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REVIEW

Proteomic responses to environmentally induced oxidative stress

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ABSTRACT

Environmental (acute and chronic temperature, osmotic, hypoxic and pH) stress challenges the cellular redox balance and can lead to the increased production of reactive oxygen species (ROS). This review provides an overview of the reactions producing and scavenging ROS in the mitochondria, endoplasmic reticulum (ER) and peroxisome. It then compares these reactions with the findings of a number of studies investigating the proteomic responses of marine organisms to environmentally induced oxidative stress. These responses indicate that the thioredoxin–peroxiredoxin system is possibly more frequently recruited to scavenge H_2O_2 than the glutathione system. Isoforms of superoxide dismutase (SOD) are not ubiquitously induced in parallel, suggesting that SOD scavenging activity is sometimes sufficient. The glutathione system plays an important role in some organisms and probably also contributes to protecting protein thiols during environmental stress. Synthesis pathways of cysteine and selenocysteine, building blocks for glutathione and glutathione peroxidase, also play an important role in scavenging ROS during stress. The increased abundance of glutaredoxin and DyP-type peroxidase suggests a need for regulating the deglutathionylation of proteins and scavenging of peroxynitrite. Reducing equivalents for these scavenging reactions are generated by proteins of the pentose phosphate pathway and by NADP-dependent isocitrate dehydrogenase. Furthermore, proteins representing reactions of the tricarboxylic acid cycle and the electron transport system generating NADH and ROS, including those of complex I, II and III, are frequently reduced in abundance with stress. Protein maturation in the ER likely represents another source of ROS during environmental stress, as indicated by simultaneous changes in ER chaperones and antioxidant proteins. Although there are still too few proteomic analyses of non-model organisms exposed to environmental stress for a general pattern to emerge, hyposaline and low pH stress show different responses from temperature and hypoxic stress. Furthermore, comparisons of closely related congeners differing in stress tolerance start to provide insights into biochemical processes contributing to adaptive differences, but more of these comparisons are needed to draw general conclusions. To fully take advantage of a systems approach, studies with longer time courses, including several tissues and more species comparisons are needed.

KEY WORDS: Endoplasmic reticulum, Environmental proteomics, Glutathione, Mitochondria, NAD(H), NADP(H), Peroxiredoxin, Peroxisome, Reactive oxygen species, Thioredoxin

Introduction

A number of cellular processes respond to environmental stress, which I define here as a change in abiotic conditions such that they are either close to the limits of the adaptive (evolutionary) niche or beyond the homeostatic response limits set by the recent (acclimatization or acclimation) history of the organism. A classic example is the synthesis of heat-shock proteins (molecular chaperones) in response to protein-denaturing conditions, e.g. during acute heat shock (Lindquist, 1981, 1986), with its evolutionary variation and phenotypic plasticity being well documented (Tomanek, 2008, 2010). A common theme of almost all stresses is that they damage macromolecular structures of the cell during the acute phase, activating the response of a common set of cellular processes – for example, those that repair DNA damage and regulate redox balance (Kültz, 2005). It is also known that many if not all of the abiotic stresses, including temperature, hypoxia, osmotic and pH stress, increase the production of reactive oxygen and nitrogen species (ROS and RNS), in part causing damage to macromolecular cellular structures (Halliwell and Gutteridge, 2007). The cellular sources of ROS during stressful and non-stressful conditions depend on the tissue, but generally involve the mitochondria, endoplasmic reticulum (ER) and peroxisome (Csala et al., 2010; Mailloux et al., 2013; Murphy, 2009; Nordgren and Fransen, 2014).

In addition, a number of studies have looked at how organisms respond to the challenge of increased ROS production in response to environmental stress by quantifying changes of a limited set of indicators of oxidative stress, e.g. ROS and antioxidant proteins (for an excellent collection of reviews, see Abele et al., 2012). While these approaches were successful in highlighting the common occurrence of the increased production of ROS with environmental stress, a systems perspective, using transcriptomic, proteomic or metabolomic approaches, which can take a broader range of transcripts, proteins or metabolites into account, possibly provides novel insights into several questions. For example, which of the many antioxidant proteins might be more important in scavenging ROS during stress, i.e. is there a tiered response? What is the response of different organelles to environmentally induced oxidative stress? Are all environmental stresses triggering the same response to oxidative stress? How do differently adapted species vary in their response to environmentally induced oxidative stress? To begin to answer some of these questions, I want to focus on a number of proteomic studies and review what they might tell us about the possible role of the different antioxidant proteins during environmental stress.

As there is no current review covering oxidative stress of the mitochondria, the ER and peroxisomes, I have tried to provide a general overview of this topic. Several organelle-specific reviews have gone into more detail covering the production and scavenging of ROS in these organelles (Csala et al., 2010; Mailloux et al., 2013; Murphy, 2009; Nordgren and Fransen, 2014). These introductory sections are meant to set the stage for a subsequent focus on how the proteomes of marine organisms respond to environmental conditions causing oxidative stress, focusing on temperature, hypoxia, pH and osmotic (salinity) stress. While a proteomic approach comes with its own limitations, together these proteomic studies generate novel insights about the response of the cell to ROS

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List of symbols and abbreviations

ALDH	aldehyde dehydrogenase
BiP	binding immunoglobulin protein
$CoQH_2$/CoQ	coenzyme Q (reduced/oxidized form)
Cyp	cyclophilin
DLDH	dihydrolipoamide dehydrogenase
ER	endoplasmic reticulum
Ero1α	endoplasmic reticulum oxidase 1α
ETS	electron transport system
FAD(H)	flavin adenine dinucleotide (oxidized/reduced form)
FKBP	FK-binding protein
FMN	flavin mononucleotide
GPx	glutathione peroxidase
GR	glutathione reductase
Grp78/94	glucose-regulated protein 78 or 94 kDa
Grx	glutaredoxin
GSH/GSSG	glutathione (reduced/oxidized)
GST	glutathione S-transferase
Hsp70/90	heat-shock protein 70 or 90 kDa
ICDH	isocitrate dehydrogenase
MDH	malate dehydrogenase
NAD(H)	nicotinamide adenine dinucleotide (oxidized/reduced form)
NADP(H)	nicotinamide adenine dinucleotide phosphate (oxidized/reduced)
$NO^{\bullet}$	nitric oxide
NOS	nitric oxide synthase
$O_2^{-\bullet}$	superoxide anion
$ONOO^-$	peroxynitrite
PDH	pyruvate dehydrogenase
PDI	protein disulfide isomerase
PPIase	prolyl peptidyl isomerase
PPP	pentose phosphate pathway
Prx	peroxiredoxin
PTM	post-translational modification
RCHO	carbonyl
RCOOH	carboxyl
RET	reverse electron transfer
RNS	reactive nitrogen species
ROS	reactive oxygen species
SDH	succinate dehydrogenase
SOD	superoxide dismutase [manganese (Mn)- or copper–zinc (Cu–Zn)-SOD]
TCA	tricarboxylic acid
Trx	thioredoxin
TrxR	thioredoxin reductase
XOx	xanthine oxidase
αKGDH	α-ketoglutarate dehydrogenase
Δp	proton gradient

from a systems perspective, the plasticity of the antioxidant proteome and the evolutionary variation of the stress proteome. For example, while early work on the enzymatic responses to oxidative stress in marine organisms mainly focused on superoxide dismutase (SOD), catalase and the glutathione system (Abele and Puntarulo, 2004; Abele et al., 2012), the proteomic analyses reviewed herein point to an important role for the thioredoxin–peroxiredoxin (Trx–Prx) system, in accordance with the finding that, depending on tissue and protein concentrations, up to 90% of the ROS produced in the mitochondria are scavenged through the reactions of Prx (Cox et al., 2010).

Importantly, proteomic analyses come with some important limitations. For example, most of the studies cited herein did not set out to analyze the response to oxidative stress, and thus often did not assess ROS production, as they were pursuing a discovery approach. Thus, the increase or change in abundance of an oxidative stress protein was taken as a signal for the temporary increase in the production of ROS. Most likely, an increase in ROS served as a signal, which triggered a biochemical response to re-establish the redox balance of the cell. Furthermore, an important aspect of the proteomic response to oxidative stress involves a number of post-translational modifications (PTMs), which have been reviewed comprehensively elsewhere (Silva et al., 2013; Walsh, 2006; Walsh et al., 2005), but have rarely been the focus of studies investigating the response to environmental stress. This review will only touch on them. In addition, changes in protein abundance do not necessarily translate into changes in enzyme activity. Furthermore, other antioxidant defenses, e.g. non-enzymatic antioxidants, also contribute to the cellular response to oxidative stress, but will not be the focus of this review, with the exception of the glutathione system (Abele et al., 2012; Estevez et al., 2002). While this review focuses on the proteomic responses in regard to oxidative stress, for a general overview of recent studies on proteomic changes in response to environmental stress, I refer to other reviews (Tomanek, 2011, 2014).

ROS

Oxygen has a unique arrangement of its outer shell electrons, which are involved in reacting (bonding) with other molecules. The two electrons of the outer orbital are unpaired and have parallel spin (i.e. paramagnetic). In order for other molecules, whose outer electrons are paired and have an opposite spin, to react with oxygen, one electron would have to be paired with an electron of the same spin, an energetically unfavorable process. This barrier can be overcome by adding the electrons one at a time (Fridovich, 1998; Grivennikova and Vinogradov, 2013; Halliwell and Gutteridge, 2007). This slows the reaction, creates chemical inertness and contributes to the accumulation of oxygen in the atmosphere. The reduction of oxygen therefore proceeds through single electron transfer reactions that generate the most common ROS.

The first of these electron transfers generates the superoxide anion ($O_2^{-\bullet}$), followed by hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and finally water (H_2O) (Fig. 1). Superoxide anion has a short lifespan, probably due to both the scavenging activity of mitochondrial manganese (Mn)-SOD (Fig. 1) and its reactive nature with other cellular molecules (Halliwell and Gutteridge, 2007). Because hydrogen peroxide is not a radical, it is less reactive by itself. However, this is also the reason why it is more stable than $O_2^{-\bullet}$ and diffuses easily across the inner mitochondrial, ER and peroxisomal membranes, a process that is facilitated by aquaporins (Bienert and Chaumont, 2014). It can therefore affect biomolecules

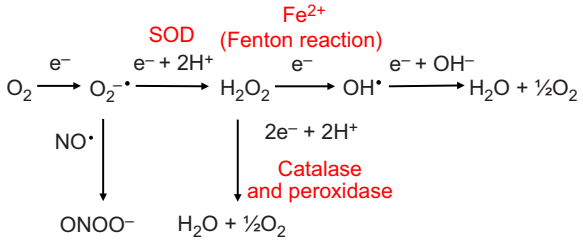

Fig. 1. Generation of reactive oxygen and nitrogen species (ROS and RNS). This overview shows the major reactions (not fully balanced) generating ROS and RNS (only peroxynitrite) and the antioxidant (scavenging) proteins catalyzing the reduction of superoxide anion ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2). It also indicates that ferrous iron (Fe^{2+}) contributes to catalyzing the formation of hydroxyl radicals. SOD, superoxide dismutase; $OH^{\bullet}$, hydroxyl radical; $NO^{\bullet}$, nitric oxide; $ONOO^-$, peroxynitrite.

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far from its origin. Importantly, when it reacts with ferrous iron (Fe^{2+}), it is converted to the hydroxyl radical (known as the Fenton reaction), which is the most reactive ROS, reacting immediately with biomolecules around the site of its cellular origin (Fig. 1). It is therefore not surprising that the main goal for the major ROS-scavenging systems, the glutathione and Trx–Prx systems as well as catalase, is to scavenge hydrogen peroxide before it can diffuse across membranes into other cellular compartments and generate hydroxyl radicals. In addition, ferrous iron can be chelated by ferritin, which prevents Fe^{2+} from reacting with hydrogen peroxide (Finazzi and Arosio, 2014).

Oxidative stress in the mitochondria

Oxidative stress, an increase in ROS above an established baseline, is the net result of a number of cellular processes that contribute to the production of ROS and their scavenging. Both are potential sites of regulation for reducing ROS during stress. While the overall reduction of mitochondrial activity, e.g. metabolic depression, as a response to environmental stress has received some attention (Podrabsky and Hand, 2015), the response of the specific biochemical reactions producing ROS to stress have not been the focus of many studies to date. In contrast, both the enzymatic and non-enzymatic ROS-scavenging systems of mitochondria have been the focus of numerous studies investigating the effect of stress (Abele et al., 2012; Halliwell and Gutteridge, 2007). Here, I will first give some background for both ROS-producing and -scavenging reactions. Then, I will review the proteomic responses of marine organisms.

Sites of ROS production in mitochondria

Insights into the rate of ROS production are based mainly on isolated mitochondria and the measurement of H_2O_2 efflux across the membrane (Andreyev et al., 2005; Murphy, 2009). Several conditions contribute to an increase in ROS production from complex I and III of the electron transport system (ETS). In mode 1 (Fig. 2), a high NADH/NAD^+ (nicotinamide adenine dinucleotide reduced/oxidized form) ratio in the matrix contributes to an increase in ROS production by complex I (NADH dehydrogenase). Complex I accepts the electrons of NADH at the site of its cofactor flavin mononucleotide (FMN), from where it is passed across several iron–sulfur centers to coenzyme Q (CoQ). Damage to the ETS or low ATP demand (and low respiration rates), which cause an increase in NADH/NAD^+, inhibits the transfer of electrons. This leads to a backup of electrons and the direct transfer of electrons from NADH via FMN to O_2 and the formation of $O_2^{-\bullet}$ (Murphy, 2009). Low ATP production leading to a low ratio of ATP/[ADP+AMP] in turn will affect the activity of a number of glycolytic enzymes and pyruvate dehydrogenase (PDH).

In mode 2 (Fig. 2), a high pool of reduced coenzyme Q ($CoQH_2$/CoQ), a high proton gradient (Δp) and low rate of ATP synthesis all contribute to the flow of electrons back from $CoQH_2$ to complex I (RET, reverse electron transfer). High $CoQH_2$/CoQ ratios can be formed when succinate, α-glycerophosphate and fatty acid oxidation provide electrons to the ETS through complex II, causing RET towards complex I. This process is highly sensitive to the proton gradient across the inner mitochondrial membrane, with a greater gradient causing higher rates of $O_2^{-\bullet}$ production (Murphy, 2009). While the exact site of ROS production (FMN or the CoQ-binding site on complex I) is still debated, mode 2 generates the highest rates of ROS production.

Mode 3 (not illustrated in Fig. 2, but see Murphy, 2009) prevails during normal conditions: high rates of respiration combined with high rates of ATP synthesis and a small proton gradient. These conditions generate much lower rates of H_2O_2 efflux than mode 1 and 2. Complex III (bc1 complex or cytochrome *c* reductase) produces $O_2^{-\bullet}$ at a high rate when the exchange of electrons between the Q_i and Q_o sites of complex III is inhibited, e.g. by antimycin, during the Q-cycle (Andreyev et al., 2005). This stabilizes the ubisemiquinone bound to the Q_o site for long enough to interact with O_2 to form $O_2^{-\bullet}$, which is released both towards the intermembrane as well as at the matrix site of the inner membrane. To what extent changes in cytochrome *c* reductase or Δp contribute to the stabilization of the ubisemiquinone and thus the production of $O_2^{-\bullet}$ by complex III under *in vivo* conditions is unclear. While mode 3 prevails through most of life, modes 1 and 2 are likely to occur under environmental stress.

Other sources of ROS in mitochondria are either linked to the NADH pool of the mitochondrial matrix or CoQ in the inner membrane. Any perturbation of the ETS might generate higher NADH/NAD^+ ratios, e.g. mode 1. Subsequently, high levels of reduced NADH (low levels of NAD^+) and 2-oxoglutarate can increase ROS production by α-ketoglutarate (2-oxoglutarate) dehydrogenase (αKGDH) (Starkov, 2013). One of the three

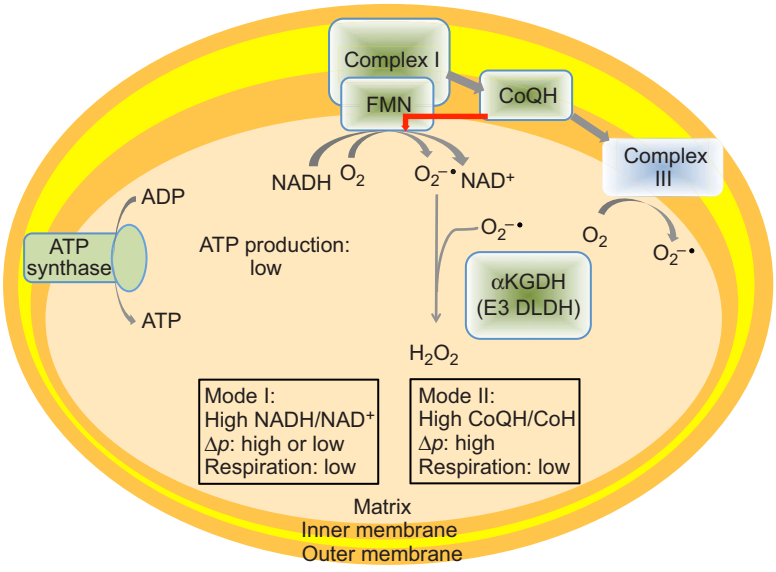

Fig. 2. Sources of ROS in the mitochondria. Stress-induced interruption of the electron transport system (ETS) and high levels of nicotinamide adenine dinucleotide (reduced/oxidized form; NADH/NAD^+) (as well as low rates of ATP production) lead to the generation of superoxide anion ($O_2^{-\bullet}$) at complex I (mode 1), most likely at its cofactor flavin mononucleotide (FMN). A high ratio of coenzyme Q reduced/oxidized form ($CoQH_2$/CoQ) generates $O_2^{-\bullet}$ at complex I and III (mode 2). The E3 subunit of α-ketoglutarate dehydrogenase (α-KGDH), called dihydrolipoamide dehydrogenase (DLDH) also contributes to the generation of $O_2^{-\bullet}$. Δp, proton gradient. Thick gray arrows indicate the normal flow of electrons; the red arrow indicates the flow of electrons during reverse electron transfer (RET) (after Murphy, 2009).

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components of αKGDH, dihydrolipoamide dehydrogenase (E3 component; DLDH), reoxidizes the reduced lipoyl residues of the E2 component, requiring NAD^+ as a cofactor and producing NADH as a result (Starkov, 2013). High ratios of NADH/NAD^+ favor the reverse reaction and lead to the transfer of electrons to oxygen, resulting in the formation of $O_2^{-\bullet}$. Importantly, αKGDH activity is repressed in a number of pathological conditions that are associated with high levels of ROS (Starkov, 2013). Two factors could be responsible for this. First, the redox state of the lipoyl group (E2) is controlled in part by Trx through Prx, generating a possible trade-off during oxidative stress (Bunik, 2003). Second, DLDH generates $O_2^{-\bullet}$ and H_2O_2 when the NADH/NAD^+ ratio is high, as NAD^+ is a co-factor for DLDH. Thus, decreasing the activity of DLDH in response to stress-causing conditions similar to mode 1 or 2 could decrease the production of ROS directly as well as the production of $O_2^{-\bullet}$ at complex I during mode 1 by decreasing the production of NADH. Earlier studies indicate that the DLDH component of αKGDH is a major contributor of ROS in functionally normal mitochondria (Starkov et al., 2004). Importantly, DLDH is also a component of the PDH complex. The *in vivo* contribution to the production of ROS by other mitochondrial dehydrogenases, e.g. α-glycerophosphate dehydrogenase, is also linked to the ratio of NADH/NAD^+ (Adam-Vizi and Tretter, 2013; Grivennikova and Vinogradov, 2013). As far as I know, the role of DLDH or any of the other dehydrogenases in responding directly to environmentally induced oxidative stress has not been the focus of any study to date.

A comprehensive picture of the sources of ROS in mitochondria needs to also consider the role of fatty acids in uncoupling the inner mitochondrial membrane, interacting with ETS complexes and the transfer of electrons from fatty acid β-oxidation via flavin adenine dinucleotide (reduced form; FADH) to the ETS (Schönfeld and Wojtczak, 2008; Tahara et al., 2009). Importantly, recent evidence suggests that a transfer of electrons (as FADH) to CoQ via electron transfer flavoprotein, originating from β-oxidation, does not cause RET and therefore ROS production from complex I, opposite to the action of succinate dehydrogenase (SDH), which transfers electrons (as FADH) from succinate, emphasizing that the substrate-specific entry of electrons to the ETS affects ROS production (Chouchani et al., 2014; Schönfeld et al., 2010).

Finally, closely linked to any possible changes in enzyme activity in response to oxidative stress are possible changes in metabolites that act as antioxidants. There is evidence that pyruvate protects neurons from H_2O_2 and this might also be the case for other α-ketoacids (Desagher et al., 1997), including α-ketoglutarate (α-KG) (Mailloux et al., 2007). A decrease in the activity of PDH and the DLDH component of αKGDH might therefore lower the production of ROS through the reduced production of NADH from the citric acid cycle and scavenge ROS through the accumulation of metabolites like pyruvate and αKG that function as antioxidants (Mailloux et al., 2007). However, while they may serve as antioxidants of last resort, their relative importance to the Trx–Prx and glutathione systems is considered small.

ROS scavenging in mitochondria

Superoxide anion is produced in the mitochondrial matrix as well as the intermembrane space of mitochondria. As a first line of defense, $O_2^{-\bullet}$ is converted to H_2O_2 by the mitochondrial Mn-SOD or the intermembrane (which also occurs in the cytosol) copper–zinc (Cu–Zn)-SOD (Fig. 3). The two SOD isoforms are distant orthologs (Fridovich, 1998) and can occur in other cellular compartments, e.g. both SODs can occur in peroxisomes (Bonekamp et al., 2009).

The second line of defense includes the dismutation of H_2O_2 by a number of enzymes, including catalase, glutathione peroxidase (GPx) and Prx. Catalase is mainly localized in the peroxisome, and mitochondria and the ER have only a little catalase activity in most tissues (Halliwell and Gutteridge, 2007). The two other major scavenging reactions in the mitochondria include GPx and Prx (Fig. 3) (Mailloux et al., 2013; Murphy, 2012). GPx couples the reduction of H_2O_2 to the oxidation of glutathione, a tripeptide consisting of glutamine, cysteine and glycine, and the main cellular non-enzymatic antioxidant. The oxidized form of glutathione (GSSG) is then reduced (GSH) by glutathione reductase (GR), a reaction that requires nicotinamide adenine dinucleotide phosphate (reduced form; NADPH) as a reducing equivalent. Two GPx isoforms play an important role in scavenging peroxides in mitochondria: GPx1 degrades hydrogen peroxides and GPx4 catalyzes the conversion of lipid hydroperoxides to alcohols, thereby slowing down lipid peroxidation. Two other enzymes use GSH as an electron donor; glutathione S-transferase (GST) and

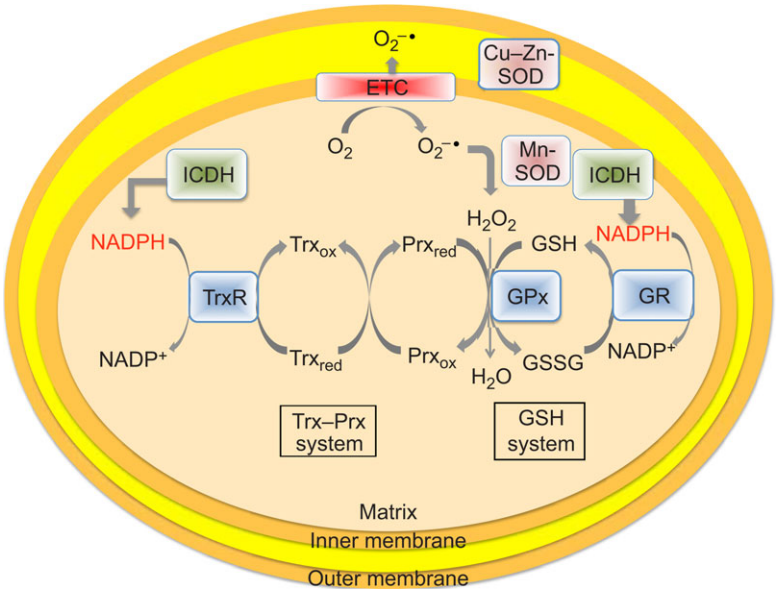

Fig. 3. ROS-scavenging reactions in mitochondria. Antioxidant reactions in the intermembrane space (between the inner and outer membrane) and matrix of mitochondria. Superoxide anions ($O_2^{-\bullet}$) are produced by the ETS and released into the intermembrane space and matrix where they are reduced to hydrogen peroxide (H_2O_2) by Cu–Zn-superoxide dismutase (SOD) (which is also localized in the cytosol and peroxisomes) and Mn-SOD, respectively. Hydrogen peroxide is reduced to water by either glutathione peroxidase (GPx) or peroxiredoxin (Prx); the oxidized form of both is reduced by glutathione (GSH) and thioredoxin (Trx), respectively. The oxidized forms of GSH and Trx are reduced by thioredoxin reductase (TrxR) and glutathione reductase (GR), respectively, with NADPH as reducing equivalents produced by NADP-dependent isocitrate dehydrogenases (ICDH). Transhydrogenase, mitochondrial and cytosolic isoforms of NADP-dependent malic enzyme and aldehyde dehydrogenase might also contribute to the production of NADPH but are not shown (after Mailloux et al., 2013; Murphy, 2012).

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glutaredoxin (Grx) (Fig. 4) (Murphy, 2012). GSTs are historically associated with detoxification reactions in which GSH is conjugated to a compound through a thioester linkage (Tew and Townsend, 2012). These conjugates increase water solubility and are excreted through efflux transporters. However, some GST isoforms can also catalyze the formation of mixed disulfides between proteins and GSH, called S-glutathionylation, a process that protects thiol groups from ROS (Townsend et al., 2009). There is also evidence that S-glutathionylation affects the activity of various metabolic proteins (Dalle-Donne et al., 2009; Fratelli et al., 2003). Grxs catalyze the deglutathionylation (and possibly glutathionylation as well) of glutathionylated proteins (Murphy, 2012).

The Trx–Prx system is the other major hydrogen peroxide-scavenging system (Figs 2, 3) (Mailloux et al., 2013; Murphy, 2012). Trx catalyzes the reduction of disulfide bonds on other proteins, specifically those of Prxs, which reside not only in mitochondria (Prx3 and 5) but also in the cytoplasm (Prx1, 2 and 6), the ER (Prx4) and peroxisomes (Prx5) (Cox et al., 2010). While all Prxs scavenge hydrogen peroxide, Prx5 targets organic peroxides and peroxynitrite, a highly reactive RNS, which forms from the reaction between $O_2^{-\bullet}$ and $NO^{\bullet}$ (Trujillo et al., 2008). A kinetic analysis for mice, taking into account rate constants and published protein concentrations, estimated that 90% of the H_2O_2 produced in the mitochondria is scavenged by Prx3, the main mitochondrial Prx, an estimate that will likely vary with tissue and cell type (Cox et al., 2010).

Abundance changes in ETS and tricarboxylic acid cycle enzymes during environmental stress

Organisms could respond to acute environmental stress by decreasing the activity of pathways and reactions that produce ROS, as outlined above. A number of recent proteomic studies indeed showed changes in the abundance of either NADH dehydrogenase or cytochrome *c* reductase isoforms, or both, in gill of several marine invertebrate species in response to acute and chronic temperature and hyposalinity stress (Dilly et al., 2012; Fields et al., 2012b; Tomanek and Zuzow, 2010; Tomanek et al., 2012), suggesting that decreasing abundance, composition or PTM of both complex I and III of the ETS are potential sites for regulation.

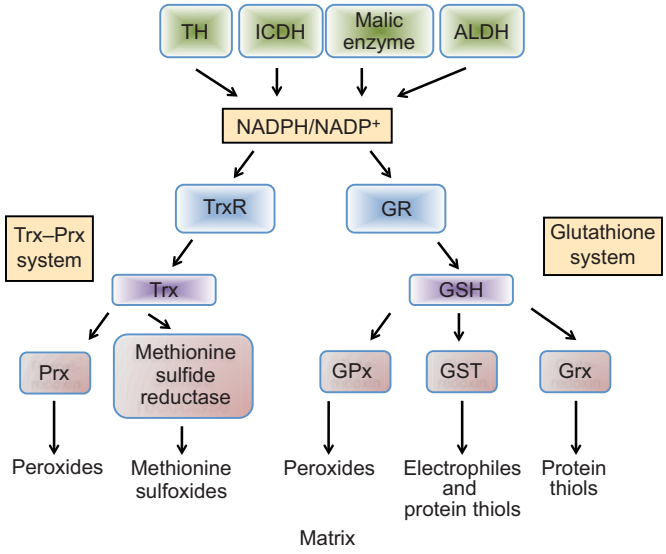

Fig. 4. Overview of major elements of the cellular ROS-scavenging system. A schematic overview of the glutathione and thioredoxin–peroxiredoxin (Trx–Prx) system and the enzymatic reactions affecting various thiol modifications (after Murphy, 2012; Mailloux et al., 2013). While this perspective suggests a strict separation between the two systems, they do interact with each other to some extent (Casagrande et al., 2002). NAPD-dependent ICDH, malic enzyme and aldehyde dehydrogenase (ALDH) can have separate isoforms for the mitochondria, cytosol, endoplasmic reticulum (ER) and peroxisome (see text for details). TH, transhydrogenase; GPx, glutathione peroxidase; Grx, glutaredoxin; GST, glutathione S-transferase.

One study compared the proteomic response of gill to acute heat stress in the heat-sensitive blue mussel *Mytilus trossulus*, a native to the Pacific coast of North America, with that of the more heat-tolerant Mediterranean *Mytilus galloprovincialis*, a species that invaded and replaced the native species in the southern part of its original distribution range (Tomanek and Zuzow, 2010). Acute heat stress at 24, 28 and 32°C for 1 h following a heating rate of 6°C h^{-1} from 13°C (an acclimation temperature in the middle and low end of the temperature range for *M. trossulus* and *M. galloprovincialis*, respectively) followed by a 24 h recovery period at 13°C showed a decrease in abundance of complex I and III with acute heat stress in the more heat-sensitive *M. trossulus* (Fig. 5).

A comparison of the proteomic responses to acute heat stress in gill between two vent polychaetes that differ in thermal tolerance showed that the relatively more heat tolerant *Paralvinella sulfincola*, in contrast to the less heat tolerant *Paralvinella palmiformis*, decreased the abundance of complex I (Dilly et al., 2012).

While both complexes decreased in the less heat tolerant *Mytilus* congener, suggesting a potential cellular injury, in *Paralavinella* it was the more heat-tolerant congener in which complex I decreased, suggesting that these changes constitute a strategy to maintain homeostasis by decreasing ROS production in response to acute heat stress. Based on two species comparisons measuring these differences at one and two different time points (immediately and 24 h after heat shock in *Paralvinella* and *Mytilus*, respectively), we cannot conclude that either of the interpretations is the more general one, as the heat shock response differs greatly depending on the recovery time in closely related congeners varying in thermal tolerance (Tomanek and Somero, 2000). The phylogenetic distance between the two genera further precludes a simple comparison between these two contradictory results.

Isoforms of PDH (E1) as well as DLDH decreased in response to acute and chronic warm temperature as well as during recovery from acute hyposalinity stress in *Mytilus* congeners, suggesting that their abundance is closely regulated during environmental stress (Tomanek, 2012). Together, theses studies point to ROS-producing reactions of the tricarboxylic acid (TCA) cycle and the ETS as sites of regulation during stress. They warrant and require more specific investigations to clarify the role of these reactions in helping organisms to cope with environmental stress.

SDH, by delivering reducing equivalents (FADH) to the ETS, is a potential source for RET and increased ROS production, especially under conditions of high ratios of NADH/NAD^+ and $CoQH_2$/CoQ, and thus might be an enzyme undergoing changes during environmental stress. SDH decreased in response to acute heat stress in the more heat-tolerant *P. sulfincola*, while several antioxidant proteins increased (Dilly et al., 2012). The reason for a decrease in SDH might be to counteract the potentially pro-oxidant effects of succinate accumulation (Chouchani et al., 2014). SDH also decreased in abundance in an opposite fashion to some antioxidant proteins during chronic temperature stress in *M. trossulus* (Fields et al., 2012b). However, highlighting the multiple roles of this enzyme, it increased in gill in response to hypoxia in *Geukensia* (Fields et al., 2014), most likely as part of an anaerobic metabolic pathway typical of bivalves (Muller et al., 2012).

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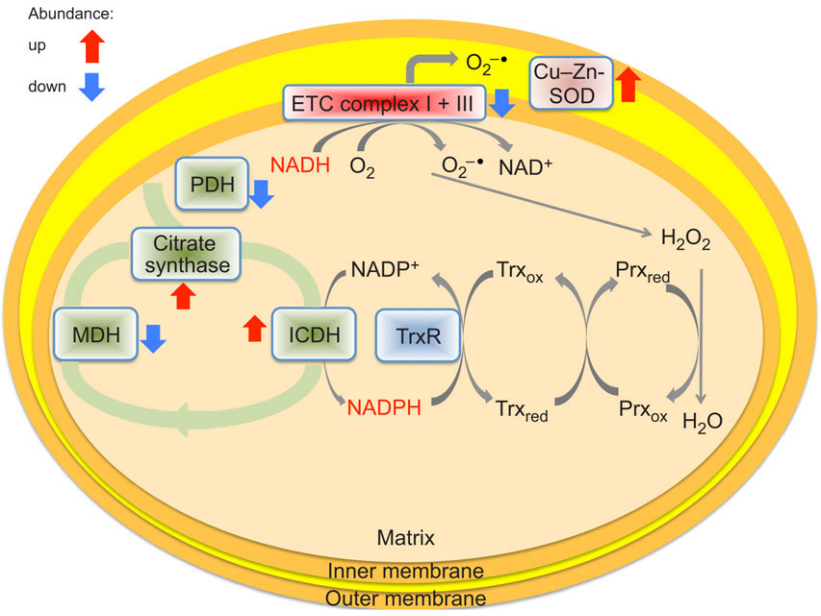

Fig. 5. Putative changes in the mitochondrial proteome in gill of the heat-sensitive mussel *Mytilus trossulus* and heat-tolerant *M. galloprovincialis* detected in response to acute heat stress. While pro-oxidant NADH-producing enzymes (pyruvate dehydrogenase, PDH; and malate dehydrogenase, MDH) and subunits of complex I and III decreased at the highest exposure temperatures, antioxidant NADPH-producing enzymes (citrate synthase and NADP–ICDH) increased in *M. trossulus*; in *M. galloprovincialis*, one Cu–Zn-SOD increased with acute heat stress (after Tomanek and Zuzow, 2010). The connection to the Trx–Prx system is putative (it could also be the glutathione system or both).

ROS scavenging during environmental stress in mitochondria

ROS-scavenging reactions have been the focus of studies investigating the effect of environmental stress on oxidative stress in marine organisms (Abele et al., 2012). However, few of these studies used a systems approach to evaluate the role of reactions other than the ones assumed to be contributing to the organism's scavenging capacity *a priori*. While having its own limitations, a proteomics approach provides an assessment of which of these major enzymatic ROS-scavenging systems is activated in marine organisms during environmental stress, e.g. during heat, cold, hypoxic and hyposaline stress. Furthermore, species comparisons, although still the exception, provide insight into the adaptive differences in responding to environmentally induced oxidative stress and thus the role of the various ROS-scavenging systems in setting stress tolerance limits.

Temperature-induced antioxidant responses

One caveat of a proteomics-only approach is that an increase in enzymatic antioxidants is needed to serve as the signal indicating an imbalance of ROS production to scavenging, necessitating an increase in the abundance of scavenging enzymes decreasing ROS. Thus, this proteomic signal serves as a measure of oxidative stress, as few of these studies measured changes in ROS directly. In some cases, non-enzymatic antioxidants were quantified, which can also contribute to the scavenging of ROS.

First, I will consider changes in ROS-scavenging reactions to acute heat stress. Before doing so, I want to point out that most proteomic studies of non-model organisms only identified proteins that changed in response to stress, not those that did not change. And to remind the reader, most proteomic analyses are limited to the most abundant proteins, often limited to a narrow pH range and the most soluble proteins, frequently missing membrane proteins. Nevertheless, by detecting hundreds to thousands of proteins, these analyses provide novel insights into the dynamics of the proteome that have hitherto been unexplored.

Previous studies, using other methodological approaches, showed that acute heat stress increased the production of ROS, oxidative damage and antioxidant enzymes, and produced a more oxidized cellular redox potential in marine organisms (Abele et al., 2002; Heise et al., 2003; Keller et al., 2004). However, it is unclear how these ROS affected the proteome.

The aforementioned comparison of the gill proteome of two vent polychaetes in response to acute heat stress showed that the *Paralvinella* congeners differed in changes in abundance of SOD isoforms, enzymes of the glutathione system and the glutathione biosynthesis pathway (Dilly et al., 2012). Specifically, the more heat-tolerant *P. sulfincola* did not change abundance of either SOD isoform with increasing heat stress (Fig. 6). In contrast, the less heat-tolerant *P. palmiformis* increased the abundance of the cytosolic Cu–Zn-SOD and the mitochondrial Mn-SOD isoform. Two enzymes that are part of the cysteine (and selenocysteine) and therefore the glutathione synthesis pathway, cystathione β-synthase and γ-glutamylcysteine synthetase (Fig. 6), increased in abundance with heat stress in both congeners (Dilly et al., 2012). However, *P. sulfincola* increased the abundance of both enzymes of the glutathione scavenging system, GPx (GPx3; cytosolic) and GR, while *P. palmiformis* showed an increase in GPx3 and a decrease in GR. As a result, *P. sulfincola* was able to maintain total levels of GSH as well as the ratio of GSH/GSSG in contrast to the less tolerant *P. palmiformis*, which decreased by about half the total GSH as well as decreased its GSH/GSSG ratio to one-third (Dilly et al., 2012). The synthesis of selenocysteine, which shares the enzymes of the cysteine pathway, could be as important as the one that leads first to cysteine and then to GSH, as it is incorporated into TR and GPx (Dilly et al., 2012). Interestingly, selenide water dikinase, which catalyzes the formation of selenophosphate, a universal selenium donor, to selenocysteinyl t-RNA, the t-RNA necessary for the incorporation of selenocysteines into selenoproteins, increased in *P. sulfincola* but not *P. palmiformis* (not shown in Fig. 6; Dilly et al., 2012).

The comparison of the proteomic responses to acute heat stress of the two *Mytilus* congeners showed opposite changes in the abundance of two Cu–Zn-SOD isoforms varying in their isoelectric point, indicating the occurrence of a PTM, in the heat-tolerant *M. galloprovincialis* (Tomanek and Zuzow, 2010). The heat-sensitive *M. trossulus* showed first a slight increase in abundance with an increase in temperature to 28°C followed by an abrupt steep decrease in Trx at the highest exposure temperature

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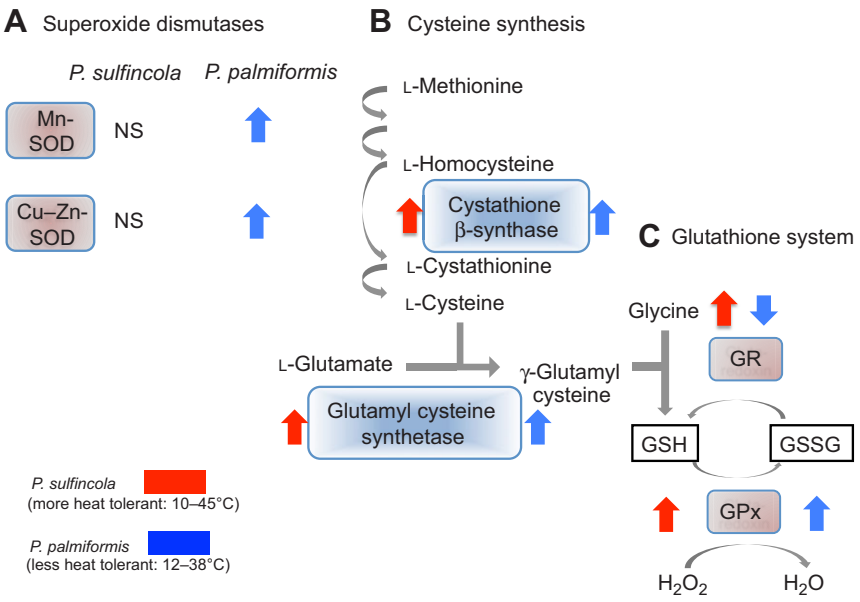

Fig. 6. Changes in abundance of several antioxidant proteins in response to acute heat stress in gill tissue of two vent polychaete species, the more heat-tolerant *Paralvinella sulfincola* and the less heat-tolerant *P. palmiformis*. Arrow up: increase; arrow down: decrease relative to the control. The differences shown are a summary of the interspecific differences (NS, non-significant) in abundance changes of (A) two isoforms of superoxide dismutase (SOD) and (B) proteins representing cysteine (cystathione β-synthase) and glutathione (glutamyl cysteine synthase) synthesis pathways and the glutathione (peroxidase and reductase) ROS-scavenging system (based on Dilly et al., 2012).

(32°C). Additionally, DyP-type peroxidase also increased at 28°C in this species and subsequently decreased at 32°C in *M. trossulus*, possibly indicating a lower antioxidant capacity with exposure to this temperature. DyP-type peroxidases represent a novel family of heme-containing peroxidases whose function has yet to be determined (Colpa et al., 2014), although it is possible that they play a role in decreasing peroxynitrite (Trujillo et al., 2008). Thus, *M. trossulus* was unable to activate an antioxidant response at the highest temperature, a limitation likely contributing to its lower thermal tolerance. There was no indication of a change in enzymes of the glutathione system in either of the *Mytilus* species.

Second, a comparison of the proteomic responses of gill of the Salt Marsh mussel *Geukensia demissa* from several populations along a latitudinal gradient to acute heat stress (40°C) for 1 h during emersion following acclimation to 18°C (for 4 weeks) showed that Grx was the only oxidative stress protein that differed in abundance and this occurred in the most northern population (Fields et al., 2012a). The main function of Grx is the removal of glutathione (deglutathionylation) from proteins (Meyer et al., 2009). However, it might also play a role in the recruitment of glutathione from glutathionylated protein thiols during oxidative stress, while protein thiols can act as sinks for ROS until they are recycled by both Grx and Trx (Meyer et al., 2009; Murphy, 2012; Thomas et al., 1995). While other chaperones, cytoskeletal and signaling proteins as well as a translation initiation factor changed, Grx was the only antioxidant protein detected responding to acute heat stress (Fields et al., 2012a), suggesting that the constitutive proteome is robust enough not to require a broad response to heat-shock-induced oxidative stress. However, a time course of the proteomic changes may have given greater insight into an earlier response to oxidative stress, immediately following the acute heat stress.

Finally, acclimation to cold (7°C), moderate (13°C) and warm (19°C) temperatures for 4 weeks increased Prx at cold to moderate temperatures in gill of the warm-adapted *M. galloprovincialis* for which 7 and 13°C are at the low end of the temperature range it prefers (Fields et al., 2012b). In contrast, cold to moderate temperatures in comparison to warm temperatures caused a change in abundance in an opposite fashion of two isoforms of DyP-type peroxidase in the cold-adapted *M. trossulus*, indicating a PTM as the reason for this change of abundance from cold and moderate to warm temperatures.

In summary, the proteome of gill tissue of several marine organisms increased the abundance of SOD, proteins of the Trx–Prx and glutathione-scavenging system and the glutathione (cysteine and selenocysteine) biosynthesis pathway in response to acute and chronic temperature stress. In some cases, homologous isoforms increased and decreased simultaneously, indicating a switch in PTMs, e.g. DyP-type peroxidase. Both cold and warm temperature stress increased the abundance of antioxidant proteins, depending on the adaptation temperature of the congener in the case of *Paralvinella* and *Mytilus*, with partly contradictory results (e.g. SOD) that could be due to differences in sampling times following stress. Additional antioxidant measures included the increase in Grx and DyP-type peroxidase with acute and chronic temperature stress. Thus, the full complement of antioxidant proteins includes a broad range of reactions and pathways, which will have to be considered when assessing the response of organisms to environmentally induced oxidative stress.

Hypoxia-induced antioxidant response

Life in the intertidal zone is characterized by periods of aerial emersion that cause hypoxic conditions for a number of sessile organisms, especially bivalves (i.e. *Mytilus*) that close their shells, although some bivalves (i.e. *Geukensia*) may temporarily switch to aerial oxygen exchange during low tide through shell gaping. The ribbed mussel, *G. demissa*, is found buried in mud at the upper fringes of tidal flats along the east coast of North America (Blackwell et al., 1977), where it experiences long periods of emersion and extreme temperatures. A time course of the proteomic response of gill tissue to prolonged periods of aerial exposure [but decreasing times of recovery (emersion/recovery under immersion: 6 h/18 h, 12 h/12 h, 18 h/6 h and 24 h recovery all at 15°C)], e.g. hypoxic conditions (during shell closure), was characterized by an early increase in proteins involved in an anaerobic pathway towards succinate and propionate (Fields et al., 2014). Subsequently, there was an increase in oxidative stress proteins and, finally, changes in the abundance of cytoskeletal proteins (Fields et al., 2014).

Short time periods of aerial exposure but long immersion (emersion/recovery 6 h/18 h) increased the abundance of three

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isoforms of GST (GST1–3) and Prx6. GST isoforms perform a range of functions, including the detoxification of small molecules and the transfer of glutathione to proteins (Tew and Townsend, 2012). Unlike other Prxs, the cytosolic Prx6 uses GSH rather than Trx to decrease H_2O_2 (Fisher, 2011), and GST catalyzes the transfer of GSH to Prx6 (Zhou et al., 2013). While these results point to an important role of GST during hypoxia, given the design of the experiment, it is not clear whether aerial exposure or subsequent re-immersion (re-oxygenation) caused the increase in antioxidant proteins.

Studies on hypoxia-induced proteins in brain tissue of male medaka (*Oryzias latipes*) also showed an increase in GST (Oehlers et al., 2007), but brain and skeletal muscle in carp (*Carasssius carassius*) and zebrafish (*Danio rerio*), respectively, did not identify GST among proteins changing in response to anoxia/hypoxia (Bosworth et al., 2005; Smith et al., 2009).

Thus, it seems that hypoxia or re-oxygenation following hypoxic conditions causes an increased need for the glutathionylation of proteins, at least in mollusks, possibly to protect thiol groups from forming irreversible sulfinic and sulfonic acids (Murphy, 2012).

pH-induced antioxidant response

Estuaries are characterized by great fluctuations in CO_2 production due to the respiratory activity of the resident biota and temporarily limited exchange with open ocean water. CO_2 levels in estuaries can reach 1.3–4.7 kPa and pH values frequently reach as low as 7.5–6.0 (Cochran and Burnett, 1996; Ringwood and Keppler, 2002). Mantle tissue of a typical estuarine inhabitant, the eastern oyster, *Crassostrea virginica*, responded to a 2 week exposure to a P_{CO_2} of ∼357 Pa (∼39 Pa for control), equating to a pH of 7.5 (8.3 control), by increasing the abundance of Cu–Zn-SOD, Prx2 (cytoplasm), Prx4 (ER) and Prx5 (mitochondria and peroxisome) (Tomanek et al., 2011). In addition, the Trx homolog nucleoredoxin also increased in abundance in response to low pH, suggesting that the SOD–Trx–Prx system was activated to respond to increasing levels of ROS, either as a direct effect of pH via the Fenton reaction or indirectly by CO_2 interacting with peroxynitrite, resulting in reactive carbonate, oxygen and nitrogen species (Trujillo et al., 2008). Three other studies investigating the response to low pH in marine invertebrate larvae did not identify a single antioxidant protein among those proteins that were detected and that changed significantly with low pH (Dineshram et al., 2013, 2012; Wong et al., 2011).

Sources of reducing power (NADPH)

The source of protons (reducing equivalents) for the scavenging reactions and thus for the antioxidant responses of the cell is NADPH and not NADH (Mailloux et al., 2013; Murphy, 2012). Both GR and thioredoxin reductase (TrxR) require reducing equivalents in the form of NADPH to maintain levels of reduced glutathione and Trx, respectively (Figs 3, 4). Thus, the reactions producing NADPH are potentially important in contributing to an effective antioxidant response (Fig. 4), but they have only received scant attention as part of investigations into the antioxidant response to environmental stress.

While the cytosolic pentose phosphate pathway (PPP), specifically the reaction of glucose 6-phosphate dehydrogenase, was long thought to be the major source of NADPH, other cytosolic and mitochondrial NADP-dependent dehydrogenase reactions have emerged as sources of reducing power during oxidative stress (Pollak et al., 2007). Specifically, isoforms of NADP-dependent isocitrate dehydrogenase (NADP–ICDH) are found in the mitochondria, cytosol and peroxisome (Jo et al., 2001; Margittai and Banhegyi, 2008; Yoshihara et al., 2001). Cytosolic and mitochondrial malic enzyme produces NADPH and pyruvate, using malate as a substrate (Michal and Schomburg, 2012). In addition, a cytosolic isoform of aldehyde dehydrogenase (ALDH6P) catalyzes the following reaction: $RCHO+NAD(P)^{+}+H_2O \Leftrightarrow RCOOH+NAD(P)H+H^{+}$ (where RCHO is carbonyl and RCOOH is carboxyl), and thereby contributes to the NADPH pool (Grabowska and Chelstowska, 2003). While each of these enzymes contributes to the level of NADPH, in yeast none of these enzymes is essential on its own (Pollak et al., 2007). Furthermore, a mitochondrial membrane-based transhydrogenase uses the proton motive force across the inner membrane to reduce $NADP^{+}$ to NADPH (Rydström, 2006). Finally, in the ER, reducing equivalents in the form of NADPH are provided by hexose 6-phosphate dehydrogenase (White et al., 2007).

Abundance changes of NADPH-generating enzymes during environmental stress

Of the biochemical processes providing NADPH during oxidative stress (PPP, several NADP-dependent dehydrogenases and transhydrogenase), enzymes of the oxidative part of the PPP (6-phosphogluconate dehydrogenase and lactonase) increased in abundance in response to acute heat stress in *Mytilus* (Tomanek and Zuzow, 2010) and in response to hypoxic stress in *Geukensia* (Fields et al., 2014). Both stresses also increased enzyme abundance in the non-oxidative part of the PPP (transketolase) in both genera. This is important as the non-oxidative part recycles ribulose 5-phosphate to glucose 6-phosphate, generating a cycle that leads to the conversion of glucose 6-phosphate to six CO_2 and 12 NADPH (Nelson and Cox, 2008). Transketolase was also more abundant in larvae of the barnacle *Balanus amphitrite* in response to CO_2-enriched acidic (pH 7.6) seawater (Wong et al., 2011).

Acute and chronic heat stress, as well as hyposaline stress also showed (most frequently) an increase in abundance in additional proteins generating NADPH, specifically NADP–ICDH and ALDH in *Mytilus* congeners (Fields et al., 2012b; Tomanek and Zuzow, 2010; Tomanek et al., 2012). Part of the variation in how these proteins changed is due to a shift in the abundance of PTMs due to changes in the redox status of the cell, e.g. deacetylation of NADP–ICDH (Yu et al., 2012), reminding us to be careful when interpreting proteomic results. Nevertheless, the fact that the majority of the most abundant isoforms of these enzymes showed an increase supports their contribution to an increased demand for reducing equivalents.

Malic enzyme and transhydrogenase have so far not been detected or identified as showing abundance changes with any environmental stress in any of the proteomic studies reviewed herein, which does not exclude them for having a role in providing NADPH. They may not be abundant enough or difficult to detect or they are regulated through stoichiometrically small changes in PTMs instead of overall changes in abundance. Thus, the PPP and the NADP–ICDH reactions are commonly increasing with environmental stress. Changes in the abundance of ALDH are common in response to several stresses, including acute and chronic heat and hypoxic stress (in *Mytilus* and *Geukensia*, respectively), but the ambiguous identification of the isoform types makes it difficult to assess whether the role of ALDH is one of detoxifying aldehydes, using NADH, or providing reducing equivalents in the form of NADPH.

Oxidative stress in the ER

The ER is a single-membrane enclosed compartment separate from the cytosol in which secreted and membrane proteins fold and mature, including the folding of *de novo* translated proteins,

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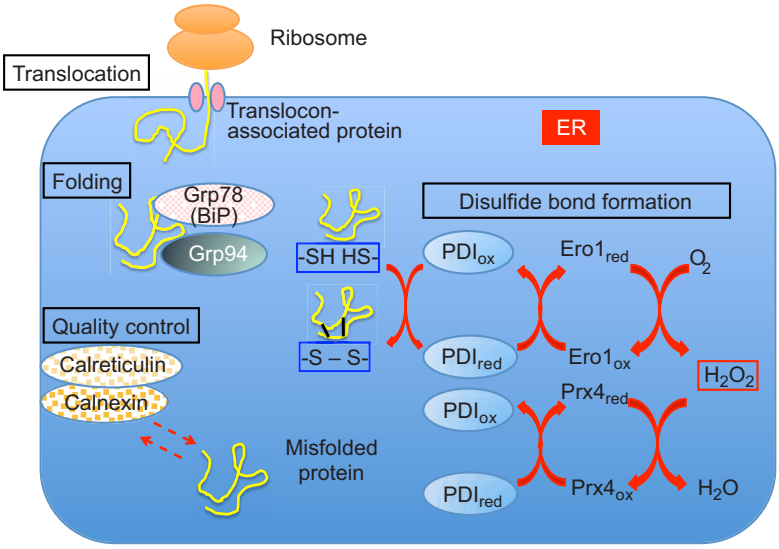

Fig. 7. Chaperones of the ER and the generation of hydrogen peroxide by ER oxidase 1 (Ero1) linking protein folding to oxidative stress. Proteins that carry a signal sequence are translocated into the lumen of the ER (by translocon-associated protein complex) after translation. While glucose-regulated proteins 78 and 94 kDa (Grp78 and Grp94) are the major chaperones of the ER during the initial phase of protein folding, protein disulfide isomerase (PDI) is involved in disulfide bond formation and calreticulin and calnexin are chaperones that conduct quality control of glycosylated proteins. The hydrogen peroxide generated is reduced by peroxiredoxin 4 (Prx4) or GPx7 and 8 (not shown), which can oxidize an additional PDI and contribute to disulfide bond formation.

formation and proofreading of N-linked glycosylation and, importantly, disulfide formation (Braakman and Hebert, 2013). The last process affects both protein structure and function and is closely linked to the production of ROS (Bulleid, 2013; Csala et al., 2010).

When newly translated proteins carry an ER signal sequence, they enter into the lumen of the ER where molecular chaperones assist their folding (Fig. 7). The ER chaperones glucose-regulated protein 78 (Grp78; or binding immunoglobulin protein, BiP) and 94 (Grp94) are members of the heat-shock protein (Hsp)70 and Hsp90 family, respectively, and are highly abundant and conserved (Braakman and Bulleid, 2011). In addition, following the glycosylation of proteins, the carbohydrate-binding chaperones malectin, calnexin and calreticulin recognize and promote the correct folding of glycoproteins (Pearse and Hebert, 2010). The initial folding process brings cysteines into close proximity and continues by catalyzing the formation of disulfide bonds by members of the protein disulfide isomerase (PDI) family (Bulleid and Ellgaard, 2011). The formation of disulfide bonds, i.e. the oxidation of two thiol groups, reduces PDI, which in turn requires an oxidase to recycle PDI back to the oxidized form, i.e. the formation of a disulfide. This oxidase is ER oxidase 1α (Ero1α), which mediates the transfer of electrons to its FAD^+ group, which, once reduced to FADH, reacts with oxygen to form H_2O_2 (Araki and Nagata, 2012; Bulleid, 2013). Thus, disulfide formation requires an oxidative environment, reflected by a much lower ratio of GSH/GSSG in the ER in comparison to the cytosol. However, the formation of non-native disulfides requires the oxidation of PDI, i.e. the reduction of protein disulfide bonds, in the same compartment and suggests that the redox state of PDI is tightly regulated to facilitate both the formation (oxidation) and reduction of disulfides. Importantly, an enzyme reducing the oxidized PDI is not known, but the process is either directly or indirectly dependent on GSH (Appenzeller-Herzog, 2011). Another folding process that occurs in the ER (not shown in Fig. 7), the conformational change from a *trans* to a *cis* isoform of proline residues, is catalyzed by prolyl peptidyl isomerases (PPIases) and is represented by cyclophilins (Cyp) and FK-binding proteins (FKBPs) (Braakman and Hebert, 2013).

While Ero1α is essential in lower eukaryotes, higher eukaryotes have additional oxidases for the reduced form of PDI. Those include Prx4 and GPx7 and 8 (Bulleid and Ellgaard, 2011). Prx4 can serve as an additional electron acceptor to PDI by using the H_2O_2 from the Ero1α reaction as the terminal receptor (Tavender et al., 2010). This results in two PDIs being oxidized, one by Ero1α, the other by Prx4. Similarly, Gpx7 and Gpx8 show high PDI peroxidase activity and can also use the H_2O_2 generated by the Ero1α reaction as a terminal electron acceptor (Nguyen et al., 2011).

Environmentally induced antioxidant response in the ER

A comparison of the time course of the proteomic responses of gill of the native more euryhaline *M. trossulus* to the more stenohaline invader *M. galloprovincialis* exposed both to an acute hyposaline challenge from 35.0 psu (control) to 29.8 psu (moderate) and 24.5 psu (extreme) for 4 h followed by a 0 and 24 h recovery under control conditions (Tomanek et al., 2012). These conditions mimic freshwater run-off in their coastal habitats following heavy precipitation events in the Eastern Pacific, which are predicted to become more extreme and more frequent in the future because of a more humid atmosphere in a warmer world (Durack et al., 2012). Based on principal component analyses, *M. trossulus* changed its proteome to the greatest extent in response to the most extreme hyposaline challenge, while *M. galloprovincialis* showed the greatest proteomic changes in response to moderate but not extreme hyposaline conditions, reflecting their tolerance limits. Specifically, *M. trossulus* and *M. galloprovincialis* increased the abundance of several ER chaperones, including Grp78, Grp94 and PDI in response to 24.5 and 29.8 psu at 0 h recovery, respectively. During the 24 h recovery under control conditions, the 24.5 and 29.8 psu groups of both species decreased the abundance of these ER chaperones. This decrease was accompanied by a parallel decrease in several antioxidant proteins, including SOD, Prx5, Trx, nucleoredoxin (a putative Trx) and DyP-peroxidases in both species. These results were interpreted as indicating an acute demand in chaperoning activity due to increased protein denaturation during hyposalinity and a subsequent reduction in protein synthesis and maturation in the ER, accompanied by a reduction in antioxidant proteins due to lower rates of production of H_2O_2, to re-establish homeostasis during recovery. However, if this hypothesis is correct, it is still unclear whether protein denaturation and the proposed subsequent reduction in protein synthesis cause an overall lower demand for (ER and cytosolic) antioxidant proteins, or whether environmental stress caused an increase in overall ROS production throughout the cell, requiring a decrease in protein maturation generating H_2O_2 and thereby also a localized decrease in antioxidant proteins. Furthermore, while acute heat stress did not

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activate changes in ER chaperones in *Mytilus* congeners, chronic cold and warm temperature stress changed the abundance of Grp78 and DyP-peroxidase in *M. trossulus* (Fields et al., 2012b; Tomanek and Zuzow, 2010).

A comparison of the proteomic responses of gill of five populations of the mussel *G. demissa* from different latitudes along the Atlantic coast of North America in response to acute heat stress showed changes in two Grp78 and one PPIase isoform (Fields et al., 2012a). The most northern population (Maine) was the only one showing a change in abundance of these proteins with acute stress, suggesting that it alone was sensitive to a heat-induced challenge to proteostasis in the ER. The only oxidative stress protein that showed a corresponding change was Grx (Fields et al., 2012a), which has not been associated with the oxidation of PDI in the ER. Furthermore, hypoxia changed the abundance of two calreticulin isoforms in *Geukensia* in complementary patterns, suggesting a switch in PTM, with a catalase isoform clustering closely with one of the calreticulin isoforms (Fields et al., 2014).

These results suggest that some environmental stresses affect protein denaturation and maturation in the ER in gill of marine mollusks, possibly mediated through a change in the redox environment of the ER. As protein maturation in the ER generates H_2O_2, a decrease in protein synthesis could lower the rate of ROS production. The importance of this link is further supported by the inclusion of the ER chaperones Grp78, PDIs and PPIases and the antioxidant protein Prx, together with SOD and ALDH, in the proposed minimal stress proteome of organisms (Kültz, 2005; Petrak et al., 2008; Wang et al., 2009).

Oxidative stress in peroxisomes

Peroxisomes are single-membrane compartments that connect to the cytosol through a porin-like channel (Rokka et al., 2009). Their functions include the α-oxidation of branched and β-oxidation of very long-chained fatty acids (e.g. acyl CoA oxidase), purine catabolism (xanthine and urate oxidase) and amino acid metabolism (amino acid oxidase) (Bonekamp et al., 2009; Schrader and Fahimi, 2006). Most of these oxidases are flavoproteins that transfer one or two electrons to oxygen, which serves as the electron acceptor in a reaction that generates $O_2^{-\bullet}$ (in case of xanthine oxidase, XOx) and H_2O_2 (hence the name peroxisome), which in turn is further reduced to H_2O and O_2 by catalase (Fig. 8). While these oxidases accept electrons from their substrates, the fate of these electrons is different from that of electrons in the mitochondria. For example, in contrast to the case in mitochondria where the electrons from β-oxidation are transported through the ETS and linked to the production of ATP, in the peroxisome, energy from the reaction of the first step of β-oxidation by acyl-CoA oxidase is dissipated as heat (Nelson and Cox, 2008). Importantly, the activities of almost all peroxisome oxidases generate H_2O_2, which, if not scavenged by catalase, will diffuse across the membrane to other cellular compartments. Furthermore, in the presence of ferrous iron (Fe^{2+}), hydrogen peroxide is reduced to the hydroxyl radical ($OH^{\bullet}$) in the Fenton reaction, which is likely to happen given the high concentration of iron-containing enzymes in peroxisomes (Nordgren and Fransen, 2014).

In addition to the oxidase reactions, nitric oxide synthase (NOS) generates nitric oxide ($NO^{\bullet}$) from arginine in an NADPH-dependent reaction (Fig. 8). In combination with $O_2^{-\bullet}$, generated by XOx following partial proteolysis or heating (Engerson et al., 1987), $NO^{\bullet}$ forms the highly reactive peroxynitrite ($ONOO^-$) (Nordgren and Fransen, 2014).

Thus, it is possible that $O_2^{-\bullet}$, H_2O_2, $OH^{\bullet}$, $NO^{\bullet}$ and $ONOO^-$ are produced in the peroxisome. A number of studies have accumulated evidence that these ROS and RNS are most likely reduced by catalase (H_2O_2), Cu–Zn-SOD ($O_2^{-\bullet}$) and Prx5 ($ONOO^-$) (Bonekamp et al., 2009; Nordgren and Fransen, 2014; Schrader and Fahimi, 2006). Proteomic studies suggested that additional antioxidative stress proteins, possibly involved in the detoxification of lipid peroxides, are active in peroxisomes, e.g. Prx1 and GSTκ (Islinger et al., 2007; Kikuchi et al., 2004).

Environmentally induced antioxidant response in peroxisomes

Studies investigating the proteomic changes in response to environmental stress have so far given few indications that peroxisome metabolism changed. However, peroxisome-enriched fractions of the digestive glands of mussels of the genus *Mytilus* showed an increasing abundance of antioxidative stress proteins (e.g. catalase and Mn-SOD), but a decreasing abundance of proteins of α- and β-oxidation (e.g. hydroxyacid oxidase and enoyl CoA hydratase) in response to marine pollutants, which often increase the production of ROS (Apraiz et al., 2009).

While several of the proteomic analyses reviewed herein showed a changing abundance of SOD isoforms and Prx5, indicating that peroxisomal metabolism might be changing with environmental stress due to increased oxidative stress in this compartment, the analyses were all based on tissue homogenates, which prevents an exact localization of the proteins in the cell and thus prevents stronger support for this hypothesis. Peroxisome-enriched samples will help to assess the contribution of this organelle to oxidative stress during environmental stress in the future.

Conclusion

I set out promising some novel insights to the study of how environmentally induced oxidative stress affects the proteome, based on studies of marine organisms. These studies showed that the Trx–Prx system is possibly more frequently recruited to scavenge ROS than the glutathione system. SOD isoforms are not ubiquitously induced in parallel, suggesting that SOD scavenging activity is either sufficient or that it is not as important a site of

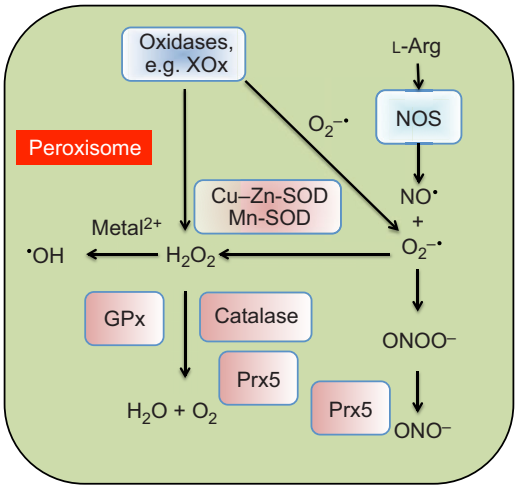

Fig. 8. Generation of ROS and RNS in the peroxisome. The production of superoxide anions ($O_2^{-\bullet}$) by xanthine oxidase (XOx) and nitric oxide ($NO^{\bullet}$) by nitric oxide synthase (NOS) leads to the formation of peroxynitrite ($ONOO^-$), a highly reactive nitrogen species. Peroxiredoxin 5 (Prx5) is the major scavenger of peroxynitrite in the peroxisome (ONO^-, nitrite). Other reactions are similar to those in other cellular compartments, but noticeable is the major role of catalase in scavenging the hydrogen peroxide formed by a number of oxidases using oxygen as an electron acceptor (after Schrader and Fahimi, 2006).

regulation as the Trx–Prx system. The glutathione system is more important for scavenging H_2O_2 in some organisms and likely plays a role in protecting protein thiols during environmental stress. Furthermore, the synthesis pathways of cysteine and selenocysteine, building blocks for glutathione and GPx, might also play a role in scavenging ROS during stress. The reactions of Grx and DyP-type peroxidase play important roles during environmentally induced oxidative stress but have not received much attention.

The proteomic analyses also highlighted the importance of down-regulating the reactions that produce ROS as part of the TCA cycle and the ETS during acute stress, specifically heat stress. While reactions generating ROS and NADH generally decreased, reactions of the PPP and NADP–ICDH, and possibly ALDH, increased to generate NADPH as a reducing equivalent for scavenging ROS.

Given the changes described in these studies, mitochondria seem to be the dominant source of ROS during stress. But the changes in ER chaperones from a compartment that produces ROS as part of protein maturation indicate that other organelles might contribute substantially to the production of ROS. This may be in part specific to gill tissue as it produces glycosylated proteins that are secreted for mucus production to trap food in bivalves.

Although I included proteomic analyses from organisms experiencing acute and chronic cold and warm temperature, hyposaline, hypoxic and pH stress in this review, it is premature to draw conclusions about stress-specific and common elements of the cellular stress response. Importantly, a full account of such differences requires more comprehensive sampling regimes during and following stress exposure and comparisons of species varying in stress tolerance. Nevertheless, two analyses stood out. The broad complement of antioxidant proteins during acclimation to low pH in mantle of the Eastern oyster *C. virginica* and the correlation of a decrease in ER chaperones and antioxidant proteins in gill of *Mytilus* congeners during hyposaline stress showed patterns that seemed stress specific (Tomanek et al., 2012, 2011).

Finally, comparisons of closely related species with differing tolerance ranges provide a unique perspective about the proteomic differences, e.g. abundance changes and shifts in PTMs, that may contribute to setting stress tolerance limits, as illustrated by the comparison of the two *Paralvinella* congeners (Dilly et al., 2012). However, deducing general patterns from species comparisons needs to take experimental design differences into account.

The proteomic responses to environmentally induced oxidative stress provide a number of novel hypotheses. More species comparisons, comprehensive sampling regimes and analyses of multiple tissues are needed to further test the general nature of these hypotheses.

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Competing interests

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