination, can be rather small. The second approach aims at determining the tautomeric form of the histidine ring through the measurement of the ^{15}N chemical shifts of the endocyclic nitrogens $N^{\delta1}$ and $N^{\varepsilon2}$ (Legge et al. 2004; Ramelot et al. 2004; Kostic et al. 2006; Liew et al. 2007; Estrada et al. 2009; Peroza et al. 2009; Eustermann et al. 2010). This is achieved via the measurement of optimized long-range ^{15}N-^{1}H HSQC or HMQC (Pelton et al. 1993). The identification of the $N^{\delta1}$ and $N^{\varepsilon2}$ chemical shifts takes advantage of the fact that $N^{\varepsilon2}$ is connected to two close proton-neighbours via a $^{2}J_{NH}$ coupling ($H^{\delta2}$ and $H^{\varepsilon1}$) whereas $N^{\delta1}$ couples to only one such neighbour ($H^{\varepsilon1}$) (Fig. 1b). As far as the $N^{\delta1}$ and $N^{\varepsilon2}$ chemical shifts can be identified, this approach is unambiguous. Indeed, the chemical shifts of the protonated nitrogen and the non-protonated nitrogen in a neutral histidine ring display an average difference of about 80 ppm, with protonated nitrogen chemical shifts being around 170 ppm and the non-protonated ones, with a lone pair, around 250 ppm (Fig. 1b). Therefore, the only possible ambiguities related to this method come from chemical shifts overlaps between different histidine residues which could render confident assignments impossible. This method requires a ^{15}N-labelled protein sample, and this definitely creates limitations in the case of samples obtained via chemical synthesis, a method commonly used for the production of small zinc-binding peptides.

Here we present a simple and reliable method for the identification of the coordination mode of histidines based on ^{13}C aromatic carbon chemical shifts. We made use of the Biological Magnetic Resonance Data Bank (BMRB) to conduct a statistical analysis on ^{13}C aromatic carbon chemical shifts of zinc-coordinated histidines and demonstrate that ^{13}C aromatic chemical shifts, which can be

easily obtained even with non-labelled protein samples, constitute an excellent marker for the determination of the coordination mode of histidines.

Methods

Data collection and analysis

A database of histidine ^{13}C aromatic carbons $C^{\delta 2}$ and $C^{\varepsilon 1}$ chemical shifts was constructed from the BMRB database (http://www.bmrb.wisc.edu/). Basic shell scripts were written to identify zinc-coordinated histidines and to extract $C^{\delta 2}$ and $C^{\varepsilon 1}$ chemical shifts. The database was divided in two groups depending on the annotation present in the BMRB file regarding the coordination mode of the corresponding histidine ($N^{\delta 1}$ or $N^{\varepsilon 2}$ coordination). Only histidines for which both $C^{\delta 2}$ and $C^{\varepsilon 1}$ chemical shifts were available were used in subsequent chemical shift analysis. Wrong annotation of the coordination mode in the BMRB entry as compared with the associated PDB coordinates was present in 3 cases (bmrb entries 10108, 6374 and 11061) and corrected in the constructed database. The coordination mode of one histidine was wrongly assigned when compared to closely related protein structures (bmrb entry 6682) and was also corrected for subsequent analysis. This selection resulted in 223 histidines coordinating zinc via $N^{\varepsilon 2}$ and 43 histidines via $N^{\delta 1}$. Statistical analysis of the chemical shifts were carried out with Gnumeric (http://projects.gnome.org/gnumeric/). Chemical shifts were grouped in 0.5 ppm bins for analyzing the statistics of occurrence.

In addition, a database with ^{13}C aromatic carbons $C^{\delta 2}$ and $C^{\varepsilon 1}$ chemical shifts of non-coordinated histidines was constructed from the Reference Corrected Database (RefDB) (http://refdb.wishartlab.com/) (Zhang et al. 2003) with similar basic shell scripts. Histidines having both $C^{\delta 2}$ and $C^{\varepsilon 1}$ chemical shifts assigned were used for analysis.

Results

Initial questions and genesis of the project

In the structure determination process of a protein domain with an unexpected zinc-binding site (Barraud et al. 2011), we noticed the presence of a downfield shifted $C^{\delta 2}$ of a histidine side chain ($C^{\delta 2} = 126.1$ ppm). This is rather unusual in proteins where most histidine $C^{\delta 2}$ have chemical shifts around 120 ppm. We wondered whether this downfield shifted $C^{\delta 2}$ was a characteristic of zinc-coordinated histidines. On one hand, downfield shifted $C^{\delta 2}$ in histidines are presented as a characteristic of zinc-coordinated histidines in some reports (De Guzman et al. 2000; Ramelot et al. 2004; Kornhaber et al. 2006). But on the other hand, we also noticed numerous examples where zinc-coordination was not associated with a particularly downfield shifted $C^{\delta 2}$ (Zeng et al. 2008; Huang et al. 2009; Eustermann et al. 2011), showing that confusion exists in the literature about this point. Interestingly, the analysis of $C^{\delta 2}$ chemical shifts from few particular enzymes with histidines involved in catalytic triads or from imidazole derivatives indicated that $C^{\delta 2}$ chemical shift would be a sensitive probe of histidines tautomeric forms (Sudmeier et al. 2003; Reynolds et al. 1973). Altogether, this suggest that a downfield shifted $C^{\delta 2}$ in histidines would not be a signature of zinc-coordination but rather a signature of the histidine tautomeric form.

Data mining and primary chemical shift analysis

In order to understand the exact correlation between zinc-coordination, tautomeric form and downfield shifted $C^{\delta 2}$ in histidines, we decided to perform a systematic survey of the chemical shifts of aromatic carbons in zinc-coordinated histidines. For this, we identified zinc-coordinated histidines in all BMRB entries and extracted their ^{13}C aromatic carbon chemical shifts, namely $C^{\delta 2}$ and $C^{\varepsilon 1}$, and retained pairs of chemical shifts for each histidines for which both $C^{\delta 2}$ and $C^{\varepsilon 1}$ were present. This resulted in a database of zinc-coordinated histidines containing a total of 266 entries (See Supplementary Table 1). We then divided the entries in two groups according to the histidine coordination mode reported in the corresponding BMRB entry. The large majority of the histidines (85 %) displayed a $N^{\varepsilon 2}$ coordination mode and the rest (15 %) a $N^{\delta 1}$ coordination mode. This preference for zinc coordination via $N^{\varepsilon 2}$ is in accordance with previous analysis of crystal structures of metalloproteins (Chakrabarti 1990). A two-dimensional landscape of $C^{\varepsilon 1}$ versus $C^{\delta 2}$ chemical shifts is shown on Fig. 2a with different symbols for the $N^{\delta 1}$ and $N^{\varepsilon 2}$ coordination modes. This shows a clear separation between the two coordination modes, the two groups differing predominantly in their $C^{\delta 2}$ chemical shift. However, few outliers seem to contradict the clear distinction between the $N^{\delta 1}$ and $N^{\varepsilon 2}$ coordination modes. For these five points (numbered 1–5 on Fig. 2a), we manually checked the consistence between the annotated coordination mode in the BMRB entry and the effective coordination mode reported in the corresponding structure deposited in the Protein Data Bank (PDB). Out of these five points, three were indeed wrongly annotated in the BMRB entry (point 1, 2 and 3 on Fig. 2a), and were then corrected in our database for the subsequent analysis. In addition, the structure corresponding to point 4

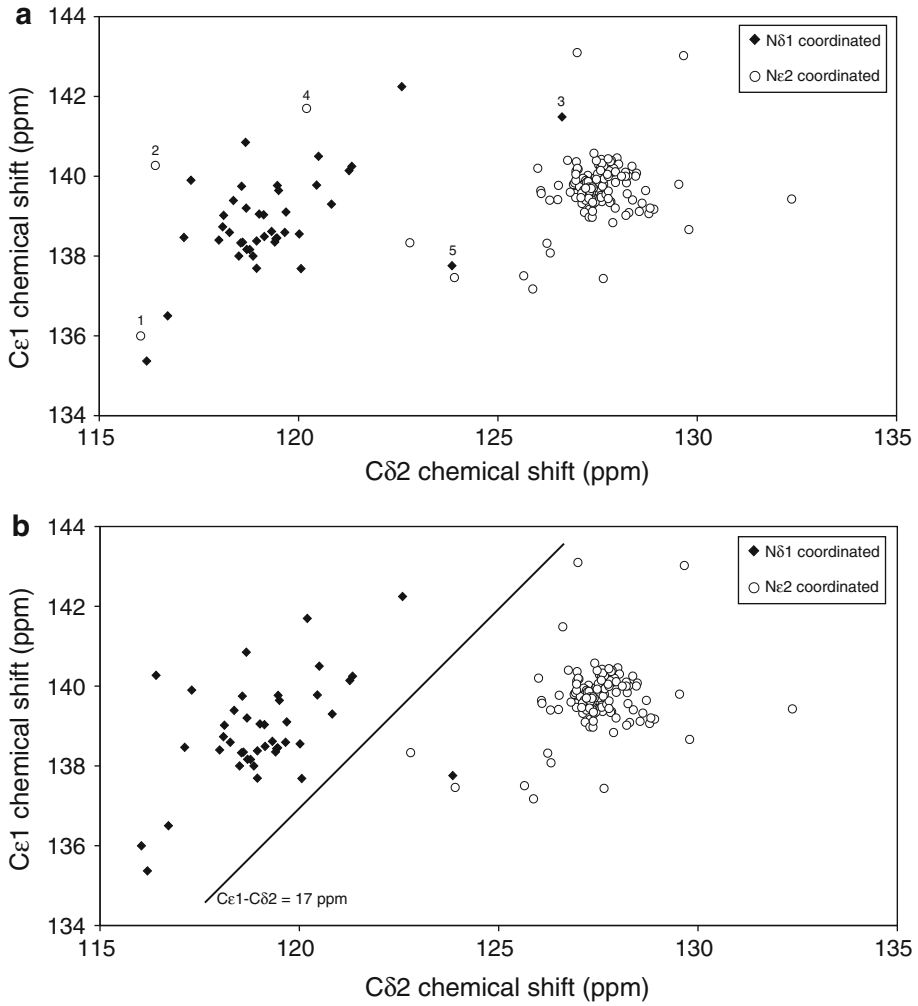

Fig. 2 Two dimensional plots of $^{13}C^{\delta 2}$ and $^{13}C^{\varepsilon 1}$ chemical shifts of zinc-coordinated histidines as a function of the coordination mode. **a** Initial database prior to any manual corrections. $N^{\delta 1}$ coordinated histidines are shown as *black diamonds* and $N^{\varepsilon 2}$ coordinated histidines as *open circles*. Numbered data points were manually checked for the consistency between the deposited structure and the annotated coordination mode in the chemical shifts database. In case of inconsistencies, these data points were corrected to give the final database represented on **b**. Five outliers corresponding to BMRB entries 6374, 11061, 10108, 6682 and 5668 are marked with numbers *1* to *5*, respectively. **b** Final corrected database showing a clear distinction between the two coordination modes of histidines. The *solid line* with its corresponding equation ($C^{\varepsilon 1}$–$C^{\delta 2}$ = 17 ppm) delineate the two clusters of the $N^{\delta 1}$ and the $N^{\varepsilon 2}$ coordination modes. The corresponding statistical values are listed in Table 1

(PDB code 1ZR9; BMRB entry 6682) was solved assuming a $N^{\varepsilon 2}$ coordination mode without any direct experimental evidence (Hayes et al. 2008) even if closely related proteins structures had shown unambiguously that zinc-coordination via this histidine was in fact occurring with its $N^{\delta 1}$ (Möller et al. 2005; Andreeva and Murzin 2008). We also corrected this entry in our database. Chemical shifts of the corrected database are presented on Fig. 2b. Finally, only a single point out of 266 (point 5 on Fig. 2a) was found further away from the average of the histidines with an $N^{\delta 1}$ coordination mode. It should be pointed out that the coordination mode of this histidine has been determined by measuring an optimized long-range ^{15}N-^{1}H HSQC (Kwon et al. 2003). This particular shift might therefore reflect either an incorrect chemical shift assignment or a very special electronic environment (two tryptophan residues are indeed present in the vicinity of this zinc-binding site; PDB code 1NKU). This point remained in the database that was used for statistical analysis.

Chemical shift of $C^{\delta 2}$ aromatic carbons is a sensitive probe of histidine coordination mode

Our statistical analysis revealed a clear separation between the histidine $C^{\delta 2}$ chemical shifts of the two coordination modes (Fig. 2b). Statistical values for the aromatic carbons chemical shifts $C^{\delta 2}$ and $C^{\varepsilon 1}$ of our entire zinc-coordinated

Table 1 Summary of the statistical values of the $^{13}C^{\delta 2}$ and $^{13}C^{\varepsilon 1}$ chemical shifts for 266 histidines coordinating zinc via $N^{\delta 1}$ or $N^{\varepsilon 2}$

	$N^{\delta 1}$ coordination		$N^{\varepsilon 2}$ coordination	
	$^{13}C^{\delta 2}$	$^{13}C^{\varepsilon 1}$	$^{13}C^{\delta 2}$	$^{13}C^{\varepsilon 1}$
Number of shifts	43	43	223	223
Average value $\overline{\delta}$ (ppm)	119.09	138.86	127.42	139.74
Min value δ_{min} (ppm)	116.04	135.37	122.79	137.18
Max value δ_{max} (ppm)	123.85	142.25	132.37	143.1
Standard deviation σ_δ (ppm)	1.54	1.30	0.75	0.59

histidine database were calculated and are given in Table 1. Whereas the $^{13}C^{\varepsilon 1}$ chemical shift is rather insensitive to the coordination mode, there is in average a significant chemical shift difference of about 8.3 ppm for the $^{13}C^{\delta 2}$ chemical shift between the $N^{\delta 1}$ coordination mode (average value $\overline{\delta} = 127.42$ ppm) and the $N^{\varepsilon 2}$ coordination mode (average value $\overline{\delta} = 119.09$ ppm). This chemical shift difference is not due to the zinc coordination *per se,* but reflects the nature of the tautomeric form of the histidine. To be precise, the $N^{\varepsilon 2}$ coordination mode is uniquely compatible with the $N^{\delta 1}$–H tautomeric form (Fig. 1) and as a consequence, the $^{13}C^{\delta 2}$ chemical shift has a characteristic value of about 127 ppm. Vice versa, the $N^{\delta 1}$ coordination mode is uniquely compatible with the $N^{\varepsilon 2}$–H tautomeric form and the $^{13}C^{\delta 2}$ chemical shift has then a typical value of about 119 ppm. This shows that $^{13}C^{\delta 2}$ chemical shift in zinc-coordinated histidines constitute an extremely potent probe for the determination of the coordination mode of histidines. However, the chemical shift is a relative measure that depends strongly on correct calibration to a

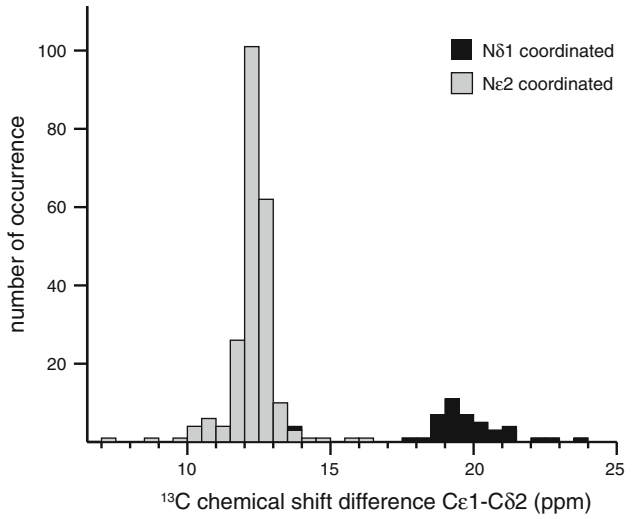

Fig. 3 Number of occurrence of each histidine–zinc coordination mode as a function of the chemical shift difference $\Delta_{\varepsilon\delta}$ ($\Delta_{\varepsilon\delta} = \delta\{^{13}C^{\varepsilon 1}\} - \delta\{^{13}C^{\delta 2}\}$), plotted in 0.5 ppm intervals. The statistical values are listed in Table 2

Table 2 Statistical value of the chemical shift difference $\Delta_{\varepsilon\delta}$ ($\Delta_{\varepsilon\delta} = \delta\{^{13}C^{\varepsilon 1}\} - \delta\{^{13}C^{\delta 2}\}$) as a function of the coordination mode of histidines

	$N^{\delta 1}$ coordination	$N^{\varepsilon 2}$ coordination
Number of shifts	43	223
Average value $\overline{\Delta}$ (ppm)	19.77	12.32
Median value $\tilde{\Delta}$ (ppm)	19.66	12.38
Standard deviation σ_Δ (ppm)	1.49	0.82

standard. Even if the standard procedure for calibrating chemical shifts of biomolecules is well documented (Wishart et al. 1995), correct calibration is not always properly conducted (Zhang et al. 2003). Incorrect chemical shift referencing could then potentially lead to a wrongly predicted coordination mode of histidines if the $^{13}C^{\delta 2}$ chemical shift were used exclusively. To circumvent this problem, we sought to establish a simple reference-independent parameter which will constitute a more robust probe for the determination of the coordination mode of histidines.

The chemical shift difference $C^{\varepsilon 1}$–$C^{\delta 2}$ is a reference independent marker of the coordination mode of histidines

As the $^{13}C^{\varepsilon 1}$ chemical shift is rather insensitive to the coordination mode of histidines (Fig. 2; Table 1), the chemical shift difference $C^{\varepsilon 1}$–$C^{\delta 2}$ retains all the information content of the $^{13}C^{\delta 2}$ chemical shift regarding the coordination mode of histidines, but in addition constitutes a reference independent marker of this coordination mode. The complete histogram showing the distribution of this chemical shift difference $\Delta_{\varepsilon\delta}$ is presented on Fig. 3. In addition, statistical values are given in Table 2. Remarkably, average and median values are very close to each other for both coordination modes showing that both distributions are rather symmetrical which is also visible on the histogram plot (Fig. 3). In case of an $N^{\varepsilon 2}$ coordination mode, $\Delta_{\varepsilon\delta}$ is distributed around an average value of 12.32 ± 0.82 ppm whereas for an $N^{\delta 1}$ coordination mode, $\Delta_{\varepsilon\delta}$ is found around 19.77 ± 1.49 ppm (Table 2). Altogether, we can formulate the following principles for the determination of the coordination mode of histidines. If the chemical shift difference between the $\varepsilon 1$ and the $\delta 2$ aromatic carbons is larger than 17 ppm, the corresponding histidine should coordinate zinc through an $N^{\delta 1}$ coordination mode whereas if $\Delta_{\varepsilon\delta}$ is less than 17 ppm it should coordinate zinc via an $N^{\varepsilon 2}$ coordination mode (Figs. 2b, 3). Remarkably, the two groups of histidines are clearly separated with almost no overlap between the $N^{\delta 1}$ and the $N^{\varepsilon 2}$ coordination modes. The present method would have

allowed the determination of the correct coordination mode in more than 99 % of the zinc-ligated histidines of the BMRB, and is therefore extremely potent. This shows that aromatic carbon chemical shifts in histidines are only slightly affected by the many different structural environments assumed to be present in our large database of zinc-ligated histidines. Precisely, the variations due to each local electronic environment remain smaller than the intrinsic chemical shift difference of the two different histidine tautomeric forms, which allow to predict the histidine coordination mode with an almost absolute confidence. Nevertheless, we want to mention that it is always possible that in case of a very special electronic environment, extremely shifted chemical shifts could be observed (see point 5 in Fig. 2a).

Discussion

We show in the present report that ^{13}C aromatic carbon chemical shifts can be used to distinguish between the $N^{\delta 1}$ and the $N^{\varepsilon 2}$ coordination mode of histidines in zinc-binding proteins. This provide an alternative method to the currently used approaches for the determination of the coordination mode of histidines. This method has several advantages that will be discussed below.

NMR structure calculations with unlabelled protein samples of small zinc-finger proteins, obtained via chemical synthesis is a competitive approach, as compared with recombinant protein production, regarding the size of these small zinc-binding domains (ranging from about 20 to 60 residues). This approach is therefore quite common in the NMR community working with small zinc-knuckles or zinc-fingers (see for example references (Sharpe et al. 2002, 2005; Isernia et al. 2003; Dempsey et al. 2004; Matsui et al. 2007; Cordier et al. 2008; Bourbigot et al. 2008; Briknarová et al. 2011; Bazzi et al. 2011; Malgieri et al. 2011)). In these cases, the measurement of long-range ^{15}N-^{1}H correlation spectra is simply not possible regarding the very low natural abundance of ^{15}N nuclei. Therefore, the determination of the coordination mode of histidines in these studies relied on either preliminary structure calculations that should then be very precise or on previously determined close homologous structures. However, structures determined without heteronuclear labelling are in general less precise which could therefore lead to incorrect evaluation of the coordination mode. The method presented in this report, which is based on the aromatic ^{13}C chemical shift, is perfectly applicable to unlabelled protein samples. The aromatic carbon assignment could easily be obtained with a natural abundance ^{13}C–^{1}H HSQC together with $H^{\delta 2}$–$H^{\varepsilon 1}$ correlations in homonuclear 2D COSY or TOCSY spectra (King and Wright 1982; Miura and Ichikawa 1991; Xia et al. 1995). The coordination mode can then be determined with a direct measurement and not with an indirect method making use of preliminary structure calculations. It also does not rely on previously published structures, therefore avoiding two approaches that could strongly bias the structure determination of new classes of zinc-binding domains.

Moreover, with the present method, the information on the histidine coordination mode is available directly after chemical shift assignment prior to any structure calculation. This could for example allow the early identification

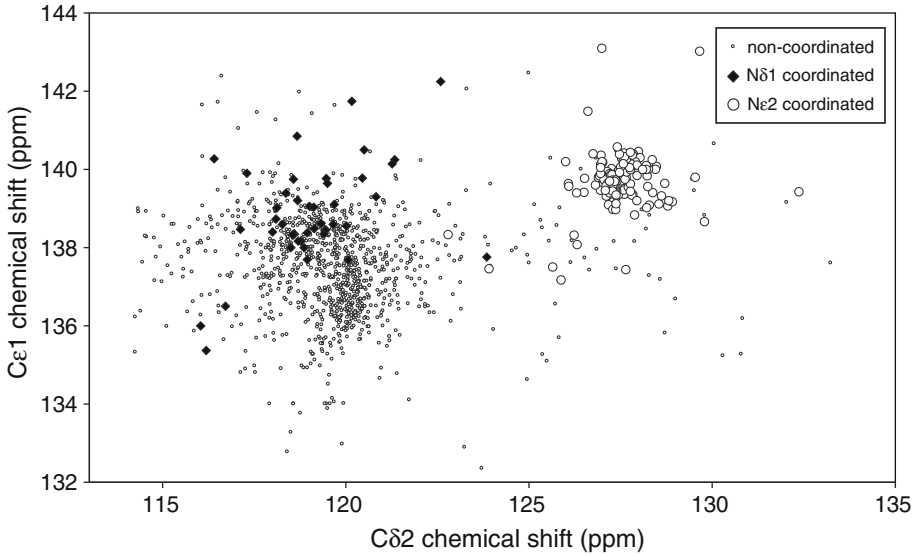

Fig. 4 Two dimensional plots of $^{13}C^{\delta 2}$ and $^{13}C^{\varepsilon 1}$ chemical shifts for non-coordinated histidines (*small open circles*), for $N^{\delta 1}$ coordinated histidines (*black diamonds*) and for $N^{\varepsilon 2}$ coordinated histidines (*large open circles*)

of an unusual zinc-binding site with an unconventional histidine coordination mode. Moreover, the discrimination revealed in this study could be used by structural and chemical shift databases as internal check for the determination of histidine coordination modes. If such a chemical shift based verification would be used for BMRB depositions, structures and chemical shifts with wrong coordination modes for zinc-ligated histidines could be prevented (see points 1–4 on Fig. 2a). Remarkably, in the database we constructed, there were very few points with a wrong annotation of their coordination mode, most of which were actually correctly determined in terms of structure but wrongly annotated in the database. Overall, this indicates the high quality of the zinc-binding protein structures determined by NMR. This point was definitely crucial to conduct such chemical shift analysis that relied on the correct determination of the histidine coordination mode in the BMRB entries used for analysis.

Histidine side-chains contain an aromatic ring that could in principle have a strong influence on the chemical shift of neighbouring nuclei. Particularly, zinc-sites with one, two or three histidines in their coordination sphere might have significantly different histidine aromatic carbon shifts due to mutual ring-current effects. We analyzed this potential effect on $C^{\delta 2}$ and $C^{\varepsilon 1}$ chemical shifts but no clear correlations between the number of histidines and these carbon chemical shifts appeared in our analysis, indicating that this effect is too small to be significant.

Finally, one should emphasize once more that the particular downfield shifted $C^{\delta 2}$ is not due to the zinc coordination per se, but reflects the nature of the tautomeric form of the histidine. The observation of a particularly downfield shifted $C^{\delta 2}$ is not a signature of the presence of a zinc-ligated histidine. However, the $N^{\delta 1}$–H tautomeric form of neutral histidine is less common and is present only in particular cases where this tautomeric form is stabilized (Pelton et al. 1993; Day et al. 2003; Sudmeier et al. 2003; Schubert et al. 2007). To illustrate the relative abundance of the $N^{\delta 1}$–H tautomeric form compared to the $N^{\varepsilon 2}$–H tautomeric form, we constructed another chemical shift database by extracting aromatic chemical shifts of non-coordinated histidines from the Reference Corrected Database (RefDB) (Zhang et al. 2003). This resulted in about 1000 histidines with both $C^{\varepsilon 1}$ and $C^{\delta 2}$ chemical shifts. These data are displayed on Fig. 4 and compared with the ^{13}C aromatic chemical shifts of the zinc-coordinated histidines. Most histidines that are not coordinated to zinc have their $C^{\delta 2}$ chemical shift around 120 ppm and only a small proportion (about 5 %) display a downfield shifted $C^{\delta 2}$ chemical shift of more than 124 ppm. Importantly, $C^{\delta 2}$ chemical shifts of protonated histidines are also found around 120 ppm, similar to the $N^{\varepsilon 2}$–H tautomer (Sudmeier et al. 2003). Therefore, whereas a downfield shifted $C^{\delta 2}$ is a true signature of the $N^{\delta 1}$–H tautomeric form, ^{13}C aromatic chemical shifts do not allow to distinguish the $N^{\varepsilon 2}$–H tautomer from the protonated form of the histidine ring (Sudmeier et al. 2003).

Moreover, ^{13}C aromatic chemical shifts cannot distinguish between coordinated and non-coordinated histidines (Fig. 4) and it is therefore important to mention that the present method allows to determine the histidine coordination mode, but the knowledge on which histidine within a protein sequence is coordinated to zinc must come from another source of information, e.g. preliminary structure calculations. Nonetheless, this information is also not provided by the other approaches aiming at determining the coordination mode of histidines, and must in any case be deduced from other data.

Remarkably, the $N^{\varepsilon 2}$ coordination mode which corresponds to the chemical shift signature of the $N^{\delta 1}$–H tautomeric form is very common in zinc-coordinated histidines (Fig. 3). As a consequence, most histidines with a downfield shifted $C^{\delta 2}$ chemical shift are actually zinc-coordinated histidines and this can therefore explain the confusion in the literature about the origin of downfield shifted $C^{\delta 2}$ chemical shift. The present report with a comprehensive survey of the ^{13}C aromatic chemical shifts of histidines should definitely help clarify the relations between zinc-coordination, tautomeric form and downfield shifted $C^{\delta 2}$ in histidines.

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Conflict of interest The authors declare no conflict of interest.

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