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Bioinorganic methods

Biophysical approaches for the study of metal-protein interactions

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ABSTRACT

Protein-protein interactions play important roles for a variety of cell functions, often involving metal ions; in fact, metal-ion binding mediates and regulates the activity of a wide range of biomolecules. Enlightening all of the specific features of metal-protein and metal-mediated protein-protein interactions can be a very challenging task; a detailed knowledge of the thermodynamic and spectroscopic parameters and the structural changes of the protein is normally required. For this purpose, many experimental techniques are employed, embracing all fields of Analytical and Bioinorganic Chemistry. In addition, the use of peptide models, reproducing the primary sequence of the metal-binding sites, is also proved to be useful.

In this paper, a review of the most useful techniques for studying ligand-protein interactions with a special emphasis on metal-protein interactions is provided, with a critical summary of their strengths and limitations.

1. Introduction

Proteins, the main workhorses of the cell, do not carry out their actions in isolation, but their biological functions and consequently the survival of living organisms often depends on proper metal-protein interactions [1–3]. Metals are present in more than one-third of all proteins [4]. Metalloproteins (and metalloenzymes) mediate some of the most complex and essential processes in life such as photosynthesis and dioxygen transport [5]. The dishomeostasis of metal ions results in or is implicated in numerous disorders, with neurological disorders being one of such examples [6,7]; high levels of metal ions are found in solid deposits as diverse as the plaques of Alzheimer's (AD) and prion (PrPD) diseases, the nuclear and cytoplasmic inclusions of Huntington disease (HD), the Lewy bodies of Parkinson's disease (PD) and also, the Bunina bodies of amyotrophic lateral sclerosis (ALS) [8,9]. Different transition metal ions are of importance in these disorders, especially those more active from a redox point of view, such as iron and copper [10]. The chemical properties of redox-active metal ions enable them to take part in many biological functions such as electron transfer, catalysis, and structural shaping; however, an excess of these metals can be toxic to cells, tissues and organisms, especially neurons [11]. All copper-binding proteins in neurons are co-regulated [12].

Metal-mediated protein interactions are also of importance for bacteria and microorganisms, which can utilize metal ions in several different enzyme systems that enable them to survive and proliferate in various environments [2,13]. Typically, the biosynthesis of metal-containing enzymes is a multi-step process, involving a number of accessory proteins, some of which are dedicated to the delivery of the proper metal ion to the active site of the enzyme [13,14]. Other accessory proteins (metal chaperones) serve as a metal reservoir utilized in the sequestration and detoxification processes.

Most proteins need to fold into unique, 3D structures to acquire their functionality [15]; their intermediate states can also play significant roles in events such as translocation across membranes and trafficking to specific cellular locations. Metal coordination requires that the binding site assumes specific geometries, thus either favouring or hindering the correct three-dimensional shape of the protein. The knowledge of the protein structure, both free and complexed (preferentially in solution), can be of great value in the understanding of the protein activity and biological role. For this purpose, peptide models, reproducing the primary sequence of the metal-binding site, can be very useful, especially if the corresponding domain of the free protein is unstructured [16,17].

In addition to the role played by metals in protein activity, the metal-mediated protein–protein interactions contribute to the correct routes for metal trafficking and act as “caretakers” to preserve the metal homeostasis inside the cell [1]. Studies on metal interactions with biomolecules are currently of great attention in both the source and prevention of various diseases. Among the many methods designed to study protein interactions, the biophysical tools deserve a large amount of attention, as they can give vast and accurate information about the stoichiometry and affinity of many protein interactions. These methods

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currently used for studies on metal-protein and metal mediated protein-protein interactions, all have their advantages and disadvantages, which will be discussed below.

2. Analytical and biophysical methods in metal-protein investigations

The following sections cover a wide range of critically important techniques in the scope of metal-protein investigation and will encompass the main themes of the area such as micro- and nanoscale analysis, kinetics, structural analysis of bio-macromolecular complexes, stoichiometry, affinity and enthalpy of these systems. Such studies can be performed by means of mass spectrometry, X-ray crystallography, NMR-spectroscopy, several spectrophotometric techniques, calorimetry and other tools, each being complementary with specific strengths and limitations. Using as many experimental methods as possible highly increases the accuracy of the conclusions [18].

3. MS-based methods

Mass spectrometry (MS) is a highly sensitive technique which enables the rapid identification of subpicomolar amounts of proteins from a variety of biological samples [19]. Moreover, it allows real-time addition of substrates or ligands while monitoring the resulting changes to the complexes. One of the most suitable methods for generating gas-phase ions of large biomolecules is electrospray ionization (ESI). The electrospray ionization spectra of intact proteins were reported for the first time by John Fenn, in 1989 [20]. ESI-MS is a powerful technique not only for protein identification and characterization, but also for studying the stoichiometry and binding strength of metal-protein complexes. This method, for instance, was used to study zinc binding properties of the amyloid fragment [21] or interactions between human serum transferrin and two nonferrous metals (indium and bismuth) [22] and even for multiprotein-metal-ligand noncovalent complexes analysis [23]. A disadvantage of the technique can be due to possible difficulties in ionization of hydrophobic proteins by the ESI interface, because of their inherent insolubility in the buffers compatible with electrospray [24]. Moreover, when using top-down tandem ESI-MS, where whole proteins are directly sprayed into the mass spectrometer, a challenge to interpret the complicated mass spectra occurs. On the other hand, top-down MS can bring valuable information on the combinatorial effect of multiple post-translational modifications in the same polypeptide chain. A significant advantage of intact protein analysis is that large protein ions exhibit a lower level of bias in their ionization efficiencies in comparison to their component peptide ions [25].

Advanced nano-ESI sources, new MS configurations, and hardware, provide new opportunities regarding the analysis of protein complexes. Recently, electrospray conditions were used together with an automated tool, called Analysis of Protein Modifications from Mass Spectra (Apm2s), to study metallodrug-protein interactions [26]. Using this tool, the authors have discovered that far more potential binding sites of cisplatin with ubiquitin exist than previous studies had identified. In another work, ESI-MS and hydrogen–deuterium exchange coupled with MS (HDX-MS) were used to examine the effects of metal and DNA binding on protein dynamics. These studies revealed mechanism by which Ni(II) and Co(II) ions induce structural changes in RcnR that affect DNA binding affinity [27]. It was shown that nickel and cobalt ions binding altered flexibility from the N-terminus through helix 1 and modulated the RcnR–DNA interaction. The ordering of the N terminus makes helix 1 more rigid and repacks helices 1 and 2, changing the distribution of surface positive charge on the protein and decreasing the affinity for DNA. Researchers conclude, that the metal-binding site reflects an adaptation that allows for detection of a larger variety of chemical signals.

Over the last years, inductively coupled plasma mass spectrometry (ICP-MS) has proved to be a very efficient and sensitive bio-imaging tool for studying metal-binding proteins (metalloproteins) in life sciences [28–30]. This method provides a multitude of useful information of metal, metalloid and isotopic distribution in biological tissues. LA (laser ablation)-ICP-MS can be also utilized for imaging metal-containing proteins in a 2D gel after electrophoretic separation of proteins. By this method a wide variety of samples can be analyzed. A big benefit of this technique for protein research is, that sample requirements are minimal. Many improvements of the technique regarding resolution and quantification have been made in the last few years. However, the large number of sample preparation steps makes the trace element distribution studies difficult. New strategies of sample preparation have recently been proposed [31].

More details on the applications of LA-ICP-MS to life science studies including applications to proteome analysis in combination with MALDI/ESI-MS to analyze metal-containing proteins have been reported by J.S Becker [32] New achievements on mass spectrometry methods offering the highest resolving power and mass accuracy have been reviewed by Nikolaev et al. [33]

4. X-ray crystallography and X-ray scattering

The increasing number of crystal structures of key individual proteins has boosted the progress in metal-protein interaction studies. The structural characterization of metalloproteins is often a starting point for enzymology. Most of the structures are deposited in the Protein Data Bank (PDB) and have been determined using X-ray crystallography (or, more rarely, by NMR spectroscopy – the technique is discussed in one of the later chapters). To obtain the 3D structure of a protein, its single crystal is frozen in a stream of liquid nitrogen, then it is placed in the X-ray beam and the diffracted rays are measured on an image plate [34].

A highly powered, tuneable, modern X-ray source is delivered by a synchrotron. The crystal is mounted on a goniometer and hit with a beam of X-rays. Synchrotron radiation is 100% linearly polarized in the plane of the electron beam orbit and elliptically polarized above and below the plane [35]. Data are collected as the crystal is rotated through a series of angles. Synchrotron sources have enabled a rapid collection of data. These data, together with sophisticated molecular modelling techniques, allows the refinement of protein structural models as well as fast estimation of conformational changes resulting from metal binding or macromolecular interactions. Such structural data can further be enriched by computational approaches [36].

The main advantage of X-ray crystallography is that it can provide a detailed picture of protein interaction with metal ions at atomic resolution. However, in order to run an X-ray diffraction experiment, one must first obtain a crystal, which is usually challenging and time consuming. This process can dictate limitations on the types of proteins that can be investigated by this method. Another challenge is the proper incorporation of the relevant metal or metallocofactor and maintaining the appropriate environment for the protein. Moreover, to entirely elucidate how proteins perform their function, one must watch their motions and dynamics and that often involve short-lived intermediates. To address that challenge, a time-resolved crystallography tool has been developed [37]. Even structural transitions which occur in the picosecond to microsecond range can be provided at atomic resolution by time-resolved crystallography. The difficulty of this method is that one can only examine protein transitions which can be triggered in the solid state and that do not interfere with the crystal packing matrix. Moreover, the intermediate of interest must be at a decent concentration within the crystal.

The application of XFEL (X-ray free electron laser) to metalloproteins can't be omitted in this chapter. The appearance of XFEL, which emits extremely intense, very brief X-ray pulses, opened the possibility of time-resolved experiments with even femtosecond time resolution on macromolecular structure, in both single crystals and solution [38]. As an example, the earliest phases of the reaction in which the CO is flashed away from Myoglobin (Mb) were investigated on femtosecond

time scales at the XFEL [39]. However, working with transition metals, care has to be taken that the metal is not photoreduced during X-ray structure analysis [40]. Although X-ray pulses from an X-ray free electron allow to acquire a signal before the sample is destroyed [41], there are some cases, where rapid configurational changes could affect the interpretation of catalytic mechanisms [42]. For more details, please refer to a recent specialized review [43].

Small-angle scattering (SAXS) is a universal low-resolution method to analyze structural changes in response to variations of external conditions. The major benefit of this method lies in its ability to provide structural information about partially or completely disordered systems [44]. SAXS can be helpful in assembling together high-resolution models of individual domains provided by protein crystallography into the model of the entire macromolecular complex. Complementary benefits of crystallography and SAXS in studies of protein dynamics have been recently reviewed [45]. Another excellent review has been recently provided showing the challenges of metalloprotein crystallography [46]. There are, for instance, problems with refinement, which, on one hand, could produce biased structures and, on the other hand, have created new developments in metalloprotein structure determination.

That development includes the incorporation of spectroscopic equipment and detectors allowing simultaneous experiments on single crystals and new advances implemented at synchrotron facilities, among others, providing a wealth of new information, which, in combination with the crystallographic structures, can shed a light on aspects that may otherwise be impossible or troublesome to resolve [45].

It is worth to note the recently published protocol, alerting researchers to potential pitfalls during the preparation and handling of metal-containing protein samples for X-ray crystallography studies [47].

5. Spectroscopic techniques

5.1. X-ray absorption spectroscopy

X-ray absorption spectroscopy (also called X-ray absorption fine structure, XAFS) is an extremely useful approach to study metal-associated proteins [48]. This technique is one of the most sensitive methods to probe the local environment of a metal ion with high spatial resolution (0.1 Å) [49]. The X-ray absorption process involves the excitation of a core electron to an empty or partially filled orbital of the absorbing atom, generating the region of the absorption spectrum called XANES (X-ray absorption near-edge structure) [50]. The XANES spectroscopy gives information on charge transfers, orbital occupancy, oxidation states and symmetry. At higher X-ray energies, when a photoelectron is formed by ejection of the core electron from the absorbing atom, the spectrum region contains important information on the environment of that atom; this region is called EXAFS (extended X-ray absorption fine structure). In other words, the EXAFS technique uses the X-ray photoelectric effect and the wave nature of the electron to determine the local structure around selected atomic species. Both XANES and EXAFS can be recorded by measuring the absorption of the X-rays by solid or solution samples that contain relatively high concentrations of the absorbing species [51]. EXAFS is used to determine the presence of metal-protein bonds, atomic distances, coordination numbers and the nature of the neighbours of the absorbing atom; XANES is instead strongly sensitive to formal oxidation states and coordination geometry [52]. Practical experimental limits on the XAFS measurements are set by the characteristics of the synchrotron source and experimental station, because they establish what energy ranges, beam sizes, and intensities are available.

A few years ago, high-throughput X-ray absorption spectroscopy was used to investigate 3879 purified proteins in order to find out their metal ion content. It was shown that 9% of these proteins contain metal ions (Zn, Cu, Ni, Co, Fe, or Mn) in stoichiometric amounts [53]. An X-ray based structural–kinetic approach was also used to study zinc-dependent metalloproteinase [54], iron enzymes [55], molybdenum-iron nitrogenase [56], and zinc farnesyltransferase [57]. Nowadays, it is possible to perform X-ray absorption spectroscopy with virtually no particular sample preparation [58].

The method can be applied to study metalloproteins and other biological systems as a ‘stand-alone’ technique, but it is especially powerful when used alongside other X-ray and spectroscopic techniques as well as computational approaches [59].

5.2. NMR (Nuclear Magnetic Resonance)

Over the last few decades, a huge number of investigations exploited different NMR approaches to study metal-protein interactions, understand their biological relevance and elucidate some of the cellular events occurring in living systems (for some excellent reviews, see [60–66]). NMR is a powerful technique, able to provide structural and metal binding features of proteins and eventually map their interactions with other biological macromolecules, including nucleic acids, drugs and protein partners. Besides providing structural and dynamic information on the protein, NMR studies are commonly used to identify the coordination environment of the metal binding domains, i.e. the type and number of metal donor atoms and metal binding affinity [60,62–65,67–69].

NMR approaches generally rely on looking at the change of NMR parameters of the protein (i.e. chemical shift, intensity, relaxation rates) upon metal addition. This is usually done by simply comparing the NMR spectra (1D, 2D or 3D according to the complexity of the system) of the metal bound and *apo* protein. Since the NMR parameters of a nucleus strongly depend on its chemical environment, any variation observed upon metal addition, allows to identify the amino acids involved in metal binding. The largest effects are generally attributed to those nuclei whose chemical environment is more perturbed by the metal coordination.

NMR titration experiments are generally performed by gradually increasing the amount of metal ions. As for any protein interaction (i.e. metal-protein, protein-protein and ligand-protein), during a titration experiment, NMR signals behave differently according to the NMR exchange regime (fast, intermediate or slow). These exchange regimes are usually explained by looking at the variation of the NMR signals upon metal addition. In fast exchange, the NMR signals correspond to the weighted average of those corresponding to the free and the bound states. Upon gradual addition of the metal ion, the NMR parameters of the *apo* protein gradually shift towards the bound form until reaching a plateau. In the intermediate exchange, the gradual addition of metal ions causes extensive line broadening of NMR resonances (usually preventing signal detection), which return visible, as long as the metal is diamagnetic, as the stoichiometry of the complex is reached. However, in some cases, signals of the bound state do not sharpen and they remain invisible even with an excess of the metal. Since the loss of NMR signals is very detrimental for any further NMR analysis, an optimization of the experimental conditions (i.e. temperature, ionic strength, solvent) should be attempted in order to make the resonance of the complex visible. In the slow exchange regime, NMR signals corresponding to both free and bound states are simultaneously present. By gradually adding the metal, the simultaneous decrease of the NMR signals of the *apo* protein and the increase of the ones belonging to the bound state are observed. The integral of each signal is linearly dependent on the population of the two states and is directly correlated to the molar ratio of both components.

As already said, the metal binding domain is identified by mapping the most perturbed protein regions: large variations in side-chain nuclei (^{1}H or ^{13}C), belonging to His, Glu, Asp, Cys and Met, strongly support their coordination to the metal ion. On the other hand, changes of backbone resonances (NH and CH groups) may also depend on structural protein rearrangements induced by the metal binding.

NMR, together with X-ray crystallography, has been successfully employed to obtain the 3D structure of metal-protein complexes. Compared to X-ray crystallography, solution NMR does not require sample manipulations and protein crystallization. Moreover, recent progress on solid state (SSNMR) and sedimented solute (SedNMR) improved resolution and sensitivity to study complex systems like amyloid fibrils [70–73].

Structural determination by NMR requires two different approaches according to the paramagnetic or diamagnetic nature of the metal ion [62,64,65]. If the metal ion is diamagnetic, the NMR analysis strongly resembles the one used for the *apo* protein whose conformation is obtained by using the conventional NOE based analysis [74–76]. On the other hand, the presence of unpaired electrons causes a large line broadening of the NMR signals belonging to nuclei nearby the metal ion and more dramatically hamper the success of experiments based on either NOEs or scalar couplings. Nevertheless, the use of paramagnetic based structural restraints, such as paramagnetic relaxation enhancements, pseudocontact shifts and residual dipolar couplings are commonly applied to get structural information of paramagnetic metalloproteins [77–80].

A detailed multidimensional NMR study was associated with UV–visible spectroscopy in order to elucidate the way of nickel(II) binding in the histone-H4 fragment, in order to shed light on the complex mechanism of nickel carcinogenesis [81]. NMR experiments identified the specific donor atoms for that binding.

The successful applications of NMR used together with ultraviolet-visible (UV–Vis), circular dichroism (CD), EXAFS and potentiometry for the studies on nickel binding sites in histone proteins has been reviewed by M. Peana et al. [82] The work shows that the spectroscopic and potentiometric analysis of nickel complexes using model peptides have given useful information such as structural characterization of metal complexes, chemical speciation and binding constants. A similar set of techniques was used in studies on the coordination environment of Cu (II) ions bound to angiogenin [83]. UV–Vis and CD parameters helped to discriminate the copper(II) coordination environment. Moreover, confocal images of actin organization, when peptides and the whole angiogenin protein were supplemented to the cells together with the copper(II) ions, were recorded. The results showed how important is to use the wild type angiogenin present in the human plasma, to analyze the pathways involving the protein in angiogenesis processes, especially in the presence of the metal ions. There are numerous other examples of papers in which NMR was employed together with potentiometric, UV–Vis, and circular dichroism measurements in the studies on Ni(II) complexes [84,85]. These studies were carried on to get more insight in the molecular mechanism of nickel hypersensitivity.

NMR is exceptionally powerful when coupled with complementary observations also from other techniques, such as X-ray crystallography, cryo-electron microscopy or molecular dynamic (MD) simulations. Indeed, it is often necessary to investigate biological macromolecules using multiple orthogonal experimental techniques in order to obtain robust conclusions [86].

5.3. Fluorescence-based methods

In the process of fluorescence, an incident light beam of a suitable wavelength is absorbed by the protein or its metal adduct which is transferred to an excited state; after a short lifetime, deactivation and return to the ground state occurs through the emission of a photon of an energy somewhat lower compared to that absorbed. Using classical fluorescence spectroscopy, metal-protein interactions can be studied by monitoring changes in the tryptophan (endogenous fluorophore) environment detected by changes in its intrinsic fluorescence [87]. Tyrosine and phenylalanine are both much less fluorescent than tryptophan; tyrosine fluorescence can only be of some use in the absence of tryptophan, as it is usually quenched in the presence of Trp; phenylalanine is very weakly fluorescent and can only be observed in the absence of both Tyr and Trp.

In the presence of a complex-formation reaction, these changes in the fluorescence emission spectrum can involve either a shift in the wavelength of maximum emission or a shift in the fluorescence intensity. As a result, the fluorescence intensity at a particular wavelength can be used to evaluate the complex-formation constants.

Nowadays, a variety of fluorescence-based methods are used [88,89]. These include methods based on fluorescence resonance energy transfer (FRET) [90,91], time-integrated fluorescence cumulant analysis (TIFCA) [92], fluorescence correlation spectroscopy (FCS) [93] or fluorescence lifetime imaging microscopy (FLIM) [94], to name a few. These technologies are among the most promising approaches for visualizing the metal-protein and protein-protein interactions occurring in living cells. In 2013 the FCS method was used by researchers to determine the size of protein aggregation induced by metal ions [95]. Fluorescence based experiments (with the support of other analytical methods) were also conducted to provide the basis for understanding Cu(I) binding to the bacterial protein CopA [96].

5.4. CD (circular dichroism)

Circular dichroism (CD) is one of the most popular spectroscopic techniques used to investigate the structure and conformational changes of proteins, which can, in turn, provide information about their biological functions. It enables protein stability determinations, detection of protein folding and unfolding, formation of macromolecular complexes and characterization of ligand–protein and metal-protein interactions. Theoretically, it is based on the chirality of proteins. Chiral molecules absorb right and left handed circularly polarized light to different extents; that differential absorption can be measured [97]. The chromophores either possess intrinsic chirality or can be located in chiral environments [98]. CD as a low resolution technique gives less specific structural information than X-ray crystallography or NMR spectroscopy, nonetheless, it is a fast and straightforward tool that does not require large amounts of protein or extensive data processing. Most of the measurements can be made on samples containing less than 20 μg of protein in physiological buffers in a short time [99]. CD can be used to examine a large number of solvent conditions, varying temperature, pH, salinity and the presence of various cofactors [100]. Analysis of CD spectra in the far-UV wavelength region (below 240 nm), where polarized light is absorbed by peptide bonds, provides information about the secondary structure of the protein [101]. On the other hand, near-UV (260–350 nm) CD spectroscopy is sensitive to protein tertiary structure including changes coming from metal-protein interactions. Changes in CD spectra directly depend on the amount of the metal-protein complexes formed and can be used to estimate binding constants [102], in particular, when a coordination bond between a transition metal ion and a chiral molecule (like a peptide or a protein) is formed. In such case, different CD bands can appear in the spectrum, due either to the characteristic d-d absorption of the metal or to ligand-metal charge-transfer transitions (LMCT) deriving from the new metal-ligand bond [103]. For instance, the onset of a typical CD band is observed at 300–320 nm when a Cu(II) ion binds an amide nitrogen of the protein backbone [104–106]. CD spectroscopy has been widely used to study metal-protein complexes, like: cadmium binding by metallothioneins [107], conformational changes induced by carcinogenic nickel on the Histone H4 protein [108] or Cu^{2+} and Ni^{2+} interactions with bacterial proteins and their fragments [109,110].

It is also worth mentioning that CD could be used to evaluate the structure and stability of a protein under conditions used for NMR and/or to adjust the conditions, chosen for crystallization trials, to the more physiologically relevant [111].

The utility of conventional CD spectroscopy is broadened by the synchrotron radiation circular dichroism (SRCD) technique which can extend the analysis range to lower wavelengths, thus increasing the information contents and improving the signal-to-noise ratio. The

developments in instrumentation, methodologies and bioinformatics that have enabled applications of the SRCD technique for the study of proteins and protein interactions have been discussed by Wallace and Janes [112] and Kumaqai et al. [113]

5.5. FT-IR (Fourier Transform Infrared Spectroscopy) and Raman spectroscopy

FT-IR spectroscopy provides several advantages compared to other spectroscopic techniques. As mentioned above, the lack of ability of some proteins to crystallize or the large molecular weights of proteins are the main obstacles for X-ray and NMR techniques, respectively. Moreover, the crystallographic data of a protein cannot be easily extrapolated to the dynamic properties of the proteins in solutions. FT-IR is a fast and sensitive technique, widely used for the investigation of secondary structure of proteins. In order to deduce information on reaction mechanisms, the infrared bands have to be assigned to molecular groups within the protein. Analysis of the difference spectra sorts out the absorptions of respective protein groups involved in an interaction against the background of the whole sample [114]. The problem of strong attenuation of the IR signal in highly absorbing media can be avoided by the ATR (attenuated total reflection) technique due to limited path length into the sample. Two dimensional IR spectroscopy and ^{13}C labelling can be useful for structural and kinetic studies of many protein systems, such as membrane and amyloidogenic proteins [115]. Specific isotope labelling, site-directed mutagenesis and hydrogen/deuterium exchange can be very helpful in the proper identification of the chemical groups [116].

In contrast to far-ultraviolet CD, IR spectroscopy is insensitive to light scattering, thus providing a valuable tool for studying protein secondary structure [117]. The combination of CD and FT-IR analysis proved to be very helpful in the characterization of the conformational changes induced by ligand binding by proteins [118]. After CD studies, samples can be recovered, and then deposited on films for infrared analysis. FT-IR spectroscopy has been applied to structural characterization of the interaction of *Lactobacillus kefiri*'s S-layer proteins with metal ions (Cd^{2+}, Zn^{2+}, Pb^{2+} and Ni^{2+}) [119]. It was also used, together with UV–Vis absorption, fluorescence and CD spectroscopy, to investigate the influence of metal ions (Zn^{2+}, K^{+}, Cu^{2+}, Ni^{2+}, Mn^{2+} and Co^{2+}) on the binding of hesperitin (HES) to bovine serum albumin (BSA) [120].

The Raman scattering technique, which is a vibrational molecular spectroscopy derived from an inelastic light scattering process, can also be a very useful tool to determine secondary conformations of proteins and interaction structures [121,122]. Inelastic scattering means that the frequency of a monochromatic light-beam changes upon interaction with a sample. There are some advantages when using Raman in comparison to FT-IR: for instance (i) sample preparation is very easy and non-destructive, (ii) concentration of a species is directly proportional to the intensity of its spectral features, (iii) Raman can be used to analyze aqueous solutions, since it does not suffer from water absorption effects, (iv) a high level of spatial resolution can be achieved by using a Raman microscope [123]. Another asset is that samples can often be investigated without taking them out of the vial. Raman spectroscopy was used to reveal the effects caused by Zn^{2+} and Cu^{2+} ions on the thermal aggregation process of beta-lactoglobulin [124].

It is worth noting that Raman scattering is rather a weak effect. The disadvantage of the small cross section of Raman spectroscopy is overcome by the development of surface-enhanced Raman spectroscopy (SERS) which has cross sections approximately 8 orders of magnitude greater than ordinary Raman scattering [125]. For a current overview of SERS, see ref. [126]

5.6. Electron paramagnetic resonance (EPR)

The interaction of an external magnetic field with the magnetic moments of unpaired electrons, called Zeeman effect, is the basis of the EPR method [127].

Over the years, EPR remains a key technique for studying any system involving a transition metal ion that can occur in a paramagnetic oxidation state. It provides information on the chemical environment around unpaired electron(s) and therefore on the electronic structure and geometry of the metal–protein complex [128]. EPR can also examine interactions between the electron spin and nuclear spins in the vicinity, yielding valuable information on some metal-ligand interactions. This technique has been used in such investigations as: copper interactions with Amyloid-β aggregates [129], the metal-binding affinities of MntR (Manganese transport regulator) [130] or Cu (II) and Ni(II) binding to bovine serum albumin [131], to name a few.

It had been initially suggested that complex of metal binding site A of human albumin (HSA) with Zn(II) has a distorted trigonal bipyramidal coordination geometry [132]. However, the recent X-ray crystallographic structures showed site A to be essentially tetrahedral with the fourth ligand being a water molecule [133]. These findings are supported by a study, in which using CD and EPR techniques, the Cu(II) coordination at site A of HSA has also been suggested to have a tetrahedral geometry [134].

In other studies, also with the use of EPR, it has been revealed that human serum albumin closely interacts with the first 14 amino acids of the copper transporter, Ctr1 [135]. Based on information from this kind of spectroscopy in combination with UV–Vis and CD, the nature of copper complexes of the Ctr1's N-terminal sequence was displayed [136]. With the help of potentiometry, authors were able to demonstrate that the N-terminal sequence of human CTR1 is an efficient Cu(II) chelator, with the affinity of the 4 N complex around 13 times higher from that of HSA.

In bi- and polymetallic complexes, the unpaired electrons may couple through the ligands, resulting in either a paramagnetic or diamagnetic total spin system. Examples comprise the active site of cytochrome *c* oxidase [137] or diiron proteins [138]. The accurate distance over even long distances can be measured in situ, using Pulsed Electron–Electron Double Resonance (PELDOR/DEER) spectroscopy. By using PELDOR dipole–dipole interactions between paramagnetic pairs are measured in the nanometer range, and these pairs are commonly either nitroxide–nitroxide, or nitroxide–metal or metal–metal centers [139]. For more information please see rev. by G. Jeschke [140].

There are also other spectroscopic techniques, which can be useful for metal-protein investigations, such as: Mossbauer spectroscopy, widely applied for the study of iron-containing proteins and enzymes [141,142]; UV–Vis spectroscopy [103] For details, please refer to the cited reviews and references therein.

6. Calorimetric methods

In order to fully understand the thermodynamics of metal-protein interactions, a number of parameters should be determined, namely: the stoichiometry (n), the association or dissociation constant (K or K_d), the changes in Gibbs free energy (ΔG°), enthalpy (ΔH°), entropy (ΔS°), and heat capacity of binding (ΔC_p). Isothermal titration calorimetry (ITC) is the most efficient quantitative method to determine the thermodynamic properties related to a complex-formation equilibrium between a protein and a metal ion [143,144]. In fact, ITC directly measures the heat generated by the association of two binding partners and it can derive, from a single experiment, (i) the stoichiometry, (ii) the affinity constant and (iii) the enthalpy of binding. The corresponding values of free energy and entropy can be successively calculated from the affinity constant. In addition, it is also possible to determine the temperature dependence of ΔH° and the parameter ΔC_p performing measurements at variable temperature [145]. Typically, a solution of the protein is titrated with a solution of the metal (protein or other ligand), at a given pH and temperature.

The heat flow is measured and the binding isotherm is registered,

from which the stoichiometry, the association constant and enthalpy can be derived. By this technique, it is also possible to elucidate the binding of different ligands to metalloenzymes carrying two active sites, with the help of mutagenesis [146]. ITC goes beyond binding affinities and can enlighten the mechanisms underlying biomolecular interactions. To do so, proper designing of appropriate mutants and experiments is needed. Many experimental parameters, such as buffer type, concentration, purity, pH and reagent concentrations should be carefully checked and taken into account [147,148].

In addition to the heat associated with the binding reaction, the thermal output of ITC includes other contributions that must be taken into account, i.e. the heat of dilution of any solute in both the cell and the syringe and the heat coming from stirring. Moreover, when metal ions are involved, other important factors should be considered, such as: (i) multiple metal-protein equilibria; (ii) redox reactions, precipitation or hydrolysis; (iii) complex-formation with the buffer, which is present in high concentration; (iv) displacement of protons from residues of the protein and consequent protonation of the buffer. The latter contribution can be minimized by using a buffer with a low enthalpy of protonation, such as phosphate [149]. The fitting of the experimental titration curve can be accomplished though software implemented; however, further computer programs have been developed to analyze the most complicated systems [150–152].

A deep description of the theoretical aspects involved in ITC experiments is beyond the aims of this review; excellent papers that detail these aspects are available [153,154].

An attractive feature of ITC is that it allows direct, label-free measurements in water or buffer solutions. The use of titration calorimetry has been initially limited by poor sensitivity, but modern ITC instruments make it possible to measure heat effects as small as fractions of a μJ and can analyze weak to high affinity binders.

However, an important limitation of the technique has to be taken into account: for an optimum outcome, the parameter c ($c = nK$[P], where [P] is the protein concentration) should fall between 5 and 500, thus limiting the measurable binding constants into the range 10^3–$10^8\ M^{-1}$ [155]. The lower value practically depends on protein solubility; the upper limit can be extended through the use of a competitor [156].

Applications of calorimetric techniques on the role of metal binding to protein structure and activity concern studies on: neurodegenerative diseases [157–159]; zinc fingers [160]; human carbonic anhydrase II [161,162]; chaperone proteins of *Helicobacter pylori* [163]; two-site EF-hand proteins [164]; human cardiac troponin C [165]; *Escherichia coli* metal-efflux accessory protein RcnB [166]; insulin [167,168]; HIV-1 virion infectivity factor [169]; keratin [170]; manganese transporter MntC from the *Staphylococcus aureus* [171]; and membrane proteins [172]. In the last few years, metal-binding sites and metal–protein interactions in the RC-LH1 complexes from *Thermochromatium tepidum* bacteria [173] and in Vps29 subunit of metallo-phosphatase from *Entamoeba histolytica* [174] were investigated by ITC and other techniques. More information on the earlier applications of ITC in the study of ligand-protein interactions can be found in ref. [175] and references therein.

A thermodynamic description of bioinorganic systems can also be given by potentiometry, a technique complementary to ITC – overall stability constants calculated on the basis of potentiometric titrations of a complex system are useful in determining not only the strength of binding, but also in estimating the metal binding donors at various pH values [176–178]. This method is limited not by the size of the protein/peptide, but by the number of dissociating amino acid side chains; the presence of more than 20 will most likely make the system very challenging to study via potentiometry [179,180].

Another technique, widely used for the analysis of the thermal stability of proteins and their metal complexes, is differential scanning calorimetry (DSC) [181]. The instrumentation is equipped with a sample cell (which contains a solution of the protein, free or complexed, in a suitable buffer) and a reference cell (containing only buffer). The cells are heated together at a constant speed and the heat, required for the sample cell to maintain the same temperature as the reference cell, is measured. The unfolding of the protein produces one or more peaks in the thermogram at a characteristic temperature, called the thermal melting temperature (T_m) [182]. The T_m value of the protein can change as a consequence of metal complexation. In addition, DSC can provide information on other thermodynamic parameters, such as the changes of enthalpy, entropy, Gibbs free energy and heat capacity.

Some recent applications of DSC to metal interactions with proteins concern: the effect of a Cr(III) gallate complex on the thermal stability and conformation of collagen [183]; the folding behaviour of two homologous proteins, Ros87 and Ml4 (52-151), that differ in the presence of zinc ions [184]; characterization of the antigen-manganese transporter MntC of *Staphylococcus aureus* [167]; and the role of Zn(II) and Ca(II) ions in stabilization of the insulin hexamer [163].

7. SPR (Surface Plasmon Resonance)

Another technique, bringing quantitative information on binding affinities, and allowing for the study of molecular mechanisms of conformational changes, is Surface Plasmon Resonance (SPR). With this tool, kinetics and aggregation states of proteins can be investigated. Since its development in the 1980s, SPR has been applied mainly to the interrogation of protein-ligand interactions [185,186].

To run an SPR experiment, a solution of the label-free protein is immobilized on a gold surface. Gold sensor surfaces have been utilized almost exclusively. Then a sample containing a potential interacting partner in solution is injected over the surface. The changes in refractive index occurring at the gold surface (covered by dextran layer in modern devices) upon interaction of the protein with a specific ligand or a metal ion can be measured, obtaining information on the binding itself [187]. Compared to ITC techniques, SPR requires less sample, however it is more expensive (instrument and sensors) and an operationally more sophisticated technique.

In recent years, SPR has been employed to study the interaction between metal ions and proteins immobilized on surfaces [188]. However, the direct binding of metal ions to an immobilized protein is usually detectable by this technique only if it causes a major change in: *i*) protein conformation [189–191]; *ii*) protein aggregation properties [192,193]; *iii*) protein binding properties [194–201]; *iv*) or if the mass of the metal ion is very large. On the other hand, SPR proved to be a vital experimental approach to obtain the kinetic parameters of Aβ aggregation in the presence of different metal ions, which gave an insight into the pathogenesis of Alzheimer's disease (AD) [202] By applying different immobilization procedures to the protein on the metal surface it is possible to obtain specifically oriented anchored protein [203]. This approach can mimic, in a more realistic way, biological environments and opens new perspectives not accessible otherwise. For more applications of experimental approaches based on plasmonics in metal- interactions studies, please see ref. [204].

8. Summary and outlook

Metal ions play central roles in protein structure, function and stability, and metal cofactors are involved in all basic chemical processes that sustain life.

The work provides a comprehensive overview of the current state of the art of the analytical and biophysical methods utilized for studies on metal-protein interactions. Applications of these methods range from fundamental research such as the understanding and regulation of biological pathways to applied biochemistry, e.g. in studies on metallodrug-protein investigations. Metalloproteomics is gaining more and more interest in human disease research.

There are many other important tools in metalloproteomics investigations, i.e.: metalloprotein separation techniques [205,206],

Table 1
The main strengths and weaknesses of the described techniques.

Technique		Strengths	Weaknesses
MS-based methods	ESI-MS (Electrospray Ionization Mass Spectrometry)	Compatibility with liquid chromatography and tandem mass analysis. Enables rapid identification of subpicomolar amounts of proteins. ESI is well suited for dynamic studies. It allows the analysis of heavy proteins with limited m/z range analyzers due to the multiple charging realizing during ionization. High sensitivity.	Complicated spectra. A high intensity peak can eclipse smaller intensity peaks. This technique needs fine tuning work: solvent/sample ratios, flow rate, etc. Some salts, used for sample preparation, drown the signals and take time to be removed from the machine. MS is a destructive technique – the sample cannot be reused after the analysis.
X-ray crystallography and SAXS (Small-Angle X-ray Scattering)		Advantage of providing a detailed picture of how protein interacts with metals at atomic level. Crystallography can solve structures of arbitrarily large molecules. SAXS enables measurements in liquids and has short response times, for this reason it can be used to follow biological processes in real time.	The information is related to the solid state, not to the solution. Obtaining crystals is challenging. This process can dictate limitations on the types of proteins that can be investigated. Via time-resolved X-ray methods, one can only examine protein transitions that do not interfere with the crystal packing matrix. SAXS has a modest resolution.
Spectrosc. techniques	XAS (X-ray absorption spectroscopy)	Sample preparation is often relatively simple. It is possible to measure XAS on a wide variety of sample types and in a wide variety of environmental conditions. Extremely useful approach to study metal-associated proteins. Quite high resolution.	A full interpretation of the XAS data may require substantial training. The samples should contain relatively high concentrations of the absorbing atom. Practical experimental limits are set by the characteristics of synchrotron sources (what energy ranges, beam sizes and intensities are available).
	NMR (Nuclear Magnetic Resonance) Spectroscopy	One of the most informative techniques. Compared to X-ray crystallography, solution NMR does not require sample manipulations and protein crystallization.	It often needs protein labeling, which is expensive, especially for large systems. Requires a relatively high concentration of sample, with the possibility of protein precipitation under these conditions. Protein dynamics make structural analysis very challenging.
	CD (circular dichroism) Spectroscopy	Quick technique that does not require large amounts of protein or extensive data processing. Gives information about overall structure. Changes in CD spectra are directly proportional to the amount of the metal-protein complex formed and can be used to estimate binding constants. CD can be performed in a large number of solvent conditions, varying temperature, pH, salinity and the presence of various cofactors.	CD gives less specific structural information than X-ray crystallography and NMR spectroscopy. In order to record data for the wavelength below 175 nm, vacuum UV techniques or the use of high intensity synchrotron radiation sources are necessary. It is essential to minimize absorption due to other components (buffers, supporting electrolytes, solvents etc.) in the mixture.
	FT-IR (Fourier Transform Infrared) Spectroscopy	Fast and sensitive technique. It is possible to provide detailed understanding of the detailed molecular reaction mechanism by using time-resolved FT-IR. In contrast to far-UV CD, IR spectroscopy is insensitive to light scattering. Spectra can be obtained for proteins in a wide range of environments.	More restricted range of experimental conditions than in CD technique. Problems of strong attenuation of IR signal in highly absorbing media. It is essentially impossible to resolve the spectral signals when two protein subunits containing the same type of secondary structure react with each other or with metal ion.
	Electron paramagnetic resonance (EPR)	Very useful for studying paramagnetic complexes and for their quantification. Sensitive, requires relatively low amounts of sample. Spectra can be recorded in a few minutes, depending on the precise experiment.	Requires unpaired electrons, which limits the use of the technique. Most paramagnetic complexes need to be measured in very low temperatures (ca. 20 K), which increases the costs of the experiments.
	Raman spectroscopy	Requires little sample preparation. The intensity of spectral features is directly proportional to the concentration of the particular species. Provides reproducible measurements on biological samples. The spectra are generally unaffected by temperature changes.	Raman scattering is a very weak effect. The disadvantage of the small cross section is overcome by the Surface-Enhanced Raman Spectroscopy (SERS). The availability of powerful software is needed to overcome problems with interpreting Raman data.
	FCS/FCCS (Fluorescence Cross Correlation Spectroscopy)	It needs only nanomolar concentration, what can yield information closely mimicking physiological conditions. FCCS seems a very promising technique for the investigations in living cells.	For multiplexed assays, some problems emerge because the presence of many intrinsic and extrinsic fluorophores requires multiple excitation sources and detectors.
	FRET (fluorescence resonance energy transfer)	Enables quantitative analysis of metal mediated protein-protein interactions and analysis of protein conformational changes. Allows for in vivo studies of molecular interactions. Can be easily linked to other instrumentation.	Interacting proteins should be labeled, what is time consuming and expensive. Common limitations: auto-fluorescence and short fluorescence lifetime of fluorophores. It alters the natural state of the cell and therefore may not represent its real structure.
	SPR (Surface Plasmon Resonance)	It is a label-free analytical tool to study and quantify biomolecular interactions. It is possible to characterize very weak or transient interactions. Direct analysis in buffer, as well as in complex media such a serum or cellular extracts. Experiments performed at different temperatures can determine the thermodynamic properties of an interaction.	Care must be taken to ensure that data interpretation is not hampered by nonspecific binding and adsorption of the analyte to the sensor surface and instrument. It needs precise control over the delicate interplay between surface architectures, functionalization and the chosen sensor transducer principle.
ITC (isothermal titration calorimetry)		The most quantitative technique available for measuring thermodynamic properties. It is fast and label-free technique. Also catalytic reactions, conformational changes, protonation and dissociations constants can be readily quantified by ITC experiments.	Small molecule impurities and pH mismatches can cause artifacts in the thermogram. Sample concentration needs to be within a proper range to get reliable data. Requires a soluble sample.

analytical methods for imaging metals in biology [207], coordination chemistry of metals and protein fragments [208], electrochemical methods [209,210], and computational approaches for prediction of metal ion-binding sites [211,212], which have to be mentioned.

Nowadays, most researchers agree that the blending of a few independent experimental methods of investigation, joined into one concerted approach is both preferred and essential [213]. Linking time-resolved spectroscopic and structural–kinetic tools provides broad insights into specific molecular events over a wide range of timescales. The advantages and disadvantages of the discussed methods are summarized in Table 1.

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