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Redox-Related Chemical Shift Perturbations on Backbone Nuclei of High-Potential Iron–Sulfur Proteins

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Electron-transfer proteins such as cytochromes[1−5] and, to a smaller extent, blue copper proteins[6,7] as well as redox enzymes such as Fe superoxide dismutase[8] have shown differential effects in chemical shifts for backbone nuclei for the two redox states that could not be attributed to paramagnetic effects and were termed "redox shifts".[9] These redox shifts, not yet fully understood, might have implications in the electron-transfer process. Similar effects have been reported for Fe^{n+}(Cys4) rubredoxins,[10] as well as for ferredoxins[11] and putidaredoxins,[12,13] which contain an $[Fe_2S_2]^{n+}$ cluster, whereas, to our knowledge, no such effects have been reported so far for any protein of the $[Fe_4S_4]^{n+}$ family. We report here the observation of redox shifts for the two forms of *Chromatium vinosum* HiPIP, an iron−sulfur electron-transfer protein containing an $[Fe_4S_4]^{n+}$ cluster coordinated by four cysteine residues.[14−16] The presently reported data, which were collected not only for backbone amide groups (^1H and ^{15}N) but also for $^{13}C\alpha$ and $^{13}C'$ resonances, will provide another piece of information in the current debate on redox shifts and will provide some further insights toward the understanding of such effects.

The NMR signals of the ^{15}N, ^1H_N, $^{13}C_\alpha$, and $^{13}C'$ backbone nuclei of reduced and oxidized forms of *C. vinosum* HiPIP, prepared as previously reported,[17] have been assigned through $^{15}N-^1H$ Heteronuclear Single Quantum Coherence (HSQC),[18] 2D and 3D HNCA, 2D and 3D HNCO experiments.[19] All experiments have been performed at 298 K and at 600 MHz proton frequency using either normal or tailored experiments previously described.[20,21] HiPIP samples were from the same batch under the same pH and buffer conditions (pH 5.7, 50 mM phosphate buffer) and differed only for the addition of a 5 mM excess of potassium ferricyanide in the oxidized case or of sodium dithionite in the reduced case. The differential chemical shift data are summarized in graphical form in Figure 1A−D, and collected as Supporting Information in Table S1.

It is immediately apparent from Figure 1 that in some regions of the sequence sizable chemical shift differences are observed for all four backbone nuclei. These regions encompass the cluster binding residues (Figure 2), two of the shorter loops formed by the coordinated cysteines (43−46 and 63−77) as well as the stretch of sequence 14−20,

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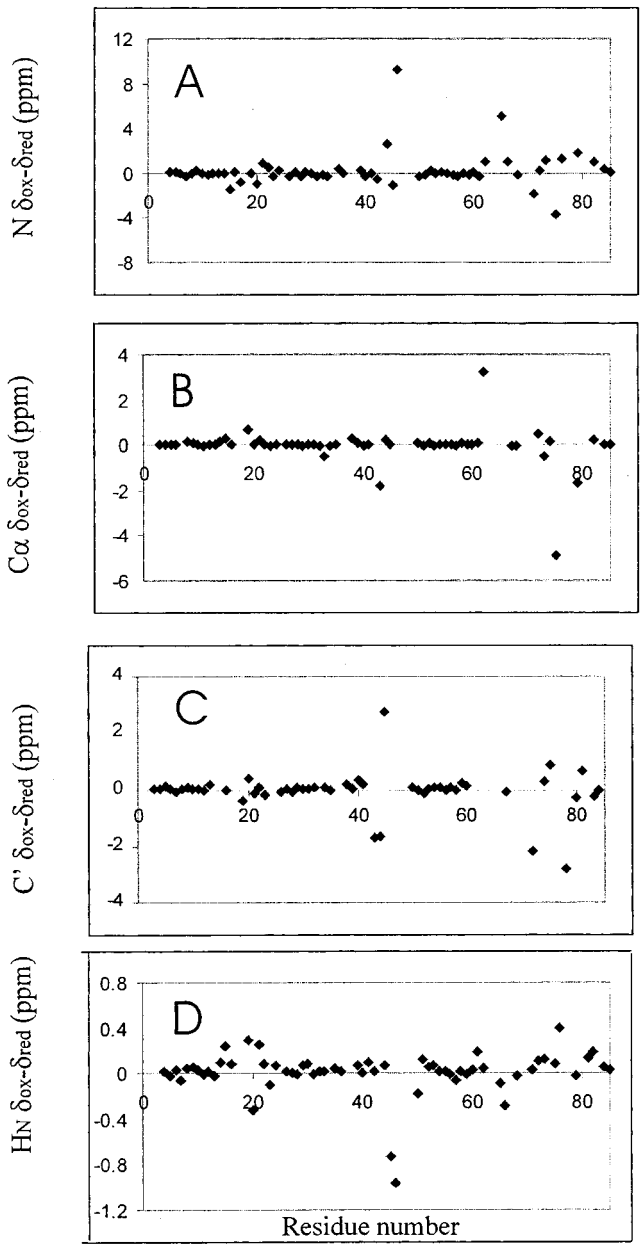

Figure 1. Chemical shift differences between the oxidized and reduced forms of *Chromatium vinosum* HiPIP at pH 5.7 measured at 600 MHz and 298 K for the backbone nitrogen (A), α-carbon (B), carbonyl carbon (C), and NH proton (D).

which is also close to the iron−sulfur cluster.[22] Therefore, paramagnetic effects can be responsible for these differential shifts. These have to be recognized before attempting the detection of the redox shifts on this system.

Paramagnetic effects on nuclear shifts are contact or pseudocontact in origin.[23] The former effect arises from unpaired electron spin density that is transferred from the metal centers to s-type orbitals of the nuclei usually through covalent bonds. The latter is a through-space effect that is sensed by nuclei in isotropic solutions only in the presence

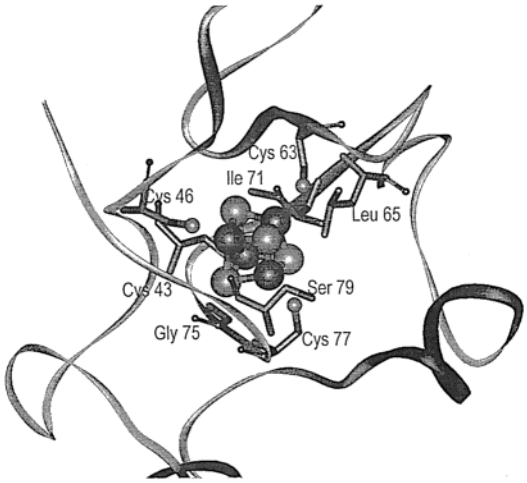

Figure 2. Schematic structure of *Chromatium vinosum* HiPIP[22] showing the protein regions that may experience contact shift contributions through either covalent bonds or orbital overlap with cluster donor atoms.

of anisotropy of the magnetic susceptibility tensor. Quantitation of these effects in systems containing iron−sulfur clusters is not easy.[24] The oxidized state of the protein contains an $[Fe_4S_4]^{3+}$ cluster that has an average magnetic moment corresponding to one unpaired electron per iron ion,[25] and a modest magnetic anisotropy.[26−31] On the other hand, the reduced state contains an $[Fe_4S_4]^{2+}$ cluster that has a diamagnetic ground state and only experiences a residual paramagnetism due to population of paramagnetic excited states.[32,33] The residual magnetic moment of the reduced state corresponds to less than half of that of the oxidized protein.[34] This state has virtually no magnetic anisotropy. Therefore, the largest paramagnetic effects are expected to arise from short-range contact interactions.

As a matter of fact, sizable contact shifts have been already reported for the cysteine ligand nuclei in both oxidation states of HiPIPs and successfully accounted[35] for in the case of HiPIP I from *E. halophila*.[36] The contact shifts are, on average, sizably larger for the oxidized than for the reduced protein,[20] as expected from the larger magnetic moment of the former state.

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The differential shifts of the backbone nuclei of the four coordinated cysteines are easily accounted for on the grounds of the previous analysis:[36] Cys43 Cα and C′ carbon atoms show negative shifts, while Cys46 N shows a positive differential shift. This is the expected pattern for the coordinated cysteines in oxidized HiPIPs.[37] Contact shift contributions are likely to be present also on the backbone nuclei of the preceding and following residues. In addition, there are a few residues that, although farther in sequence from the coordinating cysteines, are so close to one or more cluster atoms that some kind of orbital overlap cannot be ruled out (Figure 2). This is the case of the N nuclei of Leu65 and Ser79, which are involved in hydrogen bonds to the sulfur atoms of Cys63 and Cys77. Direct spin transfer through hydrogen bonds of this type has been described earlier for other iron−sulfur proteins.[38] The strong differential shift of Asn72 carbonyl carbon is easily accounted for by the very close proximity of the carbonyl oxygen to the sulfur of Cys43. The differential shift of N71 can be explained by multiple van der Waals contacts between its side chain and the sulfur atom of Cys43, the iron ion to which it is coordinated, and two of the three sulfide atoms that complete its coordination polyhedron (Figure 2).[22] Likewise, the shifts of N75 and Cα75 are explained by a close van der Waals interaction with the sulfur of Cys43.

As contact shifts attenuate rapidly with the number of covalent bonds intervening between the metal ion and the nucleus under consideration, no sizable effects should be expected a few bonds away from the cysteine sulfur donors, except for the few orbital overlaps described previously. Apparently, however, several more backbone nuclei than expected experience differential shifts, which are hardly ascribed to orbital overlap of any kind. Figure 3 shows the differential shifts for the four backbone nuclei as a function of the average distance from the cluster ions, after exclusion of residues 42−47, 62−64, and 76−78, as well as residues 65, 71, 72, 75, and 79. A possible contribution from pseudocontact shifts is thus considered. As anticipated, oxidized HiPIPs have an $S = {}^1/_2$ ground state possessing a modest *g*-anisotropy (around 6%). Therefore, the pseudocontact shifts are expected to be already below 1 ppm at 6 Å from the paramagnetic center. Population of the excited states may introduce further contributions, which, however, are not expected to be much different.[39] In any case, two additional observations rule out pseudocontact contributions as relevant for the observed intermediate and long range shifts: (1) Nuclei very close to one another and relatively far from the cluster should have similar pseudocontact shifts, whereas often even sign inversions are observed between, for instance, the nitrogen and its proton, or the nitrogen and the carbonyl carbon of the same peptide bond. (2) As it is apparent from Figure 3, of the four backbone nuclei, only the nitrogen atoms show marked effects, while carbons and NH protons do not; pseudocontact shifts should not depend on the nuclear species and should be, on average, the same for NH protons and α and carbonyl carbons. We can thus conclude that redox shifts not attributable to contact or pseudocontact shifts are operative in HiPIPs, as they are in other electron-transfer proteins. Therefore, either more subtle and as yet unknown paramagnetic effects, or electrostatic

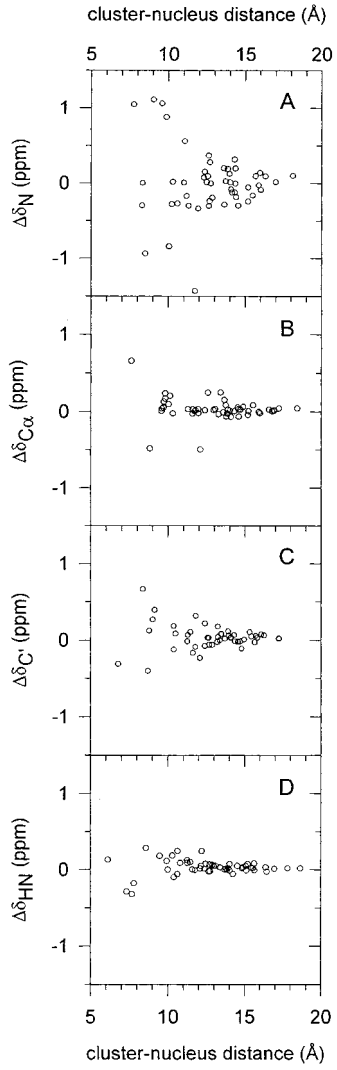

Figure 3. Plots of the chemical shift differences observed for the backbone nitrogen (A), α-carbon (B), carbonyl carbon (C), and NH proton (D) as a function of their average distance from the cluster iron ions. Residues 42−47, 62−64, 75−77, 65, 71, 75, and 79 are not included in the plots.

(36) Positive spin density on an iron ion is transferred with the same sign onto the σ and π orbitals of the coordinated cysteine γ-sulfur and onto the cysteine β-carbon. The latter has been shown to experience very large downfield contact shifts. With an increasing number of covalent bonds from the β-carbon, the σ contribution should attenuate and alternate in sign. It is thus expected to be negative on the α-carbon and the β-protons. However, a positive π-contribution is also present on the α-carbon and β-protons, arising from direct overlap with a sulfur p-orbital orthogonal to the Fe−S−Cβ plane. Such overlap is maximal when the Fe−S−Cβ-nucleus dihedral angle is 90° and minimal for 0° or 180°.[43]

(37) In the oxidized form of HiPIPs, the sign of the spin density pattern on the various metal ions is not uniform.[17,44−49] While that on the metals coordinated to cysteine 63 and 77 is positive as in the reduced form (and much larger), that on the metal coordinated to Cys43 is negative, and that on the metal coordinated to Cys46 is intermediate and, therefore, positive but smaller.[17]

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effects caused by the change in charge of the redox center, must be operative.

The fact that ^{15}N nuclei experience much larger long-range effects than ^{1}H and ^{13}C nuclei is an important feature of the present system that may help in understanding the origin of redox shifts. Among various hypotheses put forward to explain redox shifts, small but long-range conformational changes due to electrostatic effects have been proposed.[8,40] If redox shifts are due to changes in conformation, they should be roughly proportional to the spreading in chemical shifts observed for each type of residue and, within each residue, for each type of nuclei observed for proteins in general.[41] To check this hypothesis, the present redox shift data have been normalized by dividing them by the standard deviation for the corresponding nucleus available in protein NMR databases.[42] The redox shifts normalized in this way are shown in Figure 4. Surprisingly, the scattering in differential shifts at large distances from the cluster is now more similar for all nuclei, being only somewhat less pronounced for α-carbons. On the other hand, attenuation with distance is even more apparent, confirming that these differential shifts are indeed redox-related and not distributed randomly. This also suggests that, consistently with previous studies,[2] H-bonds are not the main factors determining the redox shifts. This behavior does support small conformational changes as possible determinants of redox shifts.[8] At the same time, the somewhat smaller values for α-carbons than for the other backbone nuclei are also consistent with a contribution to redox shifts from electron polarization effects, which are expected to be larger for the CONH moiety than for α-carbons.[2] Polarization effects are also electrostatic in origin, and therefore, they are expected to attenuate with $1/r$ (or at most with $1/r^2$ if the microscopic dielectric of the protein is considered) at variance with the $1/r^3$ dependence of pseudocontact shift effects. Both polarization effects and conformational changes may have important implications for the preferred electron transfer pathways in redox proteins. The former are virtually instantaneous and, therefore, compatible with even the fastest elementary electron transfer step. The latter may occur over a wide range of time scales, from very slow, and therefore irrelevant for electron transfer, to very fast, that is, of the order of picoseconds, as amply demonstrated by molecular dynamics calculations.

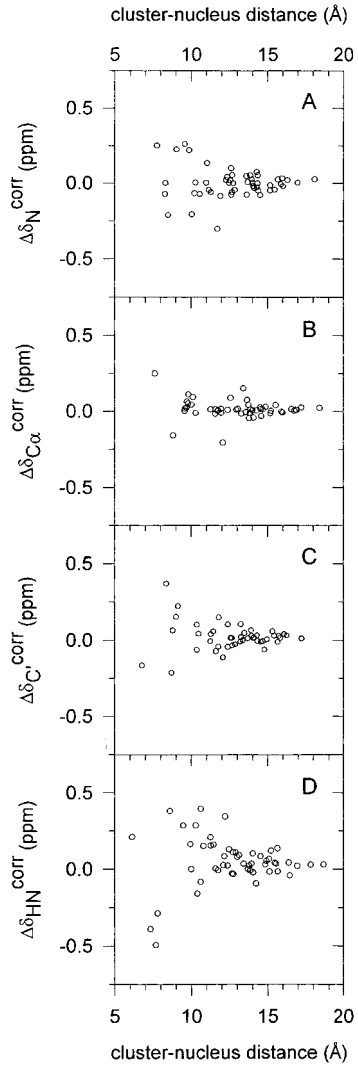

Figure 4. Plots of the normalized chemical shift observed for the backbone nitrogen (A), α-carbon (B), carbonyl carbon (C), and NH proton (D) as a function of their average distance from the cluster iron ions. The same residues as Figure 3 are shown. The normalized shifts are obtained by dividing each value by the standard deviation for the corresponding nucleus in the appropriate amino acid as reported in protein NMR shift databases.[42]

In conclusion, redox shifts seem to be a very general phenomenon whose understanding is certainly worth further investigations. The present results allow us to extend the presently available collection of redox shifts to the class of $[Fe_4S_4]$ proteins. They also provide further hints that should be considered when discussing redox shifts in other systems: (a) Hyperfine contact shifts are not only affecting the metal-coordinated residues and those residues involved in H-bonds with sulfur atoms, but also those residues that are in van der Waals contact with atoms from the cluster or from coordinating groups. (b) The pattern of "weighted" redox shifts reported in Figure 4 seems to agree with previous observation ruling out changes in H-bonds as determinants of redox shifts. Conversely, H-bonds are extremely important in the understanding of chemical shift differences in the immediate proximity of the prosthetic group, because of effects due to hyperfine coupling. (c) Although the present data seem to indicate that polarization effects are involved

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in redox shifts, also small conformational changes cannot be ruled out. In this respect, it is important to point out that, once all contributions arising from paramagnetic effects are properly recognized, there are still several observed shifts that certainly arise from the change in the oxidation state, and therefore, they should be considered as redox shifts, independently of whether the origin is conformational or electrostatic.

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Supporting Information Available: Chemical shift values observed in reduced and oxidized HiPIPs from *Ch. vinosum*, at 298 K, pH 5.7. This material is available free of charge via the Internet at http://pubs.acs.org.

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