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Cellular redox potential and the biomolecular electrochemical series: A systems hypothesis

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Abstract

The role of cellular redox potential in the regulation of protein activity is becoming increasingly appreciated and characterised. In this manuscript we put forward a new hypothesis relating to redox regulation of cellular physiology. We have exemplified our hypothesis using apoptosis since its redox phenomenology is well established, but believe that it is equally applicable to several other pathways. Our hypothesis is that since multiple proteins in the apoptosis pathway are thought to be regulated *via* oxidation/reduction reactions and since cellular redox potentials have been shown to become progressively more oxidative during apoptosis, that the proteins could be arranged in an electrochemical series where the protein's standard potential correlates with its position in the pathway. Since the most stable oxidation state of the protein is determined by its standard potential and the redox potential of its environment (in a way predictable by the Nernst Equation), a quantitative model of the redox regulation of the pathway could be developed. We have outlined our hypothesis, illustrating it using a pathway map which assembles a selection of the literature on apoptosis into a readable graphical format. We have also outlined experimental approaches suitable for testing our hypothesis.

Redox potentials in biology

Intracellular redox potential is a measure of how oxidizing the environment inside the cell is. In cells redox potential is highly regulated, compartmentalised and critically important for normal physiological processes and the dysregulation of cellular redox potential is implicated in the initiation and proliferation of several diseases. Intracellular redox potential is controlled by a variety of mechanisms including regulation of glutathione, reactive oxygen species (ROS), thioredoxin, NADH and NADPH^[1, 2]. In healthy mammalian cells, the redox potential is typically more negative than that of the surrounding environment and this state is maintained by a flow of reductive electrons *via* NADPH from the pentose phosphate pathway. While NADPH provides the thermodynamic driving force for a reductive intracellular environment, the dominant “redox buffer” is considered to be glutathione. The oxidizing power of a species is generally expressed as the standard redox potential (E^θ , the work done by electron transfer *vs.* a standard hydrogen electrode, under standard conditions: 298 K, all substrates at 1 M or 1 atm, pH 0), however for biological species it is more meaningful to use the biochemical standard redox potential ($E^\oplus$, the redox potential measured as above but at pH7). Glutathione ($E^\oplus = -0.24$ V) is considered to be the cell's dominant redox buffer since its high concentration (1-10 mM) allows it to “transform” the highly reductive potential of NADPH ($E^\oplus = -0.32$ V) into a potential more amenable to the control of biomolecular oxidation state. The ratio of the oxidised form (glutathione disulfide, GSSG) to the reduced form (glutathione, GSH) varies from 1:1

to 1:300 under physiological conditions in mammalian cells and since the biochemical standard potential ($E^\ominus$) for electron transfer between GSH and GSSG is known, the redox potential at pH7 (E) can be calculated using the Nernst equation.

$$E = E^\ominus + \frac{RT}{nF} \ln \frac{[GSSG]}{[GSH]^2}$$

While the theoretical electrochemical window of the cell (determined by NADPH and oxygen) spans more than a volt, the glutathione system ensures that under physiological conditions, redox potential is tightly controlled within a relatively narrow potential window approximately -0.15 V to -0.42 V^[3]. In any given cellular state, redox potential is a measure of the thermodynamic driving force for electron transfer and modulates cellular function through regulation of the oxidation state of key molecules such as proteins and lipids^[4]. The subcellular location and magnitude of redox potential can exert a dramatic control over the state of the cell, making the difference between (for example) proliferation and apoptosis. Measurements of intracellular redox potentials have shown that compared to the cytoplasm, the nucleus is a relatively reductive environment (presumably to protect DNA integrity) and that the endoplasmic reticulum is relatively oxidizing (to enable correct protein folding). Measurements on the compartmentalization of redox potentials in eucaryotic cells have been summarized in a recent review^[5]. An increasing number of proteins are thought to be regulated by modulation of the oxidation state of key amino acids such as cysteine and proline. Since the oxidation state of any protein is influenced by the redox potential of its environment there is an obvious interplay between the redox state of the cell and the function of its component proteins.

One of the most studied examples of a redox mediated protein is Redox Factor-1 (Ref-1 or APEX1). The protein is known to contain two critical cysteines whose oxidation state mediates the activation of transcription factors such as AP1, NFκB (NFKB1), p53 (TP53) and Hif 1α (HIF1A). As a result the redox regulated effects of Ref-1 alone intersect multiple cellular pathways^[4]. Redox regulation of protein activity can also be effected by indirect mechanisms, for example Yap-1(YAP1) and NRF2(NFE2L2) are known to undergo redox-dependent changes which lead to their translocation^[6, 7]. In the case of Yap-1 translocation to the cytoplasm from the nucleus following disulfide reduction leads to a loss of its activity as a transcription factor, turning off the expression of antioxidant proteins. Protein turnover can also be redox dependent, for example degradation of HIF1α is regulated *via* proline hydroxylation which is dependent on the redox status of the cell^[8]. Some proteins are more susceptible to oxidation than others and subtle changes in potential of as little as 30 mV in a particular organelle may cause a 10 fold difference in the ratio of oxidized/reduced forms of a protein. This sensitivity to small changes in potential (along with differences in localisation and reaction kinetics) (7) provides a basis for specificity in redox signaling which could lead to significant activation of protein or pathway activity.

Apoptosis: an oxidative phenomenon.

Apoptosis is the physiological process of programmed cell death which is thought to be driven by oxidative changes in the cell^[1]. Cells undergoing apoptosis typically exhibit a redox potential more oxidative than -190 mV which may be driven by a variety of mechanisms including GSH export^[9, 10], ROS generation by proteins such as p66^{SHC} (SHC1) or NADPH oxidases and changes in metabolic efficiency or metabolic pathway (leading to oxidation of the cell's GSH pool)^[11].

Redox regulation of apoptosis by protein redox-switches

Within the known apoptotic pathways, several redox-regulated molecular switches have been identified. For example, mTOR (MTOR) is an upstream regulator of p53 whose structure and function has been proposed to be redox regulated^[12]. p53 has been shown to change its activity depending on its redox environment^[13], and recent studies have shown that its DNA binding domain contains several reactive cysteines whose oxidation state is differentially regulated by redox potential^[14]. The turnover of HIF1 α (an important regulator of hypoxia-induced transcription) is regulated in a redox dependent manner by prolyl hydroxylase (PHD) and as a result only accumulates in significant levels under reductive conditions^[8, 15]. Proteins downstream of p53 and HIF1 α such as p66^{SHC} and Bax (BAX) are known to contain reactive cysteines whose oxidation states are critical regulators of their apoptotic function^[11, 16-18]. The regulatory role of redox potential is further underlined by the finding that a key step in the pathway, apoptosome formation, is dependant on the oxidation state of key components: both cytochrome-c (CYCS) and caspase-9 (CASP9) are required to be oxidized for formation and activity of the apoptosome. While activation of CASP9 is facilitated *via* (oxidative) disulfide mediated interaction with APAF1, excessive oxidative stress has been shown to inactivate the function of caspase 3 (CASP3) *via* oxidation of the active-site catalytic cysteine residue and at higher levels of oxidative stress, cell death occurs primarily *via* necrosis^[18-20]. This finding suggests that there is a “potential window” in which apoptosis occurs and that redox potential is a key regulator of the process.

Pathway mapping Overview

Redox regulated molecular modifications, like any biochemical interaction, do not exist in isolation. To fully understand them, they must be viewed in the context of the other interactions that occur around them. However, for proteins such as the tumour suppressor, TP53, whose activity is subject to regulation by a myriad of background processes, this is a daunting task. Visualisation of these

interactions is a key tool in our arsenal to aid understanding, and pathway mapping plays an important role.

Many early examples of pathway maps used schemes invented by a specific author which require specialist knowledge of the content to decipher. In response to this, standardized pathway mapping schemes such as KEGG (<http://www.genome.jp/kegg/>), mEPN (modified Edinburgh Pathway Notation)^[21] and, most recently, SBGN (Systems Biology Graphical Notation)^[22] have been developed. These standardized graphical notation schemes afford a reader fluent in their use the ability to interpret what is being described, regardless of whether they possess any specialist knowledge of a pathway. Furthermore, the use of standardized graphics allows the maps to be updated easily as new knowledge becomes available.

Here we use pathway mapping to aid in the visualization of how redox potentials regulate apoptosis. The map shown here uses a slightly modified version of SBGN since its generic process nodes allow the map to show transitions between cellular redox states. The scheme used here shall be referred to as mSBGN (modified SBGN). mSBGN differs from SBGN in two ways: 1) mSBGN allows phenotype nodes (hexagons) to participate in processes *via* process nodes (open squares); and 2) the colour scheme from the modified Edinburgh Pathway Notation (mEPN)^[21] was appropriated for ease of identification of compartments.

Not only do pathway maps serve as a means of visualization they also serve to illustrate dependencies (*e.g.* formation of complex AB is dependent on proteins A and B being present), thus facilitating subsequent *in silico* modeling. Broadly speaking, mathematical modeling can be done using ordinary differential rate equations, stochastic differential equations or logic modeling. Stochastic differential equations attempt to predict the behavior of each individual element in a given system and are thus extremely computationally intensive. This limits the size of the systems that can be modeled. Logic mapping reduces complexity by discretising gene expression data into binary ON/OFF states, this reduces computational overheads and allows the modeling of large systems at the expense of accuracy. Modeling using ordinary differential equations represents the midpoint between both in terms of accuracy and computational power needed^[23]. Systems modeling seems well suited to the study of redox regulation since the thermodynamically stable oxidation state of any protein can be predicted using the Nernst Equation, assuming a knowledge of the protein's $E^\ominus$ and the redox potential E in its immediate environment.

Our map (Figure 1) has been assembled using published data collated from primary research papers and each process is supported by at least 3 pieces of peer-reviewed primary literature. Where conflicts arise between published literature, a decision has been made based on: 1) number of supporting articles, 2) methodology, and 3) number of citations. Conflicts arose in two places. Firstly, pertaining

to the interactions between HIF1A and p53, Rempe et al have suggested that the HIF1A:p53 interaction might be an artifact from studying transformed cell-lines and have presented data showing that the two proteins do not interact in primary astrocytes^[24]. However, a wealth of supporting experimental data led to the inclusion of this interaction in the final map^[25-27]. Secondly, pertaining to the redox-regulation of protein kinase c (PRKC), reports suggests that PRKC is oxidatively regulated in a biphasic manner^[28], with initial oxidation of cysteines stimulating activity, while further oxidation is inhibitory. However, research from the same group suggested oxidative modification was either purely stimulatory or inhibitory^[28-31]. These differences may be due to the different isoforms studied. The biphasic mechanism was settled upon due to it explaining both stimulatory and inhibitory modifications found in other publications. However, oxidative modifications of PRKC are shown using SBGN's uncertain process node (open square containing a question mark) to illustrate that this important step merits further investigation.

Information pertaining to each interaction is recorded in an Excel spreadsheet which can be found in supporting information. Each line of this spreadsheet contains details of one interaction described in a specific primary research article. These details include: the names of the two interactants, along with Entrez gene and UniProt ID numbers; descriptions of the type and location(s) of this interaction; and the pubmed ID of the reference along with the cell-type(s) and methodology they used. Specific ontologies (found at <http://www.ebi.ac.uk/ontology-lookup/>) have been used to provide unambiguous descriptions of interaction types and locations. This avoids ambiguity from misinterpreting process descriptions. Names of interactants are given according to their HUGO (Human Genome Organisation) Gene Nomenclature Committee symbols (HGNC). HGNC represents a standardised nomenclature for naming and describing human genes. All genes are assigned a short-form symbol and a long-form description. Use of these names allows easy retrieval of information pertaining to specific genes from databases. Most importantly, using the HGNC nomenclature avoids ambiguity caused by some proteins having several different synonyms

Pathway map depicting redox regulation

The map (Figure 1) is a schematic depiction of a cell with compartments shown in different colours. The map illustrates the interactions which underpin apoptosis. The map shows 3 things: 1) the sequence of interactions involved in initiating and propagating apoptosis; 2) the components of these interactions that are regulated by oxidative modification, and thus by intracellular redox potential; and 3) the processes that regulate intracellular redox potential.

At time of writing this pathway map is the first to incorporate the role of redox potential in the apoptotic process. This is done by using SBGN's phenotype (hexagon) symbol to illustrate the broad

ranges of redox potential that exist during oxidative stress, normoxia and hypoxia. Since the map is constructed from existing literature, redox state is represented in the broad terms of oxidative stress, normoxia and hypoxia

To illustrate the use of the map, Figure 3 explains a small section of the map that deals with apoptosome assembly and CASP9 activation. Under normoxic conditions CYCS is located in the mitochondrial inner membrane and CASP9 is in an inactive form in the cytoplasm. Oxidative changes lead to mitochondrial outer membrane permeability transition (MOMP)^[32] and membrane permeabilisation due to BAX oxidation and the combination of these factors allows translocation of CYCS to the cytoplasm. Figure 3 illustrates the association between CYCS and APAF1 (indicated by black lines leading from the proteins to a filled black circle). Oxidation of CASP9 is indicated by a red line and process node linking CASP9 with the form of CASP9 in which cysteine 403 has been oxidized (indicated by ox@C403 as a state variable). The role of oxidative stress in CASP9 oxidation is indicated by a red line leading from the red hexagon which illustrates the cell's oxidative phenotype to the process node between the two CASP9 oxidation states. Oxidation of CASP9 is followed by association with the preformed CYCS/APAF1 complex and apoptosome formation (indicated by black lines leading to a filled black circle). Apoptosome formation leads to activation of CASP9, this is again illustrated by a change in its state variable. Activated CASP9 can subsequently proteolytically activate CASP3 and CASP7.

$E^{\oplus}$	Protein
-ve	KEAP
	TP53
	SHC p66
	BAX and SLC25A4
	CYCS
	CASP9
+ve	CASP3 and CASP7

Table 1. Qualitative electrochemical series of apoptotic redox proteome.

Our Hypothesis

Since our map shows the several distinct stages of apoptosis which are regulated by specific protein oxidation, and since it is known that redox potential becomes progressively more oxidative throughout the course of apoptosis, it raises an interesting hypothesis: if certain key proteins are regulated by redox potential and the proteins are ordered within the pathway depending on their

function, these redox regulated proteins must be ordered in an electrochemical series where their $E^\oplus$ correlates with their position in the pathway. In other words, proteins which are regulated early in the pathway have the most reductive redox potential (are easiest to oxidize) and those used latest in the pathway have the most oxidative potential (are the most difficult to oxidize). The repercussions of this hypothesis are that there are key “gateway potentials” that the cell must attain in order to regulate specific proteins and progress to the next stage of apoptosis and also that the cell may “step back” from apoptosis before these key potentials are reached; furthermore it suggests when the potential becomes excessively oxidative, and caspase activity is inhibited this functions as a switching point at which cell death progresses primarily *via* necrosis. A qualitative, hypothetical electrochemical series of the known redox proteome for the apoptosis pathway is suggested in Table 1. While the above map is useful for visualizing biochemical interactions, such a map has the potential to be developed into a quantitative model of redox regulation of apoptosis.

In order to quantitatively model apoptosis as an electrochemically regulated system we need to be able to measure the standard potential of the aforementioned redox regulated proteins and the redox potential of the organelles as the cell proceeds through apoptosis. With this information it would be possible to assemble a model of apoptosis in which the thermodynamic driving force for reaction of key proteins could be calculated using their standard potential and the redox potential of their environment. The following section summarises the various technical approaches which may be used in addressing this hypothesis.

Intracellular redox potential measurements.

Intracellular redox potential is traditionally calculated by measuring the concentrations of oxidised and reduced forms of glutathione^[1, 33]. To make this measurement requires cell lysis and strict environmental control to ensure that the ratio does not change as a result of environmental oxidation. Furthermore, this measurement calculates a potential that is averaged over the whole cell and over a population of cells and cannot take into account local variations between cells or compartmentalisation within a cell. The measurement is most sensitive at potentials close to the standard reduction potential for glutathione and as such does not accurately quantify potential in cases in which redox homeostasis is dysregulated to the extent that glutathione no longer controls potential (a likely cause of disease)^[34]. While other means of measurement, such as the direct cellular microinjection of glutathione reductase crystals can give information on localised redox potential the technique is slow to respond to changes in potential and difficult to use^[35].

Recently, a range of genetically encoded fluorescent reporter proteins has been engineered in order to non-invasively monitor redox potential^[2]. These proteins are based on Green Fluorescent Protein and

have been engineered such that oxidised and reduced states have different excitation wavelengths (termed roGFP). These proteins have been used for the measurement of redox potential and through fusion to targeting domains from proteins such as pyruvate dehydrogenase, cytochrome oxidase or glycerol phosphate dehydrogenase they can be site-specifically localized in mitochondria. While early versions of roGFP reporters suffered from relatively slow reaction kinetics, subsequent versions in which either cationic side chains were introduced to the immediate environment of the disulfide or roGFP was fused to an enzyme (glutaredoxin) which catalyses the reaction between glutathione and roGFP have been shown to offer improved response times^[36, 37]. Recent examples of the use of roGFP have demonstrated redox potential compartmentalization in vascular smooth muscle cells in response to hypoxia and organelle-specific differences in redox potential in plant cells undergoing senescence^[38, 39].

One drawback of the use of fluorescent protein reporters is that they are limited to measurements in a relatively narrow potential window. The original roGFP reporters and a similar variant rxYFP have standard potentials which vary between -290 and -260 mV and they have been demonstrated to be insufficiently stable to oxidation to measure the potential of endoplasmic reticulum or endosomal compartments^[40]. Efforts to increase this potential window using mutagenesis have only succeeded in extending this range to -230 mV which is unlikely to be sufficient for studies of severe oxidative stress^[33, 37].

While several fluorescent dyes (e.g. dichlorofluorescein-diacetate and hydrocyanines) have been developed which report on particular ROS, these do not report on the redox state of the cell – only on the rate of production of a particular ROS and since they report *via* covalent reaction they are not reversible, so unsuitable for truly reversible monitoring. Furthermore, they don't take into account the mechanisms that the cell naturally puts into place for detoxification of ROS (e.g. in the presence of an efficient antioxidant system, a rise in ROS may not impact the redox state of the cell).

We have recently developed a redox nanosensor which can quantitatively measure intracellular redox potential over a greater range than existing techniques. The sensor consists of small organic reporter molecules assembled on a gold nanoparticle (Figure 4A). The Raman spectrum of the reporter molecule changes depending on its oxidation state and the gold particle functions as an amplifier for the Raman signal *via* Surface Enhanced Raman Spectroscopy (SERS) (Figure 4B). Since the standard potential of the reporter can be measured and the ratio of oxidized to reduced forms of the reporter measured *via* SERS, the redox potential in the environment of the sensor can be calculated quantitatively using a simple optical technique and the Nernst Equation. Experimental conditions can be controlled such that nanosensors can be delivered to the cytoplasm and there is scope for further organelle-specific targeting (Figure 4C). A key advantage of this approach is that the standard potential of the reporter molecule can be tuned using relatively simple and predictable changes to its

structure and this allows monitoring over a larger potential range than that covered by roGFP sensors. We have demonstrated that these measurements can be made in a non-invasive manner at the single cell level. Figure 4D shows the correlation between cytoplasmic redox potential and CASP3/7 activity in mouse fibroblast cells undergoing apoptosis^[41]. These findings demonstrate the tractability of making single-cell redox potential measurements and correlating them with enzyme activity.

Redox proteomics – measuring standard potentials of protein redox modifications.

In order to measure the standard redox potential of specific protein modifications (such as disulfide bond formation or glutathionylation), accurate ratiometric measurements of reduced/oxidized disulfide must be taken at various, accurately known, redox potentials. Because of the indirect nature of this methodology, and the sensitivity of $E^{\oplus}$ measurements to pH and temperature, great care must be taken in controlling experimental conditions. Specific reduction potentials are set by careful preparation of buffers containing differing concentrations of reduced and oxidized low molecular weight thiols. Typically reagents used include glutathione/glutathione disulfide, cysteine/cystine, or dithiothreitol. By knowing the standard reduction potential of the thiol system used, and applying the Nernst equation, accurate reduction potential for each buffer can be determined.

The protein is allowed to reach equilibrium in each redox buffer before the ratio of reduced to oxidised protein disulfide is measured. Several techniques have been used to measure the reduced/oxidized ratio of protein disulfides, these include exploiting differences in the fluorescence emission properties of reduced and oxidised forms of the protein^[40, 42], gel electrophoresis, Western blot, followed by densitometric analysis^[43], SERS^[44], and NMR spectroscopy^[45]. However, mass spectrometry (MS) is perhaps the best suited technique for this analysis since it has the ability to characterise the chemical nature of redox modifications based on an accurate intact protein mass measurements. For example, disulfide bond formation is accompanied by the loss of two hydrogen ions, (a mass decrease of 2Da) and glutathionylation involves the addition of GSH to a specific Cys sidechain *via* a disulfide bond (a mass increase of 305 Da). Furthermore, tandem MS experiments can be employed to locate the site of specific redox modifications within a protein. Indeed, we have demonstrated that top-down and middle-down MS fragmentation experiments are powerful tools for analysing the molecular details of cysteine redox modifications^[14, 46-48].

Oxidation of cysteine results in a loss of reactivity of the sidechain to electrophilic alkylating reagents, allowing selective chemical derivatization of oxidation states prior to MS analysis. Recently, a differential alkylation approach coupled with MS detection has been developed and used to measure the reduction potential of the two individual active sites within human Protein Disulfide Isomerase (PDI)^[49]. This strategy uses unlabeled and isotopically labeled alkylating agents to modify the reduced

and oxidized cysteines within a protein. After this chemical labeling, the protein is digested with a specific protease and the resulting peptide fragments are analysed by MS. The labeling procedure results in a mass difference between peptides that contained cysteines in the reduced and oxidized states; thus signals deriving for the oxidized and reduced peptides are separated in the mass spectrum and an accurate ratiometric measurement can be taken. We propose a similar isotope labeling strategy using commercially available isotope-labels and *intact* protein mass spectrometry (see Figure 5).

To date, only a handful of proteins have had the $E^{\oplus}$ of their disulfide bonds quantified, many of these are from the oxidoreductase superfamily (see Table 2). The proteins in Table 2 exhibit a range of $E^{\oplus}$ values and with such a small number of proteins from a range of species it is difficult to pick out any trends. However, there is now growing appreciation that cysteine oxidation can play an important role in controlling protein function, and this redox-switching is a common regulatory mechanism used throughout the proteome. Therefore, qualitative and quantitative characterization of specific cysteine redox-switches and allosteric disulfides is of crucial importance for understanding biological pathways and systems.

Protein	Function	Disulfide	$E^{\oplus}$ (mV)	Method	Reference
DsbD	Disulfide Bond Isomerase	103-109	-229	HPLC	[50]
CD4	Co-receptor protein	130-159	-241	SDS -PAGE	[51]
GFP	Green Fluorescent Protein	149-202	-261	IF	[40]
TF	Tissue Factor	186-209	-278	SDS -PAGE	[52]
Trx1	Thioredoxin	32-35	-230	SDS -PAGE	[43]
PDI	Protein Disulfide Isomerase	53-56 397-400	-163 and - 169	MS	[49]
$\alpha 3(\text{IV})\text{NC1}_{67-85}$	Antigenic isoform of Collagen (type IV)	74-80	-200	SERS	[44]
HMGB1	Cytokine	22-44	-237	NMR	[45]
E.coli Trx	Thioredoxin	33-36	-279	HPLC	[53]
E.coli Grx 1 and 3	Glutaredoxin	11-14 12-15	-233 and -198	HPLC	[53]
E. coli DsbA	Disulfide Bond Isomerase	49-52	-124	IF	[42]

Table 2. Published values for a range of proteins. Abbreviations used: HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; MS, mass spectrometry, IF, intrinsic fluorescence; SERS, Surface enhanced Raman spectroscopy; NMR, nuclear magnetic resonance.

Conclusions

We have presented a hypothesis which proposes that a subset of the proteome is regulated in a redox-potential dependant manner. Each protein in this “redox-proteome” can be considered to be a switch whose activity depends on its oxidation state, and thus on the redox potential of its environment. Since apoptosis is a cellular event in which the cell becomes progressively more oxidative, one ramification of this hypothesis is that the components of the redox proteome are arranged in the apoptotic pathway in a manner such that those with the most reductive standard potential are utilized early in apoptosis and those with the most oxidative standard potential are those used late in the pathway – in other words the redox proteome is arranged in an “electrochemical series”. We have assembled a pathway map of apoptosis and through this process identified a subset of the apoptotic proteome which has been either proposed or shown to be regulated by redox potential. While this map is useful for representing the role of redox potential in apoptosis, it has potential to be developed into a quantitative model of the redox regulation in apoptosis. Development of such a quantitative model requires a dedicated effort both to define the standard potentials of each of the proteins in Table 1 and also to measure the potential in each cellular organelle as apoptosis progresses. We have outlined experimental approaches to test this hypothesis and ultimately to develop a quantitative model of the redox regulation of apoptosis.

Figures

Figure 1. mSBGN map illustrating redox-regulation of cellular apoptosis (see Figure 2 for key to symbols).

Figure 2. Key to symbols used in mSBGN

Figure 3. An enlarged section of the main map, showing the apoptosome assembly. Of note is the role that oxidation of CASP9 plays in stimulating its binding to CYCS/APAF1 and initiating the apoptosis cascade.

Figure 4. A – Scheme showing nanosensor oxidation states; B – Nanosensor Raman spectra at a range of redox potentials; C – Nanosensors in cells; D – Correlation between redox potential (line) and CASP3/7 activity (columns) in cells undergoing apoptosis.

Figure 5. Determination of biochemical standard potential, $E^\oplus$, of redox active cysteines within a protein by isotope-labeling and mass spectrometry. **A.** The protein is incubated in a range of buffers of known redox potentials. After the oxidation/reduction process reaches equilibrium, the resulting series of proteins will contain mixtures of differing proportion of oxidized (green) and reduced (red) cysteine residues. For each sample, the mixture is then quenched, before labeling of the reduced cysteines is performed using the light ^{12}C -labelled maleimide reagent (red circles). The remaining oxidized protein is then reduced, before labeling with heavy $^{13}\text{C}_5$ -labelled maleimide reagent (yellow circles). **B.** This procedure allows the oxidized and reduced forms of the protein to be clearly distinguished due to a ‘mass-shift label’ of 10 Da. MS analyses of all the resulting samples is then performed and ratiometric measurements of the two species present in the spectrum of each sample affords an accurate determination of the ratio of oxidized to reduced protein at each measure redox potential. **C.** The fraction of protein reduced is then plotted against the redox potential and, by fitting to the Nernst equation, the $E^\oplus$ of the protein can be calculated.

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