Applications of Structural Biology and Bioinformatics in the Investigation of Oxidative Stress-Related Processes

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Abstract

Reactive oxygen species (ROS)-mediated dysfunction of certain biological processes is implicated in different diseases in humans, including cardiovascular, cancer, or neurodegenerative disorders. Not only human cells and tissues are affected by ROS but also all other biological systems, including plants and microorganisms. Primary targets of ROS are proteins, lipids, and nucleic acids. Modifications of these macromolecules result mostly in the start of signalling cascades between proteins, proteins and DNA, DNA and RNA, proteins and RNA, proteins and lipids within single cell compartments, entire cells, or tissues. In this chapter, basics of tools of structural biology (i.e., X-ray crystallography, NMR, and EPR spectroscopy) as well as bioinformatics are presented. These tools are explained as well as how they can be applied in the analysis of ROS-mediated modifications within macromolecules and systems, and perspectives are discussed.

Keywords

Bioinformatics • In vivo imaging • Oxidative stress • Signalling • Structural modifications

Abbreviations

EPR	Electron paramagnetic resonance
MX	Macromolecular X-ray crystallography
NMR	Nuclear magnetic resonance
PDB	Protein Data Bank
ROS	Reactive oxygen species
SDSL	Side-directed spin labelling

General Introduction

Depending on their ecological niche, biological systems are constantly exposed to endogenously or exogenously generated ROS. These include free radicals, oxides, and peroxides (Stadtman and Levine 2003; Groves and Ortiz de Orué Lucana 2010). Under conditions of oxidative stress, these reactive species cause oxidative modifications to macromolecules – proteins, DNA, and lipids – leading to an increased rate of mutagenesis and cell death. In humans, for example, oxidative stress is involved in many diseases such as atherosclerosis, Parkinson's disease, heart failure, myocardial infarction, and Alzheimer's disease. One might also note that postmortem analyses of brains from patients with Alzheimer's disease often reveal a high number of oxidatively modified proteins showing excessive carbonylation, 3-nitrotyrosines, or disulfide bridges (Butterfield et al. 2006; Butterfield and Dalle-Donne 2012; Walker et al. 2012). These modifications are implicated in the formation of cross-linked proteins and hence in the protein aggregation that is an important biomarker in pathogenesis and progression of this disease.

Disruption of metal homeostasis leads to oxidative stress via uncontrolled, metal-mediated formation of deleterious free radicals (Jomova and Valko 2011). Thus, metal-binding proteins are major targets for ROS attack. For example, ferrous iron generates highly reactive hydroxyl radicals ($^{\bullet}$OH) in the presence of hydrogen peroxide. This is referred to as the Fenton reaction (Fenton 1986). Due to its high reactivity, the hydroxyl radical has a half-life of less than 1 ns in aqueous solution and therefore reacts in close proximity to its site of formation (Jomova and Valko 2011). Proteins exposed to $^{\bullet}$OH undergo a set of oxidative modifications, including hydroxylation of aromatic groups and aliphatic amino acid side chains, nitration of aromatic amino acid residues, sulfoxidation of methionine residues, and conversion of some amino acid residues to carbonyl derivatives (Stadtman and Levine 2003; Siedenburg et al. 2012). Cysteine residues in proteins are additional targets for protein oxidation. Reversible oxidation of cysteines refers to a set of complex chemical reactions and can lead to multiple sulfur oxidation states, including thiols, sulfenic and sulfinic acids, thiyl radicals, disulfide *S*-oxides, or disulfides (Jacob et al. 2006; Ortiz de Orué Lucana et al. 2012). Under unstressed conditions, protein oxidation also serves as sensory mechanism that leads to the start of a cell signalling cascade resulting in a coordinated response to oxidative stress. Moreover, ROS chemistry is even exploited by certain cells. Parts of the human immune system use these species to attack and kill pathogens.

To understand complex molecular processes during the interaction between free radicals and biological systems, it is absolutely necessary to characterize the ROS-induced modifications and to obtain detailed insights into structures and dynamics of the biological macromolecules involved in ROS chemistry. Structural biology approaches such as X-ray crystallography or NMR can provide highly resolved three-dimensional structures of macromolecules. These also allow one to characterize molecular complexes, protein dynamics, and conformational changes.

Together with EPR, they can provide basic information about the environment of interplaying radicals and macromolecules. Computational approaches can also be useful in the prediction and identification of metal centers in macromolecules and in modelling dynamics.

In this chapter, we focus on the application of bioinformatics and structural biology (i.e., macromolecular X-ray crystallography, NMR, and EPR) to the characterization of ROS- induced protein modifications. In the first section, we discuss some bioinformatics approaches to identify proteins related to oxidative stress. In this, the readers will be introduced to strategies used in bioinformatics screening, data interpretation, and the need for careful planning of the working schedule and analysis. The section "X-Ray Crystallography" provides a very general overview as to how this technique revolutionized the investigations of proteins at the atomic level. It also provides examples about its application in the analysis of proteins related to oxidative stress. The sections "NMR Methods for the Investigation of Oxidative Stress-Related Processes" and "EPR Spectroscopy" provide a detailed overview about the basics of these techniques. They provide also numerous and specific examples as how they are currently applied in the investigation of oxidative stress-related processes.

Bioinformatics

If you are dealing with oxidative stress, the generation of free radicals and how biology avoids or exploits these processes, you have a choice of experimental techniques. Everything from absorption spectroscopy to X-ray crystallography might be helpful, and these methods are treated in the subsequent sections of this chapter. At the same time, you may well postpone donning a lab coat as long as possible and see what can be found out, without leaving your desk. One can also assume that one prefers the use of web services and only installs software locally, when really necessary. The questions you may ask are whether one can recognize a protein involved in free radical chemistry, what parts of the protein are important, and perhaps, how its chemistry is regulated. What we consider here are the techniques you might use to answer these questions or how best to avoid putting on a lab coat.

Philosophy

There is a broad philosophy that you might find disappointing or inelegant. Imagine you had a perfect model for physics and infinite computer time. Given a protein's sequence, you would be able to predict its structure, simply by simulating and watching the molecule fold to its final shape (conformation). You would be able to predict the chemistry by introducing reactants and seeing what happens. Unfortunately, one has neither perfect models for physics nor unlimited computer time. It is debatable whether one can expect to fold even a small protein computationally, and if you ask two quantum chemists about reaction simulation methods, you may get three suggestions giving at least four different predictions. This means that physics

and real chemistry may not be the best weapons to wield. One must accept that many predictions will be wrong and prepare excuses and explanations early on.

If you are going to make wrong guesses, there are two things to remember. First, methods such as simple sequence database searches give good estimates of significance. You might be wrong, but you can say how likely it is that you are correct. Secondly, the best excuse is to say that you are using computational methods as a source of hypotheses and you know that everything should be experimentally tested.

In the field generously termed bioinformatics, most so-called predictions are really just a matter of recognizing similarities. You might predict that your protein is involved in oxidative stress, but you are probably only saying that it is similar to a protein that is involved in oxidative stress. One may claim that some residues are involved in ligand binding, because the corresponding residues in a related protein have been mutated and found to affect ligand binding. This requires no research in the classic sense of natural sciences. It is the victory of pragmatism and the exploitation of archived data.

Obviously, the methods rely on public databases, but these are far from perfect. In 2011, it was noted that more than a fifth of the non-primate trace archives contained stretches of human DNA sequence (Longo et al. 2011). This had the entertaining result that various human functions had been ascribed to various kinds of bacteria. In 2007, the term "penta-retraction" was coined. Five papers had to be retracted when it was found that the structure they were based on contained an egregious error (Miller 2007). These examples have nothing to do with radical biology specifically, but they are a reminder that the databases are full of small mistakes that are rarely noticed or corrected. If one treads in the area of functional annotations, the ground can be even more slippery. A gene may have been labelled "putative haem-binding protein." This does not mean it binds haem groups. It means there is a similarity to a protein that probably binds haem groups. Even the original annotations are not necessarily reliable. A protein may have shown some enzymatic activity in a standard assay or screen, but this is not a guarantee that this is the primary function of the protein in vivo.

Given these caveats, one can consider what one should be doing computationally. The course of action depends on what information you have and how speculative one is willing to be. Maybe you have a protein sequence, the underlying DNA sequence, and biochemical data from wild-type and mutated versions of the protein. In that case, you should not read this section. At the other extreme, maybe you have almost no information except for a genomic sequence. We consider the cases going from very little information to the extreme of having a crystal structure for a protein involved in oxidative stress or radical biology.

Generalities

In the worst case, you do not even know exactly what protein you should be looking at, but perhaps you have a genomic sequence and you would like to find the relevant encoded proteins. If a genome has been published, it is extremely likely that it

comes with some annotation information. This means that somebody has already taken all confirmed or suspected open reading frames and done some form of database search, looking for similarities to proteins with annotated functions. The reliability of this information depends on how closely related your organism is to others which have been well investigated. If you are lucky, the database containing your genomic sequence may have direct links to a searchable genome browser. One should also check if one's organism is or was the subject of a structural genomics project. These projects usually maintain a web site with their best guess as to the function of every protein they might be tackling. Whatever source of information you find, there will still be a small text searching problem. It might be obvious to you that "radical" or "oxidative" is the relevant keyword, but a previous author may have felt that "haem-binding protein" is the crucial phrase. You have to be imaginative in your choice of keywords, and it might be wise to look for related terms in the enzyme classification scheme (EC numbers) or perhaps the gene ontology (GO) database.

Working from a Specific Sequence

Given a candidate sequence at the DNA or protein level, there is not only a good chance of making predictions (recognizing similarities). There is also the chance of estimating the plausibility of a claim or the probability of a similarity. You can work at the DNA or protein level, but the protein sequence is usually much more informative. If we just consider sequence database searches, there are major advantages to protein sequences. Firstly, using the protein sequence automatically accounts for similarities between amino acids, and uninteresting synonymous mutations have no effect. Secondly, there is a much better model for the treatment of deletions and insertions.

There is a constant trickle of new methods for sequence database searches, each of which claims some improvement in search sensitivity or speed. In practice, the workhorse method, BLAST and its variations, completely dominates the field (Altschul et al. 1990). Whether this is because of the method's established reputation or the easy access provided by web interfaces to various databases and analysis tools is irrelevant. A site such as the National Center for Biotechnology Information (NCBI) lets one quickly choose between databases and easily follow links to other databases when sequence similarities are found. It should also be clear that, if one finds the information one is looking for in a simple blast search, one should not worry about more sophisticated methods. If two proteins are sufficiently similar to be found by a simple search, one can trust that there really is an evolutionary connection between the proteins. The other important feature of blast is the simple estimate of reliability it provides. The *e*-values estimate the likelihood that your database hit has been found by chance and is not of biological significance. The theory behind these *e*-values has been well described and the small approximations used to get these values are well documented. The only real problem is the temptation of overinterpretation. An *e*-value may indicate that something in your

database is unusually similar to your query protein. It is possible that this relationship is statistically significant but reflects a long evolutionary time. In this case, it is possible for two related proteins to have different biochemical roles or even the same role in different pathways. Exactly how you interpret small *e*-values depends on how enthusiastic or desperate you are. Do not forget that discussing an *e*-value is always preferable to simply looking at the sequence identity. The significance of sequence similarity depends on the length of the alignment and the types of residues in the aligned regions. An *e*-value accounts for these factors.

Depending on how one does a database search, one may also find other information, essentially for free. The NCBI provides links to conserved domains. The European Centre for Bioinformatics web site has its own types of links to additional information about specific proteins.

If your protein sequence is somewhat more unusual, a simple database search may not retrieve any interesting candidates for homologous proteins. You may find only proteins without clear annotations. In this case, one should move to more sensitive methods. In this context, sensitive means being able to detect proteins linked by more remote evolutionary relationships. Methods such as PSI-BLAST and hidden Markov models differ very much in the details, but there is a common philosophy. One has proteins A, B, and C. The A-B and B-C relationships are significant, but the A-C relationship is too weak to be detected directly. Starting from sequence A, one may well find protein C if one is allowed to use the indirect relationships that go via protein B. Searching against a protein family database such as Pfam is based on a similar idea. One looks to see if your protein is similar to a family of proteins, rather than any specific member of the family. In a sense, this is like averaging over statistical noise. The disadvantage is that the information they provide will be less reliable than that provided by closely related proteins.

If there really is an evolutionary relationship involving your protein and there are homologues in the databases, you would expect to find these relationships using a simple sequence-based method. You may, however, feel that evolution has cheated you and standard methods are not finding similarities you expect. In desperation, you might feel entitled to look for even weaker hints to the function. If you believe that evolution has whittled away all similarity, except for a few key residues, you may want to look for sequence motifs. The term refers to a stretch of sequence between a few residues and maybe 100 residues long where some residues have very high probability at some sites. For example, some would claim that the C-X-X-C pattern (Cys-something-something-Cys) is diagnostic of disulfide oxidoreductases (Ortiz de Orué Lucana et al. 2012). Of course, without an estimate of statistical significance, this is meaningless. Any pattern can and will occur by chance in random sequences. The better web services do offer reasonably sophisticated estimates of significance. At the same time, one must be more than careful. If you test for the presence of a motif you are interested in, you can interpret the statistical significance. If you test for the presence of 1,000 motifs, you will find some by chance. If you blindly feed your sequence to a web site that checks for the presence of many thousands of motifs, you will certainly find a few with high probability, even if they arise purely by chance.

Information on Residues Within Your Sequence

Given a candidate sequence, there are many calculations one can do, each of which will help postpone the moment when you have to wear a lab coat. As always, the greater the similarity of your sequence to well-characterized proteins in the databases, the more information one will find. Usually one is interested in finding out which residues are involved in either catalysis or regulation. By far the fastest and most effective method to identify important sites is a simple multiple sequence alignment. Even if nothing is known about the biochemistry of your system, high conservation of a residue indicates that the particular site may be functionally important. A residue that is critical for catalytic chemistry or binding a ligand or cofactor can rarely mutate without disturbing the protein's function. Unfortunately, a residue such as a proline or glycine that is essential for the protein's structure may well be equally conserved, even if it is not involved in any interesting chemistry.

A simple blast search suffices to identify completely conserved residues, but this is not really sufficient. A good sequence editor such as Jalview (Clamp et al. 2004) will help to identify sites with certain properties (hydrophobic, acidic, basic). It will also calculate a more objective and general property known as sequence entropy. This is usually something like

$$S = -\sum_{i=1}^{n_{typ}} p_i \ln p_i \tag{24.1}$$

where p_i is the fraction of times (probability) amino acid type i seen in that column of the alignment and the summation runs over all $n_{typ} = 20$ types of amino acid. A value near zero means the site is very conserved. This procedure sounds very automatic and objective, but there is at least one arbitrary decision to be made – how many homologues does one consider? If your multiple sequence alignment is based on closely related species, every site will seem to be conserved. If your multiple sequence alignment ranges from bacteria to eukaryotes, almost nothing will seem to be conserved. Finally, one should note that this approach would not satisfy a true phylogenist. Counting mutations in a multiple sequence alignment assumes that mutations are independent events. If one were to put sequences on a phylogenetic tree, one would see that different sequences are correlated with each other. If one has enough patience, the correct way to look at conservation is to count mutations per site on a phylogenetic tree.

Structural Models

In a set of homologous proteins, there may be a crystal or NMR structure for one or more of them. If the primary sequence similarity is sufficiently high, this structure can serve as a template for a three-dimensional model for one's sequence. Basically, this simply means the residues from your sequence are placed on the

corresponding residues of the template. The resulting model may not be Ångstrom accurate, but it may well be sufficient to explain why a residue is strongly conserved or perhaps totally variable. It may also allow one to take advantage of information deposited with the structure. Since we are concerned with the biology and chemistry of radicals, the biochemistry will often involve cofactors, metal ions, or other ligands. If any of these have been co-crystallized with the protein and if your model is of reasonable quality, you can find the corresponding residues in your sequence.

Structural biologists spend years learning how to look at protein structures, but there are a few features you might be able to observe relatively quickly. There are many powerful, free protein-viewing programs such as Chimera, PyMOL, Coot, RasMol, or SWISS-PdbViewer, and they can be used to color a structure according to various properties (Pettersen et al. 2004; Emsley et al. 2010; Bernstein 2000; Guex and Peitsch 1997). Mapping sequence conservation onto a structure often lets one quickly recognize residues involved in the protein's specific biochemistry. Mapping hydrophobicity onto a protein surface of a soluble protein indicates some kind of interaction and perhaps the site where monomers join to form a higher-order structure (Jones and Thornton 1996; Kellis et al. 1988).

When looking at a model for a protein, one must always remember that the quality of the coordinates cannot be better than the template structure. They can only be worse. This means that if you want to look at structural properties, you may well be better off looking at the template coordinates directly. That means, you should temporarily forget your protein. Work with the homologue for which you have a structure. For example, you may want to use a program that looks for clefts in the protein surface in the belief that they are involved in ligand binding. You could apply the program to your model, but it would be far better to apply the program to the template structure since it has smaller coordinate errors. You can then take any interesting residues you identify and find the corresponding residues in your protein, using the sequence alignment.

The last interesting aspect of model building is that the good automatic web servers such as SWISS-MODEL (Schwede et al. 2003) are as good as competent human modellers. They also provide some estimate of confidence. If SWISS-MODEL does report a low confidence in a model, it is unlikely that a typical human modeller will do much better.

Structural Homologues

Sometimes, one may even have a structure for one's protein. Obviously, one can do all the analyses one could do with the model but now with the confidence that the coordinates are as correct as the structure is. If, for example, you find residues in a cleft on the protein surface, then these really are residues in a cleft and you are dealing with uncertainties in the active site prediction, but not with uncertainties in your coordinates.

Having a structure opens possibilities for yet more kinds of similarities. Remember that in evolutionary terms, structure is conserved more than the sequence.

This means that you can find evolutionarily related proteins even when a pair of proteins is so weakly related and that a sequence search cannot disclose any homologues. Servers such as SALAMI or Dali can take a set of coordinates and look for similar structures in the Protein Data Bank based solely on structural similarity, completely ignoring sequence (Schenk et al. 2008). This is only going to be helpful under certain circumstances such as when one wants to transfer information based on a structure alignment. Imagine, for example, you would like to find the residues involved in binding a cofactor such as NAD, FMN, or perhaps a haem group, but none of your sequence homologues provide a clue. A structure search may turn up candidate coordinates co-crystallized with a relevant ligand. From the structural alignment, you can find the corresponding residues in your protein. Do remember that one is now operating in the regime where sequence similarity is very weak and evolutionary distance is a rather large. This means one should be extremely careful in interpreting the results.

In this section, we have deliberately avoided de novo methods that operate from first principles. Instead, we have only considered a pragmatic approach where one tries to exploit existing data. There is one overriding principle that should guide all such work. One begins with the most reliable methods in which one has the most confidence. Only when necessary does one go to more speculative and unreliable approaches. One always starts with a simple sequence search. If one finds some information here, it is based on short evolutionary distances and the similarities you find will be plausible. If necessary, one looks at more remote sequence similarities with methods such as HHsearch, or even protein threading servers. Finally, inferences drawn from structural similarity in the absence of sequence similarity do reflect evolutionary similarity, but over much longer timescales over which protein functions to begin to change. Ultimately, if you make interesting predictions, nobody will believe you and you will have to test them experimentally, for example, using techniques such as those described in the next sections.

X-Ray Crystallography

Introduction

The diffraction of X-rays by macromolecular crystals remains the primary method to obtain high-resolution information on protein structure. The Protein Data Bank (PDB; Bernstein et al. 1977) currently contains 84,381 protein structures (11 September 2012), of which 87.9 % are the result of a macromolecular crystal diffraction experiment (MX; Table 24.1). The structures available range from those of small proteins (e.g., crambin) to large protein complexes (e.g., the ribosome) and include both soluble proteins and membrane proteins (Schmidt et al. 2011; Ben-Shem et al. 2011). Indeed, the value of crystal structures, and MX in particular, to the life sciences is reflected in the awarding of Nobel Prizes to individuals or teams of scientists for their pioneering work in structural biology methods, the analysis of membrane proteins, or large macromolecular

Table 24.1 Structure determination method of macromolecules contained in the PDB (11 September 2012)

Macromolecular X-ray crystallography (MX)	74,131
Solution nuclear magnetic resonance spectroscopy (NMR)	9,530
Electron microscopy (EM)	453
Solid-state NMR	53
Hybrid methods (i.e., combinations of MX, NMR, and EM)	51
Neutron diffraction	38
Fiber diffraction	37
Electron crystallography	32
Small angle X-ray solution scattering (SAXS)	32
Others	23

assemblies (Table 24.2). The critical advantage of MX is the potential it holds to reveal structural information at high resolution, such that individual atomic positions of all atoms within a macromolecule are known to a high accuracy. The current world record is held by the protein crambin (PDBID: 3NIR), for which diffraction data is available to 0.48 Å (1 Å $= 10^{-10}$ m). This level of information and detail allows the location of individual hydrogen atoms of side chains to be accurately located, which in turn allows an understanding of the chemical processes performed by macromolecules on the atomic level. This provides essential information in the design of drugs, the understanding of enzymatic mechanisms, as well as the basis of protein targeting, interactions, and function.

The essential component of any diffraction experiment is the availability of a macromolecular crystal, which can be produced firstly through the purification of the macromolecule of interest. The purified protein (or protein complex) can be crystallized through various methods to produce regular arrays of molecules that can be probed by X-ray radiation. Here, the goal is to "persuade" the protein molecules to adopt an ordered arrangement (a crystal lattice) by manipulating the behavior of the protein through changes in the chemical nature of the solution it is in. Typically, this is performed by adding a variety of different biochemical reagents to a protein solution that affect its surface chemistry (e.g., shielding its surface exposed electronic charges or water-repulsive (hydrophobic) surfaces). However, while this stage is the critical portion in any macromolecular structure determination project, the nature of the conditions necessary to support crystal growth cannot be easily predicted and relies primarily on the concept of "sparse matrix screening" (Jancarik and Kim 1991) – a trial-and-error approach that attempts to screen through the essentially infinite possibilities offered in chemistry in a limited number of experiments and (ideally) identify conditions that result in protein crystals. The regular assembly of proteins within a crystal (the lattice) is the reason why MX can produce such high-resolution information, primarily through an effect known as "constructive interference."

In essence these crystals can be exposed to X-ray beams and the ordered nature of the crystals gives rise to a diffraction pattern through an interaction of the

Table 24.2 Nobel Prizes awarded in field of structural biology

James Batcheller Sumner, John Howard Northrop, and Wendell Meredith Stanley (Nobel Prize in Chemistry, 1946)	Preparation and crystallization of enzymes and virus in a pure form
Linus Carl Pauling (Nobel Prize in Chemistry 1954)	Nature of the chemical bond and its application to the elucidation of the structure of complex substances (alpha helix)
Max Ferdinand Perutz and John Cowdery Kendrew (Nobel Prize in Chemistry, 1962)	Studies of the structures of globular proteins (development of isomorphous replacement techniques)
Francis Crick, James Watson, and Maurice Wilkins (Nobel Prize in Physiology or Medicine, 1962)	Discoveries concerning the molecular structure of nucleic acids
Dorothy Crowfoot Hodgkin (Nobel Prize in Chemistry, 1964)	Determination by X-ray techniques of the structures of important biochemical substances (structure of vitamin B12)
Aaron Klug (Nobel Prize in Chemistry, 1982)	Development of crystallographic electron microscopy and structural elucidation of biologically important nucleic acid-protein complexes
Johann Deisenhofer, Robert Huber, and Hartmut Michel (Nobel Prize in Chemistry, 1988)	X-ray determination of the structure of a photosynthetic reaction center
Richard Ernst (Nobel Prize in Chemistry, 1991)	Development of the methodology of high-resolution nuclear magnetic resonance (NMR) spectroscopy
Paul D. Boyer, John E. Walker, and Jens C. Skou (Nobel Prize in Chemistry, 1997)	Discovery and study of the enzymatic mechanism underlying the synthesis of adenosine triphosphate (ATP)
John B. Fenn, Koichi Tanaka, and Kurt Wüthrich (Nobel Prize in Chemistry, 2002)	Development of nuclear magnetic resonance spectroscopy and mass spectrometry
Peter Agre and Roderick MacKinnon (Nobel prize in Chemistry, 2003)	Studies of the structure and function of membrane protein ion and water channels
Roger Kornberg (Nobel Prize in Chemistry, 2006)	Studies of the molecular basis of eukaryotic transcription
Venkatraman Ramakrishnan, Thomas A. Steitz, and Ada Yonath (Nobel Prize in Chemistry, 2009)	Studies of the structure and function of the ribosome
Brian Kobilka and Robert Lefkowitz (Nobel Prize in Chemistry, 2012)	Studies of the G protein-coupled receptors (GPCRs)

electrons of the molecules in the crystal with X-rays (Fig. 24.1). Establishing the basis of diffraction was deemed such a significant breakthrough that Sir William Henry Bragg and William Lawrence Bragg were jointly awarded with the Nobel Prize in Physics (1915) "for their services in the analysis of crystal structures by means of X-rays." The diffraction "spots" seen in Fig. 24.1 contain information on the electron distribution of the atoms contained within the crystalline repeating unit

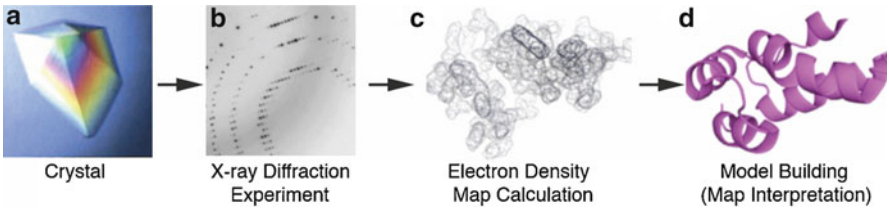

Fig. 24.1 A stucture determination project performed by X-ray crystallography requires crystals to be grown (**a**) and the intensities of the diffracted spots to be collected (**b**). Structure factor amplitudes ($|F_{(hkl)}|$) are calculated from these intensities. The Fourier transform (Eq. 24.2) is then used to calculate an electron density map (**c**) from these structure factor amplitudes, with the missing "phase" information either recovered mathematically using either "phases" from a previously known structure (molecular replacement) or "phases" that are determined experimentally (**d**)

(also known as the "asymmetric unit"). Each diffraction spot in the diffraction pattern can be identified by a unique identifier, its Miller index (h,k,l). The important parameter is the intensity (or strength) of the diffractions spots, as this is correlated with the structure factor amplitude ($|F(h,k,l)|$) of that reflection. These structure factor amplitudes in turn allow the calculation of the electron density distribution within the crystal – an electron density map (Fig. 24.1), through a Fourier transform (Eq. 24.2):

$$\rho(x,y,z) = \Sigma_{(h,k,l)} \left| F_{(hkl)} \right| \exp^{-2\pi i(hx+ky+lz)} \tag{24.2}$$

The electron density at position x,y,z ($\rho(x,y,z)$) is related to a summation of all the structure factor amplitudes of the reflection with Miller indices h,k,l ($\Sigma_{(h,k,l)}|F(hkl)|$). As the scattering of X-rays occurs only from the atoms of the macromolecule within the crystals, the structure factor amplitudes are convolved (modified by) an exponential term ($\exp^{-2\pi i(hx+ky+lz)}$) that describes the relation between each reflection and all atomic positions. These positions are not generally known a priori, giving rise to an unknown in the equation. This exponential term has the physical meaning of describing the relative "phase" of the diffracted X-rays – leading to a term commonly used in X-ray crystallography: "the phase problem."

However, while modern X-ray diffraction experiments can collect highly accurate intensities, the second parameter (the "phases" of the individual diffraction spots) needed to calculate the electron density map is not directly measured. A number of methodologies exist to recover this lost information – including molecular replacement, isomorphous replacement, and anomalous scattering.

The remaining challenge in MX is then to interpret the map in terms of an atomic model of the contents of the asymmetric unit. This is classically done by building a model that obeys the chemical and geometric constraints that are known to be present in the constituents of macromolecules – including bond lengths and bond angles. The resulting model usually contains highly accurate positions (coordinates) for each atom of the macromolecule.

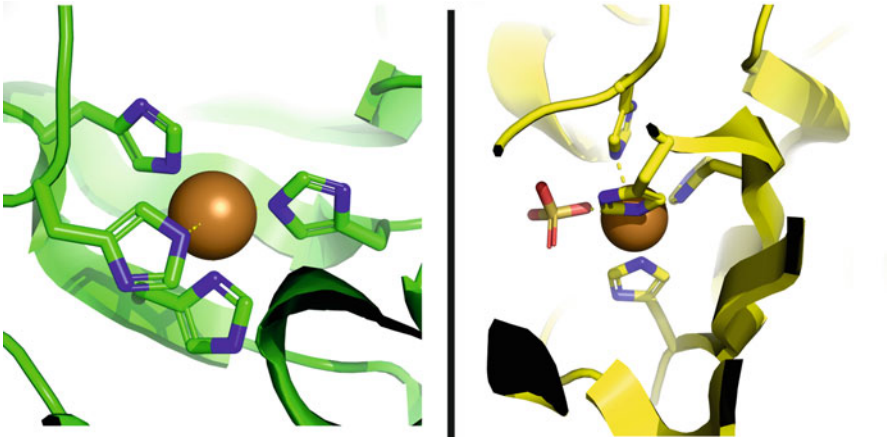

Fig. 24.2 Examples of Cu(I) and Cu(II) coordination in protein structures. The oxidation state of the copper atom can be inferred from the geometry of the residues that coordinate (interact) with the Cu atom. Cu(I) is inferred from the ∼120 ° angles between coordinating residues (*left*; PDB: 2AQN). Cu(II) is inferred from the ∼90 ° angles between the coordinating residues (*right*; PDB: 3kbf)

Crystallographic Models in the Analysis of Oxidative Stress

The result of a crystallographic structure determination is a highly accurate model of the atoms within a macromolecule. In addition to the overall structure of the molecule, these models can also be used to provide information on the oxidative state of any components in the macromolecule. For example, the resulting structures can be used to assess global structural rearrangements that may be seen in some oxidative stress signalling molecules, such as two-component sensor kinases, in which a transfer of a phosphate moiety leads to a structural rearrangement that triggers the release of the signalling component (the response regulator) to activate gene transcription (Casino et al. 2010). Structural information can also be used to assess how oxidative signalling molecules are recognized by their cognate receptors. The model can also be used to assess the oxidation state of any bound metals present within the macromolecule. Any change in the oxidation state of a metal center will be accompanied by a change in the electron structure of the metal ion(s). In favorable cases, the coordinate state of this metal can be established by examining the geometry of interaction between the metal ion(s) and the surrounding residues (Fig. 24.2). Otherwise, the crystallographic study can be completed by spectroscopic studies such as EPR and XAS (X-ray absorption spectroscopy). As shown below, EPR provides information on the electronic structure of the metal site, whereas XAS provides information on the type of ligands involved and on the geometry of the metal site (Strange et al. 2005). Crystallographic studies on redox sensor HbpS allowed the elucidation of molecular mechanisms involved in sensing of oxidative stress-based signals and also the interaction with the membrane histidine kinase SenS (Ortiz de Orué Lucana et al. 2009; Ortiz de Orué Lucana et al. 2010; Klare and Ortiz de Orué Lucana 2012).

The structures obtained can also be used to generate models of catalytic mechanisms whereby detoxifying enzymes degrade oxidative compounds, such as the breakdown of hydrogen peroxide by catalase. In this example, the knowledge of the exact position of the catalytic residues provides a starting point to understand how the transfer of electrons and/or protons allows the enzyme to catalyze the reaction (Zámocky et al. 2012).

In summary, structural information can provide a wealth of information on how biology has evolved mechanisms to sense and combat oxidative stress conditions. The models can also be used as starting points in computational drug design.

NMR Methods for the Investigation of Oxidative Stress-Related Processes

Introduction

Basics Concepts of NMR

Nuclear magnetic resonance (NMR) is a versatile technique widely applied in physics, chemistry, biology, and medicine. NMR exploits a quantum mechanical property of atomic nuclei called spin (for an introduction, see Hornak, http://www.cis.rit.edu/htbooks/nmr/ or Zheltikov 2006). In a very simplistic representation, nuclear spin can be considered as a small bar magnet that, when exposed to an external magnetic field, will take discrete orientations with respect to the magnetic field. These orientations differ in energy depending on the strength of the external field and on a physical constant, the gyromagnetic ratio (γ). Differences in energy levels are the basis for any spectroscopy. The principle of NMR relies on the interaction of the bulk or net magnetization, resulting from the slight excess of spins in the energetically more favorite orientation, with a second radio-frequency field. This field is applied during a short pulse perpendicular to the external, static field, commonly called B_0. When the frequency of the oscillating field fulfils the resonance condition (Eq. 24.3), energy is absorbed leading to the rotation of the bulk magnetization to a plane perpendicular to B_0 where it rotates with a characteristic angular frequency ω_0, called the Larmor or precession frequency. This frequency is of course also related to the energy difference between spin states and therefore to the strength of the external magnetic field (Eq. 24.3).

$$\Delta E = h\nu_0 = \gamma h B_0/2\pi \text{ and } \omega_0 = \gamma B_0 \quad (\text{with} \quad \omega = 2\pi\nu) \tag{24.3}$$

h is the Planck's constant (6.626×10^{-34} J s). NMR machines are named with respect to the Larmor frequency of the proton: a 600 MHz magnet has a static magnetic field of 14 T according to Eq. 24.3 with $\gamma_H = 2.675 \times 10^8$ rad $T^{-1}s^{-1}$. The precession of the bulk magnetization induces a small current in a receiver coil and can be measured as a time-dependent signal. A mathematical function called Fourier transformation then allows transforming the time-dependent signal back into frequency space. The different precession frequencies of the nuclei present in the

sample provide initial *structural information*. More sophisticated NMR experiments also allow to monitor spin-spin interactions in multidimensional experiments. These interactions are mainly through-bond or through-space and are the basis for resonance assignment (which signal belongs to which nucleus in the sample) and structure calculation (which nucleus is close in space to which other nucleus). In biomolecular NMR, one records signals from ^{1}H, ^{15}N, ^{13}C, and ^{31}P. However, in the case of nitrogen and carbon, the most abundant nuclei are ^{14}N and ^{12}C and these cannot be observed. This has led to much effort developing protocols for uniform or specific isotopic labelling of biomolecules (Lian and Middleton 2001; Takeda and Kainosho 2012). For example, recombinant ^{15}N- and ^{13}C-labelled proteins can easily and cost-efficiently be produced from bacteria cultured in isotopically labelled media.

Along with X-ray crystallography, NMR is a powerful technique for biomolecular structure determination (9,530 solution NMR structures in the Protein Data Bank; see also Table 24.1). Besides well-established liquid-state NMR in which molecules are studied in aqueous solution, solid-state NMR emerges in the field of structural biology. This approach allows addressing larger systems, such as protein fibrils and membrane proteins (Baldus 2007). Detailed introduction on how structural information is obtained from NMR data is beyond the scope of this review, and the reader is referred to a recently published review (Breukels et al. 2011). Along with information on 3D structure, NMR offers exciting possibilities to investigate features closely related to protein function, as, for example, study of protein modifications, intermolecular interactions (Zuiderweg 2002; Campagne et al. 2011), ligand binding (Skinner and Laurence 2008), and protein dynamics (Göbl and Tjandra 2012). Therefore, NMR can contribute important information not only for the structure determination of proteins involved in ROS-related processes but also on the physiological consequences of oxidative stress on a molecular or even cellular level.

The Chemical Shift: A Sensitive Probe for the Electronic Environment

As described above, the NMR signal corresponds to the precession frequency of a nuclear spin in a given magnetic field. A typical proton spectrum of a biomolecule (Fig. 24.3a) shows many signals, each one originating from a single proton in the molecule. These protons are characterized by different *chemical shifts* (δ). The chemical shift is expressed in ppm (Eq. 24.4) and is therefore independent of the spectrometer used.

$$\delta = \frac{\nu_{\text{substance}} - \nu_{\text{reference}}}{\nu_0} \text{ with } \nu_0 \text{ the operating frequency of the spectrometer} \tag{24.4}$$

At the origin of the chemical shift are the moving charges of the electrons that induce a local magnetic field that adds or opposes to the external static field. The effective field is therefore slightly different for each proton, resulting in different resonance frequencies (http://www.cis.rit.edu/htbooks/nmr/; Zheltikov 2006).

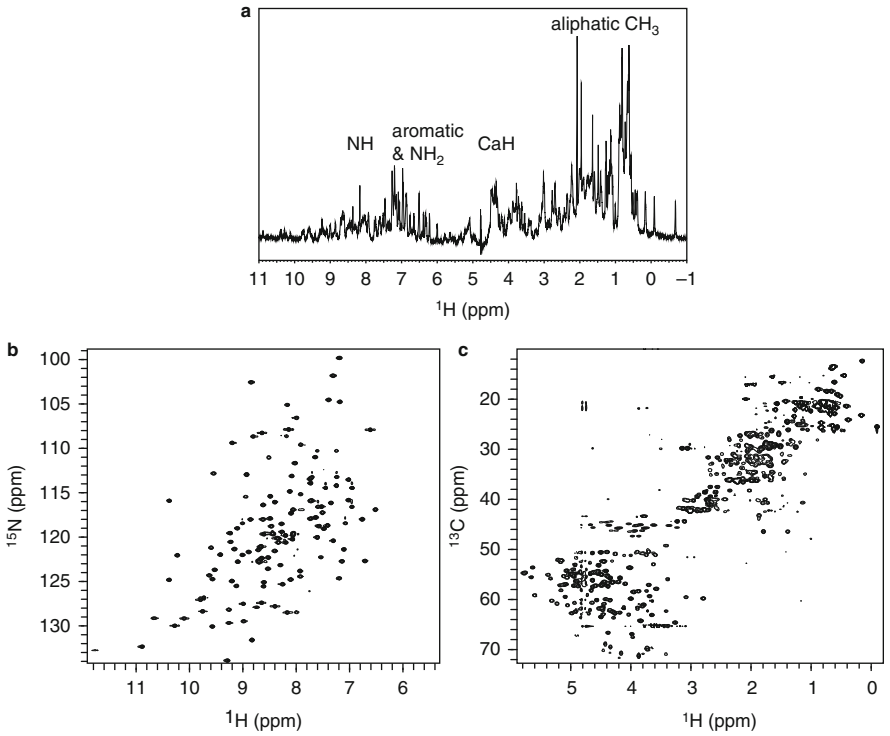

Fig. 24.3 Protein NMR spectra. (**a**) The one-dimensional ^{1}H spectrum of a 15 kDa protein. Different types of protons provide signals in different spectral regions indicated on top of the spectrum. (**b**) The two-dimensional ^{1}H,^{15}N-HSQC spectrum obtained for the same protein. Covalently bound ^{1}H and ^{15}N nuclei from the amide group give rise to a single peak by residue. (**c**) The two-dimensional ^{1}H,^{13}C-HSQC spectrum showing ^{1}H, ^{13}C correlations within the side chains of the protein (Courtesy of L. Lecoq)

Structural or chemical modifications can therefore easily be detected by comparing NMR spectra. For less complex compounds or compound mixtures, 1D ^{1}H spectra are generally recorded (Fig. 24.3a). In the case of complex compounds, resonances are separated or resolved in *multidimensional* spectra, such as ^{1}H,^{15}N- or ^{1}H,^{13}C-HSQC (heteronuclear single-quantum correlation) spectra in which a peak appears at the interception of the ^{1}H frequency and the directly attached ^{15}N or ^{13}C atom (see Fig. 24.3b, c).

Identification of Oxidative Stress-Induced Protein Modifications: Methionine Sulfoxides, Sulfenic Acids, and Cysteine Disulfide Bonds

Chemical modifications of amino acids can easily be located by inspection of the chemical shifts of nuclei next to the modified group. As proton chemical shifts are very sensitive to the electronic environment and show large variations, chemical

shifts of heteronuclei such as ^{13}C and ^{15}N are more reliable. The advantage of NMR compared to other analytical techniques is that the obtained information is *sequence specific*, once a sequential resonance assignment (Breukels et al. 2011; Ferella et al. 2012) has been performed. This means that not only the type of modified residues is determined but also their position in the protein sequence. However, in order to obtain more detailed information on the *nature* of the chemical modification, additional techniques, such as mass spectrometry, may be considered.

The most convenient way to detect these chemical modifications by NMR is the analysis of two-dimensional $^{1}H,^{13}C$-HSQC spectra. Methionine oxidation in proteins can be detected by inspecting the carbon chemical shift of the methionine methyl group. In proteins, this shift is 17.7 ± 1.49 ppm (http://www.bmrb.wisc.edu/ref_info/statsel.htm; Markley et al. 2012), whereas for isolated methionine sulfoxide, it has been determined to be 26.6 ppm (http://www.bmrb.wisc.edu/metabolomics/; Markley et al. 2012). In addition, formation of two diastereoisomers can yield two separate peaks in the NMR spectrum (Wang et al. 2001), which allows monitoring stereoselective reactions. Another example is the chemical shift of the C^{β} carbon in cysteine residues. Chemical shift analysis of 132 proteins revealed significant differences between oxidized ($^{13}C^{\beta}$: 40.7 ± 3.8 ppm) and reduced cysteines ($^{13}C^{\beta}$: 28.3 ± 2.2 ppm) (Sharma and Rajarathnam 2000). NMR can therefore identify the oxidation state of cysteines without need for any chemical modification. A recent example for the application of NMR to the determination of cysteine redox states is the investigation of human haem oxygenase-2 that is an enzyme degrading haem in the presence of oxygen and NADPH-cytochrome P450 reductase (Varfaj et al. 2012). NMR characterization of other cysteine modifications related to oxidative stress such as *S*-nitrosylation or sulfenic acid formation is less well documented in the literature. An interesting perspective could be the characterization of the reaction kinetics of oxidative stress-induced protein modifications using time-resolved NMR techniques (Schanda et al. 2007).

Identification of Metal Sites: Diamagnetic and Paramagnetic Metals

Metals can be essential for protein structure (structural sites) and function (catalytic sites) and many proteins with functions related to oxidative stress contain metal sites. Whereas for diamagnetic metals, the sequence-specific identification of protein ligands by chemical shift analysis is well established (e.g., Kostic et al. 2006; Kornhaber et al. 2006; Bersch et al. 2011), paramagnetic metals are more challenging. In paramagnetic metals, unpaired electrons are at the origin of an electronic spin (see section "EPR Spectroscopy") that interacts with the nuclear spin observed by NMR. This affects the NMR spectra leading to changes in peak position, peak broadening, and disappearance of resonances corresponding to nuclei close to the paramagnetic metal center. The magnitude of these effects depends on the electronic properties of paramagnetic metals that differ as a function of the atomic number and the particular occupancy of the molecular orbitals. As a consequence, protein ligands of paramagnetic metals cannot always be "seen" by NMR, but in favorable cases,

sequence-specific information can be obtained (Bertini et al. 1993). Replacement of a paramagnetic by a diamagnetic metal however should be considered with caution as it can result in significantly different coordination spheres.

Detection of Conformational Changes in Proteins

Metal-binding and oxidative stress-related protein modifications can induce conformational changes in proteins. This leads to variations in the observable NMR parameters related to protein structure and dynamics. The most easily accessible parameter is the chemical shift. Backbone conformational changes are most widely identified from the comparison of $^{1}H,^{15}N$-HSQC spectra in which a single peak is observed for each backbone amide group (Fig. 24.3). Simple comparison of $^{1}H,^{15}N$-HSQC spectra yields qualitative information, whereas sequence-specific resonance assignments of the unmodified and the modified protein are required for a quantitative analysis of the chemical shift perturbation. Mapping the chemical shift perturbation on the protein sequence or structure provides information on the protein regions involved in the conformational change. If the entire protein changes conformation, then all shifts are affected. Chemical shift information can be completed by additional parameters related to protein structure and dynamics (Breukels et al. 2011). As discussed above, the presence of stable paramagnetic centers in the protein such as metal ions or spin labels induces additional perturbations that can provide structural information. More details on these parameters and the information they provide can be found in review articles on NMR of paramagnetic molecules (Bertini et al. 2005; Clore and Iwahara 2009; Otting 2010). As a noninvasive technique, NMR also offers the possibility to study proteins in a more physiological environment such as living cells (in-cell NMR, Ito and Selenko 2010; Maldonado et al. 2011) or cell extracts. Recently, the maturation of human copper, zinc superoxide dismutase 1 has been studied in living *E. coli* cells and cell lysates (Banci et al. 2011). This study nicely illustrates the possibilities NMR offers for the in vivo study of protein conformational changes and ligand binding. In-cell NMR is however limited by the need for selective detection of the targeted molecule. In case of proteins to be studied, this can be achieved by either introducing isotopically labelled proteins into living cells or cell extracts or by inducing overexpression of the desired protein in appropriate cell cultures that are supplemented with isotopically labelled metabolites or amino acids (Serber et al. 2006). In any case, the amount of the protein within the cell or cell extract may be far from physiological conditions, which should be borne in mind when interpreting the experimental data.

In Vivo Magnetic Resonance Imaging (MRI) and Magnetic Resonance Spectroscopy (MRS)

In MRI, the spatial distribution of hydrogen atoms is detected using magnetic field gradients that modulate the signal's phase and frequency in a space-dependent way.

This produces cross-sectional images of the object studied, for example, the human body. The signal detected originates mostly from water or fat, which has the highest proton densities. An illustrative introduction to this technique can be found in (Hornak, http://www.cis.rit.edu/htbooks/mri/; Sands and Levitin 2004). Contrast agents are used in MRI in order to modify spectral parameters (the longitudinal relaxation rate, R_1) and thereby enhance the contrast of the image. This is achieved by paramagnetic metal complexes (mainly of Gd^{2+} or Mn^{2+} ions) that are stable and nontoxic. Their ability to affect the longitudinal relaxation rate of water protons is called relaxivity. *Structural* MRI allows determination of disease-related morphological characteristics, such as atrophy of specific brain regions related to Alzheimer's disease (Frisoni et al. 2010). Recently, the use of activatable contrast agents emerged as a tool to monitor changes in specific physiological parameters such as pH, metal ion concentration, and oxidative stress. In this approach, contrast agents are used that change their relaxivity in response to specific parameters of the microenvironment (Tu et al. 2011). An introduction to the use of activatable contrast agents in the assessment of tissue redox status by EPRI (see section "EPR Imaging") and MRI is found in the work of Hyodo et al. (2008). *Magnetic resonance spectroscopy* (MRS) allows acquiring 1D NMR spectra for individual *voxels* (volume elements) in an image (Drost et al. 2002). Because the same magnet is used for MRI and MRS, subtle metabolic changes can be detected together with morphologic information. Recent examples for the use of MRS for detection and quantification of the antioxidant glutathione in schizophrenia and neurodegenerative conditions like Alzheimer's disease and mild cognitive impairment can be found in Matsuzawa and Hashimoto (2011) and Mandal et al. (2012).

EPR Spectroscopy

Introduction

Electron paramagnetic resonance (EPR) – or electron spin resonance (ESR) – spectroscopy is the resonance spectroscopy of systems with unpaired electrons, rendering it the method of choice for studying free radicals and other paramagnetic species like transition metal ions. EPR spectroscopy therefore is a valuable tool for investigations of ROS and ROS-mediated modifications within macromolecules, as well as on redox processes involving transition metal ions either being converted from or to a paramagnetic state – a multitude of events being causative or at least closely related to oxidative stress-related processes. Its use is not limited to naturally occurring paramagnetic species. The approach of site-directed spin labelling (SDSL) – the site-specific covalent introduction of a paramagnetic reporter group into proteins or nucleic acids – combined with the application of various experimental EPR techniques can provide structural and dynamic information about the spin-labelled biomolecule. The technique is applicable to soluble molecules as well as to membrane proteins. The size and complexity of the system under investigation

is almost arbitrary, rendering this approach as a very flexible and a widely applicable tool for investigation of protein structure and conformational dynamics, complementary to X-ray crystallography and NMR techniques.

ROS-mediated processes including signalling events strongly depend on the specific properties of the system under investigation. Especially when talking about the physiological significance of ROS-mediated processes, it is necessary to investigate their impact – also from a systems biology point of view – in living cells, that is, to perform experiments in vivo. EPR spectroscopy has been established as a very powerful technique to measure ROS also in vivo, and – especially during the last years – also spin labelling approaches have been adapted to provide structural and dynamic information from experiments on living organisms.

The Basic Concept of EPR Spectroscopy

EPR spectroscopy is based on the absorption of electromagnetic radiation by paramagnetic species, that is, systems with one or more unpaired electrons, in an external magnetic field. Analogous to atomic nuclei, electrons possess an intrinsic angular momentum, the electron spin S being associated with a magnetic dipole moment with the same orientation. Consequently, for EPR a resonance condition exists, $hv = g\beta B$, with the splitting factor g ($g = 2.0023$ for a free electron), the Bohr magneton β and the magnetic field strength B, and transitions between the two energy levels of the electron in the magnetic field can be induced by applying microwave radiation of the energy hv, where h is Planck's constant and v is the microwave frequency. Interaction of the electron spin with other spins in its environment, being either other electron spins (zero-field interactions) or nuclear spins (hyperfine interactions), gives rise to further splitting of the energy levels resulting in the appearance of multiple resonance lines and/or line broadening and narrowing effects. Two basic approaches are used in EPR spectroscopy: In continuous wave (cw) EPR, radiation with constant frequency and constant power level (μW-mW) is used and the magnetic field is swept to record the resonance lines. In pulsed EPR, short (∼1–100 ns) high-power (W-kW) microwave pulses are applied, analogous to techniques used in NMR spectroscopy (Schweiger and Jeschke 2001). For introductions into the concepts of EPR spectroscopy, see, for example, Brustolon and Giamello (2009) and Hagen (2009) and references therein.

Detection of Free Radicals

Direct Detection

Most straightforward is the direct detection of stable paramagnetic species like transition metal ions and organic radicals. Especially for transition metal ions, detailed information about their nature, their coordination within a protein, and how this coordination might change, for example, under oxidative stress conditions,

can be obtained from analyses of the hyperfine interactions resulting from the close vicinity of other nuclei to the ion (Hagen 2006). For this purpose, numerous pulse EPR techniques (Schweiger and Jeschke 2001) have been developed, like electron spin-echo envelope modulation (ESEEM) (Dikanov and Tsvetkov 1992) and hyperfine sublevel correlation (HYSCORE) spectroscopy (Höfer et al. 1986), or double resonance techniques like electron nuclear double resonance (ENDOR) or pulsed ENDOR (Kulik and Lubitz 2009). For more detailed information about EPR on transition metals and also organic radicals, see Brustolon and Giamello (2009) and Hagen (2009) and references therein.

Spin Trapping

Especially in vivo most free radicals, including ROS like superoxide ($O_2^{\bullet -}$) and the hydroxyl radical ($^{\bullet}OH$), are extremely short-lived, making them no longer observable by EPR. *Spin traps* are compounds that rapidly react with such radicals to form (more) stable paramagnetic *spin adducts* that can be investigated by EPR. The number of EPR lines and their spacing results from hyperfine interactions within the spin adduct. Spectra simulations and/or comparison with hyperfine couplings obtained from known samples allow identification of the spin adducts and hence the trapped radical species. Quantification of radicals can be achieved by double integration of the EPR spectra and comparison to samples of known concentration. Commonly used spin traps for ROS are, for example, DMPO, DEPMPO, or POBN, but a variety of spin traps exhibiting different reactivities towards various radicals, for example, $Fe(DETC)_2$ for trapping of nitric oxide, are available. A comprehensive introduction into spin trapping and the properties of different spin traps can be found in Alberti and Macciantelli (2009).

SDSL EPR on Redox-Active Proteins

In site-directed spin labelling (SDSL), a paramagnetic reporter group, usually a stable nitroxide radical, is covalently attached at selected positions within proteins by cysteine substitution mutagenesis followed by modification of the sulfhydryl group (s) with a spin label, for example the most commonly used methanethiosulfonate spin label (MTSSL) (Fig. 24.4) (Altenbach et al. 1989, 1990). Similar approaches exist for spin labelling of nucleic acids. Numerous alternative spin labels with different structural, dynamic, and chemical properties and different coupling chemistry (e.g., maleimide or iodoacetamide spin labels) are available. Furthermore, several approaches to optimize the spin labelling technique for in vivo applications and for investigations under oxidative stress conditions have been developed or are under development (see section "EPR with Living Cells"). For recent, detailed reviews about the SDSL technique, see Klare and Steinhoff (2009) and references therein.

SDSL EPR can provide structural and dynamic information about the spin-labelled biomolecule. Information about the spin-label side-chain mobility, solvent

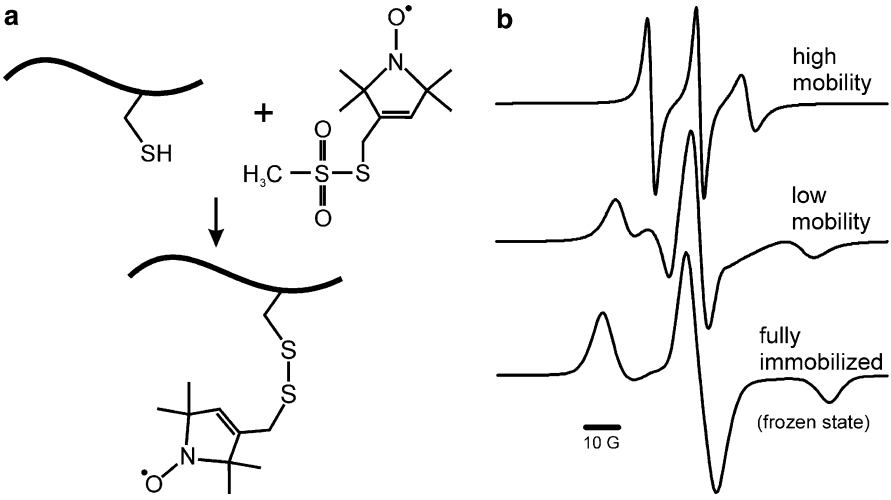

Fig. 24.4 SDSL EPR. (**a**) Reaction of MTSSL with the sulfhydryl group of a cysteine side chain. (**b**) Representative EPR spectra recorded at room temperature on a cw X-band (~9.5 GHz, ~0.34 mT) EPR spectrometer of protein-bound MTSSL with different mobilities

accessibility, the polarity of its immediate environment, and intra- or intermolecular distances between two labels or a single label and another paramagnetic center in the system can be obtained (Klare and Steinhoff 2009, 2010). Combining cw and pulsed EPR techniques like double electron–electron resonance (DEER) spectroscopy (Martin et al. 1998; Pannier et al. 2000; Klare and Steinhoff 2010) allows determining inter-spin distances in the range from ~5 to 80 Å that can be directly related to structural models (Klose et al. 2012). EPR analysis of a series of spin-labelled protein variants allows modelling of protein structures with a spatial resolution at the level of the backbone fold, and conformational changes in proteins, nucleic acids, and complexes thereof can be followed using time-resolved cw detection or be characterized by trapping the molecules in activated or intermediate states.

SDSL in combination with cw and pulsed EPR spectroscopy has recently been used to characterize conformational changes in the octomeric redox sensor protein HbpS that governs up- and downregulation of a novel type of signalling pathway. Upon iron-mediated stress, movements of subunits within the HbpS octomeric assembly could be recorded; these include a motion of the C-terminal α-helix towards the preceding helical segment (Klare and Ortiz de Orué Lucana 2012) (Fig. 24.5). Other recent examples for the use of SDSL EPR for investigations on redox-active proteins are investigation on structural and functional effects of site-directed methionine oxidation in myosin (Klein et al. 2011) and spin labelling of the *Escherichia coli* NADH-ubiquinone oxidoreductase (Pohl et al. 2010).

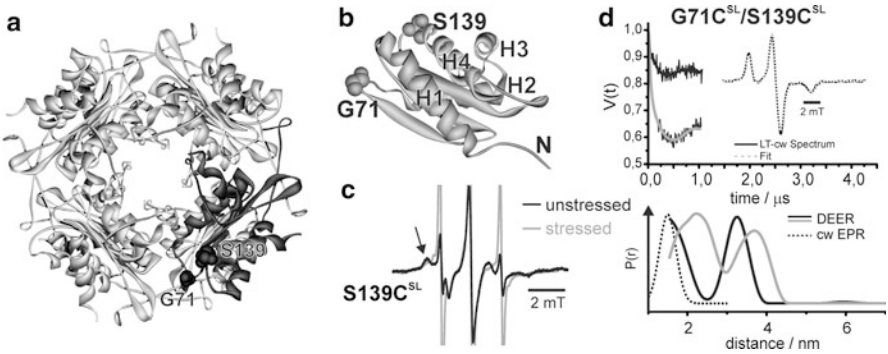

Fig. 24.5 EPR analysis of conformational changes in HbpS. (**a**) Octomeric HbpS assembly viewing down the fourfold axis. (**b**) Amino acid residues S139 and G71 on the crystal structure of a single HbpS. α-Helices are numbered H1–H4. The N-terminus is indicated. (**c**) cw EPR analysis for HbpS-S139C^{SL} showing the changes in spin-label side-chain mobility upon oxidative stress. (**d**) DEER/cw distance analysis of doubly labelled HbpS-G71C^{SL}/S139C^{SL} showing a clear distance decrease between the two spin-label side chains under oxidative stress. For distances $<$1.5 nm, the DEER data has been supplemented with low temperature cw measurements

EPR With Living Cells

To obtain definite information about the functional properties of a biomolecule and its interaction with cell components, it has to be studied in living cells. EPR methods for the in vivo detection of intrinsic paramagnetic centers in proteins like paramagnetic transition metal ions, organic enzyme cofactors, or free radical species like ROS are well established, and increasing instrumental sensitivity and spin-trapping techniques allow studying such systems despite their possibly low concentrations or short lifetimes. Contrarily, the application of spin-labelling EPR in vivo still remains challenging due to complications, like how spin labels can either be site-specifically attached to molecules in vivo or how spin-labelled moieties can be incorporated into living cells (Berliner 2010). In two recent studies in vitro labelled nucleic acids have been injected into *Xenopus laevis* oocytes, and DEER inter-spin distance measurements on the cells have been performed (Azarkh et al. 2011, Krstic et al., 2011). Alternative labelling approaches for such purposes are the so-called click reaction (Hong et al. 2010) and enzymatic labelling of proteins by means of the introduction of a peptide sequence recognized by certain phosphopantetheinyl transferases that covalently attach the desired probe to a specific serine residue within this sequence (Yin et al. 2006). A second complication is that the commonly used nitroxide spin labels are sensitive towards reducing or oxidizing conditions, limiting their lifetime at room temperature to only several minutes. Alternatives are trityl compounds also used for EPR imaging and EPR-based oximetry that have been shown to exhibit great stability in the presence of reducing or oxidizing agents (Rizzi et al. 2003) and in biological fluids (Grucker 2000), as well as complexes of the gadolinium ion (Gd^{3+}) that exhibits paramagnetic properties (Raitsimring et al. 2007; Potapov et al. 2010; Yagi et al. 2011).

EPR Imaging

Especially in the background of systems biology approaches to oxidative stress, the spatial distribution of radicals – or more general of the redox status – in specific organs or whole organisms is of utmost interest. EPR imaging (EPRI) permits the three-dimensional mapping of the spatial distribution of paramagnetic species like free radicals in tissue samples (Vikram et al. 2010), whole animals, or even parts of the human body that can be placed in the imaging devices, for example, to map tissue oxygenation, and recent developments also enable time-resolved imaging (Chen et al., 2012). EPRI can also be combined with other imaging techniques like in EPR/NMR co-imaging (Caia et al. 2012). The biomedical applicability of EPRI in general, and specifically for oxidative stress research, significantly improved during the last years, finding its applications, besides the imaging of tissue oxygenation in general, in cardiac research (Kuppusamy and Zweier 2004; Elas et al. 2008a); in brain research for, for example, three-dimensional mapping of the redox status (Liu et al. 2004; Fujii et al. 2011); in drug delivery research (Kempe et al. 2012); in dermatology, specifically in melanoma research (Vanea et al. 2008; Godechal et al. 2012), for potential monitoring of infection pathways (Tsai et al. 2008); and also in general in cancer research, for example, by monitoring tumor oxygenation (Krishna et al. 2012; Elas et al. 2008b). EPRI can also be combined with spin-trapping approaches, for example as reported already in 1996 by Yoshimura et al., for the in vivo EPRI of endogenous NO, trapped by an Fe-DTCS complex, in the abdomen of a live mouse (Yoshimura et al. 1996). The use of EPRI techniques specifically for imaging of oxidative stress in live animals has recently been reviewed by Elas et al. (2012).

In summary, NMR and EPR are powerful techniques to investigate dynamics of free radicals and proteins in solutions. They additionally allow the analysis of these dynamics in living cells, and hence, they can provide useful insights in the complex networks within biological systems during oxidative stress-related processes.

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