Amino Acids (2015) 47:1421–1432
DOI 10.1007/s00726-015-1955-8

ORIGINAL ARTICLE

Protein-borne methionine residues as structural antioxidants in mitochondria

Mario Schindeldecker[1] · **Bernd Moosmann**[1]

Received: 19 October 2014 / Accepted: 3 March 2015 / Published online: 10 April 2015
© Springer-Verlag Wien 2015

Abstract Methionine is an oxidant-labile amino acid whose major oxidation products, methionine sulfoxides, can be readily repaired by various NADPH-dependent methionine sulfoxide reductases. Formally, the methionine oxidation–reduction circuit could act as a cellular antioxidant system, by providing a safe sink for oxidants that might cause much more damage if reacting otherwise. This concept is supported by focal experimental evidence; however, the global importance, scope and biochemical role of protein-borne methionine as an inbuilt macromolecular antioxidant have remained incompletely defined. In analyzing proteomic methionine usage on different levels of comparison, we find that protein methionine (i) is primarily an antioxidant of mitochondria, especially of the inner mitochondrial membrane, (ii) responds strongly to respiratory demands on an evolutionary timescale, (iii) acts locally, by selectively protecting its carrier protein, and (iv) might be utilized as a molecular predictor of aerobic metabolic rate in animals, to complement traditional markers like the presence of a respiratory pigment. Our data support the idea that proteins in need of a long lifespan or acting in dangerous environments may acquire massive structural alterations aimed at increasing their resistance to oxidation. Counterintuitively though, they sometimes do so by accumulating particularly labile rather than particularly stable building blocks, illustrating that the technical concept of cathodic protection is also employed by the animate nature.

Keywords Antioxidant · Cathodic protection · Metabolic rate · Methionine oxidation · Oxidative stress · Respiratory chain complex

Introduction

Antioxidants have once been defined as substances "that, when present at low concentrations compared to those of an oxidizable substrate, significantly delay or prevent oxidation of that substrate" (Halliwell 1990). This classic definition, compelling as it may be in many cases, has yet experienced various modifications in recent years to gain validity also for biological contexts. Primarily, the comparison of concentrations had to be replaced by a quality rating, because an antioxidative advantage for the cell might equally be achieved when a (low-molecular mass) compound whose oxidation is "harmless" is present in larger concentrations than a (macromolecular) oxidizable substrate whose oxidation is "harmful". Regarding the protection of proteins or nucleic acids, the latter scenario actually seems to be the most common case of antioxidant protection. Hence, it is foremost the quality of the consequences of each oxidative event that needs to be assessed.

The latter consideration has become particularly relevant in the context of the concept that macromolecules like proteins may themselves act as antioxidants by means of specific redox-active groups on their surface, built in to protect other parts of the same macromolecule (*cis*-acting) or its surroundings (*trans*-acting) from oxidation. To be biologically meaningful, this concept requires that the protected part would have to be of a higher quality rating, i.e. more

Handling Editor: F. Galli.

✉ Bernd Moosmann
moosmann@uni-mainz.de

[1] Evolutionary Biochemistry and Redox Medicine, Institute for Pathobiochemistry, University Medical Center of the Johannes Gutenberg University, Duesbergweg 6, 55099 Mainz, Germany

functionally relevant or more difficult to replace or repair, than the sacrificial antioxidant group. Initially, this conclusion has been elaborated on the example of methionine on protein surfaces (Levine et al. 1996) and, later, on the functional role of the widely observed tyrosine and tryptophan accumulation in membrane proteins (Moosmann and Behl 2000), followed by the recent speculation that even protein-borne cysteine, a classical target of irreversible oxidative destruction, might sometimes find an even higher-rated counterpart which to protect from oxidation (Requejo et al. 2010). In fact, a major point in any argument that certain protein moieties might act as antioxidants for more highly rated others was that the oxidation of the protector was readily reversible and thus basically harmless. For instance, the existence and functional characterization of diverse methionine sulfoxide reductases and the infrequence of irreversible methionine oxidation beyond the sulfoxide level have provided support for the methionine concept (Levine et al. 1996; Weissbach et al. 2005; Oien and Moskovitz 2008; Kim et al. 2014), whereas the tyrosine/tryptophan concept was corroborated by the demonstration of rapid non-enzymatic repair through ascorbate and trolox, a tocopherol analogue (Bisby et al. 1984; Jovanovic and Simic 1985), as well as by experiments with membrane-targeted amino acid derivatives (Moosmann and Behl 2000; Hajieva et al. 2015) and hydrophobic peptides (Moosmann and Behl 2002).

The question whether protein-borne methionine indeed acts as an antioxidant whose insertion provides a net benefit to the cell has never been probed directly. However, two approximative experimental approaches have provided evidence that it may indeed do so. Most notably, in partially replacing protein methionine by norleucine in bacteria, the capacity of *Escherichia coli* to survive different forms of oxidative stress was impaired (Luo and Levine 2009). Second, administration of lipophilic *N*-acyl-methionine to primary midbrain neurons has been found to protect these cells from prooxidative toxicity (Bender et al. 2008). In both cases, though, it cannot be excluded that redox-independent secondary effects, obviously the potential unfolding of proteins when statistically replacing methionine for norleucine, or the potential alteration of membrane dynamics after addition of *N*-acyl-methionine, contributed to the observed effects.

In an attempt to exploit the power of bioinformatics for this experimentally refractory question, we have analyzed a series of experiments of nature, i.e. cases in which significant quantitative alterations of protein methionine content have been realized, and compare them with their often well-established redox biochemistry. On several independent levels, we show that whenever protein methionine deviates significantly from background levels, this deviation is found in a prooxidant environment or in the vicinity of very highly ranked targets of oxidation. Applying the evolutionary principle that non-fitness is not selected for, and the biochemical principle that rapid oxidative decomposition of methionine regarded alone would be a molecular embodiment of non-fitness, it is concluded that methionine is in fact a biologically selected, locally acting antioxidant.

Materials and methods

Human protein sequences

A number of 20,233 reviewed sequences were retrieved from the Uniprot database (http://www.uniprot.org) as of December 2012. Subcellular locations were assigned following the classification in Uniprot. A final number of 19,806 sequences were included for analysis after the exclusion of olfactory receptors; this amplified family of 427 very closely related sequences that are yet hardly expressed in the organism was excluded as its consideration would have introduced a large bias into the "cytoplasmic membrane" analytical subset.

The following were the final numbers of sampled human proteins: mitochondrion 985; nucleus 4901; endoplasmic reticulum 886; Golgi apparatus 719; peroxisome 94; cytoplasm 4195; mitochondrion inner membrane 228; mitochondrion outer membrane 94; nuclear membrane 116; endoplasmic reticulum membrane 637; Golgi apparatus membrane 508; peroxisome membrane 37; cytoplasmic membrane 2256.

Nuclear and mitochondrially encoded respiratory chain complex subunits

Seventy-five human sequences were collected as described (Schindeldecker et al. 2011), encompassing 42 complex I subunits, 9 complex III subunits, 10 complex IV subunits and 14 complex V subunits. The human "respiratory chain" protein set was assembled from these 75 sequences plus the 13 proteins encoded by human mitochondrial DNA.

The sampling of 361 mitochondrially encoded proteomes from ten animal phyla was done by predefined criteria based on sequence availability or, for chordates, on species occurrence in an arbitrarily chosen reference book as detailed (Bender et al. 2008). In particular, the proteomes of 3 sponges, 7 cnidarians, 10 platyhelminthes, 16 echinoderms, 55 insects, 2 bees, 6 whiteflies, and 95 mammals were sampled and compared with the average metabolic rate of each clade.

Sources of metabolic rates

Mass-specific basal or active metabolic rates were assembled for selected phylogenetic groups. The sampled numbers of species and their literature sources were as follows:

7 sponges (Lanfear et al. 2007; Hadas et al. 2008); 6 cnidarians (Lanfear et al. 2007); 4 platyhelminthes (von Brand and Bowman 1961; Lanfear et al. 2007; Mouton et al. 2011); 20 echinoderms (Lanfear et al. 2007); 402 insects (Makarieva et al. 2008); 4 bees (Wolf et al. 1996; Harrison and Roberts 2000; Darveau et al. 2005; Makarieva et al. 2008); 1 whitefly (Salvucci and Crafts-Brandner 2000) and 625 mammals (Makarieva et al. 2008).

Numeric analyses

Proteomic methionine distributions and ranks were determined using customized scripts within the framework of the Bioperl software suite (Stajich et al. 2002). Regarding all ranking analyses, a reference flat file database was assembled containing the selected basis set of 19,806 human protein sequences. Similar flat file databases were created for each of the different organelles and compartments chosen to be analyzed separately; all database entries were sorted by descending methionine frequency. Then, each protein from each of the compartment databases was tested iteratively for its occurrence in the reference database. For each consecutive match, the assigned rank of the protein in the reference database was returned as result. The obtained rank lists were then analyzed for their averages and medians and visualized with the scientific graphing and statistical analysis software package SigmaPlot (Systat Software, San Jose, CA, USA). Statistical comparison of the different human protein subsets (rank lists) was done by one-way ANOVA on ranks, followed by Scheffe's post hoc test. Here, p values of 0.001 or less were considered significant. All other statistical analyses were also conducted nonparametrically (i.e. on ranks), as in a number of cases the assumption of normality of the assembled data was not met. Statistical calculations were performed with SPSS 21 (IBM SPSS, Armonk, NY, USA).

Identification of transmembrane domains

Helical transmembrane sequence segments were identified with TMHMM 2.0, a software algorithm that employs a hidden Markov model trained on 160 protein structures with respect to seven character states such as helix core, helix caps, or perihelical loops. TMHMM has been shown to be suitable for the reliable transmembrane domain identification in complete proteomes (Krogh et al. 2001).

Molecular modelling

Bona fide protein structures of eight NADH dehydrogenase transmembrane domains from disparate animal families were obtained by homology modelling. Modelling was restricted to the transmembrane domains as only the seven conserved intramembrane subunits of this enzyme are encoded by mitochondrial DNA, whose sequence is available for a large number of unrelated animal species. The animals whose NADH dehydrogenases were to be modeled were selected on the basis of essentially neutral criteria: from each of the predefined clades sponges, cnidarians, platyhelminthes, echinoderms, insects, bees, and whiteflies, the species representing the clade's average methionine content most closely was chosen. In case of poor protein sequence quality, i.e. unknown amino acid identities within the coding sequence, the next best representative of a clade's mean was picked instead. Representing mammals, *Homo sapiens* was analyzed on arbitrary grounds, to connect the molecular modelling studies with the other analyses conducted in this work.

For the homology modelling, sequences of the seven mitochondrially encoded NADH dehydrogenase subunits were collected from UniProt for each animal species to be analyzed. The crystal structure of *Thermus thermophilus* NADH dehydrogenase (Baradaran et al. 2013) was used as 3D modelling template (PDB entry 4HE8; http://www.rcsb.org/pdb) as it contains homologues to all mitochondrially encoded NADH dehydrogenase subunits of metazoans. Modelling was done sequentially for each subunit and animal species using the SWISS-MODEL homology modelling server (Biasini et al. 2014). The resulting single-subunit models were then superimposed and visualized with UCSF Chimera (Pettersen et al. 2004). Surface exposures of all amino acids in the final quaternary structures were calculated with the WHAT IF webservice (Vriend 1990).

Results and discussion

Methionine enrichment primarily occurs in the vicinity of the respiratory chain

If protein-borne methionine was of significant antioxidant value to the eukaryotic cell, it might be selectively enriched at sites in specific need of protection. Following this reasoning, global methionine usage ranks for the annotated human proteome were analyzed by subcellular location as shown in Fig. 1. Mitochondrial proteins exhibited a clear bias towards possessing higher methionine contents than the cellular average. This effect became increasingly pronounced when the analysis was refined towards the physical vicinity of the major site of free radical production in the cell (Brand 2010), i.e. the mitochondrial inner membrane, the respiratory chain, and finally, the mitochondrially encoded core subunits of the respiratory chain. The latter proteins have already been described to accumulate methionine in animal species that use a non-standard genetic code for mitochondrial translation (Bender

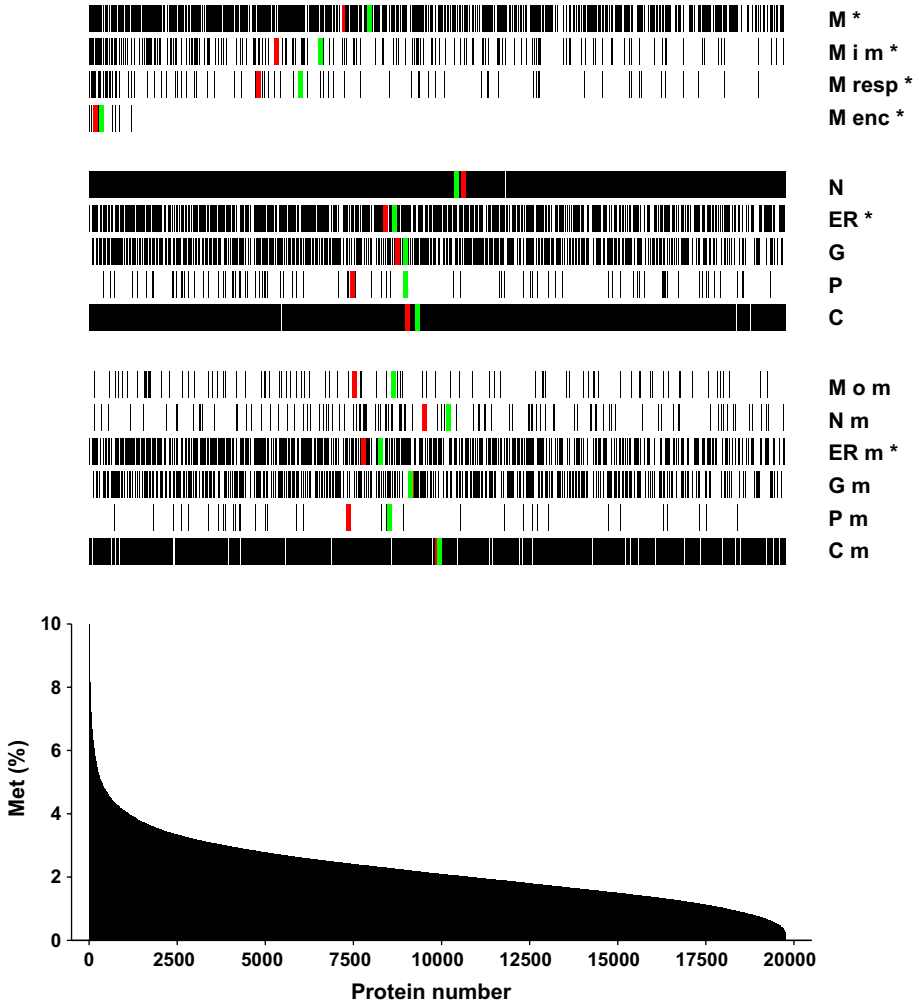

Fig. 1 Methionine usage in human proteins locating to different subcellular compartments. Each *vertical dash* corresponds to a single protein having the indicated rank and methionine content readable from the distribution plotted in the *lower part* of the figure. *Green dashes* indicate the mean, *red dashes* the median protein methionine content of each subcellular site. The abbreviations denote: *M* mitochondrion, *M i m* mitochondrial inner membrane, *M o m* mitochondrial outer membrane, *M resp* respiratory chain (of the inner mitochondrial membrane), *M enc* mitochondrially encoded proteins (of the respiratory chain), *N* nucleus, *ER* endoplasmic reticulum, *G* Golgi apparatus, *P* peroxisome, *C* cytoplasm. In the last five cases, a *lower case* "*m*" represents the corresponding membrane protein subset. *Asterisks* denote a significantly increased methionine usage rank compared to the cellular average ($p < 0.001$; exact p values are given in Table 1)

et al. 2008). In the present study, they exhibited a median methionine usage rank of 184 out of 19,806 proteins that were included in the analysis (Fig. 1). Beyond the mitochondrial protein subsets, only endoplasmic reticulum proteins and the corresponding membrane proteins exhibited a statistically significant bias towards higher methionine contents (Table 1, column e).

Transmembrane domains generally possess approximately 50 % higher methionine contents than aqueous protein domains. Accordingly, to verify the validity of the rank graphic shown in Fig. 1, the transmembrane domain contents of all depicted protein subsets were calculated, and methionine contents of the cumulated transmembrane domains were analyzed separately (Table 1). In fact, even among the different membrane protein subsets, actual transmembrane domain contents varied substantially (Table 1, column a). However, this effect was of minor relevance to the observed phenomenon of mitochondrial methionine accumulation, as the latter was similarly pronounced in transmembrane domains and non-transmembrane domains (Table 1, column b versus c). Hence, there appears to be a general preference for increased methionine contents in mitochondrially localized proteins.

In an alternative approach to detect methionine enrichment on the individual protein level, all sampled human proteins were listed by descending methionine content. Proteins possessing very high methionine contents of 6 % or more, thus deviating by more than 3.5 standard deviations from the mean, were further analyzed. After exclusion of proteins with <150 amino acids to avoid statistical artifacts, 54 functionally annotated proteins were sampled (Table 2). Beyond respiratory chain complex subunits, both mitochondrially imported and mitochondrially encoded, a second group of likely candidates for directed antioxidant protection stood out, namely proteins involved in single-stranded RNA or DNA binding and processing (Table 2). Some proteins recovered in this group were U1 small nuclear ribonucleoproteins (snRNP) A and C, heterogeneous nuclear ribonucleoprotein M, and cleavage stimulation factor subunit 2 (all directly binding to snRNA or nascent mRNA and involved in splicing or mRNA processing). Moreover, the signal recognition particle 54 kDa protein

Table 1 Transmembrane domain content and relative methionine usage in human protein sets sorted by cellular compartment

Compartment[a]	TM domain content (a) (%)	Met in TM domains (b) (%)	Met in non-TM domains (c) (%)	Met total (d) (%)	Statistical significance on ranks (e)**
Total proteome	3.41	100 (2.94 % absolute)	100 (2.08 % absolute)	100 (2.11 % absolute)	
M	2.84	125	117	117	9×10^{-17}
M i m	7.53	140	130	133	9×10^{-11}
M resp	14.36	187	144	159	2×10^{-04}
M enc	52.63	203	239	261	1×10^{-03}
N	0.23	90	100	98	2×10^{-03}
ER	8.85	95	109	110	4×10^{-04}
G	4.68	104	106	106	2×10^{-01}
P	2.15	86	109	108	1×10^{-00}
C	0.32	91	104	103	2×10^{-03}
M o m	4.12	94	117	116	1×10^{-00}
N m	3.94	84	100	100	1×10^{-00}
ER m	11.55	96	111	112	5×10^{-06}
G m	6.28	104	105	107	8×10^{-01}
P m	4.57	84	114	112	1×10^{-00}
C m	8.99	96	96	98	1×10^{-00}

** By post hoc test, accounting for multiple comparisons

[a] Abbreviations are used as in Fig. 1

[directly binding to RNA, with many surface-exposed methionines in the binding domain (Gowda et al. 1998)] and a number of single-stranded DNA-binding proteins essential for genome maintenance (Ashton et al. 2013) were retrieved (Table 2). It is easily conceivable that the high methionine contents in these proteins function to shield the notoriously vulnerable single-stranded nucleic acids from oxidative attack. To explore this idea on a structural level, the architecture of the spliceosomal U4 snRNP core domain (Leung et al. 2011) was inspected, containing a fragment of the snRNP-associated proteins B and B' from Table 2 and several other high-methionine snRNPs. In this highly expressed (Yong et al. 2004) structure, the clustering of the methionine side chains around the central RNA passage was striking (Fig. 2). Notably, one of the contained proteins, SmD3, has recently been described as a functional switch responsive to lipotoxic and oxidative stress, potentially enhancing cell survival (Scruggs et al. 2012).

Hence, methionine accumulation is not restricted to mitochondrially encoded proteins with their obvious exposure to very high oxidant fluxes (Bender et al. 2008; Brand 2010); it is similarly found in mitochondrially imported proteins, or in single-stranded nucleic acid-binding proteins that appear to be in particular need of antioxidant protection (Johansen et al. 2005). An additional factor selecting these proteins for internal antioxidant protection may have been their long half-life, which has been recognized for the respiratory chain complexes (Price et al. 2010) and the snRNPs (Yong et al. 2004).

The peaking methionine accumulation in complex I rather than complex IV indicates a *cis*-acting protective function of this amino acid in proteins

The observed, local accumulation of methionine in proteins residing in high-oxidant subcellular compartments immediately indicates a limited radius of antioxidant operation of this amino acid. In order to define this radius more precisely, an interspecies comparison of methionine usage in the respiratory chain complexes of 361 animals was conducted (Fig. 3). The sampled species represent ten different phyla and a large variety of lifestyles and metabolic rates. We have found that especially complex I and complex V proteins were characterized by exceptionally high methionine contents, exceeding 10 % in many species, whereas complex IV subunits showed more homogeneous methionine distribution. Focusing on the direct comparison between complex I and IV with their larger mitochondrially encoded sequence lengths of ~2100 and ~1000 amino acids, respectively, the higher average and peak methionine contents in complex I provide a direct indication that methionine is a *cis*-acting factor to protect its carrier protein and its immediate surroundings, rather than a *trans*-acting factor to protect, as an example, the whole inner mitochondrial membrane. This conclusion can be drawn from the different expression levels of the two complexes: complex IV is at least sixfold higher expressed than complex I in almost all animal tissues that have been analyzed (Lenaz and Genova 2009). If the selective advantage of methionine

Table 2 Human proteins with exceptionally high methionine contents

Identifier	Size (AA)	Met (%)	Name	Function
RU1C_HUMAN	159	13.84	U1 small nuclear ribonucleoprotein C	NAB
RGAG1_HUMAN	1388	10.45	Retrotransposon gag domain-containing protein 1	
COPT1_HUMAN	190	8.95	High affinity copper uptake protein 1	
HNRPM_HUMAN	730	8.63	Heterogeneous nuclear ribonucleoprotein M	NAB
MED18_HUMAN	208	8.17	Mediator of RNA polymerase II transcription subunit 18	
SSXT_HUMAN	418	8.13	Protein SSXT	
SMAP2_HUMAN	429	7.93	Stromal membrane-associated protein 2	
SOX2_HUMAN	317	7.89	Transcription factor SOX-2	
MLF2_HUMAN	248	7.66	Myeloid leukemia factor 2	
CHMP3_HUMAN	222	7.66	Charged multivesicular body protein 3	
CHM2A_HUMAN	222	7.66	Charged multivesicular body protein 2a	
CABP5_HUMAN	173	7.51	Calcium-binding protein 5	
RSMB_HUMAN	240	7.50	Small nuclear ribonucleoprotein-associated proteins B and B−	NAB
AIF1L_HUMAN	150	7.33	Allograft inflammatory factor 1-like	
PSB3_HUMAN1	205	7.32	Proteasome subunit beta type-3	
NU2M_HUMAN	347	7.20	NADH-ubiquinone oxidoreductase chain 2	RCC
M4A4A_HUMAN	239	7.11	Membrane-spanning 4-domains subfamily A member 4A	
EMC4_HUMAN	183	7.10	ER membrane protein complex subunit 4	
CHM2B_HUMAN	213	7.04	Charged multivesicular body protein 2b	
CHM1B_HUMAN	199	7.04	Charged multivesicular body protein 1b	
CPLX4_HUMAN	160	6.88	Complexin-4	
TNNC2_HUMAN	160	6.88	Troponin C, skeletal muscle	
BCL9L_HUMAN	1499	6.87	B cell CLL/lymphoma 9-like protein	
TNNC1_HUMAN	161	6.83	Troponin C, slow skeletal and cardiac muscles	
SSBP4_HUMAN	385	6.75	Single-stranded DNA-binding protein 4	NAB
CITE2_HUMAN	270	6.67	Cbp/p300-interacting transactivator 2	
SSBP2_HUMAN	361	6.65	Single-stranded DNA-binding protein 2	NAB
CS012_HUMAN	152	6.58	Protein C19orf12	
RPE_HUMAN	228	6.58	Ribulose-phosphate 3-epimerase	
FOXA2_HUMAN	457	6.56	Hepatocyte nuclear factor 3-beta	
SRP54_HUMAN	504	6.55	Signal recognition particle 54 kDa protein	NAB
ZN207_HUMAN	478	6.49	Zinc finger protein 207	
NDUB8_HUMAN	186	6.45	NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 8, mitochondrial	RCC
CP078_HUMAN	265	6.42	Uncharacterized protein C16orf78	
PRAF3_HUMAN	188	6.38	PRA1 family protein 3	
IFN14_HUMAN	189	6.35	Interferon alpha-14	
BCL9_HUMAN	1426	6.31	B-cell CLL/lymphoma 9 protein	
SNP25_HUMAN	206	6.31	Synaptosomal-associated protein 25	
SATL1_HUMAN	508	6.30	Spermidine/spermine *N*(1)-acetyltransferase-like protein 1	
BIK_HUMAN	160	6.25	Bcl-2-interacting killer	
EPN4_HUMAN	625	6.24	Clathrin interactor 1	
COX1_HUMAN	513	6.24	Cytochrome *c* oxidase subunit 1	RCC
DYDC1_HUMAN	177	6.21	DPY30 domain-containing protein 1	
SSBP3_HUMAN	388	6.19	Single-stranded DNA-binding protein 3	NAB
CLD7_HUMAN	211	6.16	Claudin-7	
SELT_HUMAN	195	6.15	Selenoprotein T	
EMC3_HUMAN	261	6.13	ER membrane protein complex subunit 3	
CREB5_HUMAN	508	6.10	Cyclic AMP-responsive element-binding protein 5	

Table 2 continued

Identifier	Size (AA)	Met (%)	Name	Function
CALL6_HUMAN	181	6.08	Calmodulin-like protein 6	
IRGM_HUMAN	181	6.08	Immunity-related GTPase family M protein	
CSTF2_HUMAN	577	6.07	Cleavage stimulation factor subunit 2	NAB
ELOV6_HUMAN	265	6.04	Elongation of very long chain fatty acids protein 6	
SNRPA_HUMAN	282	6.03	U1 small nuclear ribonucleoprotein A	NAB
FATE1_HUMAN	183	6.01	Fetal and adult testis-expressed transcript protein	

Of 19,806 annotated human proteins, those with methionine contents of 6 % or more were assembled, without considering short proteins of <150 amino acids to avoid potential artifacts. This procedure led to the assembly of 54 proteins. The 6 % cutoff value corresponds to approximately 3.5 standard deviations in the methionine distribution. Respiratory chain complex proteins (RCC) and single-stranded nucleic acid binding proteins (NAB) are indicated

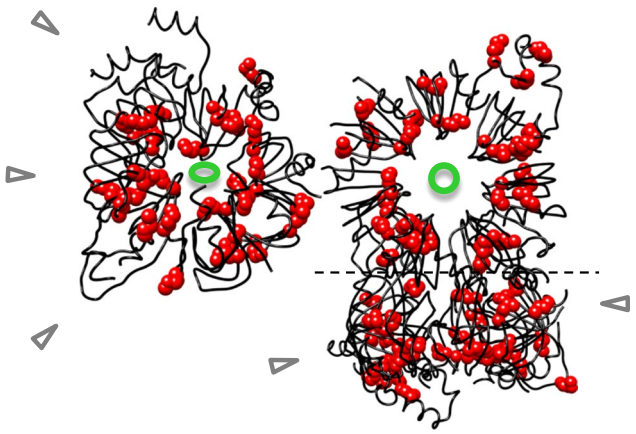

Fig. 2 Methionine distribution in the spliceosomal core domain. The ring-shaped core domain of the U4 snRNP particle (seen from three angles as in the original crystal structure) consists of seven high-methionine Sm proteins assembled around the central, single-stranded U4 RNA (omitted for clarity). The outer surfaces of these heptameric rings are largely devoid of methionine (*arrowheads*), whereas the surfaces facing the central U4 RNA passage (*green ovals*) are covered with methionine. To the *lower right*, one ring structure is seen in lateral view (demarcated by a *dashed line*). The displayed structure has a methionine content of 5.3 %

accumulation was related to a *trans*-action pertaining to the whole inner mitochondrial membrane, proteins with high expression levels would be expected to show higher levels of accumulation, because each mutation in the mitochondrial DNA towards an additional methionine codon would translate into an approximately sixfold higher methionine gain in the inner mitochondrial membrane if it happened in a complex IV gene rather than in a complex I gene. The fact that the opposite is the case (Fig. 3) clearly indicates that the precise site of methionine accumulation strongly matters. In other words, a (surface) complex IV methionine cannot substitute a (surface) complex I methionine, which denotes that the range of action of a complex I methionine is smaller than the distance between the two complexes [2–5 nm in bovine supercomplexes (Althoff et al. 2011)], and thus smaller than the diffusion path length of the majority of oxidants (Pryor 1986; Schindeldecker et al. 2011). Hence, the antioxidant actions of methionine residues are related to their direct vicinity and do not extend to distances of several nanometers or more. Therefore, the structure to be protected by methionine incorporation is the protein itself or one of its immediate binding partners. This conclusion stands to reason as complex I is also the probably most prominent source of superoxide in mitochondria (St-Pierre et al. 2002; Brand 2010) and thus inevitably exposed to significant radical fluxes. Moreover, it validates studies that have investigated the redox effects of protein methionine using isolated proteins in test tubes (Reddy et al. 1994; Levine et al. 1996; Kim et al. 2014). Necessarily, only *cis*-acting protective functions can be identified in such approaches. Finally, it supports the postulated role of this amino acid in the methionine-loaded nucleic acid-binding proteins (Fig. 2).

Regarding the apparently very specific demand of respiratory chain proteins for local antioxidant protection, our results are in agreement with calculations that have demonstrated a rapid decrease of oxidant flux with increasing distance from the inner mitochondrial membrane (Kirkwood and Kowald 2012). In fact, manganese superoxide dismutase (MnSOD), the primary and essential SOD of mitochondria, has been reported to be directly attached to mitochondrial respiratory chain supercomplex I:III:IV, to locally protect these membrane proteins from superoxide toxicity (Suthammarak et al. 2013). Thus, the unprecedentedly high methionine contents of complex I in certain insects (Fig. 3) are not only surprising, but also consistent with other formerly unexpected mechanisms of respiratory chain autoprotection.

Mitochondrial methionine usage in ectotherms mirrors aerobic metabolic rate

The interphyletic comparison of methionine usage in the mitochondrially encoded subunits of complex I indicates

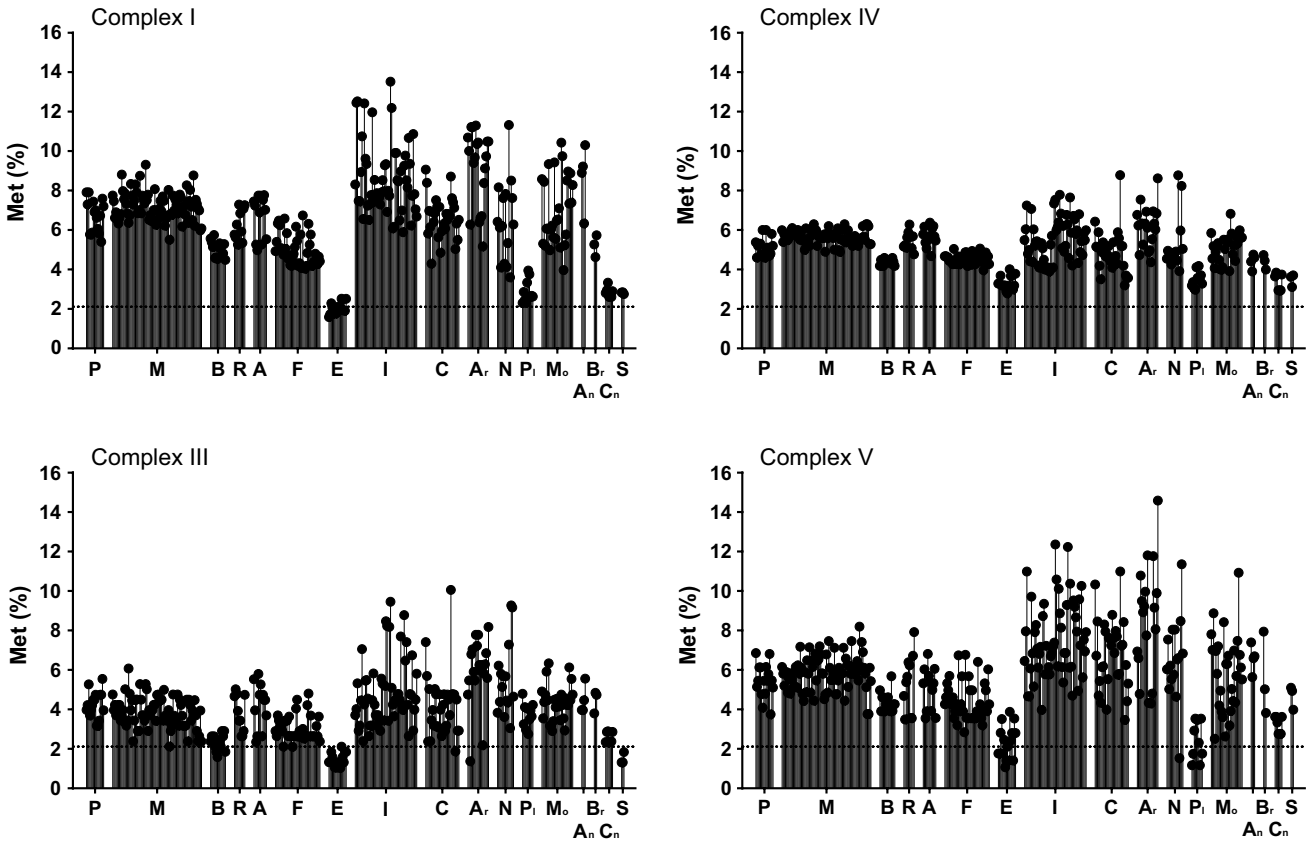

Fig. 3 Methionine contents of mitochondrially encoded respiratory chain proteins in 361 animal species. The abbreviations signify phylogenetic clades as follows: *P* primates, *M* other mammals, *B* birds, *R* reptiles, *A* amphibians, *F* fish, *E* echinoderms, *I* insects, *C* crustaceans, *Ar* arachnids, *N* nematodes, *Pl* platyhelminthes, *Mo* molluscs, *An* annelids, *Br* brachiopods, *Cn* cnidarians, *S* sponges. The *dotted reference line* indicates the average methionine content of nuclear encoded proteins in humans

a very variable content of this amino acid, ranging from <2 % in many echinoderms to more than 13 % in some insects (Fig. 3). Otherwise, complex I is known to show a relatively high level of sequence conservation (Baradaran et al. 2013). To explore this apparent discrepancy, a number of characteristic features related to aerobic metabolism and oxygen toxicity were assembled for the potentially most revealing phylogenetic groups (Table 3). Methionine contents in complex I of 12 % or more were exclusively reached by two families of insects, bees (Apidae) and whiteflies (Aleyrodidae). Remarkably, both families have been highlighted in the zoological literature for their exceptionally high metabolic rates of up to ~100 mW/g in connection with their flying lifestyle (Wolf et al. 1996; Salvucci and Crafts-Brandner 2000; Darveau et al. 2005). This value is approximately 100-fold higher than what would be expected for a typical resting invertebrate, and it also exceeds the values reported for most other flying insects, or for birds, bats, and other endothermic vertebrates (Harrison and Roberts 2000). Beyond these extremes, high methionine contents are generally realized in insects and mammals (Fig. 3; Table 3). Concomitantly, both groups exhibit relatively high aerobic metabolic rates (Harrison and Roberts 2000; Makarieva et al. 2008), which is reflected by their possession of remarkably specialized respiratory systems (Table 3). In connection, their high absolute rates of reactive oxygen species production compared to marine invertebrates have been noted (Abele and Puntarulo 2004). In sharp contrast, the four animal phyla using only a single codon in mitochondria to encode methionine (Bender et al. 2008) were not only characterized by conspicuously lower methionine contents in the encoded proteins, but were also found to consistently encompass species recognized for their low metabolic rates, as exemplified by minimal motor activity, slow growth, high anoxia tolerance or, in case of the analyzed platyhelminthes, extensive anaerobiosis in relation to a parasitic lifestyle (Tielens 1994; Halton 1997; Moosmann and Behl 2008). Concordantly, these phyla also share an insufficiently evolved or even absent anatomical and biochemical endowment for high oxygen consumption (Table 3). Comparing the subsuming metabolic classification of the different groups in Table 3 with

Table 3 Aerobic metabolic rate, metabolic rate markers, and methionine usage in the mitochondrially encoded subunits of complex I

Clade	Met in complex I (%)	Met codons	Aerobic metabolic rate	Specialized respiratory system	Respiratory pigment	Comments and references
Sponges	2.81	1	Very low	No	No	a
Cnidarians	2.83	1	Very low	No	No	b
Platyhelminthes	2.88	1	Low	No	Partly (not circulating)	c
Echinoderms	2.03	1	Very low	Water-vascular system	Partly	d
Insects	8.57	2	High	Yes	Partly	e
Bees (apidae)	12.13	2	Very high	Yes	Yes (not circulating)	f
Whiteflies (aleyrodidae)	11.85	2	Very high	Yes	Yes (not circulating)	g
Mammals	7.11	2	High	Yes	Yes	h

[a] Generally low metabolic rates in both tropical and Antarctic habitats (Gatti et al. 2002; Hadas et al. 2008)

[b] Very low metabolic rates and pronounced hypoxia tolerance (Childress and Seibel 1998). Different examples exhibit ~10× lower O_2 consumption than crustaceans from the same habitat irrespectively of O_2 availability (Thuesen and Childress 1994)

[c] All examples analyzed for this table pursue a parasitic lifestyle as adults, which is generally believed to involve anaerobiosis irrespectively of O_2 availability (Tielens 1994; Halton 1997)

[d] Very low metabolic rates. Examples exhibit ~8× lower O_2 consumption than "standard" invertebrates of the same size (Takemae et al. 2009)

[e] Generally high metabolic rates as compared to similarly sized aquatic invertebrates or ectothermic vertebrates (Lighton 1996; Makarieva et al. 2008)

[f] Exceptionally high metabolic rates. Different examples exhibit ~30× higher metabolic rates than "running insects" of the same size (Wolf et al. 1996; Harrison and Roberts 2000)

[g] Very high metabolic rates. Examples exhibit ~10× higher metabolic rates than ants of the same body mass (Salvucci and Crafts-Brandner 2000)

[h] Generally high metabolic rates, commonly much higher than in ectothermic species (Makarieva et al. 2008), yet comparable to those of flying insects (Harrison and Roberts 2000)

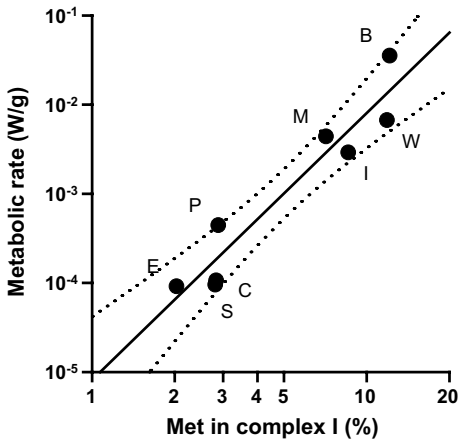

Fig. 4 Correlation of complex I methionine content with mass-specific metabolic rate. Average values for the clades defined in Table 3 were plotted in double-logarithmic form. The abbreviations denote: *S* sponges, *C* cnidarians, *P* platyhelminthes, *E* echinoderms, *I* insects, *B* bees, *W* whiteflies, *M* mammals; the *dotted lines* indicate the 95 % confidence interval. Methionine contents represent arithmetic means as shown in Table 3, metabolic rate averages were geometric means of log-transformed data, to comply with a relevant literature source of metabolic rates (Makarieva et al. 2008). In control calculations, arithmetic means gave very similar results (data not shown). Platyhelminth metabolic rates were primarily from freshwater species, which might have a higher metabolic rate than the anaerobic–parasitic animals on which the calculated methionine average is based. Bee and whitefly metabolic rates were determined during flight; activity of the other animals during the measurements varied or was not reported. Data sources are given in the "Materials and methods"

the listed traditional predictors of metabolic rate, complex I methionine content seems to be of at least the same predictive power as the traditional markers, respiratory pigment and oxygen-transporting tubular system. This conclusion is supported by the quantitative correlation of metabolic rates and complex I methionine contents depicted in Fig. 4, which was highly significant (rank order correlation coefficient: $r = 0.98$; $p = 2 \times 10^{-7}$; $n = 8$) across the investigated clades.

To analyze the biochemical deployment of methionine in the respiratory chain in more detail, complex I structural models of consensus representatives of the eight phylogenetic groups highlighted in Table 3 were calculated. The resulting structures reveal a very unusual accumulation of this amino acid in animals with high metabolic rate throughout the protein and, in particular, on its surfaces (Fig. 5). Quantitative analysis of the displayed structures (Table 4) confirmed that in high-methionine proteins, methionine was not only more frequent, but also more surface-exposed (rank order correlation coefficient: $r = 0.79$; $p = 0.02$; $n = 8$). In the strongest accumulator, the whitefly *Neomaskellia andropogonis*, methionine occupied more than 14 % of the protein surface, which comes close to the result for leucine, the otherwise by far most frequent amino acid in transmembrane domains [~20 % in humans, versus ~3 % for methionine (Table 1)]. Thus, in ectothermic animals, there seems to be a close relationship between

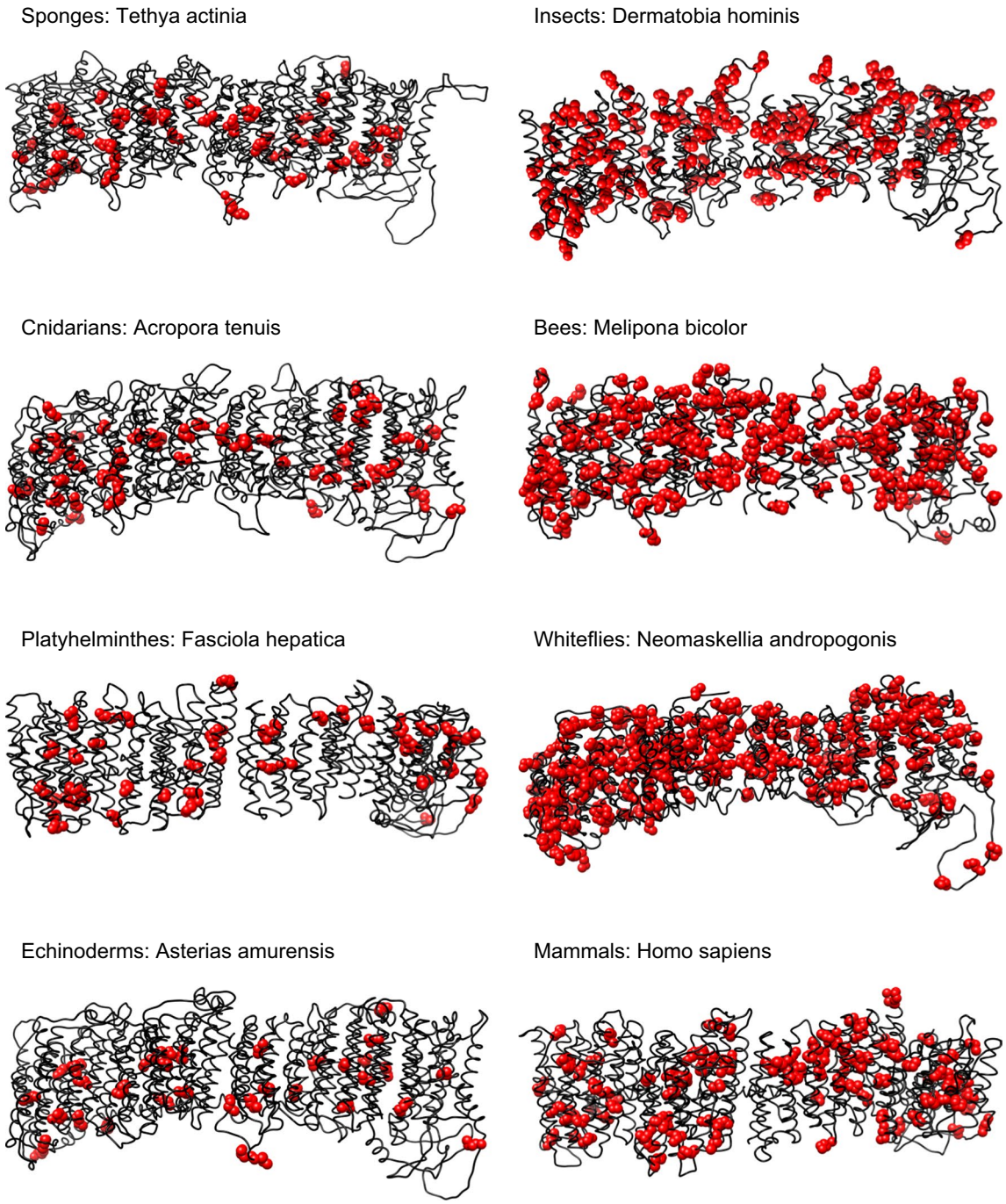

Fig. 5 Structural view of eight modelled complex I transmembrane domains. Models were generated by aligning all mitochondrially encoded sequences of complex I from eight representative animal species, with the homologous sequences from *Thermus thermophilus*, whose complex I crystal structure was used as modelling template. Methionine residues are shown in *space-filling* representation. A quantitative analysis of the displayed structures is given in Table 4

increased methionine usage in complex I and increased usage of oxygen for respiratory purposes, with the ultimately inevitable consequence of reactive oxygen species formation (Boveris and Chance 1973; St-Pierre et al. 2002; Brand 2010).

Conclusion

The oxidant-sensitive amino acid methionine (Brot and Weissbach 1983) exhibits a very unusual distributional pattern in proteins: contrary to chemical intuition, it is

Table 4 Methionine frequency and surface exposure in structural models of the complex I transmembrane domain

Species	Met number	Average exposure per Met (Å^2)	Total Met surface area (Å^2)	Total protein surface area (Å^2)	Met surface fraction (%)	Leu surface fraction (%)
Sponges: *Tethya actinia*	56	15.48	867	35,980	2.4	17.0
Cnidarians: *Acropora tenuis*	56	17.22	964	35,381	2.7	17.8
Platyhelminthes: *Fasciola hepatica*	43	17.91	770	27,436	2.8	18.1
Echinoderms: *Asterias amurensis*	35	14.22	498	33,184	1.5	19.7
Insects: *Dermatobia hominis*	140	18.45	2583	30,633	8.4	19.2
Bees: *Melipona bicolor*	214	18.17	3888	29,749	13.1	15.2
Whiteflies: *Neomaskellia andropogonis*	239	19.04	4551	32,329	14.1	14.6
Mammals: *Homo sapiens*	103	16.43	1692	30,728	5.5	22.8

The indicated numbers were derived from the structural models shown in Fig. 4. With the exception of *Homo sapiens*, all species were chosen for their representativeness of their phylogenetic clade regarding complex I methionine usage as listed in Table 3. The calculated surface fraction of leucine, the universally most frequent amino acid in animals, is shown for comparison

increasingly used in the proximity to sites of oxidant generation. This pattern is found in intracellular as well as interspecies comparisons, indicating that local redox demands exert significant positive control over methionine usage. High aerobic metabolic rate, which is generally associated with high levels of oxidants (Boveris and Chance 1973; Abele and Puntarulo 2004; Hoffman and Brookes 2009), appears to be a primary demanding factor for high methionine contents in the respiratory chain. This behavior of methionine is in diametrical opposition to the behavior of cysteine, which is sometimes equated with methionine for the apparent overlap in many of their reaction pathways and regulatory functions (Kim et al. 2014). However, cysteine usage in proteins is globally disfavored whenever there is an increased chance of uncontrolled oxidation, as in aerobic versus anaerobic bacteria, archaea, protists, and helminthes, or as seen in various intracellular comparisons (Moosmann and Behl 2008; Vieira-Silva and Rocha 2008; Schindeldecker et al. 2011). The current findings on methionine are intriguing as they describe a systematic biological accumulation of an exceedingly oxidant-labile structural building block in high-oxidant microenvironments, which marks methionine as a compelling biological embodiment of a cathodic protection device so often encountered in engineering.

Conflict of interest The authors declare that they have no conflict of interest.

References

Abele D, Puntarulo S (2004) Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. Comp Biochem Physiol A Mol Integr Physiol 138:405–415

Althoff T, Mills DJ, Popot JL, Kühlbrandt W (2011) Arrangement of electron transport chain components in bovine mitochondrial supercomplex $I_1III_2IV_1$. EMBO J 30:4652–4664

Ashton NW, Bolderson E, Cubeddu L, O'Byrne KJ, Richard DJ (2013) Human single-stranded DNA binding proteins are essential for maintaining genomic stability. BMC Mol Biol 14:9

Baradaran R, Berrisford JM, Minhas GS, Sazanov LA (2013) Crystal structure of the entire respiratory complex I. Nature 494:443–448

Bender A, Hajieva P, Moosmann B (2008) Adaptive antioxidant methionine accumulation in respiratory chain complexes explains the use of a deviant genetic code in mitochondria. Proc Natl Acad Sci USA 105:16496–16501

Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Cassarino TG, Bertoni M, Bordoli L, Schwede T (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Res 42:W252–W258

Bisby RH, Ahmed S, Cundall RB (1984) Repair of amino acid radicals by a vitamin E analogue. Biochem Biophys Res Commun 119:245–251

Boveris A, Chance B (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem J 134:707–716

Brand MD (2010) The sites and topology of mitochondrial superoxide production. Exp Gerontol 45:466–472

Brot N, Weissbach H (1983) Biochemistry and physiological role of methionine sulfoxide residues in proteins. Arch Biochem Biophys 223:271–281

Childress JJ, Seibel BA (1998) Life at stable low oxygen levels: adaptations of animals to oceanic oxygen minimum layers. J Exp Biol 201:1223–1232

Darveau CA, Hochachka PW, Welch KC, Roubik DW, Suarez RK (2005) Allometric scaling of flight energetics in Panamanian orchid bees: a comparative phylogenetic approach. J Exp Biol 208:3581–3591

Gatti S, Brey T, Müller WE, Heilmayer O, Holst G (2002) Oxygen microoptodes: a new tool for oxygen measurements in aquatic animal ecology. Mar Biol 140:1075–1085

Gowda K, Black SD, Moeller I, Sakakibara Y, Liu MC, Zwieb C (1998) Protein SRP54 of human signal recognition particle: cloning expression, and comparative analysis of functional sites. Gene 207:197–207

Hadas E, Ilan M, Shpigel M (2008) Oxygen consumption by a coral reef sponge. J Exp Biol 211:2185–2190

Hajieva P, Bayatti N, Granold M, Behl C, Moosmann B (2015) Membrane protein oxidation determines neuronal degeneration. J Neurochem (in press)

Halliwell B (1990) How to characterize a biological antioxidant. Free Radic Res Commun 9:1–32

Halton DW (1997) Nutritional adaptations to parasitism within the platyhelminthes. Int J Parasitol 27:693–704

Harrison JF, Roberts SP (2000) Flight respiration and energetics. Annu Rev Physiol 62:179–205

Hoffman DL, Brookes PS (2009) Oxygen sensitivity of mitochondrial reactive oxygen species generation depends on metabolic conditions. J Biol Chem 284:16236–16245

Johansen ME, Muller JG, Xu X, Burrows CJ (2005) Oxidatively induced DNA-protein cross-linking between single-stranded binding protein and oligodeoxynucleotides containing 8-oxo-7,8-dihydro-2′-deoxyguanosine. Biochemistry 44:5660–5671

Jovanovic SV, Simic MG (1985) Repair of tryptophan radicals by antioxidants. J Free Radic Biol Med 1:125–129

Kim G, Weiss SJ, Levine RL (2014) Methionine oxidation and reduction in proteins. Biochim Biophys Acta 1840:901–905

Kirkwood TB, Kowald A (2012) The free-radical theory of ageing—older, wiser and still alive: modelling positional effects of the primary targets of ROS reveals new support. BioEssays 34:692–700

Krogh A, Larsson B, von Heijne G, Sonnhammer EL (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol 305:567–580

Lanfear R, Thomas JA, Welch JJ, Brey T, Bromham L (2007) Metabolic rate does not calibrate the molecular clock. Proc Natl Acad Sci USA 104:15388–15393

Lenaz G, Genova ML (2009) Structural and functional organization of the mitochondrial respiratory chain: a dynamic super-assembly. Int J Biochem Cell Biol 41:1750–1772

Leung AK, Nagai K, Li J (2011) Structure of the spliceosomal U4 snRNP core domain and its implication for snRNP biogenesis. Nature 473:536–539

Levine RL, Mosoni L, Berlett BS, Stadtman ER (1996) Methionine residues as endogenous antioxidants in proteins. Proc Natl Acad Sci USA 93:15036–15040

Lighton JR (1996) Discontinuous gas exchange in insects. Annu Rev Entomol 41:309–324

Luo S, Levine RL (2009) Methionine in proteins defends against oxidative stress. FASEB J 23:464–472

Makarieva AM, Gorshkov VG, Li BL, Chown SL, Reich PB, Gavrilov VM (2008) Mean mass-specific metabolic rates are strikingly similar across life's major domains: evidence for life's metabolic optimum. Proc Natl Acad Sci USA 105:16994–16999

Moosmann B, Behl C (2000) Cytoprotective antioxidant function of tyrosine and tryptophan residues in transmembrane proteins. Eur J Biochem 267:5687–5692

Moosmann B, Behl C (2002) Secretory peptide hormones are biochemical antioxidants: structure-activity relationship. Mol Pharmacol 61:260–268

Moosmann B, Behl C (2008) Mitochondrially encoded cysteine predicts animal lifespan. Aging Cell 7:32–46

Mouton S, Willems M, Houthoofd W, Bert W, Braeckman BP (2011) Lack of metabolic ageing in the long-lived flatworm *Schmidtea polychroa*. Exp Gerontol 46:755–761

Oien DB, Moskovitz J (2008) Substrates of the methionine sulfoxide reductase system and their physiological relevance. Curr Top Dev Biol 80:93–133

Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE (2004) UCSF Chimera—a visualization system for exploratory research and analysis. J Comput Chem 25:1605–1612

Price JC, Guan S, Burlingame A, Prusiner SB, Ghaemmaghami S (2010) Analysis of proteome dynamics in the mouse brain. Proc Natl Acad Sci USA 107:14508–14513

Pryor WA (1986) Oxy-radicals and related species: their formation, lifetimes, and reactions. Annu Rev Physiol 48:657–667

Reddy VY, Desorchers PE, Pizzo SV, Gonias SL, Sahakian JA, Levine RL, Weiss SJ (1994) Oxidative dissociation of human alpha 2-macroglobulin tetramers into dysfunctional dimers. J Biol Chem 269:4683–4691

Requejo R, Hurd TR, Costa NJ, Murphy MP (2010) Cysteine residues exposed on protein surfaces are the dominant intramitochondrial thiol and may protect against oxidative damage. FEBS J 277:1465–1480

Salvucci ME, Crafts-Brandner SJ (2000) Effects of temperature and dietary sucrose concentration on respiration in the silverleaf whitefly, *Bemisia argentifolii*. J Insect Physiol 46:1461–1467

Schindeldecker M, Stark M, Behl C, Moosmann B (2011) Differential cysteine depletion in respiratory chain complexes enables the distinction of longevity from aerobicity. Mech Ageing Dev 132:171–179

Scruggs BS, Michel CI, Ory DS, Schaffer JE (2012) SmD3 regulates intronic noncoding RNA biogenesis. Mol Cell Biol 32:4092–4103

Stajich JE, Block D, Boulez K, Brenner SE, Chervitz SA, Dagdigian C, Fuellen G, Gilbert JG, Korf I, Lapp H, Lehvaslaiho H, Matsalla C, Mungall CJ, Osborne BI, Pocock MR, Schattner P, Senger M, Stein LD, Stupka E, Wilkinson MD, Birney E (2002) The Bioperl toolkit: Perl modules for the life sciences. Genome Res 12:1611–1618

St-Pierre J, Buckingham JA, Roebuck SJ, Brand MD (2002) Topology of superoxide production from different sites in the mitochondrial electron transport chain. J Biol Chem 277:44784–44790

Suthammarak W, Somerlot BH, Opheim E, Sedensky M, Morgan PG (2013) Novel interactions between mitochondrial superoxide dismutases and the electron transport chain. Aging Cell 12:1132–1140

Takemae N, Nakaya F, Motokawa T (2009) Low oxygen consumption and high body content of catch connective tissue contribute to low metabolic rate of sea cucumbers. Biol Bull 216:45–54

Thuesen EV, Childress JJ (1994) Oxygen consumption rates and metabolic enzyme activities of oceanic California medusae in relation to body size and habitat depth. Biol Bull 187:84–98

Tielens AG (1994) Energy generation in parasitic helminthes. Parasitol Today 10:346–352

Vieira-Silva S, Rocha EP (2008) An assessment of the impacts of molecular oxygen on the evolution of proteomes. Mol Biol Evol 25:1931–1942

Von Brand T, Bowman IB (1961) Studies on the aerobic and anerobic metabolism of larval and adult *Taenia taeniaeformis*. Exp Parasitol 11:276–297

Vriend G (1990) WHAT IF: a molecular modeling and drug design program. J Mol Graph 8:52–56

Weissbach H, Resnick L, Brot N (2005) Methionine sulfoxide reductases: history and cellular role in protecting against oxidative damage. Biochim Biophys Acta 1703:203–212

Wolf T, Ellington C, Davis S, Feltham M (1996) Validation of the doubly labelled water technique for bumblebees *Bombus terrestris* (L.). J Exp Biol 199:959–972

Yong J, Wan L, Dreyfuss G (2004) Why do cells need an assembly machine for RNA-protein complexes? Trends Cell Biol 14:226–232