CHAPTER 2

Techniques to monitor disulphide-bond formation and the reduction potential of cysteine–cystine couples *in vitro* and *in vivo*

Christian Appenzeller-Herzog^{a*} and Jan Riemer^b

a) UNIVERSITÄT BASEL, Universitätsbibliothek UB Medizin, Spiegelgasse 5
4051 Basel, Switzerland

b) Universität zu Köln, Mathematisch-Naturwissenschaftliche Fakultät, 50674,
Institut für Biochemie, Zulpicher Str. 47, Köln, Germany

* christian.appenzeller@unibas.ch

Chapter Abstract

Among many biological reduction–oxidation (redox) couples, the cysteine–cystine couple (where cystine denotes two disulfide-linked cysteines) is of central importance for cellular processes. It can serve both in low molecular weight diffusible redox cofactors and in conserved reactive motifs that are integrated into peptides and proteins. Protein-bound cysteine–cystine couples exhibit highly context-specific thermodynamic and kinetic features and serve a host of structural and functional purposes. In this chapter, we review the methodological repertoire to monitor redox state and specific reactivity of biological cysteine pairs. Determination of thermodynamic parameters such as standard reduction potential of cysteine–cystine couples or pKa of individual cysteines requires classical biochemical approaches using purified components. In their native environment, however, redox state analyses of cysteine pairs are complicated by *ex vivo* artifacts, which must be minimized using appropriate thiol quenching methods. Special attention is given to fluorescent protein-based real-time sensors. These reporter proteins can be applied to virtually any living specimen to monitor biological redox data with ample spatial and temporal resolution and, in some cases, high specificity for a single redox couple.

Chapter Text

2. Techniques to monitor disulphide-bond formation and the reduction potential of cysteine–cystine couples in vitro and in vivo

2.1 Introduction

Electron transfer or reduction–oxidation (redox) reactions lead to reduction of the electron acceptor and oxidation of the electron donor compound. Such redox-active compounds are characterized by their ability to adopt a reduced or an oxidized state, which together form a so-called redox couple. Every redox couple displays a specific and quantitative tendency towards its oxidized or reduced state. This tendency is expressed in millivolts (mV) and referred to as reduction potential. Thus, the reduction potential is a thermodynamic parameter that describes the electrophilicity of a given redox couple and determines the direction of electron transfer in a redox reaction with another redox couple.

Standard reduction potentials, which express a redox couple's reduction potential under biological standard conditions (298 K and pH 7.0) and equal concentrations of reduced and oxidized species, rank different redox couples on a millivolt scale (Fig. 2.1). The effective reduction potential of a redox couple is determined by entering the standard reduction potential and the effective concentrations of reduced and oxidized species into the Nernst equation (Fig. 2.2). By definition, electron transfer from a redox couple with relatively negative effective reduction potential to a redox couple with relatively positive effective reduction potential is thermodynamically favorable.

There are many different biologically relevant redox couples and electron cascades that are composed of such redox couples. This chapter will focus on one of these, the cysteine–cystine couple. Oxidation, i.e. removal of two electrons and two protons from a cysteine pair leads to covalent fusion of the pair by a disulphide bond to form cystine (Fig. 2.3). In the case of protein-bound cysteines, the introduction of disulphide crosslinks can occur within a polypeptide chain (i.e. intramolecularly) or between two polypeptide chains (i.e. intermolecularly). Both types of disulphide bonds can serve structural or functional purposes (see chapter DISULPHIDE BONDS IN PEPTIDES AND PROTEINS: STRUCTURE, FUNCTION AND EVOLUTION).

Disulphide bonds can move from one cysteine-cystine couple to another. This reaction is called a thiol-disulfide exchange reaction (Fig. 2.4). It is initiated by the deprotonation of a cysteine to a thiolate anion nucleophile, followed by nucleophilic attack of one of the sulphur atoms in a disulphide bond. The resulting “mixed disulphide” can again be attacked by a second deprotonated cysteine to complete the thiol-disulphide exchange. The amount of deprotonated cysteine residues in a protein population at a given pH can be calculated from their pKa values. This value represents the pH at which half of all cysteine residues in a certain pool is present in the deprotonated state. While free cysteine residues have a pKa of about 8.5, pKa values of cysteine residues in protein contexts can dramatically vary between 3.5 and 10⁻¹. Thus, the determination of the pKa value of a given cysteine residue provides critical insights into its reactivity in thiol-disulfide exchange reactions.

The movement of a disulphide bond from a cysteine-cystine couple with a higher effective reduction potential to a cysteine-cystine couple with a lower effective

reduction potential is thermodynamically favorable. Standard reduction potentials of protein-bound cysteine-cystine couples vary significantly. They are influenced by both the polypeptide fold (spacing of cysteine side chains or molecular strain on the disulphide crosslink) and the chemical microenvironment ². For instance, flanking positively charged amino acids that stabilize the thiolate anion form and lower the pKa will also lower the reduction potential of a cysteine-cystine couple ³.

This chapter will provide an overview of techniques that allow the determination of the thiol-disulphide state and the reduction potential of biological cysteine-cystine couples. These techniques include in vitro methods using purified proteins, cell culture methods, and the use of fluorescent redox reporters in living organisms.

2.2 Examples of biological cysteine-cystine couples

A great number of physiological cysteine-cystine couples exist throughout the kingdoms of life. Moreover, physiological thiol-disulphide exchange is not restricted to disulphide-rich cell compartments (such as the endoplasmic reticulum, ER) and the extracellular space, but extends to virtually every subcellular location ². It is therefore beyond our scope to approach a comprehensive list of biological cysteine-cystine couples. Instead, this section will describe thiol-disulphide buffering molecules as well as selected catalysts for thiol-disulphide exchange, which are critical for the “global thiol-disulphide poise” in a cell compartment.

The probably most important and universal thiol-disulphide buffering molecule is glutathione (GSH) ⁴. Together with its dimeric form glutathione disulphide (GSSG) it forms a cysteine-cystine couple that can participate in thiol-disulphide exchange reactions. GSH is a non-conventional peptide composed of the three amino acids glycine, cysteine, and glutamic acid (Fig. 2.5). The GSH-GSSG redox couple displays a standard reduction potential of -240 mV ⁵ (Fig. 2.1).

GSH is synthesized in the cytosol and present in millimolar concentrations. Both GSH and GSSG can be exported to other compartments ⁶. The high abundance and omnipresence of GSH-GSSG explain its significant impact on virtually any other cysteine-cystine couple. Thus, the effective reduction potential of GSH-GSSG (E_{GSH}) is a characteristic indicator and determinant of the local thiol-disulphide milieu in a compartment ⁷. Apart from this global buffering function, GSH-GSSG is also specifically involved in the direct control of some redox enzymes including glutaredoxins ⁸ and glutathione peroxidases ⁹.

In addition to redox buffers, thiol-disulphide catalysts are important in cysteine-cystine biology, because even thermodynamically favorable reactions hardly take place unless they are specifically catalyzed. Thus, the flow of electrons along biological cysteine-cystine cascades is not primarily determined by the slope of their effective reduction potentials but rather by a specific set of thiol-disulphide catalysts. The most critical biological thiol-disulphide catalysts are the members of the thioredoxin (Trx) superfamily ¹⁰.

The founding member of the Trx superfamily is the cytosolic thiol-disulphide oxidoreductase thioredoxin 1 (Trx1), which also localizes to the nucleus and the extracellular space ^{11, 12}. It harbors a Cys-Gly-Pro-Cys active site motif. The Trx1 active site constitutes a cysteine-cystine couple with a particularly reducing standard reduction potential of -270 mV ¹³. It participates in various redox reactions where it commonly acts as a strong reductant. Although this activity would be expected to keep the Trx1 active site in a predominantly disulphide-bound state, the steady-state redox distribution of Trx1 is strongly shifted to the dithiol state ¹⁴. This is explained by the activity of thioredoxin reductase, which uses the reducing power of NADPH to specifically break the active-site disulphide in Trx1 and regenerate the dithiol form of the enzyme ¹⁵.

All thiol-disulphide oxidoreductases of the Trx superfamily are evolutionarily and/or structurally related to Trx1. They harbor at least one domain with a Trx1-like fold and a Cys-X-X-Cys active site (where X denotes any amino acid) that serves as catalytic cysteine-cystine couple. The Trx-like enzymes in the compartments of the eukaryotic secretory pathway are known as protein disulphide isomerases (PDIs) ¹⁶. They are all closely related to the archetypal enzyme PDI. Similarly, the periplasm of gram-negative bacteria harbors

compartment-specific thiol-disulphide oxidoreductases of the Dsb family as the structural and functional homologues of Trx family enzymes ¹⁷. As a notable exception, the major thiol-disulphide oxidoreductase in the mitochondrial intermembrane space of eukaryotes, Mia40/CHCHD4, does not harbor a Trx-like fold ¹⁸.

In contrast to Trx1, PDIs, Mia40/CHCHD4, and some Dsb family members often act as catalysts of disulphide-bond formation. This is explained by the activity of specific thiol oxidases or electron transport chains, which use the oxidizing power of molecular oxygen to catalyze the formation of the active-site disulphide in the Trx-like oxidoreductases ¹⁹.

2.3 Determination of standard reduction potentials

Important protein-intrinsic determinants of the thermodynamics of thiol-disulphide exchange reactions are the pKa values of the cysteine residues performing nucleophilic attacks and the standard reduction potentials of the involved cysteine-cystine couples. These parameters must typically be determined with purified proteins. Experimental approaches frequently rely on redox-dependent changes in protein conformation, which are monitored by changes in protein-intrinsic fluorescence or by differential accessibility of cysteine residues to alkylating agents (Figure 2.6). Fluorescence changes in the investigated proteins can take place, if the microenvironment of naturally occurring or engineered tryptophan residues is shifted in response to changes in the redox or protonation state of juxtaposed cysteine residues. Alternatively, cysteine residues that become protonated or oxidized may exhibit altered accessibilities towards alkylating agents. Since these alkylating agents can be coupled to fluorescent dyes or a bulky spacer molecule, their addition can be detected by classical SDS-PAGE followed by gel staining or fluorescence imaging. To determine the pKa value of a given cysteine residue in a purified protein, it is titrated against buffers with a given pH. Changes in cysteine protonation states during the titration are then monitored by detecting fluorescence changes or by testing their reactivity towards alkylating agents (Figure 2.6).

To determine the reduction potential of a cysteine redox couple in a protein context, samples of the protein are incubated with a model redox couple adjusted in a range of defined reduction potentials. The reaction mixes are given sufficient time to reach thermodynamic equilibrium and then the thiol-disulphide ratio of the cysteine-cystine couple in question can be read out using

a gel-based or a fluorescence approach. Knowing the reduction potential of the model redox couple and the thiol-disulphide ratio of the investigated cysteine couple, the reduction potential of the investigated cysteine couple can be calculated by using the Nernst equation. Model redox couples include the GSH-GSSG redox couple, oxidized/reduced DTT and reduced/oxidized thioredoxin. Importantly, model redox couples can only be used to determine cysteine-cystine reduction potentials in a suitable range of the millivolt scale. These problems can in principle also be circumvented by direct electrochemical measurements. In these measurements, highly concentrated protein solutions are brought into thin-layer electrochemical cells (essentially two quartz windows with a distance of about 150 μm that sandwich an optically transparent minigrid electrode) that allow on the one hand continuous changes in the applied electrochemical potential and at the same time the monitoring of spectral changes. These approaches, however, require that changes in cysteine redox states can be detected by fluorescence and also unusually high protein concentrations.

A further caveat of the described approaches is that proteins with multiple cysteine residues, which can pair in multiple ways to form disulphide bonds can only be understood by mutational analyses. However, various studies demonstrated that cysteine mutations can strongly influence pKa values and reduction potentials of other cysteines and cysteine redox couples, respectively²⁰. A way around this caveat is the parallel assignment of all cysteines in a protein by either nuclear magnetic resonance or mass spectrometric approaches upon redox titration as described above. Although this requires bigger instrumental equipment for the experiment and can bring about difficulties in

assigning specific cysteines and lack of proteolytic cleavage sites in too close-by cysteine residues, it has already proven successful in the analysis of different proteins ²¹.

2.4 In situ cysteine-cystine distributions

In contrast to the situation in vitro with highly defined test tube contents, the monitoring of a cysteine-cystine couple is more difficult in its native cellular context. Owing to the presence of enzymes that can rapidly upon cell lysis transfer disulphide bonds to non-physiological loci, a thiol quenching method must either trap the thiol-disulphide status in situ or denature proteins concomitantly to the trapping process. In addition, controlled trapping of thiols must prevent artificial air oxidation during cell lysis. Quenching of unwanted post-lysis thiol reactions can be achieved by two alternative approaches, the blocking of thiol groups with a cell-permeable alkylating agent such as N-ethylmaleimide or the sudden acidification and denaturation of the cells ²². Whereas the former method is usually irreversible but somewhat hampered in efficiency, the latter is considered to be highly efficient but must usually be followed by sample neutralization and solubilization in the presence of a thiol-alkylating agent ^{22, 23}.

In many cases, the presence or absence of intramolecular disulphide bonds in a protein can be determined by non-reducing SDS-PAGE. Since disulphide crosslinks diminish the hydrodynamic radius of an SDS-denatured protein, the disulphide-bound species can migrate more rapidly in the gel. This approach has, for instance, been applied to monitor the oxidative maturation of viral proteins in the ER of infected cells by pulse-chase analysis ^{24, 25}. Similarly, it revealed the folding kinetics of various endogenous disulphide acceptors including low-density lipoprotein receptor, α 1-integrin, albumin, and α 1-antitrypsin ²⁶⁻²⁸. As a further example, different redox species of the ER oxidase ER oxidoreductin 1

(Ero1 $\square$), which reflect the activation status of the enzyme, can readily be discriminated by non-reducing SDS-PAGE ^{29, 30}.

A special case of disulphide chemistry is intermolecular disulphide-bond formation that connects two or more proteins. The detection of these bonds by non-reducing SDS-PAGE is straightforward, as the “mixed-disulphide species” migrates approximately at the speed of the added molecular mass of the proteins in the complex. It is also possible to identify the species by immunoprecipitation, Western blotting, or a combination thereof using antibodies against the different proteins in the complex. Examples are the analyses of structural disulphide bonds in immunoglobulin M ³¹ or of a meta-stable complex between PDI and Ero1 $\square$ ³². Both intra- and intermolecular disulphide bonds have also been visualized by using a two-dimensional electrophoresis technique where the first dimension is run under non-reducing and the second dimension under reducing conditions ³³⁻³⁵. In this more sophisticated gel shift assay that has been termed non-reducing/reducing “diagonal” 2D PAGE, disulphide-bonded proteins are detected as they deviate from the central diagonal ³⁶.

Many disulphide bonds do, however, not cause a mobility shift in non-reducing SDS-PAGE, which is particularly true for short-range cysteine pairs, e.g. in the active sites of thiol-disulphide oxidoreductases. To circumvent this limitation in detection, it is possible to decorate trapped thiols in a subsequent in vitro reaction with a bulky alkylating agent. Two alternative protocols for such alkylation-dependent gel shift assays are available (Figure 2.7). They differ in the thiol quenching method (see above). In protocol 1, a cell-permeable alkylating agent is applied and the alkylated sample is reduced to unmask the disulphide bonds and then subjected to a second round of alkylation using a bulky reagent

such as 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) or polyethylene glycol maleimide ^{37, 38}. In protocol 2, the sample is acidified and the acid-quenched protein pellet solubilized in presence of the bulky alkylating agent ³⁹⁻⁴¹. It is important to note that protocol 1 induces gel shifts in response to oxidized cysteines, whereas protocol 2 induces gel shifts in response to free thiol groups. The same two labeling principles can also be applied in combination with alternative thiol-alkylating chemicals, which carry detectable labels such as fluorophores, isotopes, or immunoreactive groups and enable more diversified detection methods ^{23, 36, 42}.

2.5 Genetically encoded fluorescent protein sensors to monitor intracellular redox couples

2.5.1 General principles

The cysteine-cystine monitoring techniques described so far all rely on the principle of irreversible in situ trapping followed by in vitro visualization of thiols. As such, these techniques produce endpoint measurements, which are all limited by the fact that they can only picture dynamic biological processes by the collection of conglomerate endpoint data.

This limitation was overcome by the advent of genetically encoded fluorescent protein redox sensors. These sensors allow the continuous monitoring of reversible small redox molecule dynamics with high spatiotemporal resolution. Moreover, genetic encoding is associated with several major advantages. First, the delivery of indicator molecules into living cells or tissues can conveniently be achieved by transient transfection or transduction of DNA vectors or by their stable expression. Second, genetically encoded sensors can easily and specifically be targeted to subcellular compartments by the genetic engineering of appended amino acid targeting signals ⁴³. Depending on the promoter, expression can also be controlled in a temporal manner and in multicellular organisms with tissue specificity. Disadvantages of fluorescent protein sensors are the necessity to introduce a foreign redox-active protein into a specific compartment, which might disturb local redox processes, and the fact that the specimen has to be “genetically accessible”.

A potential pitfall in the use of genetically-encoded fluorescent sensor readouts can be their inadequate selectivity for the redox couple in question. Thus, a redox

sensor that reacts with a cysteine-cystine couple in vitro will potentially react with an undefined number of additional couples when expressed in the cellular context. Fortunately, a number of fluorescent sensors have been designed to monitor specific redox couples including the cysteine-cystine couple GSH-GSSG. To this end, a sensing entity (i.e. a sensitive protein that specifically reacts or associates with the desired redox substrate) is fused to a fluorescent monitoring entity. The transfer of information between both entities can be designed in three ways (Figure 2.8): (1) rearrangement of a Förster resonance energy transfer (FRET) pair, (2) conformational manipulation of circularly permuted fluorescent proteins (cpFPs), and (3) thiol-disulfide exchange. In the case of FRET (1), the sensitive protein is sandwiched between two fluorescent proteins that form a FRET pair and undergoes a conformational change upon reaction with a redox target molecule. This principle forms the basis for the broad-specificity redox sensors Redoxfluor ⁴⁴ and cyan-RL5-yellow ⁴⁵. Oxidation of the sensing entities brings the FRET pairs into proximity and leads to a FRET signal. (2) CpFPs exhibit prominent changes in their excitation spectra upon conformational strain. Thus, the fusion of suitable sensing entities to the termini of cpFPs can be used to design fluorescent redox sensors that translate redox-dependent conformational changes into a fluorescent readout. This principle was used in the design of the hydrogen peroxide (H₂O₂) sensors of the Hyper family ⁴⁶ and the NADH sensors Peredox ⁴⁷ and SoNar ⁴⁸. (3) Through the coupling of specific redox enzymes to a reduction-oxidation sensitive GFP (roGFP ^{49, 50}) or YFP (rxYFP ⁵¹) as monitoring entity, the sensor is rendered specific to a certain redox couple. In this case, the redox signal is carried by thiol-disulfide exchange between the sensing and monitoring entity, which shall be discussed in further

detail in the following section. The principle was used in the construction of the E_{GSH} sensors Grx1-roGFP2 ⁵² and rxYFP-Grx1p ⁵³ (see below) and the H2O2 sensors Orp1-roGFP2 ⁵⁴ and Tsa2□C_R-roGFP2 ⁵⁵.

2.5.1 roGFP-based sensors for the monitoring of E_{GSH}

RoGFPs (and to some extent rxYFP) have emerged as the most widely used cysteine-cystine sensors. They are derivatives of the *Aequorea victoria* green fluorescent protein (GFP) and harbor a cysteine pair in solvent-exposed position, which can form a disulphide bond ^{49, 50}. This disulphide induces strains on the β -barrel of the fluorescent proteins that changes the relative amplitude of the two fluorescence excitation maxima by raising the pKa of the fluorophore ⁴³. Accordingly, roGFP-based sensors are ratiometric sensors that are usually employed by monitoring their excitation at around 405 and 480 nm with an isosbestic point at around 425 nm. These measurements can be performed in either plate reader format or using fluorescent microscopes. It should be noted that the redox-dependent behavior of roGFPs can also be monitored by fluorescent lifetime measurements, which – depending on the context – produce a more sensitive readout than ratiometric fluorescence imaging ⁵⁶.

Two types of roGFP indicator have originally been described, roGFP1 and roGFP2. They differ by the presence of serine (roGFP1) or threonine (roGFP2) in the GFP-fluorophore (amino acid position 65), which affects its pKa and fluorescence excitation spectrum as well as the indicator's reduction potential ⁴⁹. Further changes in the reduction potential of the roGFP cysteine pair were achieved by the introduction of flanking spacer amino acids ⁵⁷. The resulting more oxidizing forms of roGFP were successfully used to monitor redox changes

in oxidizing environments such as the ER lumen ^{56, 58-60}. In addition, roGFPs with more reducing midpoint potential (roGFP3 and 4 ⁵⁰) were generated to allow for a more efficient monitoring of e.g. reducing stress in already very reducing compartments like cytosol and mitochondrial matrix.

When expressed in the cytosol or the mitochondrial matrix of diverse organisms, roGFPs and rxYFP are mainly responsive to changes in E_{GSH} . The redox selectivity is mediated by local glutaredoxins ^{52, 53, 61-63}. This connection has not only been used to acquire precise compartment-specific E_{GSH} values; it has also allowed for a deeper understanding of glutaredoxin function in different compartments ^{40, 53} and for the design of the specific and ultrafast E_{GSH} sensor Grx1-roGFP2 ⁵². It is important to consider that the fusion of a glutaredoxin sensing entity to roGFP is essential, when monitoring E_{GSH} in compartments that are devoid of endogenous glutaredoxins such as the ER. Accordingly, an ER-localized roGFP sensor lacking a Grx1 fusion reproduces a reduction potential that is significantly more oxidizing than E_{GSH} ⁶⁴, leading to the conclusion that PDIs and E_{GSH} are not in equilibrium in the ER.

The E_{GSH} sensor Grx1-roGFP2 has by now been used in almost a hundred original studies. It thereby contributed to our understanding in such diverse areas as mitochondrial E_{GSH} dynamics in mouse pancreatic β -cells ⁶⁵, redox changes during the cell cycle in the embryonic root meristem of *Arabidopsis thaliana* ⁶⁶, in vivo redox dynamics in murine cardiomyocytes ⁶⁷, development and aging of *Caenorhabditis elegans* ⁶⁸, and the redox balance inside gram-negative bacteria ⁶⁹.

2.6 E_{GSH} dynamics in multicellular organisms

Assessing redox molecule dynamics with fluorescent sensors in multicellular organisms is limited by optical accessibility. Thus, transgenic fluorescent redox sensors have so far been mainly used in animals that are either small or translucent. For larger animals like mice, direct in vivo imaging is limited to surface tissues. Still, many studies on in vivo redox dynamics in *Drosophila melanogaster*, *Caenorhabditis elegans*, *Danio rerio*, *Arabidopsis thaliana* and *Mus musculus* have been published, often with surprising outcomes.

For example, in living *C. elegans*, E_{GSH} became more negative during postembryonic development. Importantly, this effect was delayed when life span was extended by dietary restriction. Another study in *C. elegans* was in line with these findings but additionally demonstrated an early increase in H_2O_2 sensor oxidation already during larval development ⁷⁰. Strikingly, sensor oxidation dropped rapidly during early adulthood and began to increase only after the reproductive age. The drop in sensor oxidation after larval development closely correlated with life expectancy: long-lived mutants recovered faster to reducing conditions, whereas short-lived mutants retained higher oxidant levels throughout their mature life.

In *A. thaliana*, fluorescent E_{GSH} sensors were instrumental in understanding glutathione biosynthesis ^{62, 71}, stress responses ^{72, 73}, and developmental processes ⁷⁴. For example, the sensors helped to understand phenomena that were observed during periods of darkness ⁷³. In plastids, the sensor became less oxidized during the first day of darkness. During prolonged darkness, the degree of oxidation increased again. Conversely, in mitochondria the level of oxidation of the sensor rapidly increased, while in the cytoplasm no changes in probe

oxidation could be observed. These findings led to the conclusion that mitochondria-derived reactive oxygen species are excessively formed early during darkness at a presymptomatic stage and contribute to a senescence program that is induced by darkness.

In mice, an elegant study monitored redox dynamics in multiparametric approaches in neurons of the spinal cord. It was found that axonal mitochondria undergo spontaneous 'contractions' that are accompanied by reversible redox changes. Notably, these contractions were amplified by neuronal activity and different neuronal insults ⁷⁵. Since mouse tissues are hard to investigate optically, a method to freeze the in vivo redox state of genetically encoded redox sensors was recently designed ⁷⁶. This procedure relies on the treatment of cryopreserved and cryosectioned organs expressing transgenic redox sensors with the fast-acting thiol blocker N-ethylmaleimide. Using this method on fluorescent sensor transgenic mouse tissues, redox differences within tumor tissues, redox changes that occur during embryonic development, and redox changes caused by inflammation or nutrient starvation could be assessed.

2.7 Conclusions and perspectives

Different experimental approaches to detect cysteine redox states have been fundamental to uncover the mechanisms of diverse processes ranging from oxidative protein folding to iron-sulphur cluster biogenesis and H₂O₂ signalling. The future will likely bring us improved probes for the detection of even more posttranslational cysteine modification but also further tools for the specific manipulation of cellular cysteine redox states. These tools for manipulation on the one hand side might be improved chemicals that overcome the weaknesses of currently used glutathione reductase and thioredoxin reductase inhibitors. On the other hand, these tools might also include subcellularly targeted enzymes like ChaC1⁷⁷ that specifically degrades glutathione or hyper-active Ero1 variants^{30, 32} and D-amino acid oxidase⁷⁸ variants that can be used to introduce further oxidizing equivalents into specific subcellular sites.

Recent years have already brought about the developments of numerous novel probes e.g. for cysteine sulfenic acid formation⁷⁹ or cysteine persulfidation^{80, 81}. These probes already allowed new insights for example into H₂O₂ signalling or specific cysteine repair mechanisms^{82, 83}. The future will see a further improvement of detection methods in terms of specificity and reaction kinetics for the whole spectrum of cysteine chemotypes (including sulfinic and sulfonic acid and nitrosylated thiols). These probes will allow proteomic and directed approaches that will further improve our knowledge on dynamic subcellular cysteine modifications and their significance for cells and organisms. Likewise, roGFP probes that target critical non-glutathione cysteine–cystine couples with high specificity (e.g. Trx1) are expected to complement the redox toolbox.

Also non-cysteine directed genetically encoded probes will further improve and their applicability expand even further. Recent developments included improved probes for H₂O₂ ^{55, 84} but also probes for hydrogen sulphide ^{85, 86}. Future probes might not only become directed against further small molecules but also aid in understanding enzyme activities (e.g. fusion probes between redox enzymes and different fluorescent proteins). Inevitably, a more profound knowledge of all cellular redox interactions will feed back on our understanding of the versatile population of cysteine–cystine couples.

References

1. L. B. Poole, *Free Radic Biol Med*, 2015, **80**, 148-157.
2. D. Fass and C. Thorpe, *Chem Rev*, 2017.
3. A. R. Karala, A. K. Lappi and L. W. Ruddock, *J Mol Biol*, 2010, **396**, 883-892.
4. M. Deponte, *Biochim Biophys Acta*, 2013, **1830**, 3217-3266.
5. J. Rost and S. Rapoport, *Nature*, 1964, **201**, 185.
6. A. K. Bachhawat, A. Thakur, J. Kaur and M. Zulkifli, *Biochim Biophys Acta*, 2013, **1830**, 3154-3164.
7. Y. M. Go and D. P. Jones, *Biochim Biophys Acta*, 2008, **1780**, 1273-1290.
8. A. P. Fernandes and A. Holmgren, *Antioxid Redox Signal*, 2004, **6**, 63-74.
9. R. Brigelius-Flohe and M. Maiorino, *Biochim Biophys Acta*, 2013, **1830**, 3289-3303.
10. J. Lu and A. Holmgren, *Antioxid Redox Signal*, 2014, **21**, 457-470.
11. Y. M. Go and D. P. Jones, *Antioxid Redox Signal*, 2010, **13**, 489-509.
12. U. Schwertassek, Y. Balmer, M. Gutscher, L. Weingarten, M. Preuss, J. Engelhard, M. Winkler and T. P. Dick, *EMBO J*, 2007, **26**, 3086-3097.
13. F. Aslund, K. D. Berndt and A. Holmgren, *J Biol Chem*, 1997, **272**, 30780-30786.
14. Y. M. Go and D. P. Jones, *Curr Protoc Toxicol*, 2009, **Chapter 17**, Unit17 12.
15. M. Selenius, A. K. Rundlof, E. Olm, A. P. Fernandes and M. Bjornstedt, *Antioxid Redox Signal*, 2010, **12**, 867-880.
16. C. Appenzeller-Herzog and L. Ellgaard, *Biochim Biophys Acta*, 2008, **1783**, 535-548.
17. K. Denoncin and J. F. Collet, *Antioxid Redox Signal*, 2013, **19**, 63-71.
18. N. Modjtahedi, K. Tokatlidis, P. Dessen and G. Kroemer, *Trends Biochem Sci*, 2016, **41**, 245-260.
19. K. Inaba, *Genes Cells*, 2010, **15**, 935-943.
20. J. R. Koch and F. X. Schmid, *Nat Commun*, 2014, **5**, 3041.
21. L. Banci, I. Bertini, C. Cefaro, S. Ciofi-Baffoni, A. Gallo, M. Martinelli, D. P. Sideris, N. Katrakili and K. Tokatlidis, *Nat Struct Mol Biol*, 2009, **16**, 198-206.
22. R. E. Hansen and J. R. Winther, *Anal Biochem*, 2009, **394**, 147-158.
23. J. W. Nielsen, K. S. Jensen, R. E. Hansen, C. H. Gotfredsen and J. R. Winther, *Anal Biochem*, 2012, **421**, 115-120.
24. I. Braakman, H. Hoover-Litty, K. R. Wagner and A. Helenius, *J Cell Biol*, 1991, **114**, 401-411.
25. A. de Silva, I. Braakman and A. Helenius, *J Cell Biol*, 1993, **120**, 647-655.
26. C. E. Jessop, S. Chakravarthi, N. Garbi, G. J. Hammerling, S. Lovell and N. J. Bulleid, *EMBO J*, 2007, **26**, 28-40.
27. A. Jansens, E. van Duijn and I. Braakman, *Science*, 2002, **298**, 2401-2403.
28. L. A. Rutkevich, M. F. Cohen-Doyle, U. Brockmeier and D. B. Williams, *Mol Biol Cell*, 2010, **21**, 3093-3105.
29. A. M. Benham, A. Cabibbo, A. Fassio, N. Bulleid, R. Sitia and I. Braakman, *EMBO J*, 2000, **19**, 4493-4502.
30. C. Appenzeller-Herzog, J. Riemer, B. Christensen, E. S. Sorensen and L. Ellgaard, *EMBO J*, 2008, **27**, 2977-2987.

31. R. Sitia, M. Neuberger, C. Alberini, P. Bet, A. Fra, C. Valetti, G. Williams and C. Milstein, *Cell*, 1990, **60**, 781-790.
32. T. Rammung, M. Okumura, S. Kanemura, S. Baday, J. Birk, S. Moes, M. Spiess, P. Jenö, S. Berneche, K. Inaba and C. Appenzeller-Herzog, *Free Radic Biol Med*, 2015, **83**, 361-372.
33. M. Molinari and A. Helenius, *Methods Enzymol*, 2002, **348**, 35-42.
34. R. C. Cumming, N. L. Andon, P. A. Haynes, M. Park, W. H. Fischer and D. Schubert, *J Biol Chem*, 2004, **279**, 21749-21758.
35. H. Kadokura, H. Tian, T. Zander, J. C. Bardwell and J. Beckwith, *Science*, 2004, **303**, 534-537.
36. L. I. Leichert and U. Jakob, *Antioxid Redox Signal*, 2006, **8**, 763-772.
37. C. E. Jessop and N. J. Bulleid, *J Biol Chem*, 2004, **279**, 55341-55347.
38. C. Appenzeller-Herzog and L. Ellgaard, *Antioxid Redox Signal*, 2008, **10**, 55-64.
39. A. N. Antoniou and S. J. Powis, *Antioxid Redox Signal*, 2003, **5**, 375-379.
40. K. Kojer, V. Peleh, G. Calabrese, J. M. Herrmann and J. Riemer, *Mol Biol Cell*, 2015, **26**, 195-204.
41. A. R. Frand and C. A. Kaiser, *Mol Cell*, 1999, **4**, 469-477.
42. C. PetruNGaro, K. M. Zimmermann, V. Kuttner, M. Fischer, J. Dengjel, I. Bogeski and J. Riemer, *Cell Metab*, 2015, **22**, 721-733.
43. M. Schwarzlander, T. P. Dick, A. J. Meyer and B. Morgan, *Antioxid Redox Signal*, 2016, **24**, 680-712.
44. T. Yano, M. Oku, N. Akeyama, A. Itoyama, H. Yurimoto, S. Kuge, Y. Fujiki and Y. Sakai, *Mol Cell Biol*, 2010, **30**, 3758-3766.
45. V. L. Kolossov, B. Q. Spring, A. Sokolowski, J. E. Conour, R. M. Clegg, P. J. Kenis and H. R. Gaskins, *Exp Biol Med (Maywood)*, 2008, **233**, 238-248.
46. V. V. Belousov, A. F. Fradkov, K. A. Lukyanov, D. B. Staroverov, K. S. Shakhbazov, A. V. Terskikh and S. Lukyanov, *Nat Methods*, 2006, **3**, 281-286.
47. Y. P. Hung, J. G. Albeck, M. Tantama and G. Yellen, *Cell Metab*, 2011, **14**, 545-554.
48. Y. Zhao, Q. Hu, F. Cheng, N. Su, A. Wang, Y. Zou, H. Hu, X. Chen, H. M. Zhou, X. Huang, K. Yang, Q. Zhu, X. Wang, J. Yi, L. Zhu, X. Qian, L. Chen, Y. Tang, J. Loscalzo and Y. Yang, *Cell Metab*, 2015, **21**, 777-789.
49. C. T. Dooley, T. M. Dore, G. T. Hanson, W. C. Jackson, S. J. Remington and R. Y. Tsien, *J Biol Chem*, 2004, **279**, 22284-22293.
50. G. T. Hanson, R. Aggeler, D. Oglesbee, M. Cannon, R. A. Capaldi, R. Y. Tsien and S. J. Remington, *J Biol Chem*, 2004, **279**, 13044-13053.
51. H. Ostergaard, A. Henriksen, F. G. Hansen and J. R. Winther, *EMBO J*, 2001, **20**, 5853-5862.
52. M. Gutscher, A. L. Pauleau, L. Marty, T. Brach, G. H. Wabnitz, Y. Samstag, A. J. Meyer and T. P. Dick, *Nat Methods*, 2008, **5**, 553-559.
53. O. Bjornberg, H. Ostergaard and J. R. Winther, *Biochemistry*, 2006, **45**, 2362-2371.
54. M. Gutscher, M. C. Sobotta, G. H. Wabnitz, S. Ballikaya, A. J. Meyer, Y. Samstag and T. P. Dick, *J Biol Chem*, 2009, **284**, 31532-31540.
55. B. Morgan, K. Van Laer, T. N. Owusu, D. Ezerina, D. Pastor-Flores, P. S. Amponsah, A. Tursch and T. P. Dick, *Nat Chem Biol*, 2016, **12**, 437-443.

56. E. Avezov, B. C. Cross, G. S. Kaminski Schierle, M. Winters, H. P. Harding, E. P. Melo, C. F. Kaminski and D. Ron, *J Cell Biol*, 2013, **201**, 337-349.
57. J. R. Lohman and S. J. Remington, *Biochemistry*, 2008, **47**, 8678-8688.
58. M. van Lith, S. Tiwari, J. Padiani, G. Milligan and N. J. Bulleid, *J Cell Sci*, 2011, **124**, 2349-2356.
59. M. Delic, D. Mattanovich and B. Gasser, *FEMS Microbiol Lett*, 2010, **306**, 61-66.
60. J. Hoseki, A. Oishi, T. Fujimura and Y. Sakai, *Biosci Rep*, 2016, **36**.
61. H. Ostergaard, C. Tachibana and J. R. Winther, *J Cell Biol*, 2004, **166**, 337-345.
62. A. J. Meyer, T. Brach, L. Marty, S. Kreye, N. Rouhier, J. P. Jacquot and R. Hell, *Plant J*, 2007, **52**, 973-986.
63. D. Kasozi, F. Mohring, S. Rahlfs, A. J. Meyer and K. Becker, *PLoS Pathog*, 2013, **9**, e1003782.
64. J. Birk, M. Meyer, I. Aller, H. G. Hansen, A. Odermatt, T. P. Dick, A. J. Meyer and C. Appenzeller-Herzog, *J Cell Sci*, 2013, **126**, 1604-1617.
65. L. R. B. Santos, C. Muller, A. H. de Souza, H. K. Takahashi, P. Spegel, I. R. Sweet, H. Chae, H. Mulder and J. C. Jonas, *Mol Metab*, 2017, **6**, 535-547.
66. A. de Simone, R. Hubbard, N. Vinegra de la Torre, Y. Velappan, M. Wilson, M. J. Considine, W. J. J. Soppe and C. H. Foyer, *Antioxid Redox Signal*, 2017.
67. L. Swain, A. Kesemeyer, S. Meyer-Roxlau, C. Vettel, A. Zieseniss, A. Guntsch, A. Jatho, A. Becker, M. S. Nanadikar, B. Morgan, S. Dennerlein, A. M. Shah, A. El-Armouche, V. O. Nikolaev and D. M. Katschinski, *Circ Res*, 2016, **119**, 1004-1016.
68. P. Back, W. H. De Vos, G. G. Depuydt, F. Matthijssens, J. R. Vanfleteren and B. P. Braeckman, *Free Radic Biol Med*, 2012, **52**, 850-859.
69. J. van der Heijden, S. L. Vogt, L. A. Reynolds, J. Pena-Diaz, A. Tupin, L. Aussel and B. B. Finlay, *Free Radic Biol Med*, 2016, **91**, 34-44.
70. D. Knoefler, M. Thamsen, M. Koniczek, N. J. Niemuth, A. K. Diederich and U. Jakob, *Mol Cell*, 2012, **47**, 767-776.
71. K. K. Au, J. Perez-Gomez, H. Neto, C. Muller, A. J. Meyer, M. D. Fricker and I. Moore, *Plant J*, 2012, **71**, 881-894.
72. R. Brossa, M. Pinto-Marijuan, K. Jiang, L. Alegre and L. J. Feldman, *Plant Signal Behav*, 2013, **8**, e24781.
73. S. Rosenwasser, I. Rot, E. Sollner, A. J. Meyer, Y. Smith, N. Leviatan, R. Fluhr and H. Friedman, *Plant Physiol*, 2011, **156**, 185-201.
74. S. Rosenwasser, I. Rot, A. J. Meyer, L. Feldman, K. Jiang and H. Friedman, *Physiol Plant*, 2010, **138**, 493-502.
75. M. O. Breckwoldt, F. M. Pfister, P. M. Bradley, P. Marinkovic, P. R. Williams, M. S. Brill, B. Plomer, A. Schmalz, D. K. St Clair, R. Naumann, O. Griesbeck, M. Schwarzlander, L. Godinho, F. M. Bareyre, T. P. Dick, M. Kerschensteiner and T. Misgeld, *Nat Med*, 2014, **20**, 555-560.
76. Y. Fujikawa, L. P. Roma, M. C. Sobotta, A. J. Rose, M. B. Diaz, G. Locatelli, M. O. Breckwoldt, T. Misgeld, M. Kerschensteiner, S. Herzig, K. Muller-Decker and T. P. Dick, *Sci Signal*, 2016, **9**, rs1.
77. S. Tsunoda, E. Avezov, A. Zyryanova, T. Konno, L. Mendes-Silva, E. Pinho Melo, H. P. Harding and D. Ron, *Elife*, 2014, **3**, e03421.
78. M. E. Matlashov, V. V. Belousov and G. Enikolopov, *Antioxid Redox Signal*, 2014, **20**, 1039-1044.

79. V. Gupta, H. Paritala and K. S. Carroll, *Bioconjug Chem*, 2016, **27**, 1411-1418.
80. R. Wedmann, C. Onderka, S. Wei, I. A. Szijarto, J. L. Miljkovic, A. Mitrovic, M. Lange, S. Savitsky, P. K. Yadav, R. Torregrossa, E. G. Harrer, T. Harrer, I. Ishii, M. Gollasch, M. E. Wood, E. Galardon, M. Xian, M. Whiteman, R. Banerjee and M. R. Filipovic, *Chem Sci*, 2016, **7**, 3414-3426.
81. R. Greiner and T. P. Dick, *Methods Enzymol*, 2015, **555**, 57-77.
82. J. Yang, V. Gupta, K. S. Carroll and D. C. Liebler, *Nat Commun*, 2014, **5**, 4776.
83. M. Depuydt, S. E. Leonard, D. Vertommen, K. Denoncin, P. Morsomme, K. Wahni, J. Messens, K. S. Carroll and J. F. Collet, *Science*, 2009, **326**, 1109-1111.
84. D. S. Bilan and V. V. Belousov, *Antioxid Redox Signal*, 2016, **24**, 731-751.
85. N. Velusamy, A. Binoy, K. N. Bobba, D. Nedungadi, N. Mishra and S. Bhuniya, *Chem Commun (Camb)*, 2017.
86. Z. J. Chen and H. W. Ai, *Biochemistry*, 2014, **53**, 5966-5974.

FIGURE LEGENDS

FIGURE 2.1. Reduction potentials of various thiol-disulfide couples.

Different cellular compartments like endoplasmic reticulum (ER), periplasm and mitochondrial intermembrane space (IMS) contain dedicated oxidative machineries for the generation of disulphide bonds. Of the individual components of these disulphide relays, standard reduction potentials (E'^0) are indicated. Most of them have been determined by equilibration with the glutathione/glutathione disulfide couple (E'^0 -240 mV, grey vertical line) at pH 7.0. The final electron acceptor in all three relays is O_2 . The standard reduction potentials do not necessarily sort in the direction of electron flow through the disulphide relay. Local glutathione pools frequently influence disulphide formation. The effective glutathione reduction potentials (E_{GSH}) in specific compartments, which are denoted by grey vertical lines, have been determined using targeted genetically encoded fluorescent sensors.

FIGURE 2.2. The Nernst equation.

The Nernst equation relates the effective reduction potential of a redox couple (E') to the standard reduction potential (E'^0), temperature (T), and concentrations of the oxidized and reduced species of the redox couple (Ox and Red). R , gas constant; F , Faraday constant; n , #electrons in Red – #electrons in Ox.

FIGURE 2.3. Disulphide-bond formation.

The formation of a disulphide bond from two cysteine residues is a redox half reaction. Thus, it requires electron receptors that can take up the released electrons.

FIGURE 2.4. The thiol-disulfide exchange reaction.

Given appropriate conditions (*e.g.* suitable reduction potentials and kinetic constraints) disulphide bonds can be transferred between proteins (indicated by the grey polypeptide chains). To this end, a cysteine residue in the reduced protein is deprotonated. The resulting thiolate anion can then perform a nucleophilic attack on the disulphide bond to form a mixed disulphide intermediate. Subsequently, the second cysteine residue in the reduced protein is deprotonated and can resolve the mixed disulfide intermediate by a second nucleophilic attack. Note that the pK_a value of a cysteine residue is a key determinant of its reactivity.

FIGURE 2.5. The non-conventional tripeptide glutathione.

Glutathione is an essential compound composed of the amino acids glycine, cysteine and glutamate. Between cysteine and glutamate, an unusual peptide

bond between the γ -carboxyl group of glutamate and the α -amino group of cysteine is present. This peptide bond renders the tripeptide stable inside cells. The two peptide bonds in glutathione are synthesized by dedicated ATP-dependent enzymes in the cytosol and not by the ribosome. Via their thiol groups (see the circled sulphur atom), two reduced glutathione molecules (GSH) can form the dimerized oxidized form glutathione disulphide (GSSG, not depicted).

FIGURE 2.6. Alkylating compounds to modify reduced cysteines for the detection of their redox state.

Maleimide and haloalkyl derivatives are the predominantly used chemicals for the specific modification of cysteines. Both compounds react exclusively with the thiolate anion in deprotonated cysteine residues. They can be coupled to different groups including polyethyleneglycols (PEGs) to give them additional weight for the induction of mass shifts in SDS-PAGE or biotin for the subsequent isolation of modified cysteines by streptavidin beads (not depicted). Examples of frequently used alkylating chemicals and their molecular weights (M_w) are indicated.

FIGURE 2.7. Protocols for the identification of cysteine redox states.

In general, two types of approaches can be distinguished. Protocol 1 labels previously oxidized cysteines (inverse shift). Protocol 2 labels reduced cysteine residues (direct shift). *See main text for details.*

FIGURE 2.8. Genetically encoded fluorescent sensors for the quantitative monitoring of small redox metabolites.

Sensors are characterized by their composition of two entities, a sensing entity that interacts with the metabolite (ideally in a highly specific manner) and a fluorescent monitoring entity that allows in online detection of the metabolite. Three principal types of sensors have been developed. Left panel: Sensor oxidation by the analyte causes a detectable FRET event in the monitoring entity. Middle panel: Redox reaction with the analyte (top) or analyte binding (bottom) changes the fluorescent properties of the cpFP monitoring entity by a conformational change. Right panel: Sensor oxidation by the analyte is transferred to the redox-sensitive monitoring entity (roGFP or rxYFP) by thiol-disulphide exchange. The sensor proteins can be equipped with targeting signals for different compartments and membranes, thereby allowing the monitoring of cellular subdomains. *See main text for details.*

Figure 2.1

Nernst equation

$$E' = E^0 - \frac{RT}{nF} \ln \frac{[\text{Ox}]}{[\text{Red}]}$$

Figure 2.2

Figure 2.3

Figure 2.4

Figure 2.5

Figure 2.6

Figure 2.7

Figure 2.8