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Review

An Italian contribution to structural genomics: Understanding metalloproteins

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Contents

Abstract

In this review, the contribution of the Magnetic Resonance Center of the University of Florence (CERM) to structural genomics is described. This contribution focuses on metalloproteins. Particular emphasis is given to those metalloproteins that belong to the same biochemical pathway, such as the proteins involved in copper trafficking, in metal detoxification, in the assembly of cytochrome *c* oxidase, and in the assembly of the urease nickel cofactor. Calcium-dependent signalling proteins studied at CERM are also reviewed, for their peculiar conformational features that are at the basis of the signalling process as well as for the importance of the methodological NMR approach based on the substitution of calcium with lanthanide ions. The structural determination in solution of iron–sulfur proteins and cytochromes was pioneered by us in a pre-genomic era and constitutes the methodological basis of the subsequent studies. Our methodological approach is indeed largely based on NMR, even if

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not exclusively, with important contributions deriving also from X-ray crystallography and X-ray absorption spectroscopy. The use of NMR is particularly useful for the characterization of proteins that occur in vivo as largely or completely unfolded, or that can become unfolded under extreme solution conditions or upon chemical manipulations. Examples in this sense are provided for the different classes of metal binding proteins and in particular for cytochromes and CuZn superoxide dismutase.

Within the worldwide frame of structural genomics, we are pursuing also the characterization of known naturally occurring pathogenic mutations. As the three-dimensional structures provided by structural genomics projects constitute a starting database for post-genomic drug design, mention is also made to the perspectives in this field by reviewing our activity.

Keywords: Structural genomics; Metalloproteins; Protein structure

1. Introduction

We like to refer to a genomic revolution having occurred between the year 1995, when the first genome was sequenced, i.e., that of *Haemophilus influenzae* [1] and the year 2000, when the first draft of the human genome was published [2,3]. At the turn of the century a number of consortia were established in North America (http://www.nigms.nih.gov/psi/) and a major program started at Riken in Japan (http://www.rsgi.riken.go.jp/) with the long term aim of covering with the three-dimensional structures all the proteins discovered through gene sequences. Subsequently, many other countries pursued their own national projects (an extensive list is available at: http://sg.rcsb.org/). At the same time several workshops and international series of conferences took place. In Florence a workshop was organized in 1999 by the Organization for Economic Co-operation and Development (OECD) on this subject (http://www.oecd.org/dataoecd/24/31/2105118.pdf). The discussions at the various meetings concerned protein targeting, protein production, crystallography, labelling for NMR experiments, bioinformatics, publication policies and intellectual property rights. The new concept of high throughput entered into the research strategies in order to give quick responses to the scientific demands.

In Italy, researchers in Florence, Siena and Bologna coordinated their efforts to pursue a project on structural genomics. Their expertise in bioinorganic chemistry guided their decision to contribute to the field through the structure determination of metalloproteins. A first report of their activity is available in a special volume of Accounts of Chemical Research dedicated to Structural Genomics [4].

The field of metalloproteins is very broad, and prioritarization of the proteins to be investigated was rather arbitrary. Then the financial support at the national level in Italy was not just on protein structures, but on the coupling between the structure and the protein function. Also the European Project Structural Proteomics in Europe (SPINE) (http://www.spineurope.org/) reintroduced the concept of prioritarization through the function. As a result, we started to: (i) reorganize our work by aiming at completeness in the structure determination of groups of related metalloproteins, using also modelling tools, and (ii) develop structural projects on proteins involved in specific functions, e.g., determination of the structures of all proteins involved in copper trafficking, etc.

This progress in the definition of the goals of our research led us to tackle entire biochemical processes, and in perspective to design small molecules capable of modulating such processes. Culturally, the genomic vision of the proteins led us towards the field of drug design (see Section 4). Finally, in order to frame our research, the problem arose of knowing whether a protein needs a metal ion to perform its function. This is a fascinating problem that will be discussed below.

In 2005 the NIH confirmed a second round of projects within the protein structure initiative (PSI) (http://www.nigms.nih.gov/psi/). While four centers shall be financed to generate thousands of structures by taking advantage of the methodological tools developed in the first phase of PSI, other centers should afford the structure determination of systems that are not yet amenable to high-throughput approaches, i.e.: (i) small protein complexes; (ii) membrane proteins; (iii) eukaryotic proteins. We feel that our research in metalloproteins should develop along these future directions, and prodromes of our first steps in these topics are emerging from the work described in the present review.

2. The occurrence of metalloproteins

Genome sequencing projects provide researchers with the list and the amino acid sequence of all the proteins that the investigated organism can produce (called the proteome of the organism). The question then arises of which proteins need a metal ion to perform their physiological function. This is a fundamental question for inorganic structural genomics [5].

A general approach to infer the function of a protein from the sequence is to look for similar proteins that have been experimentally characterized. In particular, to infer metal-binding capabilities one needs to identify proteins similar to others that are known metalloproteins. Here, “similar” necessarily means “similar in sequence”, as the primary structure is all that is known from genome sequencing. In this respect, the most used approach is probably that of detecting (possible) homologies by aligning the sequence of each protein lacking experimental characterization in a newly available proteome against a database of sequences of proteins that are already characterized as metalloproteins. The quality of the alignments is evaluated statistically, and alignments scoring better than a given threshold are then assumed to be indicative of functional homology. Database searches can efficiently be carried out through the programs FASTA [6] or BLAST [7], or some of their variants [8]. The functional homology detected is used to “annotate” (i.e., attach some information to) the protein in the proteome. For example, if the alignment of the sequence of an uncharacterized protein in a

new genome, and of a protein which is known to be a peroxidase, features 40% identical amino acids in aligned positions, then the uncharacterized protein will be dubbed as a peroxidase (sometimes with the additional label "putative"). From that moment on, the annotation will always be made available together with the protein sequence. The approach here outlined is only one of the many that can be used to annotate uncharacterised proteins whose sequence is predicted as the result of a successful genome-sequencing project. Other methods can exploit, besides direct experimental data, more complex bioinformatic tools, for example, making use of databases of protein domains [9,10]. A quite popular domain database in this respect is Pfam [10].

To further address this matter, using knowledge based on our experience in biological inorganic chemistry, we have developed a methodology for the identification of metalloproteins in genome databanks [11]. The methodology relies on the exploitation of known metal binding-patterns (MBPs), experimentally available from the three-dimensional structures deposited in the Protein Data Bank (PDB) [12]. MBPs are strings of the type AX_nBX_mC ..., where A, B, C, ... are the amino acids acting as metal ligands, and n, m, ... the number of amino acidic residues in between two subsequent ligands. An exhaustive list of presently known binding patterns for a given metal ion (e.g., copper(I)/(II)) can be built by retrieving from the PDB all structures where that metal has been experimentally observed and is bound in a physiologically relevant way (in other words, the PDB must be cleaned up from structures where a protein binds a metal because of the experimental procedure adopted for crystal preparation, but metal-binding is not relevant in vivo). Then, by measuring all distances between the metal atom(s) contained in the structure and the non-hydrogen atoms of the protein, the donor atoms are readily identified and thus the amino acids binding to the metal (i.e., the MBP). A similar approach has been implemented in the metalloprotein database, a collection of MBP's automatically extracted from the PDB [13]. To each metalloprotein in the PDB the above approach [11] therefore attaches a MBP. The primary structure of the metalloprotein (the query) from the PDB and of the corresponding MBP are used as input for a variant of BLAST, PHI-BLAST [14], to scan gene banks (or a complete genome sequence). PHI-BLAST extracts from the gene bank all proteins containing the MBP (the hits). Then, for each of these hits individually, the pattern is aligned with that contained in the query sequence, and the alignment extended around the MBP, stopping as soon as there is significant divergence between the two primary structures. A statistical evaluation of the results obtained with this method when applied to the prediction of metal-binding properties for all the proteins in the PDB (i.e., a mixture of metal-binding and non-metal-binding proteins) has yielded quality parameters and ranges of confidence [11].

The approaches described above give predictions that are directly relevant in vitro, rather than in vivo. It is also worth mentioning that metalloproteins can bind a metal only if the latter is made available by the tight control system of the cell, which thus selects the ion(s) actually being bound [4].

The methodology based on proteins in the PDB and MBP identified therein has been applied to search for copper-binding proteins in four different genomes [11]. For copper, it has been found that the copper-binding capability can be predicted with good confidence when the amino acid identity around the MBP is at least 20% [11]. With this result we estimated a lower limit for the number of human copper-binding proteins in the order of 3.5% of the proteome. The share of copper-binding proteins in bacterial proteomes is possibly somewhat lower, around 1% or less [11].

The analysis of the type described to search for copper proteins can be easily complemented by other bioinformatic tools. For example, a possible approach is that of identifying all metal-binding protein domains in other databases (e.g., Pfam [10]), and then look for these domains in the sequences of the proteome. In fact, all methods can be applied independently and their results compared. This gives both broader coverage of the potential metal-binding proteins in a proteome, and higher confidence in the results when two or more methods independently identify the same protein as a metalloprotein. The application of this combined approach to the human proteome indicates that $10.0 \pm 1.5\%$ of human proteins are zinc-binding [15].

3. Protein structures

From the ensemble of proteins located in given genomes on the basis of their ability to bind metals, we can then select those that will be the object of experimental studies, by focusing on specific protein classes and/or on proteins potentially involved in specific functions. In structural genomics projects, each selected protein is handled independently through a series of subsequent steps. This pipeline starts with cloning the gene encoding the protein of interest, proceeds through heterologous protein expression, and ends up with structural characterization. The following sections summarize some of the results obtained on the systems that have been recently characterized in our lab. We will also discuss older results by reframing them in the genomic perspective.

An exhaustive list of the protein three-dimensional structures solved by CERM researchers is reported in Tables 1–7. For completeness, several structures published before 1999 are also included in the appropriate set. The PDB codes for the single structures are provided, and the structural technique used is explicitly indicated. Most of the work has been carried out by NMR in solution, but X-ray crystallography has also been used in a number of cases. NMR has been also used to characterize largely unfolded states of some proteins, that may constitute the native form of the protein or may be obtained using extreme solution conditions (e.g., high pH values, chaotropic agents, etc.). These protein forms cannot be described in terms of a single structure or a family of conformations that only little deviates with respect to an average structure, but are rather constituted by an heterogeneous conformational ensemble. Under these conditions, NMR gains a particular importance as a method for the characterization of the properties of the ensemble. Some examples of this type of characterization are reported in the first part of Table 8. In a few special examples NMR has been also used to refine existing X-ray crystal structures or structures resulting from homology modelling (second part of Table 8).

Table 1
Structures of cytochromes and other heme-binding proteins

Protein	PDB ID	Heme iron oxidation and spin state	Experimental method	Reference
Cytochromes				
Mono-heme cytochromes *c*				
S. cerevisiae iso-1	1YFC	Low spin iron(II)	NMR	[30,32]
	1YIC	Low spin iron(III)		
Horse heart	1GIW, 2GIW	Low spin iron(II)	NMR	[31,35]
	1AKK	Low spin iron(III)		
M. braunii c_6	1CED	Low spin iron(II)	NMR	[41,42]
	1A2S	Low spin iron(III)		
B. pasteurii c_{553}	1N9C	Low spin iron(II)	NMR	[36,354]
	1K3G, 1K3H	Low spin iron(III)		
S. putrefaciens	1KX2, 1KX7	Low spin iron(II)	NMR	[355]
M80A *S. cerevisiae iso*-1	1FHB	CN^- bound low spin iron(III)	NMR	[29]
S. cerevisiae iso-1, alkaline form	1LMS	Low spin iron(III)	NMR	[63]
Imidazole-bound horse heart	1FI7, 1F19	Imidazole bound low spin iron(III)	NMR	[356]
R. palustris c_2	1FJ0	Low spin iron(III)	X-ray	[48,49]
	1HH7, 1I8P	Low spin iron(II)		
	1I8O	NH_3-bound low spin iron(III)		
B. pasteurii c_{553}	1B7V, 1C75	Low spin iron(III)	X-ray	[357]
Cladofora glomerata c_6	1LS9	Low spin iron(III)	X-ray	[358]
Cytochromes b_5				
Microsomal rat	1AQA	Low spin iron(II)	NMR	[16,18,20]
	1AW3, 1AXX, 2AXX, 1BFX	Low spin iron(III)[a]		
Microsomal rabbit	1DO9	Low spin iron(III)	NMR	[22]
DME-heme microsomal rabbit	1MNY	Low spin iron(III)	NMR	[24]
Partially unfolded microsomal rat	1BLV	Low spin iron(III)	NMR	[17]
Cytochromes b_{562}				
E. coli	1QPU	Low spin iron(III)	NMR	[28]
R98C *E. coli*	1QQ3	Low spin iron(III)	NMR	[27]
Cytochromes c'				
R. gelatinosus	1JAF	High spin iron(III)	X-ray	[40]
Three-heme *c*-type cytochromes				
D. acetoxidans c_7	1EHJ	Low spin iron(II)	NMR	[44,45]
	1NEW, 2NEW	Low spin iron(III)		
D. acetoxidans c_7	1LM2	Low spin iron(III) + chromate bound to the protein surface.	NMR	[47]
K9-10A *D. acetoxidans* c_7	1KWJ	Low spin iron(III)	NMR	[46]
Other heme-binding proteins				
CcmE	1LM0	Apo-form	NMR	[75]

[a] The heme binds to the protein in two different orientations, rotated by 180° around the α–γ meso axis and usually indicated as A and B forms. The solution structure in the iron(III) form has been solved for both of them.

The structural features relevant for the functional aspects of each class are reviewed in the corresponding section.

3.1. Cytochromes and other heme proteins

The progresses in our work on cytochromes, which started already 15 years ago, represent a good example of the impact of the *genomic revolution* on our research.

Cytochromes are relatively small heme proteins that function as electron transfer proteins in a number of fundamental biological processes. Hundreds of three-dimensional structures of cytochrome domains and their mutants are nowadays available in the PDB. In a pre-genomic approach we have contributed to this endeavour by solving the solution structures, by NMR, of cytochromes belonging to different classes (see Table 1). In particular, we have solved the structures and characterized the dynamic behavior in solution of a number of *b*-type [16–28] and *c*-type cytochromes [29–49]. Our challenge was represented by the determination of the solution structure of oxidized cytochromes by themselves, as the hyperfine coupling between unpaired electrons of the low spin iron(III) ion and the observed nuclei decreases nuclear relaxation times, thus making difficult the observation of their signals [50]. Reduction in nuclear relaxation times also causes a decreased ability to detect nuclear Overhauser effects between pairs of nuclei that are the main source of structural information in solution structure determination of proteins [51]. Thanks to the development of paramagnetism based-restraints [52–56] we have been able to obtain solution structures of paramagnetic proteins of a quality as good as that of the corresponding diamagnetic analogues (Fig. 1). As a consequence we could compare the structures of the oxidized and reduced proteins without the bias imposed by crystal packing

Table 2
Structures of copper binding proteins

Protein	PDB ID	Metal sites	Experimental method	Reference
Copper chaperons				
S. cerevisiae Atx1	1FES	Apo-form	NMR	[105]
	1FD8	Copper(I)		
Synechocystis Atx1	1SB6	Apo-form	NMR	[121]
Human Hah1	1TL5	Apo-form	NMR	[144]
	1TL4	Copper(I)		
P1-type ATPase's				
S. cerevisiae Ccc2 N-term domain	1FVQ	Apo-form	NMR	[111]
	1FVS	Copper(I)		
B. subtilis CopZ	1K0V	Copper(I)	NMR	[113]
S46V *B. subtilis* CopA	1OQ3	Apo-form, a domain	NMR	[114–116]
	1OQ6	Copper(I), a domain		
	1JWW	Apo-form, b domain		
	1KQK	Copper(I), b domain		
	1P6T	Apo-form, a and b domains		
Synechocystis PacS	Not yet deposited	Apo-form	NMR	Unpublished results
Human Menkes MNK2	1S6O	Apo-form	NMR	[140]
	1S6U	Copper(I)		
Human Menkes MNK3	Not yet deposited	Apo-form Copper(I)	NMR	Unpublished results
Human Menkes MNK5	1Y3K	Apo-form	NMR	[142]
	1Y3J	Copper(I)		
Human Menkes MNK6	1YJU	Apo-form	NMR	[141]
	1YJV	Copper(I)		
A629P Human Menkes MNK6	1YJR	Apo-form	NMR	[141]
	1YJT	Copper(I)		
Protein–protein complexes				
S. cerevisiae Atx1–Ccc2	1UV1	Copper(I)	NMR-based computational model	[126]
	1UV2			
Cytochrome *c* oxidase copper incorporation proteins				
S. cerevisiae Cox17	1Z2G	Apo-form	NMR	[158]
B. subtilis Sco1-like soluble domain	1ON4	Apo-form	NMR	[160]
D. radiodurans DR1885	1X7L	Apo-form	NMR	[166]
	1X9L	Copper(I)		
S. meliloti Cox11-like soluble domain	1SP0	Apo-form	NMR	[161]
	1SO9			
Copper resistance proteins				
P. syringae CopC	1M42	Apo-form	NMR	[173–175]
	1NM4	Copper(I)		
	1OT4	Copper(II)		
E. coli CutA1	1NAQ	Mercury(II)	X-ray	[179]
Rattus norvegicus CutA1	1OSC	Apo-form	X-ray	[179]
Blue copper proteins				
Synechocystis PCC6803 plastocyanin	1IOW, 1IOY, 1J5C, 1J5D	Copper(II)	NMR	[123,124]
	1JXD, 1JXF	Copper(I)		

Table 2 (*Continued*)

Protein	PDB ID	Metal sites	Experimental method	Reference
Spinach plastocyanin	1YLB	Copper(I)	NMR	[222,359]
Metallothioneins				
S. cerevisiae	1FMY	$Cu(I)_7$	NMR	[360]
S. cerevisiae	1RJU	$Cu(I)_8$ cluster	X-ray	[148]
Superoxide dismutases				
Q133M2 monomeric human CuZnSOD	1BA9	Copper(I) + zinc(II)	NMR	[217]
Q133M2 monomeric human CuZnSOD	1MFM	Copper(I) + zinc(II)	X-ray	[199]
M4 monomeric human CuZnSOD	1DSW	Copper(I) + zinc(II)	NMR	[361]
Dimeric human Cu_2Zn_2SOD	1L3N	Copper(I) + zinc(II)	NMR	[218]
Q133M2 monomeric human copper-free SOD	1KMG	Zinc(II)	NMR	[219]
Q133M2 monomeric human apo SOD	1RK7	Apo-form	NMR	[220]
Dimeric human copper-free SOD with reduced disulfide bridge	2AF2	Zinc(II)	NMR	[222]
SOD-like protein from *B. subtilis*	1U3N	Apo-form	NMR	[226]
SOD-like protein from *B. subtilis*	1S4I	Zinc(II)	X-ray	[226]
P104H SOD-like protein from *B. subtilis*	1XTL	Zinc(II)	X-ray	[227]
Y88H-P104H SOD-like protein from *B. subtilis*	1XTM	Copper(II) + zinc(II)	X-ray	[227]

effects. Also, X-ray radiation might alter the original oxidation state of the protein sample [57]. The solution structures of rat and rabbit microsomal cytochromes b_5 [16–18,20,22] and of cytochrome b_{562} [26–28] have been determined and analyzed with a particular emphasis on the stabilizing role of heme interactions within the protein matrix. Ample characterization of the mono-heme *c*-type cytochromes was performed upon solving the solution structures of a number of cytochromes from different origins both in their oxidized and reduced forms: (i) the mitochondrial *S. cerevisiae iso*-1 [30,32] and the horse heart cytochrome *c* [31,35]; (ii) the cytochrome c_6 from the green alga *Monoraphidium braunii* [41,42]; (iii) the cytochrome *c* from the gram-positive bacterium *Bacillus pasteurii* [36,58]. The redox-dependent conformational and dynamic changes have been analysed and rationalized in terms of the primary structure differences among the proteins from different organisms and related to the different protein stability (Fig. 2). The coordination properties in the distal site of mitochondrial cytochrome *c* have been extensively studied by substituting the native Met80 axial ligand with a non-coordinating alanine [29,59,60] (Fig. 3A) or by studying the protein under unfolding or partially unfolding conditions (Table 8) [29,59–62]. The determination of the structure of the molten globule state that corresponds to the alkaline form of cytochrome *c* (Fig. 3B) [63] represents a breakthrough,

Table 3
Structures of nickel binding proteins

Protein	PDB ID	Metal cluster	Experimental method	Reference
B. pasteurii urease, native	2UBP	Ni(OH)Ni	X-ray	[232]
B. pasteurii UreE	1EAR, 1EB0	Ni, Zn	X-ray	[251]
B. pasteurii urease, phosphate complex	1IE7	Ni(OH)Ni	X-ray	[235]
B. pasteurii urease, boric acid complex	1S3T	Ni(OH)Ni	X-ray	[236]
B. pasteurii urease, mercaptoethanol complex	1UBP	Ni(OH)Ni	X-ray	[234]
B. pasteurii urease, diamidophosphate complex	3UBP	Ni(OH)Ni	X-ray	[232]
B. pasteurii urease, acetohydroxamic acid complex	4UBP	Ni(OH)Ni	X-ray	[233]

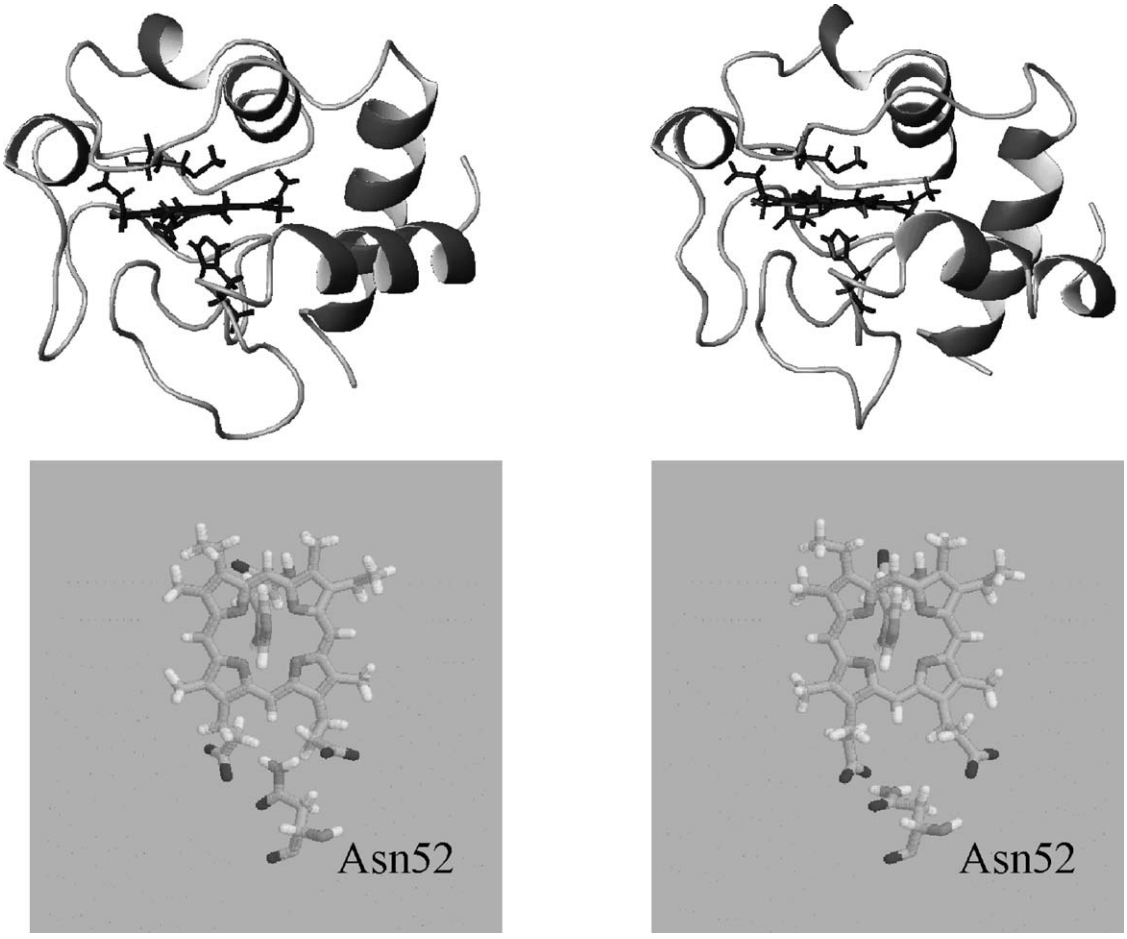

Fig. 1. The average solution structures of reduced (left) and oxidized (right) horse heart cytochrome *c* (top part). In the bottom panels local redox dependent differences in the H-bond network involving heme propionates and Asn52 are shown.

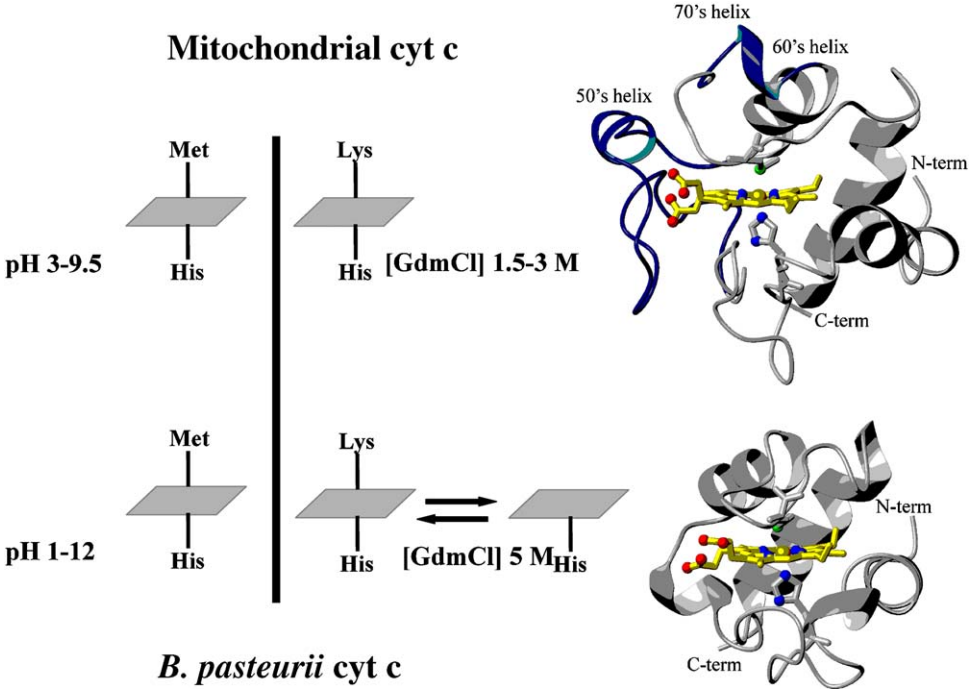

Fig. 2. Comparison of the three-dimensional structures of mitochondrial horse heart cytochrome *c* and *B. pasteurii* cytochrome *c*. The fold is essentially the same, but the mitochondrial protein is longer, with an extra helix facing the heme propionates and a longer distal loop bearing the sixth iron ligand, Met80. The ranges of existence of species with different iron(III) axial coordination are shown.

Table 4
Structures of iron–sulfur proteins

Protein	PDB ID	Metal cluster	Experimental method	Reference
HiPIP's				
E. halophila	Not deposited	Oxidized Fe_4S_4 cluster	NMR	[276,279]
	1PIH, 1PIJ	Reduced Fe_4S_4 cluster		
C. vinosum	1HRQ, 1HRR	Oxidized Fe_4S_4 cluster	NMR	[277,278]
	1NEH	Reduced Fe_4S_4 cluster		
C77S *C. vinosum*	1NOE	Reduced Fe_4S_4 cluster	NMR	[280]
H42Q *C. vinosum*	1B0Y	Oxidized Fe_4S_4 cluster	X-ray	[281]
C. vinosum	1CKU	Oxidized Fe_4S_4 cluster	X-ray	[281]
R. fermentans	1HLQ	Oxidized Fe_4S_4 cluster	X-ray	[282]
Ferredoxins				
C. pasterianum	1CLF	Oxidized 2[Fe_4S_4]	NMR	[287]
B. schlegelii	1BC6, 1BD6	Oxidized Fe_7S_8	NMR	[288]
D13C *B. schlegelii*	1BQX, 1BWE	Oxidized Fe_8S_8	NMR	[290]
Parsley	1PFD	Oxidized Fe_2S_2	NMR	[292]
PsaC from *Synechococcus sp*. PCC 7002	1K0T	Two Fe_4S_4 clusters	NMR	[289]
Rubredoxins				
C. pasterianum	1BFY	High spin iron(II)	NMR	[291]

Table 5
Structures of calcium binding proteins solved by solution NMR

Protein	PDB ID	Metal sites	Experimental method	Reference
Calcium-binding proteins				
Bovine testes calmodulin, N-term domain	1AK8	Two cerium(III) mononuclear centers, i.e., $(Ca_2)_N$	NMR	[305]
Human N60D calmodulin	1SW8	$(CaLn)_N(Ca_2)_C$ with Ln = Tb^{3+} or Tm^{3+}	NMR	[331]
P43M bovine calbindin D_{9k}	1KSM	One cerium(III) + 1 calcium(II) mononuclear centers	NMR	[56]
Human parvalbumin	1RJV, 1RK9	One calcium(II) + 1 dysprosium(III) mononuclear centers	NMR	[323]
Human oncomodulin	1TTX	One calcium(II) + 1 terbium(III) mononuclear centers	NMR	[322]
Human S100A13	1YUR	Apo-form	NMR	[334]
	1YUT	calcium(II)		

Calcium-substitution with lanthanides has been extensively used for solution structure determination as it allows to exploit the use of paramagnetism-based restraints.

Table 6
Structures of P-type ATPase's involved in zinc and cadmium detoxification

Protein	PDB ID	Metal sites	Experimental method	Reference
ATPase's				
E. coli ZntA	1MWY	Apo-form	NMR	[343]
46–118 domain	1MWZ	zinc(II)		
L. monocytogenes ATPase domain	2AJ0, 2AJ1	Apo-form	NMR	[344]

Table 7
Miscellaneous metalloprotein structures solved by either solution NMR or X-ray crystallography

Protein	PDB ID	Metal sites	Experimental method	Reference
Matrix metalloproteinanses				
Human MMP-12, catalytic domain	1YCM, 1Z3J	Two zinc(II) + 3 calcium(II) mononuclear centers; NNGH inhibitor bound to the catalytic zinc	NMR	[349]
Human MMP-12, catalytic domain	1RMZ	Two zinc(II) + 3 calcium(II) mononuclear centers; NNGH inhibitor bound to the catalytic zinc	X-ray	[350]
Human MMP-12, catalytic domain	1Y93, 1OS9, 1OS2	Two zinc(II) + 3 calcium(II) mononuclear centers; AHA inhibitor bound to the catalytic zinc	X-ray	[349]
Human MMP-10, catalytic domain	1Q3A	Two zinc(II) + 3 calcium(II) mononuclear centers; NNGH inhibitor bound to the catalytic zinc	X-ray	[352]

Table 8
Unfolded proteins[a] and structural refinement of X-ray structures[b]

Protein	Denaturing agent	Metal center	Reference
Unfolded proteins			
Cytochromes			
S. cerevisiae iso-1	GdmCl	Iron(III)	[62]
	SDS		[62]
Horse heart	GdmCl	Iron(III)	[62]
	SDS		[62]
B. pasteurii	GdmCl	Iron(III)	[58]
Rat microsomal cytochrome b_5	GdmCl	Iron(III)	[17]
Copper proteins			
Monomeric SOD	GdmCl	Copper(I), zinc(II)	[362]
Monomeric SOD	GdmCl	Apo-form	[362]
Nickel proteins			
UreG	Naturally unfolded	zinc(II)	[253]
Iron–sulfur proteins			
C. vinosum HiPIP	GdmCl	Reduced Fe_4S_4 cluster	[298]
Thermotoga maritima IscU	Naturally unfolded	Apo-form	[299]

Protein	Starting structure	Metal center	Reference
NMR-based structural refinement			
Rhodopseudomonas palustris cytochrome c_{556}	Homology modeling	Low spin heme iron(III)	[68]
Sperm whale myoglobin	X-ray	High spin heme iron(III), High spin heme iron(II)	[86]
Serratia marcescens HasA	X-ray	Equilibrium between high spin and low spin iron(III) heme	[84]
Synechocystis CtaA	Homology modeling	Apo-form	[112]

[a] The unfolded proteins that do not possess a defined tertiary structure but that have been characterized at CERM by NMR in terms of average residual intermolecular interactions and dynamics of the conformational ensemble, are listed in the top part of the table.
[b] The bottom part of the table contains a summary of structures that have been refined by NMR restraints using as a starting point structures resulting from homology modelling or available X-ray crystal structures.

showing the potentialities of the NMR approach for a deep characterization of poorly folded states of proteins, an aspect where the NMR methodology is definitely superior with respect to X-ray crystallography.

Finally, we have determined the structure of a multiheme cytochrome *c*: the solution structure of the three-heme cytochrome c_7 from *Desulfuromonas acetoxidans* in the fully reduced diamagnetic form and in the fully oxidized paramagnetic form has been solved and insights have been gained by NMR on the role of the three hemes for the protein function (Fig. 4) [44–47,64]. The three-dimensional structure of this protein was first determined in solution by NMR [43–45]. Later on, X-ray crystallography confirmed the structural details, including the presence of a long disordered loop [65]. The metal reductase activity of cytochrome c_7 was also demonstrated by NMR upon following the reduction to chromium(III) of chromate bound to the protein surface and details on the reduction mechanism involving the three heme centers were acquired [47].

In a structural genomics context, in 1999 our laboratory performed an extended bioinformatic analysis of mitochondrial

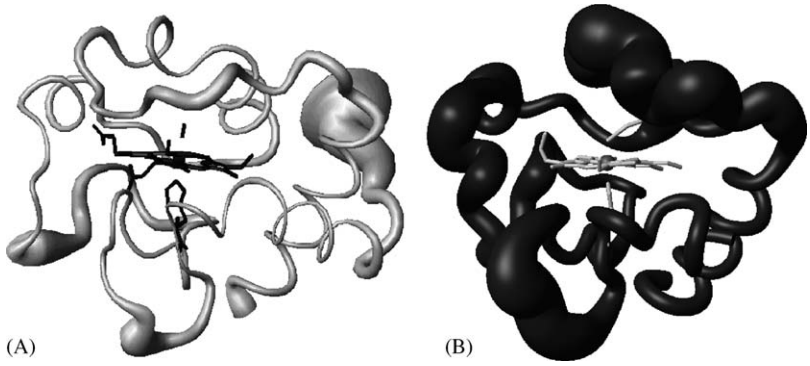

Fig. 3. Families of structures determined by NMR for cytochrome *c* forms obtained upon replacement of the native axial Met80 ligand: the structure of the cyanide adduct of Met80Ala derivative of *S. cerevisiae* protein (A); the alkaline form of the same protein determined at pH 11.1 (B). The alkaline form 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. The backbone is characterized by a large mobility, in particular in the loop regions, as it is apparent from the increased radius of the tube that is proportional to the RMSD within the family of NMR structures. Both structures have been solved for the heme iron(III) species.

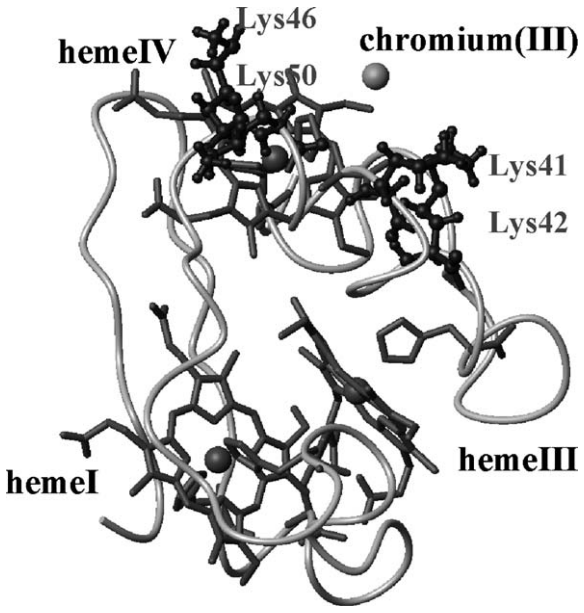

Fig. 4. The fold of the three-heme cytochrome c_7. The three heme groups are represented as black sticks. The metal reductase activity of the protein has been demonstrated by us following the reduction of chromate to chromium(III) by the fully reduced cytochrome c_7. Black ball-and-stick representation is used for the Lys residues involved in the binding of the chromate anion. The latter is then reduced to chromium(III) (grey sphere), which remains bound to the same binding site as the oxidized anion. Heme numbering follows the standard nomenclature of multiheme cytochromes.

cytochromes *c* based on the newly available genomic information, that allowed the identification of functionally relevant residues through multiple sequence alignments on mitochondrial proteins as well as the mapping of their position within the protein structure [66]. A more extensive bioinformatic search of cytochrome *c* domains starting form a much larger ensemble of quite distantly related sequences has been recently carried out [67] to provide a survey of (i) the cytochrome *c* availabilty in each organism, and (ii) the variety of proteins having a cytochrome *c* domain. The analysis of the genomic context, together with the literature data relative to the structure and function, has allowed us to put together several biochemical processes at different levels of knowledge and to focus on some interaction pathways.

The NMR validated structural model of the four-helix bundle cytochrome b_{556} can be considered an example of a post-genomic structural approach, where the homology modelling is coupled to a validation of the obtained structure by using a limited set of NMR observables and restraints [68].

The need of ^{13}C, ^{15}N labelled proteins for solution structure determination led us to optimize the procedures for cytochrome *c* expression. The problem here is the formation, during protein expression in bacteria, of the covalent and stereospecific bond between the two vinyl groups in positions 2 and 4 of the protoporphyrin IX molecule and the cysteine residues that are present in all *c*-type cytochromes in the heme-binding motif CXXCH [69,70]. A number of genes constituting the so-called cytochrome *c* maturation (Ccm) system is operating in gram-negative bacteria [71,72]. They can be efficiently be used for the production of eukaryotic *c*-type cytochromes in *Escherichia coli* [73,74]. This interest brought us to investigate the heme chaperone CcmE. We have determined the solution structure of the 132 amino acid soluble domain of a CcmE homologue from *S. oneidensis* (formerly *putrefaciens*) in its apoform. The main feature of the protein fold is the presence of a six-stranded antiparallel β-sheet that gives rise to a well packed hydrophobic core, and of unstructured N-terminal and C-terminal regions [75]. We have also performed an orthologue comparison of the sequence of this protein by alignment of the present protein with similar proteins from other organisms [75]. This approach shows that the C-terminal region is not very well conserved and is missing altogether in the *R. prowazekii* protein. We have been unable to detect heme binding to the *S. oneidensis* apoprotein. However, the analogous structural characterization of the CcmE from *E. coli* [76] has suggested that the covalent attachment of heme to an exposed histidine occurs after heme binding at the surface of the protein. A recent spectroscopic characterization of wild type and mutated proteins provided insights on the nature of the heme-binding site by pointing to possible involvement of an axial Tyr ligand for the heme iron [77]. The presence of a Tyr ligand is quite unusual in heme proteins but would be consistent with what found in another class of heme-binding proteins indicated as hemophores. The hemophore HasA (*h*eme *a*cquisition *s*ystem *A*) from *S. marcescens* has been studied by others [78–83]. We have been involved in the characterization of the structural and electronic features of the system in solution by NMR [84]. This study represents a nice example of the use of the most advanced methodological approach for the characterization of paramagnetic metalloproteins. The use of ^{13}C-direct detection allowed us to identify in NMR spectra all the residues of the protein but one and to obtain insights on the coordination environment of the heme iron(III) [84], where the binding affinity of the axial ligand Tyr74 is modulated through a hydrogen bond with the nearby His82. Breaking of this H-bond induces a change from low spin to high spin in the iron center.

The ability to refine by NMR the structural details of the available crystal structures of heme proteins taking advantage of the paramagnetism of the heme iron center was also exploited in the case of high spin iron(III) [85] and iron(II) [86] forms of myoglobin.

3.2. The case of copper

Copper is one of the metal ions essential for several fundamental biological processes in both eukaryotic and prokaryotic organisms, i.e., it is essential for life. It is used in proteins for a variety of functions from electron transfer processes to catalysis of redox reactions [87]. The entire process of copper uptake in the cell, transfer and incorporation into the final copper enzymes requires specific, selective and very efficient mechanisms [88]. The mechanisms regulating the homeostasis of copper within a cell have been the subject of many investigations in the recent past [89–92] and several intracellular copper trafficking pathways have been identified [93–96]. From unicellular organisms to specialized cells of mammals, various mechanisms have evolved to efficiently acquire and properly utilize copper. These mechanisms should meet both the requirement of copper

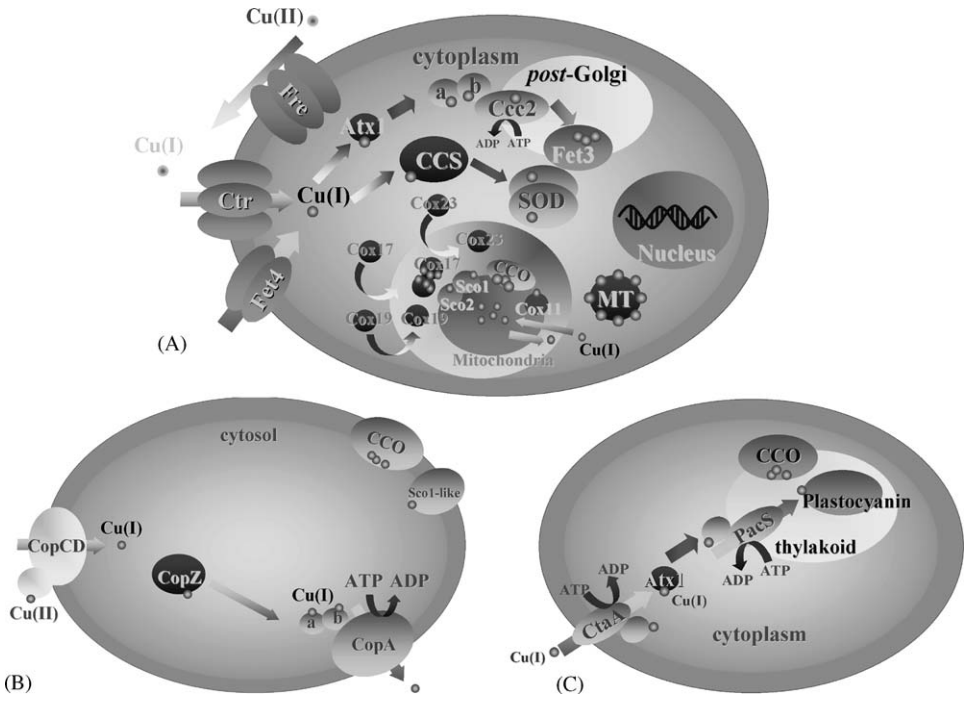

Fig. 5. Copper-trafficking pathways. (A) Known pathways for the delivery of copper in yeast are depicted. Copper is imported by the cell surface copper transporter Ctr, and is eventually deployed to mitochondrial cytochrome *c* oxidase, to cytosolic SOD, or to multicopper oxidase Fet3. There is essentially no free copper in the cytosol as the metal is scavenged by systems such as metallothioneins (MT); (B) Copper trafficking pathways in the bacterium *Bacillus subtilis* (B) and in the cyanobacterium *Synechocystis* PCC 6803 (C). In bacteria copper is imported by a specific system (CopCD or CtaA) and in the cytosol is bound by chaperones such as CopZ or Atx1. In cyanobacteria copper ATPases (PacS) are responsible for copper transfer into thylakoid organells, where copper is required for photosynthesis or aerobic respiration.

availability and easy incorporation in proteins as well as the control of the levels of free copper, which can undergo oxidative chemistry and therefore can be very toxic to the cells.

This sophisticated machinery has been deeply investigated by our group during the last few years (Fig. 5). Our projects focused on proteins involved at any level in copper homeostasis of both prokaryota and eukaryota. This definition includes essentially all copper-binding proteins in any organism. In particular, we have devoted special attention to proteins actively involved in the mechanisms of copper uptake and release, transport, and resistance. These proteins can, in fact, be regarded as the keepers of copper homeostasis in organisms, while proteins requiring copper in order to perform their function are more akin to be passive users of the system. Our structural genomic strategy consists of the structural characterization of each protein involved in a specific copper pathway, in both the metal-free and the metal-bound state, and of the study of the interaction with partner proteins in order to gain functional information on that pathway. Genome browsing and phylogenetic comparative analysis are used to identify homologous proteins of a given pathway in prokaryotic and eukaryotic organisms.

3.2.1. A copper trafficking route

A copper pathway extensively investigated in our laboratory involves copper delivery to a multicopper-ferroxidase enzyme, located in the Golgi organelle of eukaryotic cells (Fig. 5A). In a *S. cerevisiae* cell, copper transfer from the outside environment into the cytoplasm involves two membrane proteins, a high-affinity Cu transport protein (Ctr), denoted Ctr1 [97,98], and a metalloreductase Fre1, which mediates the reduction of copper(II) to copper(I) [99]. Once copper has entered the cytoplasm, soluble proteins, called metallochaperones, are used for copper ion trafficking and delivery to a number of receiving proteins [100]. The cytoplasmic metallochaperone responsible for copper transfer into the Golgi organelle is called Atx1 and it delivers copper to a P1-type ATPase, Ccc2, located in the Golgi membrane [101–103], which transfers copper to the final target Fet3, the multicopper-ferroxidase enzyme essential for high-affinity iron uptake [104].

The solution structures of Cys-reduced apo and copper(I) loaded Atx1 were solved by NMR in Florence [105] (Fig. 6A). The solution structure of *S. cerevisiae* copper(I)–Atx1 exhibits a ferredoxin-like βαββαβ-fold with the copper(I) ion coordinated by two cysteine residues, Cys15 (in loop 1) and Cys18 (in helix α1), of a conserved CXXC motif. As the copper(I) ion is not NMR accessible, we have found extremely useful to couple NMR measurements to X-ray absorption spectroscopy (XAS) [106]. The analysis of the EXAFS region of the X-ray absorption spectrum gives the type and number of metal ligands as well as bond distances. Further information about the electronic structure of the metal ion and its coordination geometry can be obtained from the analysis of the edge and pre-edge regions of the spectrum. In this way, X-ray absorption data provide the missing constraints on the metal center, which can then be used to complete the structure determination by standard NMR methods [106]. This approach has been indeed followed to obtain

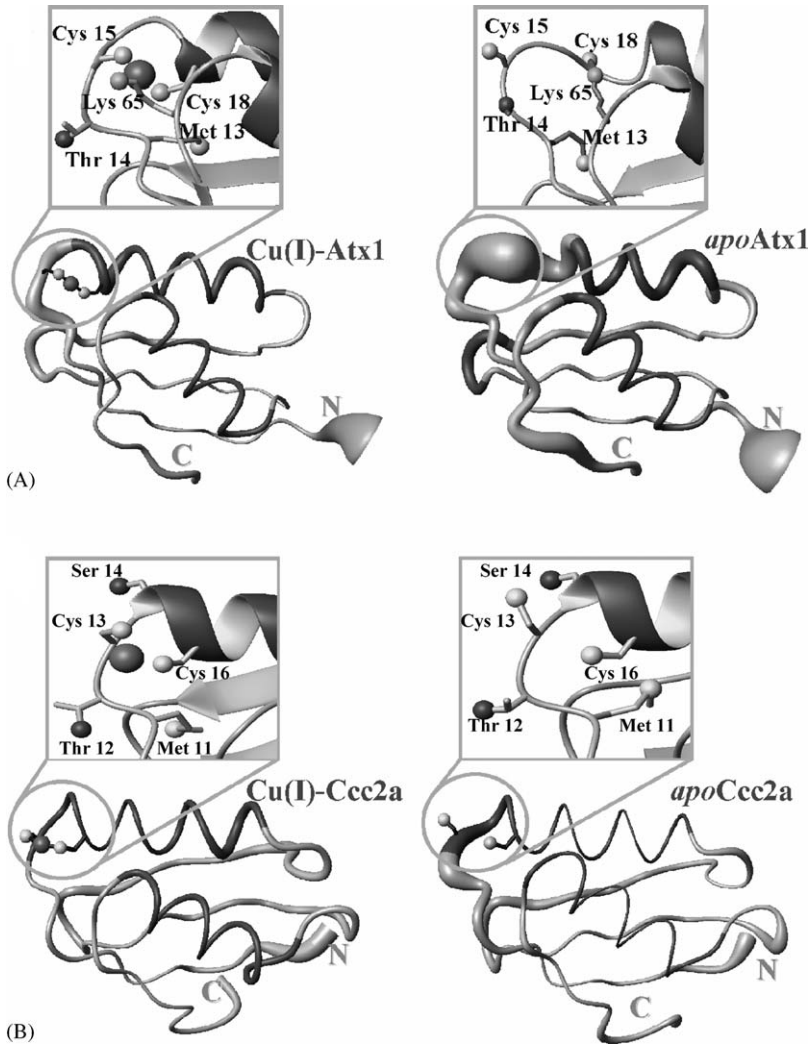

Fig. 6. Solution structures of copper(I) and apoAtx1 (A) and copper(I) and apoCcc2a (B). The structures are represented as a tube whose radius is proportional to the local backbone RMSD. The insets show the copper-binding site where copper is represented as a grey sphere. Copper-binding cysteines and other neighboring residues are also indicated.

information on the coordination sphere of copper in order to determine the structure of several copper(I) and copper(II) transport proteins reported in this review.

Once received copper(I) from Ctr1 [107], Atx1 transfers copper(I) to the N-terminal domain of Ccc2 [108,109]. This belongs to the family of P-type ATPases, i.e., membrane bound proteins that comprise eight transmembrane helices presumably forming the ionic pathway across the membrane. Among these, the 6th helix bears a CPC motif, which is thought to bind the metal to be transported [110]. In addition to the membrane domain, a cytoplasmic domain of about 400 amino acids has the ATPase activity and the cytoplasmic N-terminus comprises one or two metal binding domains in prokaryotes and up to six in eukaryotes. The N-terminal copper-binding domain of Ccc2 contains two repetitive sequences, each bearing the conserved sequence motif CXXC, capable of binding one equivalent of copper(I). The structure of the first domain of Ccc2, Ccc2a, was also solved by NMR [111] and again exhibits a ferredoxin-like βαββαβ, fold similar to that of its partner Atx1 (Fig. 6B). An ortholog search showed that proteins homologous to yeast Atx1 and Ccc2 are found in all eukaryotes and in many bacteria [112], the latter being very diversified and possibly having developed alternative routes for copper homeostasis. For example, *B. subtilis* possesses a protein, named CopZ [113], which acts as chaperone, as well as another protein, named CopA, which is an ATPase homologous to yeast Ccc2 and containing two soluble metal binding domains in the N-terminal region [114] (Fig. 5B). The solution structures of CopZ and of the N-terminal region of CopA have been also determined, showing that they have the same fold of the yeast homologues with similar copper binding properties [113–116]. However, differently from the yeast Atx1/Ccc2 copper pathway, the CopZ/CopA proteins work in a copper detoxification process [117] (Fig. 5).

Another attractive bacterial system is represented by cyanobacterial organisms, as they have peculiar cellular organization involving organelles called thylakoids [118]. The latter contain cytochrome *c* oxidase and plastocyanin, both proteins requiring copper to function (Fig. 5C). In the cyanobateria *Synechocistis*, two copper transporting ATPases homologous to Ccc2 are present, CtaA and PacS [119]. The former is located

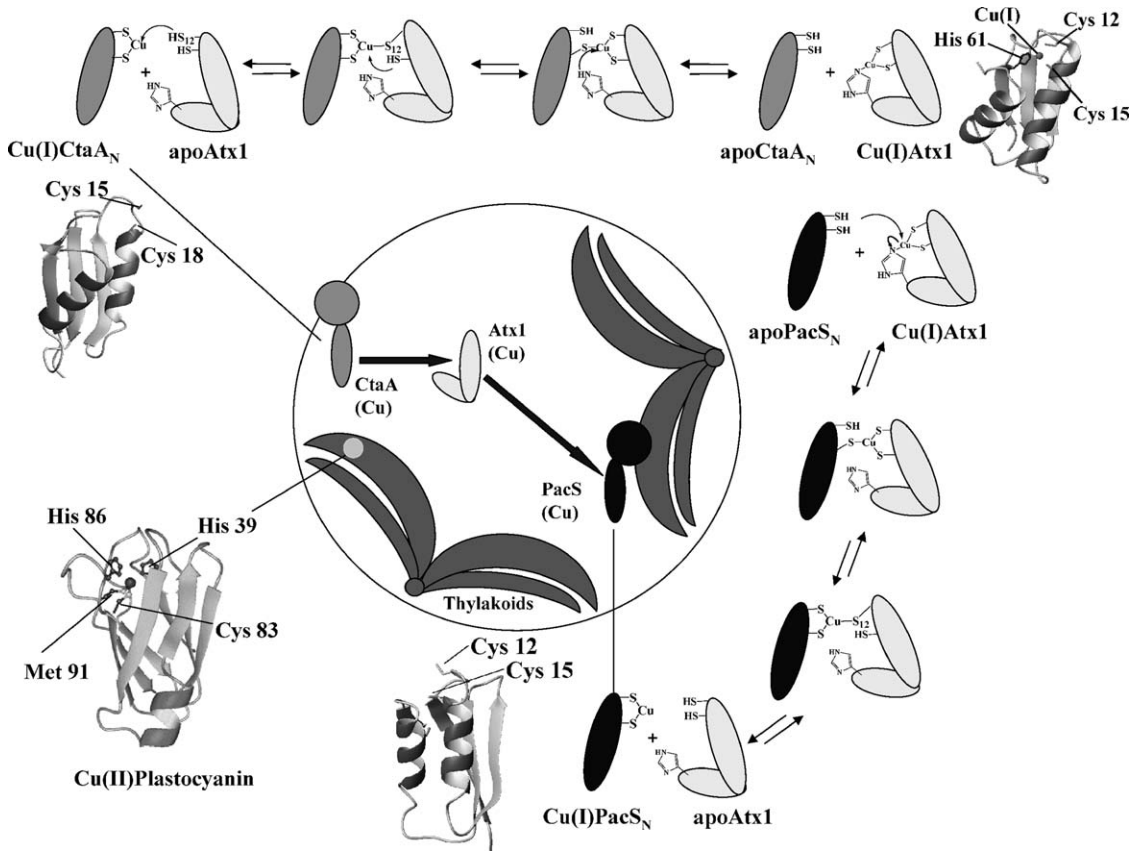

Fig. 7. Proposed pathway for copper transfer from CtaA to PacS through Atx1 in the cyanobacterium *Synechocystis*. This model implies the order of copper(I) association with Atx1 ligands to be (1) Cys12, (2) Cys15 and finally (3) His61 during acquisition from CtaA$_N$ with the converse order of ligand release upon interaction with PacS$_N$. The solution structures of copper(I)Atx1, apoPacS$_N$, copper(II)–plastocyanin and the structural model of CtaA$_N$ are shown.

on the external cellular membrane and is involved in copper uptake, while the latter is on the thylakoid membrane and transports copper inside this organelle for its delivery to copper-requiring proteins [118] (Fig. 5C). Copper is shuttled between these two ATPases by a copper chaperone, homologue to yeast Atx1, which is able to interact with the soluble copper binding domains of both ATPases [120]. Interestingly, this copper chaperone has the same ferredoxin-like fold of yeast Atx1 but with a His residue on loop 5 in a position where yeast Atx1 has a Lys residue, fully conserved in eukaryotic metallochaperones [121]. The solution structure of Atx1 from *Synechocystis* shows that the imidazole ring of the latter His protrudes from loop 5 of the cyanobacterial protein providing the third copper ligand [121,122] (Fig. 7). This residue is proposed to influence the direction of the metal transfer in the interaction of the chaperone with the amino-terminal domains of the two ATPases, PacS and CtaA [118] (Fig. 7). Once copper enters into the thylakoid lumen via PacS ATPase, it is acquired by plastocyanin, a copper-containing electron transfer protein necessary for photosynthetic electron transport of cyanobacteria [118]. In its biological function, the copper ion switches between the +1 and +2 states. The solution structure of plastocyanin from *Synechocystis* in the reduced copper(I) and oxidized copper(II) states was solved in our lab in 2001 (Fig. 7) [123,124]. The latter represents the first solution structure of a paramagnetic copper(II) protein.

In conclusion, bacterial and yeast organisms exploit structurally similar copper proteins in different copper pathways: for example, the yeast chaperone Atx1 brings copper to Ccc2 ATPase for the final copper insertion into Fet3, while *B. subtilis* chaperone CopZ brings copper to CopA ATPase in a detoxification process. These findings suggest that the structural characterization of proteins by itself is not sufficient for a full description of the copper trafficking mechanisms, but it needs to be accompanied by functional in vivo and in vitro data as well as by the understanding, at atomic detail, of how the copper ion(s) is passed from one transporter to another, until the final protein target. Therefore, the characterization of the protein–protein interactions at the atomic level is an essential step for the comprehension of these complex cellular mechanisms, and NMR spectroscopy represents an extraordinary, and probably unique, tool in this respect. Indeed, NMR can detect the often weak interactions between proteins, which give rise to short-lived protein complexes that could not be characterized by X-ray crystallography, more prone to characterize long lived complexes originated by strong intermolecular interactions. Going back to the yeast pathway and looking at the electrostatic potential surfaces of Atx1 and of Ccc2 domains, it appears that the surface of Atx1 is positively charged in the vicinity of the copper binding site, whereas, those of the Ccc2 domains are negative [105,111]. The electrostatic interaction is the long distance driving force that helps to bring the two partner

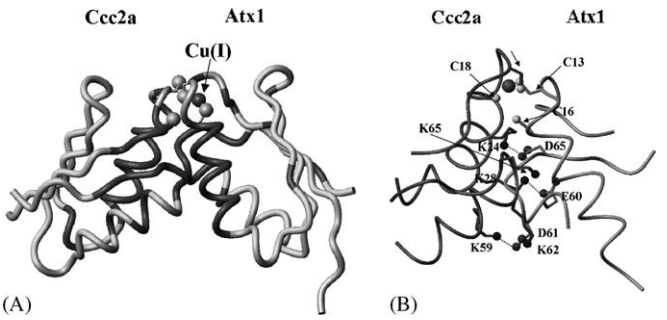

Fig. 8. Schematic drawing of the Atx1–Ccc2 adduct model. (A) The residues experiencing significant NHs chemical shift changes upon protein–protein interaction are indicated in the protein adduct. (B) Conserved hydrogen bonding interactions in the structures of the Atx1–Ccc2 complex generated by HADDOCK. The copper(I) ion is represented as a grey sphere. Residues involved in hydrogen bonding interactions and the copper binding cysteines are indicated.

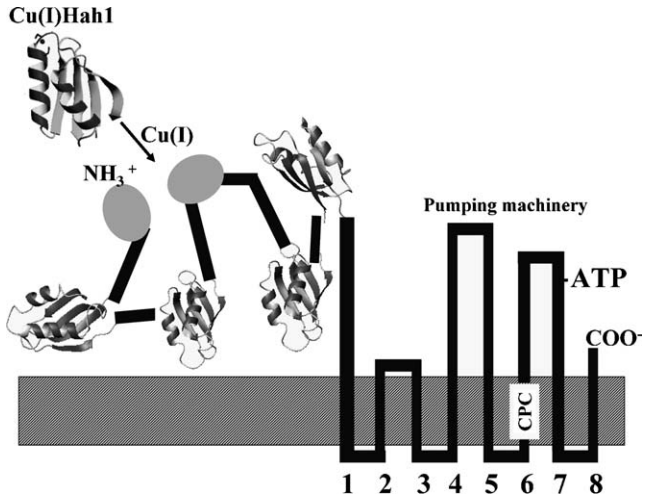

Fig. 9. Scheme of the ATP7A (Menkes disease) protein. The structures of soluble metal binding domains and of the human copper chaperone Hah1, solved in Florence, are shown. Grey circles represent soluble metal binding domains whose structures have been solved in other labs. The intra-membrane metal-binding site and the ATP-binding site are also shown. The N- and C-termini are labeled. The copper(I) ion and the copper binding cysteines of Hah1 are shown.

proteins together. NMR-based protein–protein interaction [125] and docking studies [126] on the Atx1–Ccc2 protein complex show, indeed, that three conserved Lys residues of Atx1 interact with three conserved Asp/Glu residues of Ccc2a (Fig. 8). Therefore, a low activation barrier for copper transfer between partners results from complementary electrostatic forces that orient the metal-binding loops of both interacting partners causing the formation of copper-bridged intermediates [102,109]. The exposed location of the metal binding sites of both proteins allows copper to expand its coordination sphere, thus becoming three-coordinated and efficiently allowing the copper transfer between the partners. As a final result, the rate of copper transfer between physiological partners increases with respect to that of copper transfer from small complexes and, in this sense, chaperones of metal ions work like enzymes, lowering the energetic barriers along a specific reaction pathway [109]. Analogously, NMR protein–protein interaction [127] and in vivo studies [117] show that CopZ donates copper to the N-terminal domain of CopA to be expelled outside of the cell (Fig. 5). It is peculiar that CopZ has opposite overall charge with respect to its eukaryotic homolog Atx1 and, not surprisingly, opposite charges are displayed by the two metal binding domains of the ATPase [112].

In humans, two P-type ATPases, Menkes (MNK) [128–130] and Wilson (WND) [131,132] proteins, and a copper chaperone, Hah1 [133], homologous to yeast Ccc2 and Atx1 respectively, are present. The key role of MNK and WND in the cellular copper trafficking is strongly determined by their intracellular localization mechanism by which these proteins manage to deliver copper to newly synthesized copper proteins [134,135]. At low copper concentration MNK and WND proteins are localized in the Golgi, where MNK transfers copper to a lysyl oxidase, which is responsible for cross-linking collagen and elastin. At high copper concentrations, MNK moves to the external cell membrane and pumps the excess of copper out of the cell. When the intracellular copper levels are reduced, the protein returns to the Golgi membrane. WND, also located in the Golgi membrane, incorporates copper into ceruloplasmin, a multicopper oxidase similar to Fet3, at low copper levels, while at high copper concentrations, WND moves from the Golgi membrane to a vesicular compartment where it releases copper. The copper-loaded vesicles are then discharged into the bile [134]. Therefore, humans possess both previously described yeast and bacterial copper pathways, i.e., a copper transport to a multicopper oxidase and a detoxification process. Interestingly, both human ATPase proteins have six soluble metal binding domains at the N-terminal region, while their bacterial and yeast homologous have only one or two domains, suggesting that most of the metal binding domains are not essential for the copper transport function. The soluble metal-binding domains in human proteins were indeed suggested to be involved in the copper-dependent regulation, where the 5th and 6th have a major role [136]. However, the need of six domains and their role is still not fully understood. The six metal binding domains of MNK and WND are connected by linkers of different length [137]. The longest flexible linker separates metal binding domains 1–4 from domains 5 and 6 [112]. The structures of a number of single metal binding domains of MNK [138–142] as well as of the copper chaperone Hah1 [143,144] are available (Fig. 9) and NMR interaction studies between Hah1 and single domains of the Menkes protein have been also performed [142], showing copper transfer between the two proteins without the formation of a stable protein–protein complex. The other domains of MNK and WND have been modeled [112] (and deposited at http://www.postgenomicnmr.net/Structures). All the domains share a βαββαβ fold but display different surface charges. Interestingly, it has been experimentally observed that the copper chaperone Hah1 interacts preferentially with domains 1–4 of WND [145]. It has been therefore proposed that domains 5 and 6 may receive copper from domains 1–4 [137]. From the experimental data so far available, the necessity emerges of structural studies involving multidomain constructs of the N-terminal domain as well as of interaction studies between the chaperone Hah1 and the latter constructs. They are indeed essential to understand at the molecular level the specific role of

the N-terminal domain in the structural "mechanics" of copper transport by human ATPases.

The advances in knowledge of the function of MNK and WND are particularly driven by the fact that they are involved in two human disorders of copper transport [146]. Mutations of MNK in humans cause a copper deficiency disorder, the Menkes disease, and mutations of WND result in a Cu toxicity condition, the Wilson disease. Different types of mutations have been identified which alter the copper metabolism in the Golgi (http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html). These mutations are mainly located in the transmembrane portion of the ATPases, with a few present in the soluble metal binding and ATP-binding domains. The first dynamic and structural study of a disease-causing A629P mutation, which occurs in the last of the six soluble copper(I)-binding domains of the Menkes ATPase, seems to indicate that the disease-causing A629P mutation does not significantly affect the structural properties of MNK6, but it might influence its stability with respect to protein unfolding and degradation [141].

Finally, we have also devoted some attention to the yeast copper storage/detoxification protein metallothionein (MT in Fig. 5). This very small (53 AA), cysteine-rich protein is able to bind and store seven copper(I) ions. The protein had been known for 30 years, and yet its full structural characterization was lacking. The high-resolution solution structure of the polypeptide chain was solved by us in the year 2000. However, no clues on the detailed arrangement of the seven copper ions could be obtained. The NMR structure revealed mobility of the first four and last 13 aminoacids, apparently not involved in copper binding. We then prepared a truncated form (36 AA) of the protein encompassing the whole well-structured part, showed by NMR that its fold was identical to that of the full-length protein [147], and finally succeeded in obtaining crystals and a high resolution X-ray stucture [148]. The backbone structure was almost identical to the solution structure but, surprisingly, the protein contained eight copper(II) ions. Comparison of the solution and X-ray stuctures allowed us to identify the eigth, more labile copper(I) ion, and to hypothesize a mechanism for its uptake, implying that copper(I) binding to MT is not of an "all or none" type.

3.2.2. *Cytochrome c oxidase*

Cytochrome *c* oxidase (COX) is a key component of the respiratory chain that reduces oxygen to water and generates the proton gradient driving ATP synthesis, a crucial step in cellular energy metabolism. The COX enzyme is a multi–subunit complex [149,150] that requires a large protein machinery for its assembly [151]. It also contains several metal cofactors, whose insertion and binding in the proper subunit is needed to produce the final, active enzyme [152]. Biochemical studies of COX deficient patients suggest that a failure to assemble the holoenzyme might underlie the majority of inherited COX deficiencies [151,153].

Over 30 accessory proteins are necessary for the proper assembly of the complex [154] and the final maturation of the enzyme [155]. The assembly is dependent on the insertion of metal cofactors necessary for function. The Cox1 subunit contains a binuclear iron/copper center [150], that is formed by a heme a_3 moiety coupled to a copper ion, designated Cu_B (Fig. 10) and characterized by a $HX_3YX_{44}HH$ binding motif. The heme a_3–Cu_B center is buried 13 Å below the membrane surface in a transmembrane region of Cox1. This subunit also contains another heme *a* cofactor. The Cox2 subunit contains a copper center, designated Cu_A (Fig. 10), that is formed by two copper ions bound to two His and two bridging Cys residues of the $HX_nCXEXCGX_2HX_2M$ consensus motif. This binuclear, mixed-valence copper center is located in a soluble domain of Cox2 protruding into the intermembrane space of mitochondria. The Cu_A center receives the electron from cytochrome *c* (cyt *c*) and transfers it to heme *a* of Cox1 and finally to the heme a_3–Cu_B center [156].

In order to describe the complex process of cofactor incorporation in COX, we have focused our work on proteins relevant for copper incorporation. Up to now, five accessory proteins (Cox11, Cox17, Cox19, Cox23 and Sco1) are known to be implicated in copper ion delivery and insertion into mitochondrial

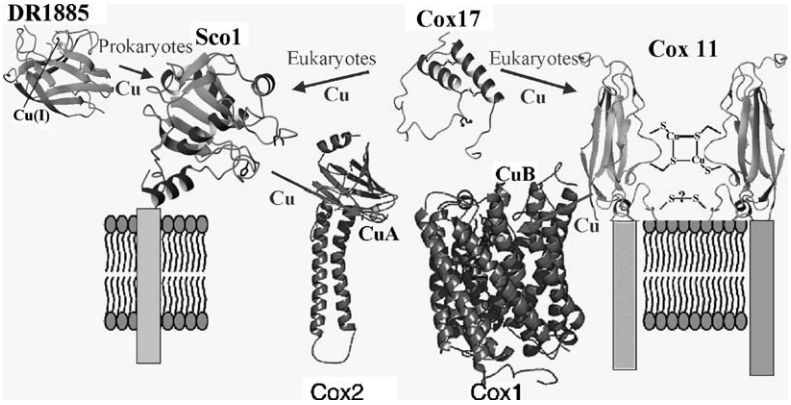

Fig. 10. Scheme of copper metallation of subunits I and II (Cox1 and Cox2, respectively) of cytochrome *c* oxidase. Cox11 and Sco1 mediate the copper ion loading in the Cu_B (within Cox1) and Cu_A (within Cox2) sites of cytocrome *c* oxidase, respectively. Cox17 has been suggested to transfer copper(I) to Sco1 as well as Cox11. A newly identified protein, called DR1885 in *Deinococcus radiodurans*, may take the role of Cox17 in bacteria and archaea donating one copper(I) ion to Sco1.

COX in eukaryotes [152]. Cox17 is a soluble protein of ∼70 residues, including six conserved cysteines able to bind copper(I). Cox17 transfers copper(I) to both Sco1 and Cox11 [157], which function downstream as membrane proteins in copper insertion into Cu_A and Cu_B, respectively. The specific role of Cox19 and Cox23 is not clear, but the sequence similarity with Cox17 suggests that they are also capable of binding copper(I). Sco1 and, to a lesser extent, Cox11 homologues are found in some prokaryotes, at variance with Cox17, Cox19 and Cox23, which have no prokaryotic homologs. These variations could be ascribed to the different copper availability, redox properties and copper trafficking routes of the cellular compartment where the soluble components of the COX enzyme are located (i.e., the periplasm in gram-negative bacteria, the outer side of the cytoplasmic membrane in gram-positive bacteria and the intermembrane space in mitochondria).

We have characterized the Cox17 protein from *S. cerevisiae* under various states, i.e., apo Cys-reduced, apo Cys-oxidized, Cu_1(I) Cys-oxidized and Cu_4(I) Cys-reduced oligomer. The NMR structure of apoCox17 is characterized by a two-helix domain stabilized by two disulfide bonds between two pairs of cysteines of the twin Cx_9C motif found also in other proteins involved in copper incorporation in COX [158], and is remarkably similar to that of the Cox12 subunit of COX. Cox12 docks to Cox2 and is involved in interactions at the dimer interface of the enzyme complex [150]. The oxidized Cox17 conformer is competent in the binding of a single copper(I) ion by a CX_2C motif in a digonal coordination [159], but this requires prior isomerization of one disulfide bond. The latter form is analogous to the recently reported structure of Cu_1Cox17 conformer [159], except that the present conformer is constrained by two disulfide bonds [158]. On the other hand, full reduction of the two disulfide bonds leads the protein, reduced apoCox17, into a molten globule state capable of binding a cluster of four copper(I) ions [158], thereby inducing protein oligomerization. We proposed that the polycuprous cluster conformer may be a storage form of copper(I) within the intermembrane space, whereas, the form that binds a single copper(I) ion may be a copper donor to Sco1 and Cox11 (Fig. 10) [158]. The two latter proteins contain a single transmembrane helix in the N-terminal segment that anchors them to the inner mitochondrial membrane (Fig. 10). The structures of the soluble portions of bacterial homologues of Sco1 [160] and Cox11 [161] from *Bacillus subtilis* and *Sinorhizobium meliloti*, respectively, have been solved by NMR. Sco1 is involved in copper delivery to Cu_A and is characterized by a thioredoxin-like fold, with eight β strands and four α helices [160] (Fig. 10). The copper(I) binding CXXXC consensus motif is located in a loop region between β4 and α1, which is structurally close to the loop between α3 and β7 containing another potential copper ligand, i.e., a conserved histidine residue. Sco1 from *Bacillus subtilis* is able to bind both copper(I) and copper(II). The latter is in a characteristic cysteinate coordination, as deduced by optical and EPR spectra [160]. On the basis of fold similarities with thioredoxins, a thiol-disulfide oxidoreductase function has been suggested for Sco1, which may have a role in the reduction of disulfide bonds of Cu_A prior to copper insertion [160]. Accordingly, the recent X-ray structure of Sco from *B. subtilis* identifies a disulfide switch demonstrating that Sco has the capability to fulfill a redox role in Cu_A assembly [162]. A redox signaling function has also been postulated for the protein on the basis of hydrogen peroxide sensitivity of yeast mutants lacking Sco1 [163].

The nature of Sco1 as a multifunctional protein is supported by a bioinformatic analysis [164]. A genome-wide search in prokaryotic genomes show that multiple Sco1-like sequences can be found in a single organism in different genomic contexts [164]. Also some eukaryotes, including humans, contain a second Sco protein, called Sco2, whose role in copper insertion into Cu_A is not yet fully elucidated [165].

Neighbor genes of prokaryotic Sco's often encode cuproenzymes and cytochrome *c* domains and, in some cases, the Sco1 gene is fused to cytochrome *c* [67,164]. This suggests a functional correlation between these proteins. From the same bioinformatic analysis we have identified a conserved bacterial protein called DR1885 in *Deinococcus radiodurans* [164,166] that possesses a cupredoxin-like fold and a $H(M)X_{10}MX_{21}HXM$ motif able to bind copper(I) [166] (Fig. 10). The surface location of the copper(I) binding site, similar to that of the copper resistance protein CopC (see next section), as well as the type of coordination, are well poised for metal transfer chemistry. As there are no prokaryotic homologues of the mitochondrial copper chaperone Cox17, the protein DR 1885 may take the role of Cox17 in bacteria and archaea and may donate one copper(I) ion to Sco1 (Fig. 10). On the basis of our structural and genomic analysis, we have drawn some hypotheses on the mechanism of copper insertion into Cu_A. This metal center is binuclear, and therefore, once Sco1 has provided one copper(I), it may be possible that copper(II), which is available in the oxidizing extracytoplasmic environment of bacteria, enters the second site of Cu_A, forming the binuclear mixed-valence units [166]. In accordance with this scheme, kinetic and copper binding studies on a Cu_A center engineered into an azurin have shown that the formation of a copper(I)–thiolate center is observed and that, following the addition of copper(II), the mononuclear intermediate converts into the final Cu_A center [167].

Formation of the Cu_B site is dependent on Cox11. This protein has an immunoglobulin-like fold consisting of ten β strands organized into two β sheets [161] (Fig. 10). The consensus motif for copper binding CFCF is located in a loop between two short β strands on one side of the barrel. In the presence of copper(I) ions and in the absence of dithiothreitol, that is commonly used as reducing agent, Cox11 becomes dimeric (Fig. 10). EXAFS data are consistent with a binuclear copper(I) cluster where the two copper(I) ions are at 2.7 Å distance from each other and coordinated by three sulfur atoms [161]. A cysteine residue of the CFCF motif of one monomer acts as a monodentate ligand to one copper(I) and the other cysteine acts as a bridging bidentate ligand to both copper(I) ions (Fig. 10). A third conserved N-terminal cysteine, which is located far from the CFCF binding site and in an unstructured region close to the transmembrane anchor, is not involved in copper binding but rather could form an intermolecular disulfide bond with the corresponding cysteine of the other monomer (Fig. 10). Some structurally related immunoglobulin proteins are known to dimerize through

a similar disulfide bond [168]. The dimeric state of Cox11 may protect the copper(I) ions before the transfer to the Cu_B center (Fig. 10).

A particularly challenging problem appears the loading of a copper ion in the Cu_B center of Cox1, as this site is buried 13 Å below the membrane surface. Evidence for the association of Cox11 with mitochondrial ribosomes [169] has recently provided a clue toward the understanding of this process. The Cu_B site might be co-translationally formed by a transient interaction between Cox11 and the nascent Cox1 in the intermembrane space. Upon transfer of a copper ion, the nascent Cox1 is pushed further into the inner membrane and the Cu_B site moves into the lipid bilayer. The dimeric state of Cox11 may be disrupted during the interaction with Cox1, and a Cox11/Cox1 heterodimer may be formed. As functional COX acts as a dimer [150], the second copper(I) ion of the Cox11 dimer could concomitantly be inserted into another nascent Cox1.

Our studies, which provided the elucidation of the molecular architecture and coordination chemistry of accessory proteins involved in copper delivery to this fundamental enzyme, are contributing to unravel the puzzling aspects of COX complex assembly. A future goal is to provide a detailed description of the molecular mechanisms underlying this process through high-resolution structural studies of protein–protein interactions occurring between the accessory components and the enzyme subunits.

3.2.3. Copper resistance in bacteria

Copper is widely used in agriculture and present around mining industry, and therefore it may exist at high levels in some environments. Even if copper is an essential element, at high concentrations it can be toxic to all branches of life including bacteria and, indeed, it is used in agriculture as bactericide [137]. Bacteria, however, have developed several mechanisms to detoxify or to tolerate this metal ion as well as other heavy metals (see Section 3.6) [170]. Several pathways for resistance to the heavy transition metal ions have been indeed determined in a wide variety of organisms. Metal resistance determinants were initially found on bacterial plasmids. Later, after the genome sequences of several bacteria and eukaryotes became available, several more metal resistance systems were found mainly in bacteria, plants and yeast [171]. Many of these detoxification mechanisms are not entirely specific towards a given metal and are used for a variety of metals. Although there are a number of possible ways in which cells can become resistant, the most frequent are those that involve transport, either through the loss of an uptake system or through the development of an efflux system for the toxic metals [171]. Another widespread resistance mechanism consists of trapping the metal in specific cell compartments, called vacuoles, or of expressing metallothioneins for metal chelation. The intake and subsequent efflux may include a redox reaction involving the metal. This is an important implication because the oxidation state of a metal ion relates to its solubility and toxicity [172]. The interactions between metal ions and bacterial systems involved in metal tolerance have important environmental and health implications, such as the use of bacteria to clean up metal-contaminated sites [172]. In an attempt to understand the structural and functional basis of copper resistance, we have studied the molecular structure and the copper binding properties of two key components of the *cop* and *cut* systems, i.e., the proteins CopC from *P. syringae* and CutA1 from *E. coli*.

The structure of the metal-free form of CopC has been solved by NMR [173]. It shows a Greek key β-barrel, similar to that of blue-copper proteins, constituted by two β sheets that involve nine strands (Fig. 11). CopC also shows a loop region containing four Met residues arranged as $[M(X)_nM]_m$ motifs, which are found in other proteins involved in copper homeostasis. The interaction with copper, studied by NMR, suggests a very

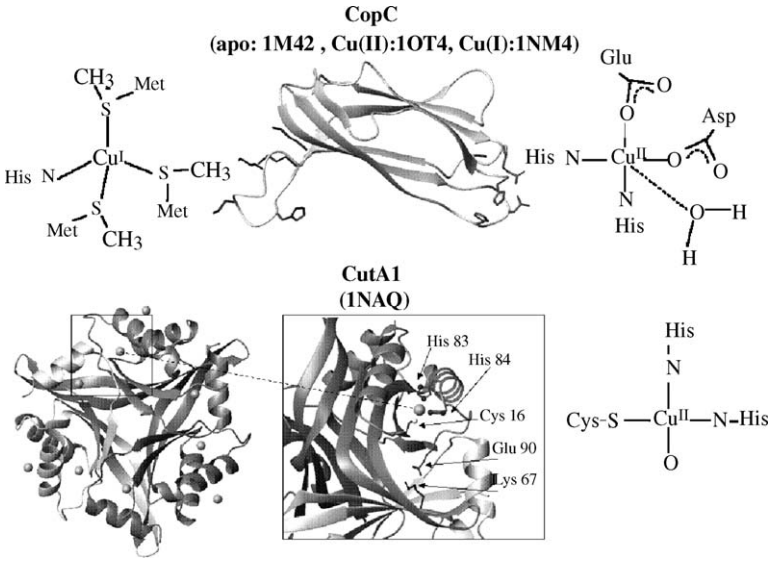

Fig. 11. Structures and copper binding sites of bacterial proteins involved in copper tolerance: solution structure of CopC from *P. syringae* (top) and crystal structure of CutA1 from *E. coli* (bottom). The PDB codes are reported in brackets. The side chains of residues involved in metal binding are shown as sticks. The potential copper(II) binding site of CutA1 is shown in the inset and the potential ligands are indicated. The side chains of Glu90 of one monomer and Lys67 of the adjacent monomer, which form an inter-subunit salt bridge, are also shown. The copper coordination for each site is schematically shown next to the corresponding structures.

peculiar and unprecedented behavior of CopC. The protein has two completely distinct copper binding sites, occupied either one at a time or simultaneously. They are specifically designed to selectively bind two copper ions, one in the reduced state and the other in the oxidized one. The copper binding sites have been characterized through X-ray absorption techniques and, in the case of copper(II), also using complementary information obtained from EPR and NMRD (nuclear magnetic relaxation dispersion) experiments [174]. The synergism of EXAFS and NMR techniques allowed us to solve the solution structure of copper(I)–CopC [175]. In the case of copper(II)–CopC extensive paramagnetic broadening of ^{1}H NMR signals occurs around the copper(II) binding site, within a sphere of about 10–11 Å centered on copper. For this reason, we have exploited purely heteronuclear ^{13}C,^{15}N-tailored experiments that reduce the limit of detectability of cross peaks in multidimensional NMR spectra to a sphere of about 5–6 Å radius, so that only residues directly bound to copper(II) still escape detection [174].

The two copper binding sites found in CopC represents two novel copper(I) and copper(II) coordination environments. Copper(I) is bound to three out of four Met residues (40, 43, 46, 51) that are clustered in the Met-rich region, and to a histidyl nitrogen of His 48, in a tetrahedral geometry [175] (Fig. 11). The copper(II) site, in solution, on the basis of EPR [173,176] and XAS [174] data, resulted to have a tetragonal ligand environment involving at least two histidine residues (1 and 91) which are fully conserved in homologous sequences. NMRD [177] measurements indicate the presence of a semicoordinated water molecule, in fast exchange with the bulk solvent, at 2.8 Å from the metal (Cu–O distance) and roughly orthogonal to the plane identified by the other four ligands [174] (Fig. 11). Possibly, Asp89 and Glu27 are two more ligands. The two copper sites are about 30 Å apart. The copper(II) site is located at the N-terminal end of the barrel, in a region encompassing some loops connecting various β-strands [173,174], similar to that of the redox site in cupredoxins, while the copper(I) site is located at the opposite end of the barrel [175]. Oxidation or reduction causes copper to be shuttled from one site to the other indicating a mechanism for copper trafficking that is activated by a redox switch [175,178].

The other investigated protein, CutA1, belongs to a family of proteins present in bacteria, plants and animals, including humans. CutA1 was originally identified in a *E. coli* gene locus, called *cutA*, which also includes two genes encoding a disulfide isomerase and an inner membrane protein, called CutA2 and CutA3 respectively. We have determined the X-ray structures of CutA1 from *E. coli* and from rat [179]. Both proteins are trimeric in the crystals, and also in solution, through inter-subunit β-sheet formation. Each subunit consists of a ferredoxin-like βαββαβ fold with an additional strand (β5), a C-terminal α-helix (α3) and an unusual extended β-hairpin involving strands β2 and β3 (Fig. 11). *E. coli* CutA1 is able to bind copper(II) in vitro in a square planar type II coordination site, as deduced by EPR and optical spectra [179]. Comparison of the EXAFS and crystallographic results suggests that copper(II) can bind Cys16, His83 and His84. The fourth candidate ligand is either a water molecule or a backbone carbonyl group or the carboxylate of Glu90, which is engaged in a salt bridge with Lys67 of another subunit (Fig. 11). In this latter hypothesis, copper(II) binding would break the salt bridge, thus destabilizing the interaction between two adjacent subunits.

Despite a very low sequence similarity, the trimeric assembly of CutA1 is remarkably similar to the architecture of PII signal transduction proteins [180], which are involved in the nitrogen signal cascade for the regulation of ammonium transport [181,182]. This structural similarity suggests an intriguing role of CutA1 proteins in signal transduction and metal regulation through allosteric communications between subunits. In *E. coli*, CutA1 is implicated in divalent cation tolerance in cooperation with CutA2 [183]. Given the role of CutA2 as disulfide isomerase [184], we have suggested that CutA1 may be involved in the tuning of a disulfide bond cascade [179], e.g., in the modulation of the redox state of thiol groups of metal binding CXXC motifs [112], which are found in copper(I)-ATPases (Section 3.2.1.) as well as in zinc(II)/cadmium(II)-ATPases (Section 3.6), thus justifying the absence of metal ion specificity of the *cut* system.

3.2.4. The world of SOD1

One of the terminal enzymes of the copper trafficking delivery system is copper, zinc superoxide dismutase (SOD1). SOD1 is a 32 kDa homodimeric protein that efficiently catalyses the dismutation of superoxide anions into oxygen and hydrogen peroxide [185–188]. The protein has been extensively characterized from the structural point of view since many years by X-ray crystallography [189–201]. Each monomer has a classical Greek key β-barrel fold and contains one copper ion and one zinc ion. In the oxidized enzyme, copper(II) is coordinated by four histidines (His46, 48, 120, and 63), one of which (His63) makes a bridge between copper and zinc. The zinc ion is bound to three histidines (His63, 71, and 80) and to an aspartate residue (Asp83). In the reduced form, the bridge between copper(I) and zinc(II) is broken at the Cu–Nε2 bond [202,203]. The copper site is accessible to the solvent through a channel, which provides the optimal electrostatic potential for the diffusion of the substrate superoxide to the reaction site. The catalytic cycle is characterized by a ping-pong mechanism in which the copper ion cycles between the copper(I) and copper(II) oxidation states [204].

SOD1 is a most studied enzyme in our lab: in the 1980s, our efforts have been devoted to the characterization of an ample ensemble of protein variants through site directed mutagenesis in the active channel with the aim to unravel the role of a number of key residues [205–214]. In addition, our lab has been able to produce a monomeric form of the protein through conversion of the two hydrophobic residues, Phe50 and Gly51, located at the subunit-subunit interface, into two negative hydrophilic Glu groups [215]. These mutations produce a protein characterized by a reduced activity with respect to the wild type protein; in order to partially recover the catalytic activity, Glu133, a residue of the active site channel, has been changed to a neutral Gln [215]. Availability of a monomeric protein of "only" 16 kDa has allowed us to afford the solution structure determination of SOD1 [216,217] (Fig. 12). With subsequent technological

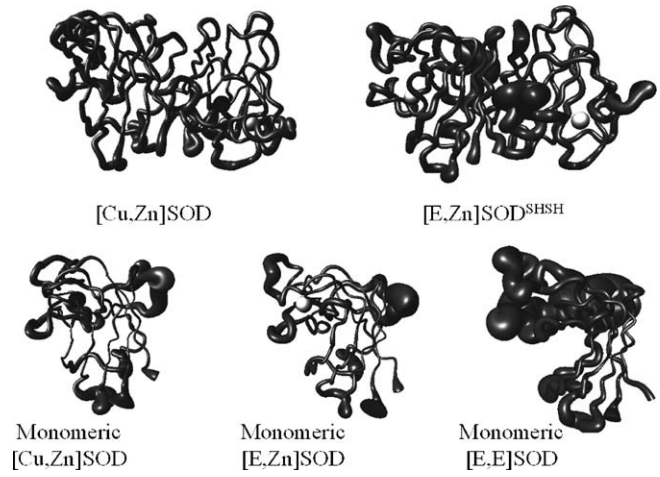

Fig. 12. The solution structures of the following forms of human SOD1 are reported in a counter-clockwise order, starting from top left: dimeric Cu_2Zn_2SOD, monomeric CuZnSOD, monomeric EZnSOD (where E indicate an empty metal binding site), monomeric EESOD, dimeric E_2Zn_2SOD with reduced disulphide bridge between Cys57 and Cys146.

advancements, we have been able to structurally characterized the dimeric enzyme as well [218] (Fig. 12).

Most recently, we have reframed our long-term research on SOD1 in a genomic approach by studying the protein under different metallation states in order to unravel the mechanism of metal cofactor assembly (Fig. 12). The ability of zinc to stabilize long loop areas (the so-called zinc and electrostatic loops) in the protein has clearly emerged from the characterization of the dynamics of the apo and zinc-only protein forms [219,220]. While the presence of copper has little effect on the conformational properties of the protein, the understanding of its insertion in the protein represents a key question. In fact, it is linked to the understanding of the functional reason for the presence of a conserved intra-subunit disulfide bond that seems not to possess any stabilizing role for the protein secondary and tertiary structure. This disulfide bridge in the reducing environment of the cytosol is an extremely conserved feature of eukaryotic and prokaryotic CuZnSOD. We have shown a correlation between the quaternary interactions between monomers and the presence of metal ions. Indeed, when in the apo form the disulfide bridge is reduced, the protein is in a monomeric state [221]. However, binding of zinc stabilizes the dimeric form even with the reduced disulfide bond [222]. The structural interplay of conserved disulfide bond and metal-site occupancy in human CuZn superoxide dismutase is of increasing interest as these post-translational modifications are known to dramatically alter the catalytic activity, the subcellular localization, and the susceptibility of the protein to aggregation. The understanding of the details of copper insertion and disulfide bond formation is most probably linked to the understanding of the role of the CCS protein in the formation of the mature form of SOD. The CCS protein, responsible for copper insertion, has been identified [223], while nothing is known on the mechanism of in vitro zinc binding.

The functional significance of these studies is gaining importance with the discovery that mutations on the SOD1 gene are responsible of the inherited form of amyotrophic lateral sclerosis (ALS) (http://alsod.org/), a fatal neurodegenerative disease characterized by progressive death of motor neurons and consequent paralysis. The understanding of the molecular determinants of such a disease is really puzzling from the chemical and structural points of view: over 100 point mutants have been shown to be toxic and to cause the same pathological effects although they are located everywhere on the protein structure. Two main hypotheses [224,225] have been formulated for the SOD1 mutants toxicity: (i) SOD1-linked fALS, like many other neurodegenerative diseases, is a protein misfolding disorder characterized by abnormal deposits of aggregated proteins in neural tissues; (ii) a new chemical reactivity of the mutated enzymes damages cell components or the protein itself. We have now undertaken the task to express, produce as well as structurally and functionally characterize a series of fALS-related mutants with the aim to verify the validity of the above working hypothesis mentioned above and to understand which of the protein forms (from apo to holoenzyme) is the main responsible for the toxicity.

Finally, we are also framing our studies on CuZnSOD in an evolutionary approach by analyzing differences and homologies between the well-characterized eukaryotic proteins and their putative prokaryotic homologues that are identified from genome browsing of an increasing number of organisms. In 138 archaea and bacteria genomes, 57 of these putative homologues were found, 11 of which lack at least one of the metal ligands [226]. Among these, *B. subtilis* SOD lacks two-copper binding histidines. Consequently, the protein can bind one zinc ion but completely lacks copper. In solution the protein was found to be monomeric, with the typical Greek key β-barrel fold but with largely unstructured loops. Such flexibility is quenched in the crystal structure obtained in the presence of an excess of zinc(II), that shows a well defined fold and is organized in symmetric dimer units, with the two monomers bridged by a zinc ion bound to two Asp–His diads on the protein surface [226]. Mutants of the *B. subtilis* SOD protein have been obtained, stepwise reintroducing the two missing copper His ligands. When both the two missing His are introduced, the metal-reconstituted mutant has a reduced activity (about 10%) with respect to hSOD1. The reduced activity has been partly related to conformational disorder that the protein experiences in solution [227].

3.3. The case of nickel

The biological role of nickel has been established only since 1975, when the enzyme urease was shown to depend on this metal ion for catalytic activity of urea hydrolysis [228]. Since then, at least five additional nickel-containing enzymes have been discovered: (i) hydrogenase, involved in the consumption and production of molecular hydrogen; (ii) carbon monoxide dehydrogenase, reversibly interconverting CO and H_2O into CO_2; (iii) acetyl coenzyme A synthase, which catalyzes the reversible formation of acetyl-CoA from CO_2, coenzyme A and a methyl group; (iv) methyl coenzyme M reductase, catalyzing the reduction of methyl-coenzyme M to methane in methanogenic bacteria; (v) superoxide dismutase, involved in the dismutation of superoxide in O_2 and H_2O_2 in fungi.

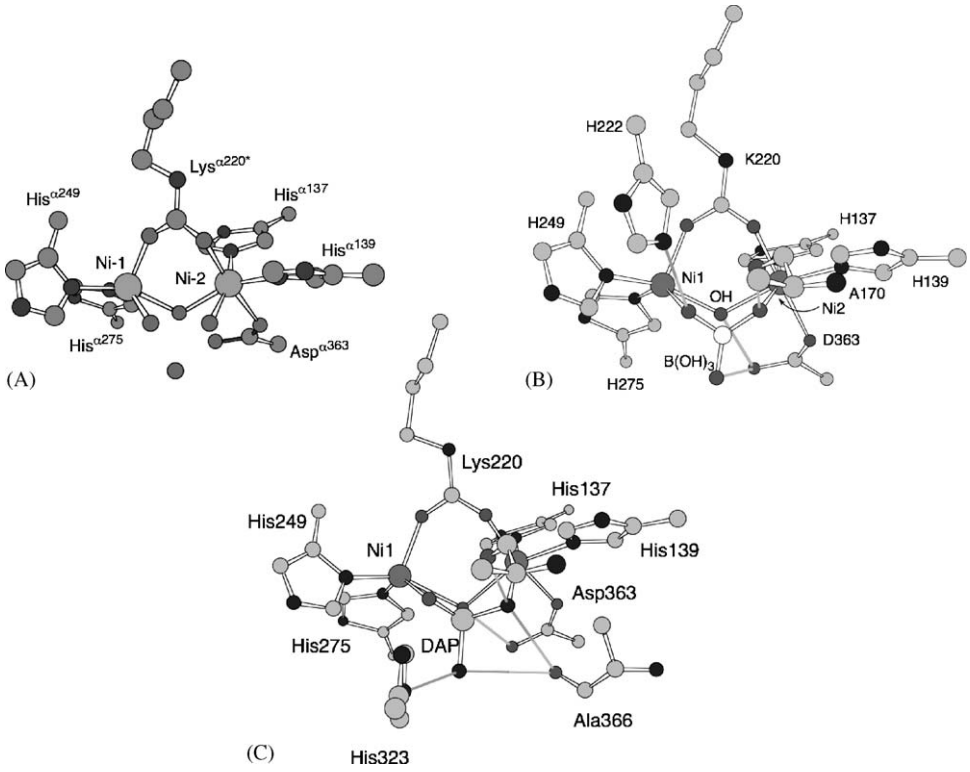

Fig. 13. The dinuclear nickel(II) center in the active site of *B. pasteurii* urease (top left), its boric acid (top right) and diamidophosphate (bottom) adducts, as they result from the corresponding X-ray structures listed in Table 3.

Additional putative Ni-dependent enzymes are peptide deformylase, glyoxalase I, and acireductone dioxygenase, and more are likely to be discovered in the near future.

In recent years, our attention has been focused on the urease system, and all efforts have been devoted to establish the molecular basis for the role of nickel in the 10^{14}-fold increase of the rate of urea hydrolysis [229–231]. The structure of urease isolated from *B. pasteurii*, a widespread soil bacterium, has been determined with high resolution and accuracy (PDB code 2UBP, 2.00 Å resolution). The structure has revealed the presence of a dinuclear nickel(II) center in the active site, with the nickel ions bridged by the carboxylate group of a carbamylated Lys and a hydroxide moiety (Fig. 13A) [232]. The two Ni ions differ in their coordination environment, with Ni(1) bound to two His N and a terminal water molecule (NiN_2O_3), and Ni(2) bound to two His N, a terminal water molecule and an additional Asp carboxylate O (NiN_2O_4).

The reactivity of the nickel ions in the active site has been probed by determining the structure of urease crystallized in the presence of several competitive inhibitors [233,234]. The capability of the urease active site to accommodate tetrahedral molecules was proven by the structure of the phosphate complex [235], that suggested a reaction mechanism that was probed using molecules mimicking the structure, but not the reactivity, of the substrate urea (boric acid, Fig. 13B) [236] and the tetrahedral transition state (diamidophosphate, Fig. 13C) [232]. Such structures prompted us to propose a novel mechanism for the enzymatic urea hydrolysis, which involves the nucleophilic attack of the Ni-bridging hydroxide onto the urea carbon atom, super-activated by the interaction with both Ni ions in the enzyme active site [232]. This mechanism resolves many of the inconsistencies present in the previous proposal, which involved the nucleophilic attack of a terminally Ni-bound hydroxide on a Ni-bound urea molecule.

Nickel is toxic to the cell, and its incorporation into the active site of enzymes that strictly depend on this metal ion for their function must involve a very specific network of proteins devoted to Ni-uptake and intracellular transport, with highly sensitive Ni-dependent expression systems. In particular, our interest concerns the elucidation of the molecular details of the in vivo assembly of urease, a process that requires four accessory proteins, named UreD, UreE, UreF, and UreG [237,238]. UreD appears to be a specific chaperone that facilitates nickel insertion into apo-urease by stabilizing a proper protein conformation [239]. UreG is possibly involved in an energy-dependent step during in vivo urease assembly [240,241]. UreG and UreF form a super-complex with the UreD–apourease complex [242], suggesting that such large aggregates could be required for in vivo activation of urease [241]. Finally, UreE binds nickel and is thought to interact with the UreDFG–apourease supercomplex facilitating nickel(II)-incorporation in the urease active site [243–248]. The contribution of the Italian bioinorganic community to the understanding of the structure-function relationships in these four metallo-chaperones has involved so far mainly UreE and UreG.

B. pasteurii UreE was cloned, and the UreE protein (*Bp*UreE) was over-expressed, purified to homogeneity, and its biochemical properties established [249]. *Bp*UreE behaves as a dimer

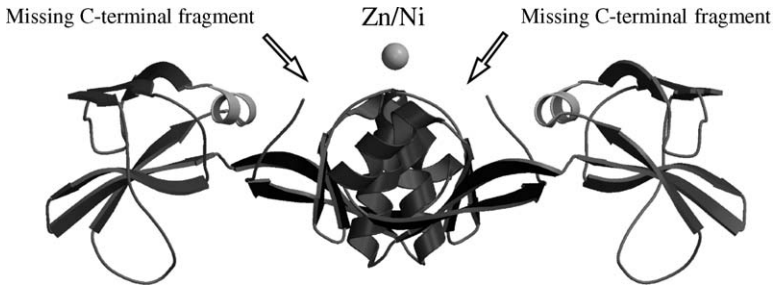

Fig. 14. The X-ray structure of *B. pasteurii* UreE.

in solution, as shown by gel filtration and mass spectrometry. *Bp*UreE has a high sequence similarity with UreE proteins isolated from different biological sources, while no sequence homology is observed with proteins belonging to different classes. A structural database of UreE proteins from a large number of different genomes was built using homology modeling. The final optimized structure-based multiple alignment and the derived model structures provided insightful information on the evolutionary conservation of key residues in the protein sequence and surface patches presumably involved in protein recognition during the urease active site assembly [250]. The X-ray structure of the protein (Fig. 14) reveals the presence of distinct N- and C-terminal domains, connected by a short flexible linker, and the topology of an elongated homodimer, formed by hydrophobic interactions of the two C-terminal domains. A single zinc(II) ion, bound to four conserved His^{100} residues, one from each monomer, connects two dimers resulting in a tetrameric *Bp*UreE, a protein form present only in highly concentrated solutions. The zinc(II) ion can be replaced by nickel(II) as shown by anomalous difference maps obtained on a crystal of *Bp*UreE soaked in a solution containing $NiCl_2$. A large hydrophobic patch, surrounding the metal ion site, is surface-exposed in the biologically relevant dimer. This patch was interpreted as the surface area involved in protein–protein interactions with the UreDFG–apourease complex, awaiting for the release of nickel(II) ions with consequent enzyme activation [251]. However, the crystal structures of *Bp*UreE is affected by severe solid-state effects that caused disorder in the C-terminal portion of the protein and do not allow to obtain a proper picture of the metal coordination environment. The C-term is a long flexible chain containing, at the end, a His^{145}–Glu^{146}–His^{147} motif, with the two conserved histidine residues potentially involved in the nickel(II) binding and release process typical for this metallo-chaperone.

B. pasteurii UreG is the second metallo-chaperone belonging to the urease assembly system that our group studied and characterized [252,253]. A combination of size exclusion chromatography, multi-angle and dynamic laser light scattering established that *Bp*UreG is present in solution as a dimer. Analysis of circular dichroism spectra indicated that the protein contains large portions of helices (15%) and strands (29%), while NMR spectroscopy indicated the presence of conformational fluxionality of the protein backbone in solution. We discovered that *Bp*UreG catalyzes the hydrolysis of GTP with a $k_{cat} = 0.04\ min^{-1}$, confirming a role of this class of proteins in coupling energy requirements and nickel incorporation into the urease active site. *Bp*UreG binds 2 zinc(II) ions per dimer with a $K_D = 42 \pm 3\ \mu M$, and has 10-fold lower affinity for nickel(II). A structural model for *Bp*UreG was calculated using threading algorithms (Fig. 15). The protein, in the fully folded state, features the typical structural architecture of GTPases, with an open β-barrel surrounded by α-helices and a P-loop at the N-terminus. The protein dynamic behavior observed in solution was interpreted using algorithms for disorder predictions. The results suggested that UreG proteins belong to the class of intrinsically unstructured proteins (IUP) that need the interaction with co-factors or other protein partners to perform their function. It was also proposed that metal ions such as zinc(II) could have important structural roles in the urease activation process, and that an interplay of nickel(II) and zinc(II) could represent a case of crossroads in the intracellular trafficking of metal ions.

The future of these studies will entail the investigation of the biochemical and structural properties of UreD and UreF, and the elucidation, at the molecular level, of the factors regulating the formation of the UreDFG–apourease super-complex and its interaction with the nickel-transported UreE. The understanding of this system will represent a potential paradigm for the in vivo nickel trafficking and active site assembly of other nickel enzymes, such as [NiFe]-hydrogenases, acetyl-CoA synthase/CO dehydrogenase, methyl coenzyme-M reductase, and Ni-superoxide dismutase, all depending on metallo-chaperones for nickel homeostasis and metabolism.

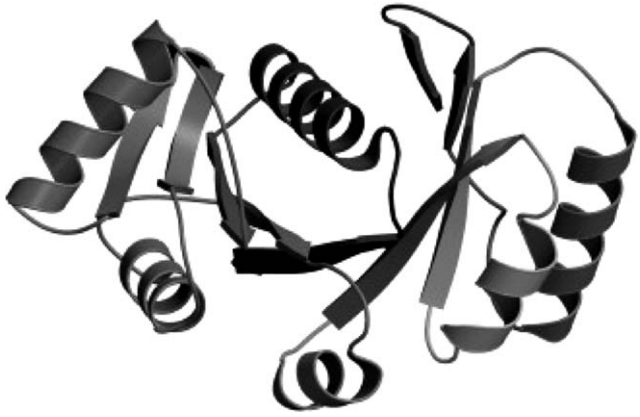

Fig. 15. The structural model for *B. pasteurii* UreG calculated using threading algorithms. The fold shown in the figure represents an "ideal" folded state. Solution studies show that the structure is highly dynamic.

3.4. FeS proteins

Our interest in iron–sulfur proteins, including high potential iron sulfur proteins (HiPIP) and low potential ferredoxins, as well as rubredoxins, dates back to 1990 and most of the early work belongs to the pre-genomic era. In retrospect, we can say that some of the strategies developed at the time to understand global structure-function relationships across protein families (see below) had already at least a genomic flavor. At the beginning of the post-genomic era, several 3D structures of representatives of all main classes were already available. However, while out of the few (23 from a recent BLAST search) high-potential Fe_4S_4 iron–sulfur proteins (HiPIP) in genomes (all protobacterial) about half of them were already structurally characterized, the ratio between solved structures and total number of genes was much lower for Fe_2S_2 ferredoxins. In 2002 there were already 179 Fe_2S_2 ferredoxin sequences in the genomes, 86 of which were plant-type sequences sharing a $CX_4CX_2CX_{29}C$ consensus sequence, and only nine of the latter were structurally characterized. This close to 1/10 ratio prompted us to undergo an extensive modeling work [254], which yielded good quality models of all 77 proteins of unknown structure. The availability of a large number of reliable structures then unraveled significant patterns of conserved charged residues on the protein surface, thus identifying the regions that are critical for the electron transfer function [254].

In the 1990s one of the issues in iron–sulfur protein research was the understanding of the electronic structure of the various iron–sulfur clusters, the distribution of valences in the mixed-valence oxidation states and, in general, the determinants responsible for the changes in reduction potential among the different protein classes and even within each class. By working out possible magnetic exchange schemes within each different FeS cluster, we realised [255] that the sign and/or the temperature dependence of the NMR hyperfine shifts induced on the β-CH_2 protons of the metal-coordinated cysteines by each different paramagnetic iron ion would have been extremely sensitive to the oxidation state of that particular ion in the cluster. Prompted by this observation, we developed the tools for the sequence-specific assignment of the iron-coordinated cysteines in these paramagnetic proteins [256], and measured their hyperfine shifts. This allowed us to perform what we called the *valence-specific* assignment of all iron ions in Fe_2S_2, Fe_4S_4 (both high and low potential) and Fe_3S_4 clusters in several iron–sulfur proteins, and to determine their microscopic reduction potentials [256–275].

To translate this new information into structure-function relationships (i.e., the influence of the protein frame on the microscopic as well as on the macroscopic potentials) we realized, even in the absence of extensive genome information, that more 3D structures were needed. We then combined the tools that we had developed to sequence-specifically assign the signals of nuclei close to the metal with the more standard tools that had been developed since 1983 to solve NMR structures of diamagnetic proteins, and tackled the first structure determination of a paramagnetic metalloprotein, that of the reduced HiPIP I from *E. shaposhnikovii* (formerly *E. halophila*), published in 1994 [276] (Fig. 16). The structures of a few other HiPIPs, in the reduced and/or oxidized states, were subsequently solved by us, mostly by NMR (Fig. 16) [277–282]. At variance with cytochromes (Section 3.1), very little changes, if any, were observed upon changing oxidation states, probably as a result of the Fe_4S_4 cluster being virtually invariant in the two states. However, the availability of an increasing number of HiPIP structures allowed us to address the structure-redox potential relationship in this protein class more confidently: we found that, probably an unique example among the many electron transfer protein families, the major determinant for the spreading of reduction potential values within HiPIPs is the number of charged residues on the protein surface [270,272,283–286].

Around the turn of the century we also solved the structures of the 2-Fe_4S_4 ferredoxins from *C. pasteurianum* and of *Synechococcus sp.* PsaC (Fig. 17), the latter an important component of photosystem I, of a $Fe_4S_4 + Fe_3S_4$ ferredoxin from *B. schlegelii* and of its 2-Fe_4S_4 artificial counterpart, of rubredoxin from *C. pasteurianum* and of a Fe_2S_2 ferredoxin from parsley [287–292]. More recently, our attention has been also focused on the use of NMR to assess the relative stability and tendency to fold/unfold of iron–sulfur proteins in the different oxidation states (Fig. 18), to characterize the different mobility of ortholog proteins from thermophilic bacteria, and to structurally characterize possible iron chaperones involved in the assembly of the iron–sulfur clusters [289,293–299].

Iron–sulfur proteins were for us also the subject of several methodological advances to exploit paramagnetic effects as constraints for structure determination. In particular, longitudinal relaxation constraints were first worked out on this class of metalloproteins, and the standard programs were modified to allow for nuclear relaxation rates being expressed as a summation over the contributions of each iron ion in the cluster, taking also into account the differences due to the different relaxation properties in the different redox states [300–303]. Another small breakthrough was the discovery of Karplus-type relationships to relate the hyperfine shifts of the β-CH_2 protons of the metal-coordinated cysteines to their Fe–S–C–H dihedral angle, and their successful use as structural constraints [263,266,267,288,300,303,304]. Some of the heteronuclear experiments that are now routinely used in structure determination of paramagnetic metalloproteins were developed in the context of these studies.

3.5. Calcium-binding proteins

Similarly to Fe–S proteins, our interest in calcium-binding proteins, and particularly the so-called EF-hand proteins, begun in the pre-genomic era [305,306], but developed through the structural genomic revolution. EF-hand proteins bind calcium(II) ions through a well defined loop that is relatively well conserved throughout the several different EF-hand protein families [307]. We had, and still have, two motivations for this research: one was our constant drive towards methodological developments in the determination of solution structures of metalloproteins; the other motivation was that EF-hand proteins possibly represent the most blatant violation of the “one gene,

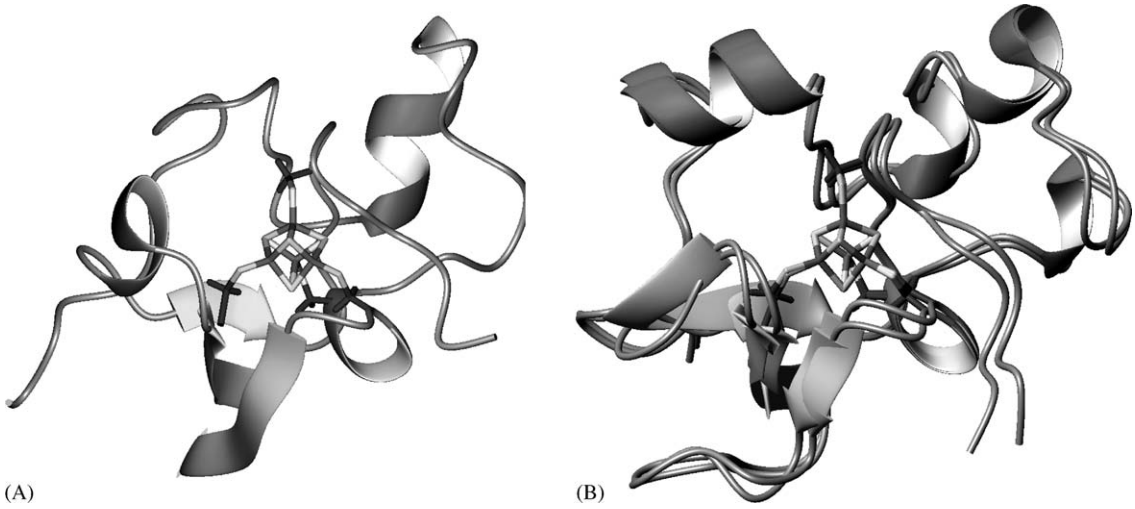

Fig. 16. Average solution structures of the reduced HiPIP iso-1 from *E. shaposhnikovii* (A), and of the reduced and the oxidized HiPIP from *C. vinosum* (B). The latter structures are superimposed in order to maximize the overlap among the cluster ions. The clusters and the sidechains of the ligand cysteines are represented as sticks.

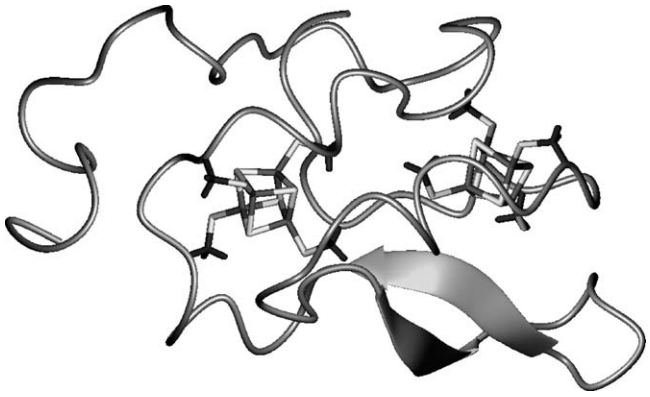

Fig. 17. Solution structure of the isolated PsaC subunit of photosystem I, containing two [4Fe–4S] clusters. The clusters and the sidechains of the ligand cysteines are represented as sticks.

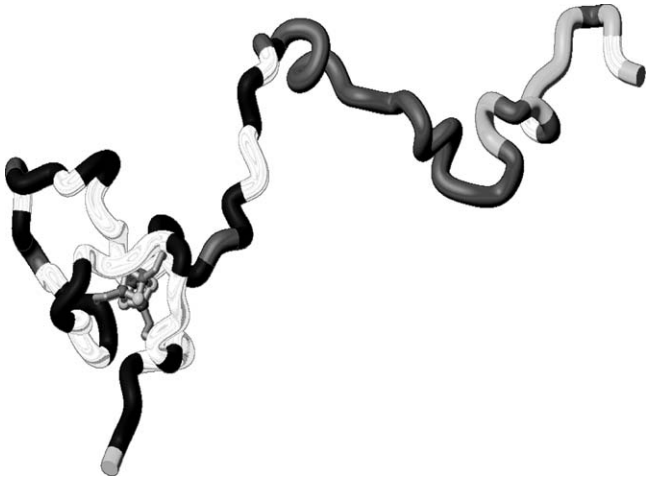

Fig. 18. Solution structure of the partially unfolded reduced HiPIP from *C. vinosum*. The backbone is colored according to the level of local disorder. The disorder increases from black to light grey. Protein regions whose degree of mobility could not be determined are colored in white. The cluster and the sidechains of the ligand cysteines are represented as sticks.

one structure" axiom. The function of a large majority of EF-hand proteins is in fact related to their ability to cycle through largely different conformations depending on the presence of calcium. At the beginning of the structural genomics projects there was a tendency to "fill the fold space", which relied on the assumption that, once a particular fold was structurally characterized, all other proteins that shared enough sequence similarity with the representative one would have a similar structure, which could be modeled using the former as a template. Although all calcium proteins belonging to the EF-hand superfamily share the same elementary folding unit (helix-calcium binding loop-helix) it was early recognized that no modeling could be safely attempted within this superfamily as a whole.

One methodological breakthrough that was provided by us was the use of lanthanide ions as substitutes for calcium. Many pioneering works since the sixties had shown that trivalent lanthanides fit very well in the calcium binding loops of EF-hand proteins [for a recent review see Ref. [308]]. We confirmed that the structural differences with respect to the native metal are very modest and limited to the immediate vicinity of the metal itself, which on average tends to a larger coordination number with respect to calcium [309]. Paramagnetic lanthanides (except gadolinium(III)) induce pseudocontact shifts and residual dipolar couplings on protein nuclei [51,54,310]. The former are long-range restraints, as they depend on the reciprocal third power of the metal-nucleus distance (as opposed to the reciprocal sixth power of the internuclear distances in the case of NOEs), and the latter are distance-independent. Long range or distance-independent restraints are ideal to monitor, for instance, global variations in the reciprocal orientations of the two helices in a EF-hand motif, or of two EF-hands in a two-EF-hand domain, or of two different EF-hand domains in multi-EF-hand domain proteins [53,54]. Most of the methodological development was, and still is, tested by us on EF-hand proteins that showed the least prominent structural variations between the apo and calcium-bound forms, such as calbindin D_{9k} [56,311–320] and parvalbumins [306,321–324] (Fig. 19). At the time, these

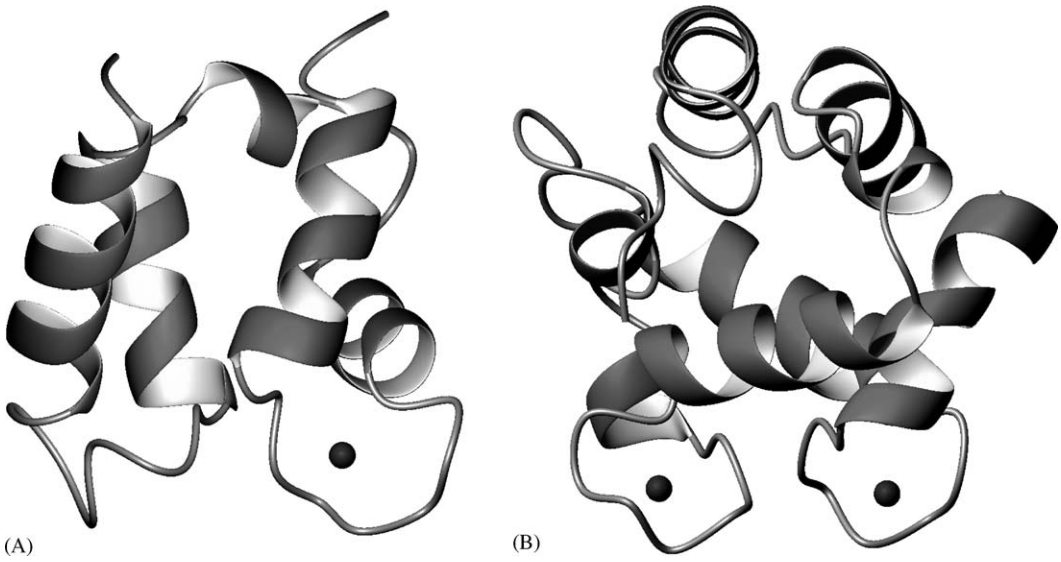

Fig. 19. (A) Average solution structure of calbindin D_{9k}, obtained using paramagnetic constraints coming from 11 different lanthanide ions substituting the native calcium(II) ion, shown as a dark sphere in the C-terminal EF-hand motif of the protein. (B) Average solution structure of calcium-loaded human α-parvalbumin. The AB helices are shown at the top, while the two pairs of CD and EF helices, forming the functional EF-hand calcium binding motifs, are shown at the bottom.

were generically called calcium buffer proteins (but see below). We could show that high quality structures, especially in terms of global arrangement of secondary structure motifs, could be obtained despite the broadening due to paramagnetism, exploiting if necessary more that one lanthanide ion to "enlighten" with pseudocontact shifts shells of nuclei at different distances from the metal ion [312,313]. In these proteins, lanthanides have different affinities for the different metal binding sites, so that selective substitution of only one lanthanide is straightforward. In other proteins like calmodulin there is little intrinsic selectivity, but engineering of appropriate point mutants still allowed us to perform selective substitutions. A successful engineering of a lanthanide site consisted in changing one asparagine into an aspartic acid residue in the calcium binding loop, thereby increasing the total negative charge of the ligand by one unit [325]. This results in an increased affinity for positively charged ions, i.e., both calcium and lanthanides, but the increase in affinity is expected, and indeed is, larger for the trivalent lanthanides than for the divalent calcium ions.

Comparison of the structures of the two strictly homologous human proteins α-parvalbumin and β-parvalbumin (50% identity), which would have been considered identical by homology modeling criteria, revealed subtle structural differences which are reflected in calcium-binding affinities, likely bearing functional implications [322,323]. Interestingly, α-parvalbumin is ubiquitous in human cells while β-parvalbumin is almost exclusively expressed in tumor cells, perhaps to respond to the needs of their accelerated metabolism.

Calmodulin is a paradigmatic example of the risks to rely on X-ray structures alone to gain functional insight (on this subject see also Section 4). The original X-ray structure showed an elongated dumbbell-shaped molecule with the two two-EF-hand domains far apart from one another [326]. NMR studies later showed that part of the helical linker between the two domains was not helical in solution but very flexible [327]. The resulting picture was that the two domains could actually move freely with respect to one another. This feature is crucial for the function of calmodulin, which is able to bind a variety of target peptides by closing up its two domains around the target [328–330]. By using selective lanthanide substitution in one domain and by measuring the differential paramagnetic effects in the other, we could provide a picture of the amplitude of the relative motion of the two domains and of the preferred regions of space occupied by one domain with respect to the other [331].

Two different calmodulin-like proteins have been structurally and functionally characterized in our lab, one (CaBP) from *Entamoeba histolytica* [332,333] and the other (CLSP) a human protein selectively expressed in skin keratinocites [Babini et al., submitted for publication]. In both proteins the relative conformational freedom of the two domains is reduced with respect to calmodulin, and the calcium/lanthanide binding sequence in the four sites is slightly altered in CaBP [333] and grossly altered in CLSP [Babini et al., submitted for publication]. The latter also shows structural disorder in the N-terminal domain. Finally, the structure of another EF-hand protein, similar to calbindin D9k but dimeric and belonging to the so-called S100 family (S100A13) has been solved [334]. Some of the members of this family bind divalent metal ions like zinc(II) or copper(II) in a site which is formed by the N-terminal portion of one monomer and the C-terminal portion of the other. S100A13, despite it does not have the "signature" residues for this additional metal binding to occur, is still able to bind copper(II) but in a different position and with a different donor atom set [334]. It is worth stressing that all of the features discovered on calmodulin-like and S100 proteins would have been hardly predictable without solving the actual structures.

Our structural genomic efforts on EF-hand proteins are now focused on the understanding of the determinants for the unique ability of each different EF-hand family to translate calcium

binding into differently pronounced conformational changes. Indeed, the EF-hand superfamily is constituted by more than 20 families that are functionally (and hence structurally) distinct. To do so, we needed to produce and analyze a large number of structures covering all relevant cases (i.e., with or without bound calcium and with or without bound target peptide). After assembling a complete database (updated to May 2005) of all available structures of EF-hand domains in various bound states (above 200), we have identified the six interhelical angles among the four helices constituting the domain as the simplest set of structural descriptors, and we have further found that two appropriate linear combinations (PC1 and PC2) of the values of these six angles retain as much as 80% of the structural information [335]. Besides simplifying the description of the structural features of the EF-hand superfamily, this analysis also allows us to attempt structural modeling of unknown EF-hands, once their belonging to one or another of the major conformational families is reliably established. Finally, based on this analysis and on a few basic rules underlying genome transformation events, we may be able to propose an evolutionary model for all EF-hand proteins that begins with the formation of a EF-hand motif that is able to bind calcium, proceeds with the duplication of this motif to yield the first functional EF-hand domain and then with a further duplication producing a prototype calmodulin, and finally evolves (or involves) into a series of proteins that are characterized by a progressive loss of symmetry and of cooperative calcium binding ability. Calmodulin, the most versatile of present days EF-hands, is closest to this symmetric ancestor, while all other EF-hand domains have downgraded to more specialized roles that took advantage of symmetry breakdown.

3.6. Detoxification from zinc and cadmium

Essential transition metals such as zinc and copper, and nonessential metals, as cadmium, lead and mercury, can be toxic at the cellular, tissue and organ levels when present in excess [336,337]. In particular, the heavy metal ions as Zn and Cd form strong coordination bonds with groups such as the thiolates of cysteine residues and the imidazolium nitrogens of histidine residues. When they react with those residues in proteins, they frequently inhibit catalytic or biological activity, producing their biological toxicity. Because these heavy metals are widespread in nature, there was pressure early in evolution to develop resistance mechanisms. Therefore, several pathways for resistance to the heavy transition metal ions have been determined in a wide variety of organisms [170]. Our contribution in this area concerns the study of the efflux system for zinc and cadmium ions in bacterial cells, in particular the P-type ATPase pumps, a superfamily of enzymes that transport cations into or out of cells or intracellular compartments [338]. Two distinct subgroups of the P-type ATPase family can be distinguished based on physiological data: those that transport metal ions such as copper(I) and silver(I), and those that predominantly handle divalent metals such as zinc(II), cadmium(II) and lead(II) [339]. Similarly to the copper(I) P-type ATPases, described in Section 3.2, each metal binding domain bears a conserved –(M,L)XCXXC– metal binding motif [112]. Again similarly to the copper(I) P-type ATPases, these domains have been shown to increase the overall catalytic rate of metal transfer [340–342] but their role in metal specificity has not clearly been addressed. Therefore, an essential topic for fully understanding the mechanism of metal detoxification concerns the metal specificity of P-type ATPases and, in particular, which is the role of this N-terminal cytoplasmic domain of P-type ATPase pumps in metal specificity. In order to address this topic, our approach started from a detailed sequence alignment study of the N-terminal cytoplasmic domains of zinc(II), cadmium(II) and copper(I) P-type ATPases. The results suggested that the presence of negatively charged residues in the vicinity of the metal binding site may modulate the affinity of the protein for divalent over monovalent metal ions. In particular, an aspartate residue, located in the metal binding motif of ZntA from *E. coli*, –GMDCXXC–, may be implicated in this role. The structural data on the N-terminal domain of ZntA from *E. coli* show indeed that this Asp residue, fully conserved in divalent cations ZntA-like P-type ATPases, facilitate the binding of a divalent cation such as zinc(II) (Fig. 20) [343]. It emerges, indeed, that a ferredoxin-like fold can accommodate a coordination site for zinc, never been reported before in proteins, constituted by two cysteine residues and a carboxylate residue. The solution structure of the N-terminus of the cadmium(II)-ATPase from *Listeria monocytogenes* also indicates that a fully conserved negatively charged residue, a glutamic acid, is involved in cadmium(II) binding (Fig. 20) [344]. In contrast to *E. coli* ZntA N-terminus, in cadmium(II)-ATPase, this acidic residue is in loop 5 (between α2 and β4), i.e., far from the CXXC motif in the sequence, thus determining a different arrangement of the metal binding residues in ZntA-like and CadA-like proteins. The different relative location of the carboxylate moiety with respect to the CXXC binding metal motif can be relevant for tuning the specificity for either zinc(II) or cadmium(II). While the carboxylate moiety is just adjacent to one Cys of the consensus motif in the ZntA-like proteins, thus creating a conformational constraint and limiting the size of the metal ion which can be preferentially bound, in the CadA-like sequences it is located far in sequence, in loop 5, thus allowing a larger flexibility in the size of the metal binding

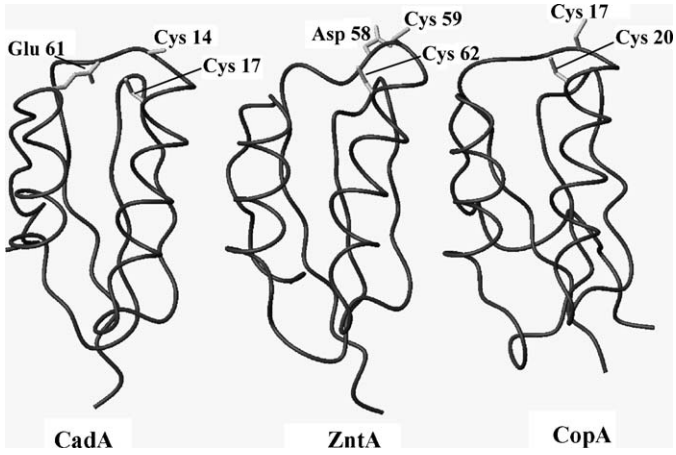

Fig. 20. The comparison of the solution structres of CadA, ZntA, and CopA. Some key residues are highlighted.

site and therefore allowing a preferential binding of ions with larger ionic radius.

In conclusion, from the structural analysis of the cytoplasmic N-terminal domains it emerges that the same ferredoxin-like fold, comprising the secondary structure elements βαββαβ, is shared by copper(I)-, zinc(II)-, and cadmium(II)-ATPases (Fig. 20) and that a single aminoacid change may represent a relevant contribution to the metal specificity of heavy metal P-type ATPases. Indeed, although the different metal binding patterns can bind several metal ions [345], a different pump efficiency for divalent cations as opposed to monovalent cations has been observed [346,347] and the presence of acidic residues at specific positions in the sequence, but spatially close to the CXXC metal binding motif, can play a role in this process. On the other hand, it is well known that the P-type ATPases ionic specificity relies on the membrane transport sites [342] and, therefore, both metal sites can be important to determine the overall efficiency of the metal detoxification mechanism. Alignments of the 8th helix of P-type ATPases show that a Met conserved among the copper(I)-ATPases is changed to an Asp in the cadmium(II)- or zinc(II)-ATPases [348], suggesting that, similarly to the N-terminal metal site, the membrane site selectivity may also be based on the presence of an acidic residue. Up to now, our data suggest a model where the metal ion specificity does not reside only in the membrane region but also in the N-terminal domain, even if the latter does not override the specificity of the membrane site [342]. From a functional point of view, the same metal binding specificity in both the soluble N-terminal site and the membrane site of the ATPase could have a key role in optimizing metal transport activity. The metal specificities in both metal sites of the ATPase pump can therefore play an essential role in order to more efficiently detoxify a cell from a deleterious heavy metal ion with respect to others, even if the ATPase can pump several heavy metal ions.

4. Perspectives

Writing a "perspectives" section is always a challenge. We feel that we should pursue methodological advancements for high throughput protein structure determination and for tackling proteins of large size. This is also due to our commitment as an infrastructure towards the European Commission and the scientific community in life sciences. From the biological side, we are still engaged in overcoming the bottlenecks of protein production. Furthermore, we like to tackle weak protein–protein complexes that represent the key steps of the biochemical processes discussed in the text. We like also to extend our characterization to membrane proteins or to their domains that contain intermembrane metal binding sites. Finally, we like to address systematically the relationship between molecular structure and pathogenic mutations.

The three-dimensional structures obtained from structural genomics research can be exploited for structure-based drug design. In this sense, a number of proteins among those described above have an interest as potential drug targets. The human calcium-binding S100A13 protein is involved in the cellular export of the pro-inflammatory interleukin-1α and of the fibroblast growth factor-1, which play a crucial role in angiogenesis, tissue regeneration, inflammation, and formation of some tumors. Molecules inhibiting the interaction of S100A13 with its protein partners can be candidate drugs. Other well-know target proteins that have been characterized by us in the last few years are matrix metalloproteinases, MMP's (Table 7), a family of proteolytic zinc-enzymes, which have been the focus of extensive research by several groups worldwide because of their involvement in serious diseases (e.g., osteoporosis, arthritis, cancer). The human genome sequence provided us with the primary structure of all MMP's. A number of them have been expressed in our lab, while the expression of others is reported in the literature. We have contributed to the structure characterization

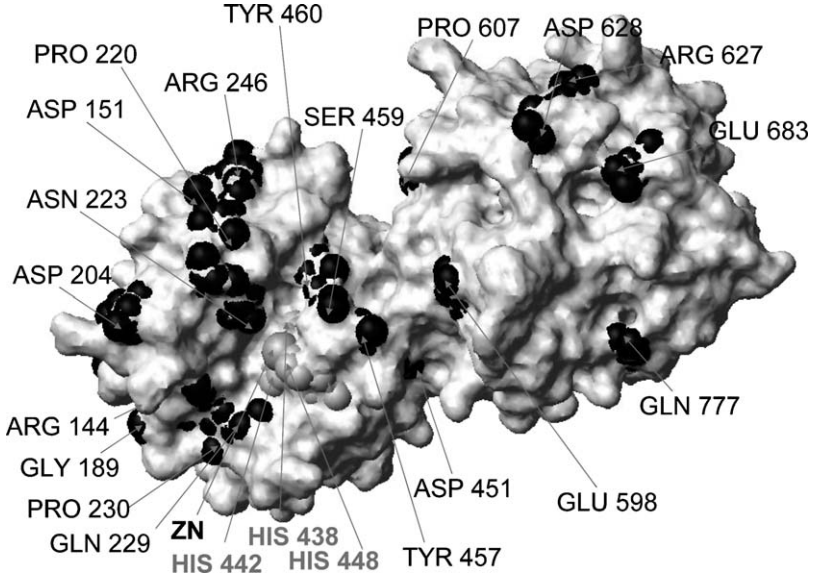

Fig. 21. Location of residues characteristic of various classes of MMPs, which have been identified through multiple sequence alignments and phylogenetic trees, mapped onto the surface of MMP1 (black spheres). The face containing the exposed catalytic zinc ion (grey sphere) is shown. The zinc ligands are also labeled.

of some catalytic domains [349–352] and coupled the structure characterization with modeling (see Fig. 21) [353]. At this point, selective ligands could be designed, synthetized and tested as inhibitors.

Concluding, while the amount of work in front of us is enormous, we must focus on the most relevant biological aspects and prioritarise our research on the basis of the expected impact on the present knowledge of the molecular mechanisms of life.

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