Oxidation State-Induced Change of Iron Ligand in the Phenylalanine-82 to Histidine Mutant of Yeast Iso-1-cytochrome *c*

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A yeast iso-1-cytochrome *c* mutant in which His substitutes for Phe at position 82 shows a unique change of iron ligand triggered by a change in the oxidation state of the metal: the His-82 imidazole is the ferric iron ligand, but upon reduction, the Met-80 side chain recovers the liganding role it has in the wild-type protein.

In mitochondrial cytochromes *c*, the iron is ligated by the His-18 imidazole and the Met-80 sulfur at the fifth and sixth coordination positions, commonly defined as the right and left sides of the heme, respectively.[1] A remarkable characteristic of these cytochromes is that the methionine has a markedly larger affinity for the Fe^{2+} than for the Fe^{3+} state of the metal.[2] This difference in stabilities is reflected in the facts that the ferric iron ligation by Met-80 is distinctly more sensitive to pH changes than that of the ferrous iron, and that, while CO does not bind ferrocytochrome *c* at neutral pH, CN^- and other complexes of the ferric iron are readily obtained.[3]

Certain changes in the primary structure of cytochrome *c* are known to result in substitutions of the sixth ligand in the ferric state. Thus, when cytochrome *c* is chemically modified at the lysyl side chains in positions 72, 73, or 79 by adding a thiopropionyl group at the ϵ-amino nitrogen,[4] the thiol sulfurs of the added groups become the Fe^{3+} iron ligands. However, the intrinsic affinity of the thioether sulfur of Met-80 for the ferrous iron is so large that the sulfur returns to the sixth ligand position when the metal is reduced.[5]

Here we describe a further example of this unique chemical property of cytochrome *c*, this time presented by a recently reported site-directed mutant of yeast iso-1-cytochrome *c*, in which Phe-82 was replaced by His.[6] This mutation was performed[7] on an already mutated gene, in which the Cys-102 codon was replaced by a Ser codon to avoid disulfide dimerization of the mutant protein. The resulting cytochrome *c*, SCc-C102S/F82H, lacks the 695 nm band that is characteristic of the Met-S-Fe^{3+} ligation in the ferric state.[2] This is in keeping with the assignment of His-82 as the sixth ligand in this mutant,[6] since imidazole binding to ferric cytochrome *c* eliminates the 695 nm band.[8] A more rigorous assignment of imidazole as the sixth ligand was provided earlier by Hawkins *et al*.,[6] based on an analysis of the magnetic circular dichroism and EPR spectrum of the ferric state.

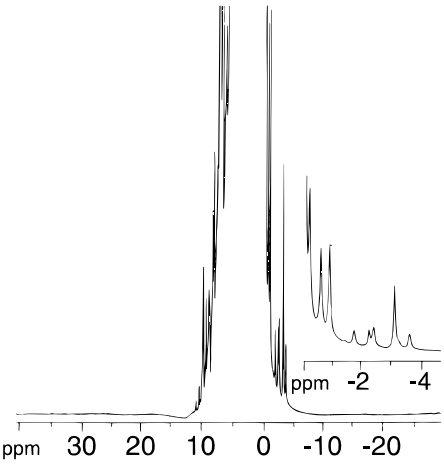

Figure 1. ^{1}H-NMR spectrum (500 MHz, 30 °C, pH 7.0) of the yeast cytochrome *c* mutant SCc-C102S/F82H in the reduced state. Chemical shifts are given in ppm from TSP. The inset shows in detail the 0 to −4 ppm region, where the displaced Met-80 shifted peaks are located.

The ligand substitution does not affect in a major way the global stability of the ferric protein, as judged by fluorometric titrations of its unfolding in urea. When analyzed in terms of the Tanford−Schellman thermodynamic model of protein denaturation,[9] the free energy of unfolding extrapolated to zero concentration of urea is 1.8 kcal/mol, as compared to the value of 1.5 kcal mol measured for SCc-C102S; the small increase does not significantly deviate from the error expected in this type of titration. The most significant effect that the F82H mutation causes on ferric cytochrome *c* is a marked decrease in the rate of reduction by ascorbate, which is 2.1×10^2 M^{-1} s^{-1}, compared to 1.6×10^3 M^{-1} s^{-1} for SCc-C102S (at 22 °C and pH 7). This change cannot be attributed solely to the substitution of the iron ligand, since exogenous imidazole affects the kinetics of reduction of ferricytochrome *c* by ascorbate only at concentrations beyond those necessary to displace the native ligand from metal coordination.[8]

It is remarkable that, in spite of the structural and chemical reactivity changes of the ferric cytochrome *c* crevice brought about by the F82H mutation, the physiological role of cytochrome *c* appears to remain unaffected, as shown by the fact that the transfected yeast grows and synthesizes the protein normally.

The iron ligation changes drastically when the protein is reduced. Prolonged sparging of the SCc-C102S/F82H solution with CO has no effect on the spectrum, indicating that ligation of the iron is as strong here as in the wild-type cytochromes *c* and prevents complex formation with CO. This suggests that the reduction of F82H results in a change of the sixth ligand, with Met-80 returning to its iron liganding role in the ferrous state.

This possibility was tested by recording the ^{1}H-NMR spectrum of ferrous SCc-C102S/F82H. Since the latter is readily oxidized by exposure to air,[10] after reduction with dithionite in a strictly anaerobic atmosphere, the protein was kept in a sealed

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(10) The rate of autoxidation increases from 1.3×10^{-2} h^{-1} for SCc-C102S to 3.7×10^{-1} h^{-1} for SCc-C102S/F82H (22 °C, pH 7.0).

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Table 1. Met-80 Proton Chemical Shifts (ppm from TSP)

	Met-80 protons				
compound	ϵ	γ	γ'	β	β'
SCc-C102S/F82H	−3.13	−3.61	−1.77	−2.44	−0.17[a]
wild-type SCc[11a]	−3.13	−3.66	−1.74	−2.39	−0.14
SCc-C102T[11b]	−3.14	−3.73	−1.76	−2.43	−0.22

[a] Tentative assignment.

NMR tube during all the operations required for measuring its ^{1}H-NMR spectrum, resulting in the complete absence of the oxidized form peaks (Figure 1). Under these conditions, the NMR spectrum indeed shows the unmistakable peaks of the methionine side-chain protons (Figure 1), with the appropriate intensities and with chemical shifts that are nearly identical to those assigned to these protons at similar pH and temperature for the wild-type protein and a SCc-C102T mutant[11] (Table 1). Our spectrum revealed an additional proton peak at −2.29 ppm, which we did not assign and which is not seen in the spectrum of the wild-type cytochrome *c*.

These observations indicate that, in SCc-C102S/F82H, the nature of the sixth iron ligand is dictated by the oxidation state of the metal. This property of the mutant protein is another consequence of the very specific strong affinity that exists between the cytochrome *c* ferrous iron and the sulfur of the Met-80 side chain, possibly because of the effects of back-donation of 3d iron electrons to the empty 3d orbitals of the sulfur.[2] The identical positions of the Met-80 proton peaks indicate that, in the mutant and wild-type proteins, the stereochemical orientations of the ligand side chain are identical,[12] thus allowing for the same maximal overlap between the d orbitals of the metal and the ligand atom. It is important to stress, however, that even if the ligation by Met-80 is structurally the same as in the wild type, the environment of the heme must have been disrupted also in the ferrous state, as implied by the large increase in the rate of autoxidation. One possible explanation of the relatively rapid oxidation by oxygen is that the presence of an imidazole group increases the polarity on the left side of the heme, lowering the redox potential of the mutant cytochrome *c*; this is currently under study, together with the question of the rates of interconversion between the two ligation states available to each oxidation state of the protein.

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