STRUCTURAL BIOLOGY OF METALLOPROTEINS:
CLUES FROM THE BIOLOGY OF METAL
COFACTORS IN PROTEINS

Mario Piccioli
Magnetic Resonance Center
CERM
University of Florence, Italy
European projects where CERM is involved

**Bio-NMR** - NMR for Structural Biology I3- Integrated Infrastructure Initiative - FP7 – n. 261863 - www.bio-nmr.net - **COORDINATED by CERM**

**BioMedBridges** - Building data bridges between biological and medical infrastructures in Europe - FP7- n. 284209

**Cosmos** - Developing an efficient e-infrastructure, standards and data-flow for metabolomics and its interface to biomedical and life science e-infrastructures in Europe and world-wide - FP7 - n. 312941

**Chance** - Low cost technologies and traditional ingredients for the production of affordable, nutritionally correct foods improving health in population groups at risk of poverty - FP7 - n. 266331- www.chancefood.eu

**IDPbyNMR** - High resolution tools to understand the functional role of protein intrinsic disorder

Network for Initial Training (ITN) - Marie Curie Action – FP7 - n. 264257 – www.idpbynmr.eu – **COORDINATED by CERM**

**EUROHyperrPOL** - MPNS COST Action TD1103 - European Network for Hyperpolarization Physics and Methodology in NMR and MRI

**pNMR** - Pushing the Envelope of Nuclear Magnetic Resonance Spectroscopy for Paramagnetic Systems. A Combined Experimental and Theoretical Approach Network for Initial Training (ITN) - Marie Curie Action - n. 317127

A complete list of projects is available at CERM website: www.cerm.unifi.it
An integrated cellular structural biology approach

- **CERM has developed a unique strategy for in-cell NMR**, based on protein expression and labelling in human cells.

- **Cutting-edge technologies are required:**
  - high magnetic field (Bruker 950 MHz)
  - latest generation of electronics
  - high expression and labelling efficiency

- **Challenging questions in cellular biology can be answered:** protein maturation; post translational modifications; chaperone interactions; redox dependent folding...

- **The technique is being integrated with other cellular techniques:**
  E.g. optical microscopy, combined with advanced techniques such as synchrotron X-ray fluorescence microscopy:

  Banci et al. Chem Biol. 2013
Integration of structural techniques
Solution and solid state approaches for high molecular weight assemblies

The ferritin nanocage: a 480 kDa 24-mer

**Solution NMR:** $^{13}\text{C} - ^{13}\text{C}$ NOESY allows to monitor the pathway of ferric products within the cage (PNAS 2010, Acc. Chem. Res. 2013)

**X-ray:** the mechanism of the catalytic oxidation of iron(II) at atomic resolution. (JACS 2012)

**Solid-state NMR:** sequence specific assignment of the cage residues (PNAS 2010, Acc. Chem. Res. 2013)
Individual metabolic phenotypes (metabotypes) 


KODAMA 
A new statistical method for knowledge discovery and clustering

HR 3.30 Metabolomics for survival prediction in Colorectal Cancer patients


Bertini, Cacciatore, Jensen, Schou, Johansen, Kruhøffer, Luchinat, Nielsen, Turano P., *Cancer Res*. 2012, 72, 356-64
By knowing the structural properties of the antigens and of the epitopes in all the variants, a chimera antigen was produced which elicits complete protective immunity.

Patent WO 2011051893 A1

Outline

Clues from Metal Cofactors in Proteins

HasA: Transient Metal sites in Proteins

Human Cyt c: Redox dependent properties: conformational dynamics

CIAPIN-1: Challenging Paramagnetic centers in (unstable and unfolded) proteins

Grx5/Isca1/Isca2: FeS Cluster maturation and protein-protein interactions
**Links**

**Biology:** Heme trafficking, signaling, 
Bacteriology: bacterial adaptation to available iron sources, understanding of bacterial transmembrane signaling

**Institute Pasteur, Paris; CNRS Marseille, Department of Chemistry North Dakota**

**Medicine:** caspase activation, apoptosis;  
Biosensors/Bioelectronics: biosensors for superoxide accumulation and antioxidant activity; bioelectronic devices

**The Beckmann Insitute, CalTech; Technical University Wildau**

**Medicine:** Apoptosis, caspase, neurodegeneration;

**Medicine:** Translational medicine and neurogenetics, Friedreich’s Ataxia, myopathy and encephalopathy,  
Cell Biology: iron-sulfur protein biogenesis and human disease  
Industries: Antimicrobial activity and industrial applications (Zinc pyrithione Antidandruf)

**FeS-Lectures, Achievements, Networking, Dissemination:** from cellular biology to molecular aspects of iron sulphur protein biogenesis
The problem with metal ions

- The information obtained by NMR are proton-proton distances or dihedral angles involving NMR active nuclei.
- No information is available to locate the metal ion in the protein frame.
The problem with paramagnetic metal ions

- Unpaired electrons:
  - Broaden the NMR lines
  - Cause shifts
  - Disturb communications between nuclei

Internuclear distances and scalar couplings may be lost within a certain sphere from the metal ion.
First solution structure of a paramagnetic protein: *Reduced HiPIP I from E. halophila*

**Cofactor**  
$[\text{Fe}_4\text{S}_4]^{2+}$  
(shortest $T_{1\text{min}} \approx 2\text{ms}$)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td><strong>AA</strong></td>
<td>73</td>
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<tr>
<td><strong>1D NOE</strong></td>
<td>40</td>
</tr>
<tr>
<td><strong>2D NOESY</strong></td>
<td>1233 (945)</td>
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</table>

**Fe-H dipolar constraints**  
58

**RMSD (Å)**  
- **BB** - $0.45 \pm 0.09$  
- **HA** - $1.09 \pm 0.17$

**Metal cluster**  
26

**Dihedral angle constraints**  
- $\phi$  
  - 45 ($^3\text{JHNH}_\alpha$, $^3\text{JHNC}'$)  
- $\chi^1$  
  - 26 ($^3\text{J}_\alpha\text{NH}_\beta$, $^3\text{JH}_\beta\text{N}$)  
**Fe-S-C-H**  
4 links

Turning limitations into advantages

**Contact shifts** may provide dihedral angle constraints

**Pseudocontact shifts** provide the coordinates of the metal ion and new structural constraints

**Nuclear relaxation** provides metal-nucleus distances

**Self orientation** provides new structural constraints to improve quality of structures

**Cross correlations** provide information of M-H-N distances and angles

*By tuning the experiments the range of systems investigated broadens*

Bertini, Luchinat, Piccioli, Methods Enzymol, 2001
HasA: Transient Metal sites in Proteins

Molecular Microbiology, 2012

HasA is secreted across the membrane

**Outer membrane**

**Cell membrane**

The Has system

extracellular

Allows heme entry and apoHasA release

Takes heme and delivers to the membrane receptor

How basic inorganic chemistry contributes to heme trafficking?
$^{13}$C NMR to investigate the Iron(III) Hemophore HasA

Shuttling the heme from a hemoprotein to a specific membrane receptor

19 kDa protein, 178 aa

Institute Pasteur, Paris
Muriel Delepierre, Anne Lecroisey, Celia Caillet-Saguy

Breaking or weakening of Y75- Fe(III) yields to High Spin Fe(III)

High spin increases at increasing temperatures
$^{13}$C as a Probe to Assess Spin State and Coordination Properties

Detachment of Y75 shifts the spin equilibrium observed in WT towards $S=5/2$

Increasing the average Hyperfine shift accounts for a pure high spin $S=5/2$ spin

Spin State is substantially different from other mutants or WT
Heme Coordination and Electronic State


Mapping the interaction between hemophore HasA and Outer Membrane Receptor HasR

HasA: 19 kDa protein
HasR: 95 kDa Protein

HasR can be solubilized in DPC Micelles

Delepierre, Lecroisey, Caillet-Saguy Institute Pasteur, Paris
CRINEPT TROSY
Spectral Profiling. A Statistical Analysis

The HasA-HasR Complex

Three different conformations for HasA:
- ApoHasA
- HoloHasA
- Complexed HasA

Complexed HasA is much more similar to ApoHasA than to HoloHasA.

The interaction surface between HasA and HasR is independent of the presence of the Heme.

Upon Complex formation, Heme group is transferred from HoloHasA to HAsR.

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CIAPIN-1: Challenging Paramagnetic centers in (unstable and unfolded) proteins

Grx5/Isca1/Isca2: FeS Cluster maturation and protein-protein interactions

In proteins, motions are as important as structure

**Structural Dynamics**

In metalloproteins, metal oxidation state is important

**Electron transfer, Catalysis**
Structural Dynamics via CSM/CSM modulations

- Global reorientation
- Water exchange
- Conformational exchanges
- Motions of sidechains
- Fast vibrations
- Folding/unfolding
- MD simulations
- Heteronuclear NOE, $T_1$ and $T_2$ relaxation
- CPMG and $R_{1p}$ experiments
- NH $\rightarrow$ ND
- Exchange rates
Chemical Shift Modulations (CSM)

Fluctuations of isotropic Chemical Shift

Slow motions affect *isotropic* properties that are not averaged out by overall tumbling.

- making or breaking of hydrogen bonds
- motions of the backbone
- large scale wobbling of a loop
- large-amplitude conformational changes of side chains

Fatiha Kateb, Daniel Abergel, Geoffrey Bodenhausen, E N S, Paris

Mori, Kateb, Bodenhausen, Piccioli, Abergel, J Am Chem Soc 2010
$R^{CSM/CSM}$ vs Secondary Structure

<table>
<thead>
<tr>
<th>ccrCSM (s$^{-1}$)</th>
<th>CaLaCb</th>
<th>c2SOD</th>
<th>MMP12</th>
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</thead>
<tbody>
<tr>
<td>$\alpha$-helix</td>
<td>-2.9</td>
<td>-6.1</td>
<td>-5</td>
</tr>
<tr>
<td>$\beta$-sheet</td>
<td>-</td>
<td>1.9</td>
<td>2.5</td>
</tr>
<tr>
<td>loop</td>
<td>-2.4</td>
<td>-2.1</td>
<td>-3.2</td>
</tr>
</tbody>
</table>

$R^{CSM/CSM} = 2p_d p_b \tau_{ex} \Delta \omega_C \Delta \omega_N$,

correlated $\beta$-sheet

anti-correlated $\alpha$-helix and loop

Mori, Kateb, Bodenhausen, Piccioli, Abergel, J Am Chem Soc 2010
Redox State dependent Dynamics

ns-ps time scale motions in Cytochrome b$_5$

Reduced Cytochrome b$_5$

Oxidized Cytochrome b$_5$

Increased Mobility

Is this finding general?
Is this finding important?

Banci, Bertini, Cavazza, Felli, Koulogliotis  Biochemistry 1998
ZQ-DQ Rates in human Cytochrome c

Reduced

![Graph showing ZQ-DQ rates for reduced form at 298 K and 280 K.](graph)

Oxidized

![Graph showing ZQ-DQ rates for oxidized form at 298 K and 280 K.](graph)

Average rates:
- Reduced form: 5.03 ± 0.80 s⁻¹ (59 res) at 298 K
- Reduced form: 8.09 ± 0.91 s⁻¹ (59 res) at 280 K
- Oxidized form: 5.07 ± 0.63 s⁻¹ (50 res) at 298 K
- Oxidized form: 8.38 ± 1.30 s⁻¹ (50 res) at 280 K

Same amount of slow motions for oxidized and reduced forms.

Lalli, Piccioli, Turano In preparation
Redox independent slow motions in Cytochrome c

Residues 48-50 interacts with heme

Residues 28-29 are in close contact with His 18 and close to heme

Conformational dynamics at the edge of some helices and within turns

Lalli, Piccioli, Turano In preparation
Redox independent slow motions in Cytochrome c

Correlated motions for loop 82-88

Low populated unfolded state

essential for protein folding

protein-protein interaction

apoptosis

electron transfer /biosensors
Redox Dependent dynamics of Cytochrome c

design mutants with improved ET properties
not only surface residues are important!
The interaction surface is not affected

Sakamoto, Kamiya, Imaid, Shinzawa-Itohe, Uchidaa, Kawano, Yoshikawa, Ishimori PNAS 2011
Monitoring Structural Dynamics

CSM/CSM gives insights on conformational equilibria on milli-microseconds time scale

Site specific information

Provides info on the nature of the exchange observed

No global differences between the two oxidation states

Low populated unfolded states are observed for loop 81-88

Redox dependent fluctuations are observed in different protein regions connected via extended network of H-bond interactions

Differences in mobility of the various protein regions need to be considered as a function of the various functions and interaction of Cytochrome c
HasA: Transient Metal sites in Proteins

Human Cyt c: Redox dependent properties: conformational dynamics

CIAPIN-1: Challenging Paramagnetic centers in (unstable and unfolded) proteins

Grx5/Isca1/Isca2: FeS Cluster maturation and protein-protein interactions

DNA polymerases, DNA helicases, and ABCE1 (Rli1), an ATPase, Biotin Sintase ...

Extra-mitochondrial pathways: nucleotide metabolism and cellular iron regulation.

Many rare diseases are linked to defects in mitochondrial Fe/S protein assembly.

Defects in Fe/S protein biogenesis affect other pathways such as lipoate synthesis.
Structural properties of Anamorsin
An essential protein for FeS cluster biosynthesis

Mia40 forms two disulfide bonds

N-terminal domain

Folded domain “standard” approach

Mia40 recognition site

[2Fe-2S] cluster

Highly paramagnetic

How unstructured is Ciapin 1?

Cytokine Induced APoptosis INhibitor
The quest for structural information in the proximity of paramagnetic Centers

15N-IR-HSQC-AP

Quench diamagnetic resonances
Observe Paramagnetic AND Unfolded

Bertini, Jimenez, Piccioli, JMR 2005
Ciofi-Baffoni, Gallo, Muzzioli, Piccioli, J Biomol NMR 2014
Structural characterization of $[\text{Fe}_2\text{S}_2]^{2+}$ -CIAPIN1

About 15 $^1\text{H}$ and $^{13}\text{C}$ $T_1$ values

The "paramagnetic" $^{13}\text{C}$ and $^1\text{H}$ $T_1$s provide structural info around the FeS cluster

Blue - residues detected in the "diamagnetic" experiments

Cyano - residues whose $^{13}\text{C}$ or $^{15}\text{N}$ signals were detected in paramagnetic-tailored $^{13}\text{C}$ or $^{15}\text{N}$ experiments
Anamorsin and its electron transfer partner, Ndor1

FMN binding domain of Ndor1

CIAPIN1 domain of anamorsin

Banci, Bertini, Calderone, Ciofi-Baffoni, Giachetti, Jaiswal, Mikolajczyk, Piccioli, Winkelmann PNAS 2013
Molecular view of an electron transfer process essential for iron-sulfur biogenesis

- Anamorsin tightly interacts with Ndor1 through its flexible linker
- The N-terminal domain of anamorsin is not involved in the Ndor1 recognition
- The [2Fe-2S]-CIAPIN1 domain of anamorsin transiently interacts with the FMN-binding domain of Ndor1 for one electron transfer from FMN to the [2Fe-2S] cluster

Banci, Bertini, Calderone, Ciofi-Baffoni, Giachetti, Jaiswal, Mikolajczyk, Piccioli, Winkelmann PNAS 2013
Iron-sulfur cluster biogenesis

The synthesis of Fe/S clusters and their insertion into apoproteins requires almost 30 proteins in the mitochondria and cytosol of eukaryotic cells.

How 4Fe4S cluster are synthetized?

![Diagram showing the synthesis of iron-sulfur clusters]

- **SUFE1**
- **ISCA1**
- **ISCA2**
- **GRX5**
- **2Fe2S**
- **4Fe4S**
- **Complex I**
Iron sulfur Clusters

Rubredoxins

Adrenodoxin
Ferredoxin
Biotin Syntase
ferrochelatase
Frataxin

High Potential Iron Proteins
Ferredoxins, Nitrogenase, Hydrongenase,
Acetylene hydratase, quinolino sinthase....
Iron sulfur Clusters

For a Synthetic Chemistry, these are extremely difficult syntheses!

Oxygen sensitive,
Highly hydrophobic
Thermodynamically and Kinetically unstable
The [2Fe-2S] clusters buried in the interior and shielded from the solvent.

Monomeric in solution:

2 x [2Fe2S], GSH

Tetrameric in the crystal structure:

GSH

[2Fe2S]

Test case in structural biology: Xray vs NMR structures

Apo hGrx5

In solution, Helix α2 is mobile and shorter than in Xray

Banci, Brancaccio, Ciofi-Baffoni, Del Conte, Gadepalli, Mikolajczyk, Neri, Piccioli, Winkelmann
PNAS, 2014
Glutathione (GSH) binding to apo Grx5

GSH is essential for FeS cluster maturation
Holo Grx5 in solution is a homodimer

117 aa each monomer

How does it work the Fe$_2$S$_2$ transfer from Grx5 to Isca Proteins?
Mapping The Cluster environment

Two set of paramagnetic signals are observed for some of the residues in close proximity to the cluster.

Two forms exist for holo-hGrx5!

Ciofi-Baffoni, Gallo, Piccioli, J. Biomol NMR 2014
The [2Fe-2S] cluster transfer process

\[ \text{GRX5-[2Fe-2S]-GRX5} \rightarrow \text{GRX5-[2Fe-2S]-GRX5} \]

50% 50%

GSH

Banci, Brancaccio, Ciofi-Baffoni, Del Conte, Gadepalli, Mikolajczyk, Neri, Piccioli, Winkelmann

PNAS 2014, 111 6203-6208
Holo GRX5 transfers the [2Fe2S] cluster to apo ISCA1 and not vice versa

UV/visible spectra of GRX5 and ISCA1 were recorded before (red) and after incubation in a ~1:1 protein ratio followed by their separation through Nickel-affinity chromatography (blue).

Banci, Brancaccio, Ciofi-Baffoni, Del Conte, Gadepalli, Mikolajczyk, Neri, Piccioli, Winkelmann PNAS, 2014
Complex formation and cluster transfer between GRX5 and ISCA1

Cluster is essential to promote protein-protein interaction. Apoproteins do not interact.

The interaction specifically involves residues surrounding GSH/[2Fe-2S] cluster binding region.

Dimeric form b transfers the [2Fe-2S] cluster to apo ISCA1.

Banci, Ciofi-Baffoni, Del Conte, Gadepalli, Mikolajczyk, Neri, Piccioli, Winkelmann *PNAS*, 2014
[2Fe-2S] cluster transfer in FeS Proteins Biogenesis
Structure Calculation vs Structure Modeling

Apo hISCA2
Using TALOS+

NMR Chemical Shifts predict Secondary Structures

Structural model of monomeric apo hISCA2
Using CS-ROSETTA
Apo hISCA2 in solution is a dimer

NMR signals of residues at the interface broaden.

Mobility studies indicate that apo hISCA2 is a dimer.

There are a number of flexible regions, including those encompassing the three conserved Cys residues.
ISCA2 is a mitochondrial protein involved in the biogenesis and assembly of iron-sulfur clusters, which play a role in electron-transfer reactions.
An Integrated spectroscopic Approach

Esi-MS

1H NMR

A [Fe₄S₄]⁺ cluster is observed!

EPR

Relative intensities between 4Fe4S and 2Fe 2S are measured

Equilibrium between different species is observed

The 4Fe4S is in the [Fe₄S₄]⁺ oxidation state

Brancaccio, Gallo, Mikolajczyk, Zovo, Palumaa, Novellino, Piccioli, Ciofi-Baffoni, Banci  Submitted
Reconstituted Isca2 binds a Fe4S4 cluster!

Brancaccio, Gallo, Mikolajczyk, Zovo, Palumaa, Novellino, Piccioli, Ciofi-Baffoni, Banci  Subn
Monitoring cluster equilibria in Holo-Isca2

Brancaccio, Gallo, Mikolajczyk, Zovo, Palumaa, Novellino, Piccioli, Ciofi-Baffoni, Banci  Submitted
Biogenesis of [4Fe-4S] clusters
Heterodimeric complex between apoIsca1/ apoIsca2

Major differences observed on Isca2
Missing residues

Heterodimer dimeric adduct is formed by substituting a subunit of homodimeric apo-Isca2 with apo-Isca1
The cluster transfer from HoloGrx5 to Isca1/Isca2 complex

1:2 ternary complex
1:2.5 ternary complex

1:2 the cluster is completely transferred
1:2.5 cluster remains on Grx5

The cluster transfer from HoloGrx5 to Isca1/Isca2 complex

2 hGRX5-[2Fe-2S]-hGRX5 + apo hISCA1/hISCA2 → 4 apo hGRX5 + hISCA1-[4Fe-4S]-hISCA2
The cluster transfer from HoloGrx5 to Isca1/Isca2 complex

1:2 ternary complex
1:2.5 ternary complex

Reconstituted
[4Fe4S]^{2+} hlISCA1/hlISCA2

15N apo Isca2/apo Isca1
1:2 ternary complex

2 hGRX5-[2Fe-2S]-hGRX5 + apo hlISCA1/hlISCA2 → 4 apo hGRX5 + hlISCA1-[4Fe-4S]-hlISCA2
The cluster transfer from HoloGrx5 to Isca1/Isca2 complex

1:2 interaction
1:2.5 interaction

15N Grx5

1:2 the cluster is completely transferred
1:2.5 cluster remains on Grx5

Reconstituted [4Fe4S]2+ hISCA1/hISCA2

15N apo Isca2/apo Isca1

1:2 interaction

2 hGRX5-[2Fe-2S]-hGRX5 + apo hISCA1/hISCA2 → 4 apo hGRX5 + hISCA1-[4Fe-4S]-hISCA2
Formation of [4Fe-4S] clusters in the mitochondrial FeS cluster assembly machinery
Acknowledgments

Iron Sulfur Clusters Assembly
Lucia Banci –CERM Director
Simone Ciofi-Baffoni
Diego Brancaccio (Naples)
Julia Winkelmann
Maciej Mikolajczyk (Novartis, Siena)
Angelo Gallo
Sara Neri
Rebecca Del Conte

Mass Spectrometry
Peep Palumaa (T.U. Tallin)
Karin Zovo (T.U. Tallin)

Paramagnetic NMR
Beatriz Jimenez (Imperial College, London)
Angelo Gallo
Mirko Mori

Hemophore HasA
Paola Turano
Muriel Delepierre (Pasteur, Paris)
Anne Lecroisey (Pasteur, Paris)
Celia Caillet-Saguy (Pasteur, Paris)

Cytochrome c/Conformational Dynamics
Paola Turano
Daniela Lalli
Geoffrey Bodenhausen (ENS, Paris)
Daniel Abergel (ENS, Paris)
Fatiha Kateb (ENS, Paris)

Acoustically Detected NMR
Stefano Cacciatore (MIT Boston)
Edoardo Saccenti (Wageningen University)
$^{15}$N labeling of a single protein to monitor Heterodimeric Complexes

$^{15}$N apo-Isla2 + apo-Isla1

$^{15}$N apo-Isla2 free + apo-Isla1

Apo hISCA2 maintains a dimeric state

Same subunit interface in involved in both homodimers and heterodimers

$^{15}$N apo-Isla1 + apo-Isla2

$^{15}$N apo-Isla1 free + apo-Isla2

apo hISCA1 passes from a homo/dimer equilibrium to a dimeric state
Paramagnetic NMR on Holo hGrx5

<table>
<thead>
<tr>
<th>Residue</th>
<th>$T_\beta$ (ms)</th>
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<tbody>
<tr>
<td>Lys-59</td>
<td>50 ± 10</td>
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<tr>
<td>Gly-60</td>
<td>84 ± 5</td>
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<tr>
<td>Glu-66</td>
<td>55 ± 5</td>
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<tr>
<td>Cys-67a</td>
<td>9 ± 5</td>
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<tr>
<td>Cys-67b</td>
<td>16 ± 4</td>
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<tr>
<td>Gly-68</td>
<td>—</td>
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<tr>
<td>Phe-69</td>
<td>—</td>
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<tr>
<td>Ser-70a</td>
<td>43 ± 3</td>
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<tr>
<td>Ser-70b</td>
<td>44 ± 4</td>
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<tr>
<td>Asn-71</td>
<td>125 ± 25</td>
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<tr>
<td>Ala-72</td>
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<tr>
<td>Leu-91</td>
<td>165 ± 35</td>
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<tr>
<td>Ile-109</td>
<td>52 ± 5</td>
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</tbody>
</table>

Residues with two set of signals

GSH

Salt bridge interaction between protein and GSH

Ciofi-Baffoni, Gallo, Piccioli, J. Biomol NMR 2014
Paramagnetic NMR and Spin Equilibrium

Up to 800 ppm hyperfine shifts observed in $^{13}$C NMR

$T_1 < 1$ ms

Two forms due to different heme orientations

Breaking or weakening of Y75-Fe(III) yields to High Spin Fe(III)

High spin increases at increasing temperatures