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Review

VDAC isoforms in mammals ☆

Angela Messina, Simona Reina, Francesca Guarino, Vito De Pinto *

Department of Biological, Geological and Environmental Sciences, Section of Molecular Biology, University of Catania, Italy
National Institute for Biomembranes and Biosystems, Section of Catania, viale A. Doria 6, 95125 Catania, Italy

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ABSTRACT

VDACs (Voltage Dependent Anion selective Channels) are a family of pore-forming proteins discovered in the mitochondrial outer membrane. In the animal kingdom, mammals show a conserved genetic organization of the VDAC genes, corresponding to a group of three active genes. Three VDAC protein isoforms thus exist. From a historically point of view most of the data collected about this protein refer to the VDAC1 isoform, the first to be identified and also the most abundant in the organisms. In this work we compare the information available about the three VDAC isoforms, with a special emphasis upon the human proteins, here considered prototypical of the group, and we try to shed some light on specific functional roles of this apparently redundant group of proteins. A new hypothesis about the VDAC(s) involvement in ROS control is proposed. This article is part of a Special Issue entitled: VDAC structure, function, and regulation of mitochondrial metabolism.

Contents

1. Introduction

VDACs (Voltage Dependent Anion selective Channels), also known as mitochondrial porins, have been discovered in the outer mitochondrial membrane about 40 years ago, when Schein et al, detected a pore-forming activity in an extract of *Paramecium tetraurelia* mitochondria [1]. In the next years a large wealth of data from practically all the most studied living beings has come out about this surprising protein, whose relevance in the cell has not yet been outlined enough. A unifying view about VDAC has sprung out from these studies. VDAC is a protein that bears extremely conserved structural and functional features (despite major difference in the sequence) and this is a clue of its important role [2,3].

A further intriguing observation is now coming in strict actuality. In the cell a set of VDAC proteins has evolved, as has been discovered

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* Corresponding author at: Department of Biological, Geological and Environmental Sciences, Section of Molecular Biology, University of Catania, Building 2, 3th floor, Campus S.Sofia, viale A. Doria 6, 95125 Catania, Italy. Tel.: +39 0957384244; fax: +39 095580138.
E-mail address: vdpbiofa@unict.it (V. De Pinto).
URL: http://www.unictbiolmol-lab.it (V. De Pinto).

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already since 1993 by the genetic screening of a cDNA library [4] after the first primary amino acid sequence was known by direct sequencing of human VDAC1 [5]. These further molecules have been simply numbered in the order of their discovery as VDAC2, VDAC3, VDACn ...., leaving to the firstly studied isoform the acronym VDAC1. The important point is that most information available on VDAC came from the isoform VDAC1, the most abundant, or from mixtures of more isoforms, as it has been proposed in [6,7], that were not resolved at the time of their studies. This point thus poses an urgent and demanding question to the audience of scientists involved in VDAC and in those willing to know the real function of these protein(s): how many of the information present in the literature are *really* due to VDAC1 and what is the influence of the other isoforms in the functions generically attributed to VDAC? In other words, why are there in the cell more VDAC isoforms? In this work we try to shed some light about this underestimated but important topic of the research about VDAC (see also [8,9]).

An additional caveat has to be added at this point: despite most of the studied organisms show a variety of VDAC genes and isoforms, and all of them have been named in their order of appearance in the scientific arena, one has to be careful about their correspondence since they evolved differently. For example the plant isoforms VDAC1, 2 and 3 do not correspond at all to the same names in mammals or insects. This review is thus limited to the eutherians, i.e. the placental mammals, since all these animals have fixed in their genomes the same number of functional VDAC genes that share the same genetic origin. Furthermore human VDACs are member of this group.

2. VDAC isoforms in mammals

The evolutionary history of VDAC isoforms has been extensively analysed. The most recent work by Young et al.[10] used a combination of Neighbour-Joining and Bayesian methods upon the largest set of sequences available at the moment. The rise of the three isoforms in the mammals was next to the separation between animals and fungi VDACs, that derive from a common ancestor.

In mammals (but also in most chordates) there are three clades corresponding to VDAC1, 2 and 3 isoforms. The assumption that VDAC3 is the oldest protein is generally accepted. The divergence between VDAC3 and VDAC1/2 has been estimated 365 ± 60 MY ago, while the divergence between VDAC1 and VDAC2 289 ± 63 MY [10,11]. VDAC1 can be considered the most recent mitochondrial porin.

It has been proposed that the duplications that gave rise to the multigene family of VDACs probably occurred independently in animals and plants after the divergence between the two kingdoms [12,13] and this conclusion explains the unrelatedness between the numbering of plant and animal isoforms.

The structure of VDAC eutherian genes is very similar. This concept is outlined in Fig. 1 where the coding structure of the human VDAC genes is reported. The genes are made of the same number of coding exons, sharing exactly the same size, with the VDAC2 gene containing an additional first exon encoding for the short pre-sequence of 11 amino acids, a peculiar feature of this isoform. The intron size varies from gene to gene but the striking point is the similarity among the exon organization that is conserved among the whole eutherian group. VDAC paralogous genes evolved quite recently and conserved similar structures for the coding region and even for intron/exon junctions [14–16]. As it was acutely observed by Young [10], the exon-intron boundary conservation means that there is intron phase correlation: this phenomenon is viewed as evidence of non-random insertion of introns [17]. Interestingly, these E–I boundaries, according to the recently proposed 19 β-strands structure of the pore [18–20], localize individual structural units corresponding to the N-terminal end and to distinct blocks of β-strands (see Fig. 7 in [8]). The introns usually do not interrupt secondary structure elements; even structural protein motifs, like the GLK motif and the "eukaryotic porin signature motif" (Prosite PS00558) are not interrupted. This observation support the exon-shuffling theory about the origin of VDAC genes from simpler units containing elementary building blocks (this hypothesis has been recently investigated with the construction of artificial pores [21]).

Trascription of the VDAC isoform genes was studied with various techniques. Northern blot and/or PCR analysis showed that the three mammalian VDAC isoforms are expressed more or less ubiquitously [4,14,22–24]. In liver the amount of VDAC transcripts is usually lower than in the other tissues. VDAC2 and expecially VDAC3 are highly expressed in testis [23,25], while mouse VDAC1 is poorly expressed in this tissue [25]. In a recent study, Real Time PCR analysis of the three isoforms in mRNA from HeLa cells showed that the VDAC1 mRNA is the most abundant and overcomes VDAC2 of one order of magnitude and VDAC3 of two o. of m. [9]. The RT-PCR dosage of transcripts after over-expression of each single isoforms showed that VDAC2 and VDAC3 mRNA levels are influenced by the other isoforms, indicating the probable presence of coordination in the expression of VDAC gene isoforms [9].

The data reported above come from teams actively involved in VDAC. If one, instead, browses the results of high throughput genome expression analysis, he will find that the picture is much more complicated and maybe confusing. For example, in the NCBI, at the address http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/index.html, the search for VDAC genes in human produces a very large amount of data. The three genes are expressed at high level, in particular VDAC1, 5.3 times the average gene in the present release of the Database. Some hundreds of GenBank accessions for each gene derived from sequenced cDNA were obtained by several tissues. Not unexpectedly, such kind of results outlines the possible presence of more exons and introns than those shown above for the canonical VDAC sequences. The potential proteins produced by these cDNA is rather high and it is useless to enumerate them until there is no indication of their presence in the cell. Two conclusions can be drawn from these information: the VDAC3 is the simplest and smallest gene among the three; alternative splicing is a reportedly common trait also for VDAC genes [6,8,26] but its functional meaning is not known [27].

2.1. VDAC isoforms distribution in mitochondria

What is the distribution of VDAC isoforms in the mitochondrial network? In a recent paper, the adoption of STED (two colors

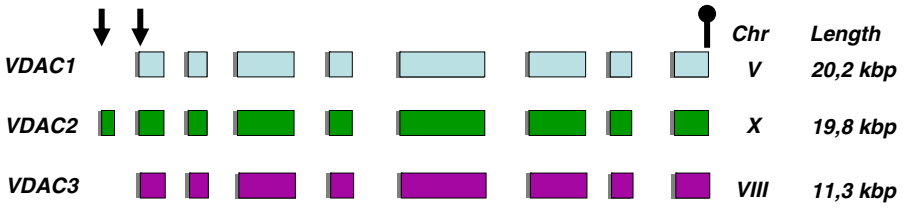

Fig. 1. Structure of genes' moieties coding for VDAC isoforms in humans. The exons coding for the proteins are shown. Their size is in scale but neither the introns nor the exons coding for untranslated transcripts are shown. The arrows show the ATG positions. The ball and stick show the Stop codon position. The chromosome (Chr) localization and the length of the gene sequences encompassed between the Start and Stop codons are reported.

stimulated emission depletion) microscopy, that enables a lateral resolution of ~40 nm, was useful to determine the detailed pattern of sub-mitochondrial distribution of VDAC isoforms [28]. While VDAC1 and VDAC2 have a common pattern of distribution, with clusters of higher abundance gapped by area relatively less populated, VDAC3 is more homogeneously distributed along the network. This is an indication of different utilization of the three isoforms.

3. VDAC isoforms: purification and structure

3.1. VDAC protein purification

Purification of VDAC from mammals and other species has been accomplished since long time and established protocols exist [29,8]. In this context, we will emphasize deductions into the cellular properties of this protein, obtained from the purification procedure. Furthermore we will update the state of art about purification of the mammalian VDAC isoforms from tissues.

The basic, but fundamental, steps for the VDAC purification are three: the detergent used, the adoption of hydroxyapatite (HTP) together with celite as the chromatographic material and the concentration of membrane phospholipids in the extract, that is empirically dependent on the ratio between the detergent and the mitochondrial membrane material during the solubilization process: i.e. the most diluted is the membrane protein to be solubilized, the lowest phospholipid concentration is obtained in the detergent extract.

The combination of these three elements gives the extraordinary result of the purification of VDAC in a single chromatographic step, with the VDAC being eluted in the not-retained or pass-through material [29]. This essential strategy was applied to any organism or tissues (Fig. 2) and it is also the basis in more complex strategies, in case of specific contaminants [8].

What does this surprising observation tell us about VDAC in the natural bilayer membrane?

In the mixture of the several integral membrane protein and phospholipids obtained during the solubilization, the hydrophobic proteins are engulfed in micelles and only protruding, hydrophilic moieties can interact with the stationary phase of chromatographic materials. If the stationary phase is only HTP, a calcium phosphate crystal, the addition to the eluent buffer of salts or, better, of phosphate, improves the elution of adsorbed proteins. On the other side, the inclusion of celite in the chromatographic material enhances the adsorption of proteins because the contribution of van der Waals forces is raised. This chromatography is thus able to adsorb all the mitochondrial membrane proteins solubilized by, for example, Triton X-100, but VDAC. This indicates that in this situation VDAC in the micelle does not expose any proteic part able to interact with the stationary phase and thus is the most membrane-embedded protein among the mitochondrial proteins. This conclusion is supported by the results obtained with different detergents. Additional moieties of membrane proteins in micelles are exposed when the hydrophilic arm of the detergent used for the solubilization is shortened. For example, when, in the same conditions, LDAO (lauryl-(dimethyl)amine-oxide) is used instead of Triton X-100, no protein at all is found in the HTP/celite pass-through and also VDAC is retained [30]. High concentration of phospholipids in the solubilized material shield the protruding parts of other membrane proteins and cause their elution in the HTP or HTP/celite pass-through. This property has been experimentally demonstrated with the purification of various metabolite carriers, whose elution is favoured by the addition in the elution buffer of externally added phospholipids, including cardiolipin [31,32].

Two questions arise at this point: is this purification procedure able also to rid the other isoforms of and, if so, how reliable are the data reported in the literature that usually refers to VDAC1?

The latter point is concerned with the electrophoretic mobility of VDAC1, lower than expected [6,32]. VDAC2, despite the higher molecular weight, is mobile as VDAC1. VDAC3 is the faster isoform [6,7]. The presence of more isoforms in HTP/celite eluates has been also shown by Menzel et al.[7]. However, they used as starting material not purified mitochondria but whole bovine spermatozoa. In their paper, the eluate was further separated by 2D-electrophoresis (Fig. 2), showing a plurality of spots that were identified as the various isoforms by Mass Spectrometry [7]. HTP/celite chromatography can be succesfully applied to all the isoforms, indicating that they have similar chemico-physical features in the context of a partitioned hydrophobic/hydrophilic environment.

The information reported by several laboratories, in the last two decades, is attributed to VDAC1. Have been these observations biased by the contamination with other isoforms? To rule out any contamination of purified proteins by others, with similar features, one should adopt either a specific labelling, to track the protein of interest, or a more powerfull analytical method, like 2-D electrophoresis. In one of the oldest work about VDAC1, this protein was identified and purified as the DCCD-binding protein [33]. DCCD (dicyclohexylcarbodiimide) binds acidic groups in an hydrophobic environment. The DCCD binding residue was found to be the glutamic acid in position 72 in mammalian VDAC1 by direct sequencing of the corresponding peptide [34]. This glutamic acid is present only in the sequences of VDAC1 and 2 (Fig. 3). The Edman degradation of the corresponding peptide should have had revealed the presence of VDAC2 contamination but it did not, indicating that the protein purified in that experiment was only VDAC1. Also the few reports showing 2-D analysis of purified VDAC1 did not show the presence of alternative spots later found to be VDAC2 or VDAC3 [35,36].

These data indicate that the protein purified and analyzed in the literature was essentially the same orthologus isoform, that has been later called VDAC1. It is in fact correct to correlate most functional information available to this protein isoform.

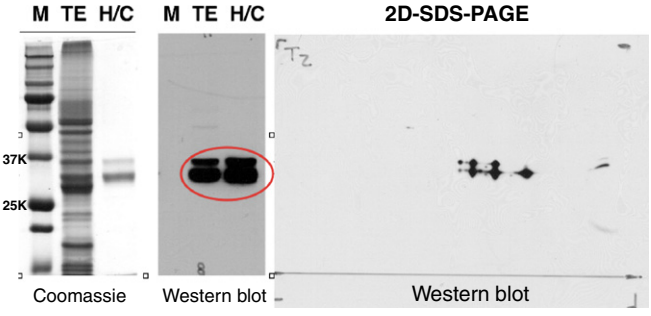

Fig. 2. Purification and analysis of VDAC2 from spermatozoa. From left to right the panels refer to the HTP/celite eluate of Triton X-100 solubilized bovine spermatozoa analysed by one-dimension SDS-PAGE (staining with Coomassie blue or Western blot with anti-VDAC2 specific Abs; TE: Triton Extract, HTP: chromatographic pass-through) or by two-dimension electrophoresis. Viviana Menzel and Elvira Hinsch (Giessen, Germany) are acknowledged for the image of 2-D gel.

3.2. VDAC protein structure

Fig. 3 shows the sequence alignment of the three human VDAC isoforms together with yeast porin1. Table 1 shows the amino acid composition of the three isoforms. Fig. 4 is a comparative secondary structure prediction obtained by the PSIPRED server. The observation of frequent, short β-strands with alternating hydrophilic/hydrophobic residues in the sequence has inspired several predictive models of the VDAC structure (for a review see Ref. [37]).

VDAC1 has been recently crystallized and the proposed structure [18–20] is formed by a large transmembrane channel, made of 19 amphipathic β-strands, with the addition of an N-terminal sequence

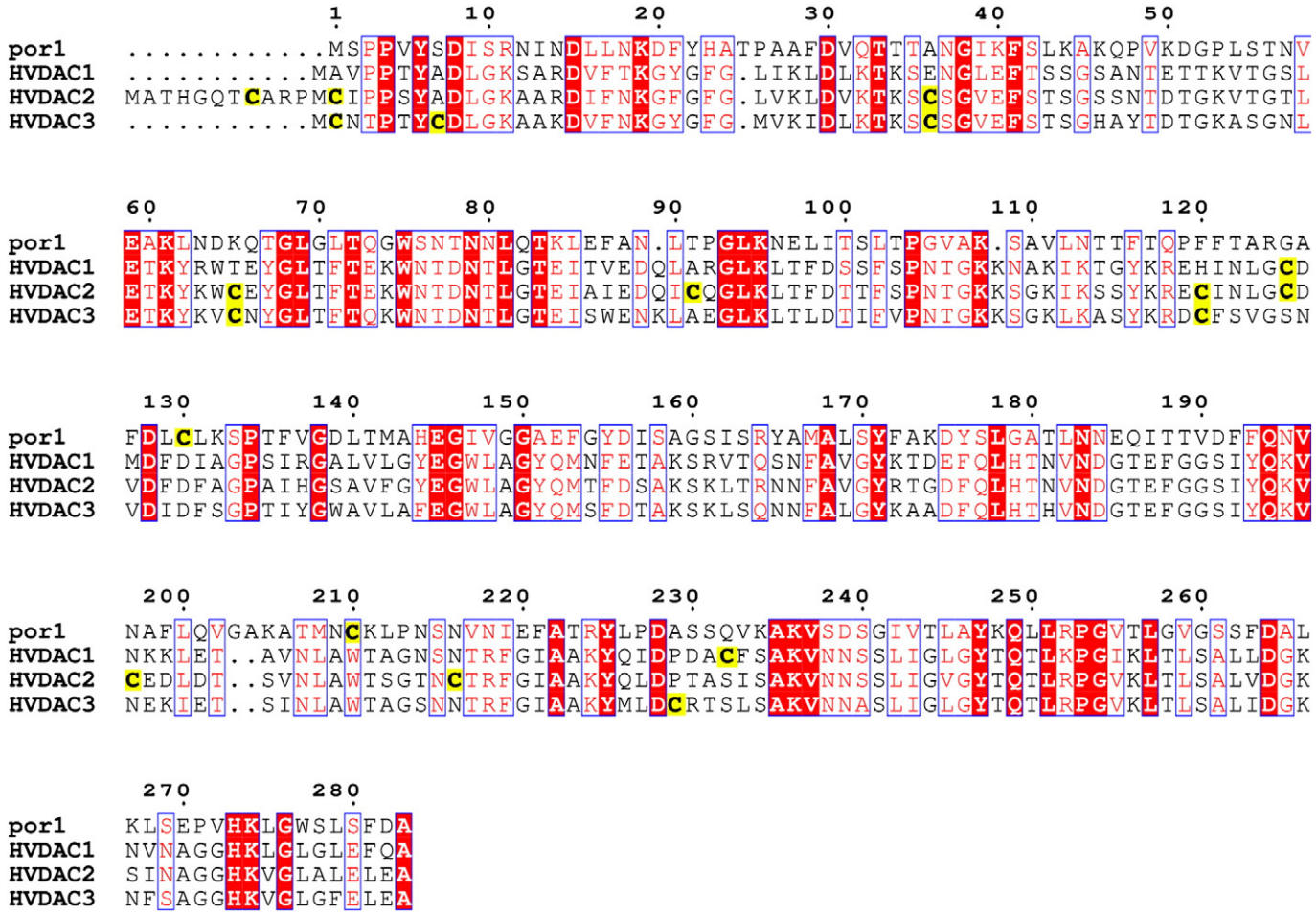

Fig. 3. Alignments of human VDAC isoforms and porin1 from yeast. In yellow the position of cysteines is outlined.

that contains the only α-helical structural segments found in the protein [38].

In this structure a wide, open barrel is formed through the regular organization of up-and-down antiparallel β-strands. This pattern is interrupted by the parallel disposition of the strands 1 and 19, which are forced to run parallel. It is not known whether this exception to the rule of even numbers strands in β-barrels, well established in bacteria [39], could have any influence on the stability of this structure. A whole criticism against this structure has been raised [40]. It is based on several, possible problems that could have led to the proposed crystallized structure. Among them, the consideration that the artificial folding procedure used to prepare enough material for the crystallization efforts might produce two (or more) populations of stable VDAC structures and the proposed crystallized structure might be just one of the available alternatives. In this respect other structural organizations of the sequence have been proposed [40,37]. This quarrel still calls for further experimental evidence, to be definitely solved: for example the crystallization of VDAC purified from mitochondria and not from recombinant DNA would give a final answer to it.

The sequence homology among VDAC isoforms (Fig. 3) justifies the ample similarity among secondary structure predictions (Fig. 4). These data converge on the clear observation that three isoforms have a very similar structure. When the mouse-VDAC1 X-ray structure (PDB: 3EMN) is used as a template to build the human VDAC2 and VDAC3 tertiary structure models, the three isoforms showed that they can be modeled to form similar barrels of 19 amphipathic β-strands and the N-terminal moiety structured with α-helix segment(s) [9]. Fig. 5 shows an overposition of the predicted structures of VDAC2 and VDAC3 isoforms upon VDAC1 coordinates (file PDB: 3EMN). Of course the structural organization in Fig. 5 is based on the PDB file (3EMN).

The N-terminal sequence is located inside the channel, partially closing the wide pore. The main distinguished difference among the three isoforms is the extension of 11 amino acids in VDAC2 sequence. It has been apposed in Fig. 5: a longer strand of the partial α-helical structure of the N-terminus protrudes outside the pore. Another difference among the VDAC isoforms are cysteines (Fig. 3 and Table 1): this point that will be treated in a next section.

4. Functional features of VDAC isoforms

VDAC1 was purified and characterized from various tissues and organisms [32]. VDAC2 has been only recently obtained from bovine spermatozoa [7]. The specific expression of VDAC3 was observed in mammalian testes [41] but its purification and functional reconstitution has not been achieved, yet. The three isoforms have also been produced in recombinant systems [42]. Their expression in yeast and further purification from mitochondria represented the first effort to compare functional VDACs [42]. Furthermore, they were cloned in *E.coli* expression vectors, where their expression produced a large amount of protein. VDAC1, in particular, was reconstituted after refolding procedures and showed the same activity pattern of the protein purified from tissues [43,44].

Table 1
Comparison of the amino acid composition of human Vdac isoforms. In yellow the Cysteine content of the proteins is highlighted.

hVDAC1			**hVDAC2**			**hVDAC3**		
Calculated Molecular Weight = 30770.05			Calculated Molecular Weight = 31563.95			Calculated Molecular Weight = 30656.04		
Estimated pI = 8.68			Estimated pI = 8.06			Estimated pI = 9.05		
Amino Acid Composition:			Amino Acid Composition:			Amino Acid Composition:		
Non-polar:	No.	Percent	Non-polar:	No.	Percent	Non-polar:	No.	Percent
A	21	7.42	A	21	7.14	A	22	7.77
V	12	4.24	V	15	5.10	V	13	4.59
L	28	9.89	L	23	7.82	L	26	9.19
I	11	3.89	I	13	4.42	I	11	3.89
P	6	2.12	P	7	2.38	P	4	1.41
M	3	1.06	M	3	1.02	M	4	1.41
F	14	4.95	F	15	5.10	F	15	5.30
W	4	1.41	W	4	1.36	W	5	1.77
Polar:	No.	Percent	Polar:	No.	Percent	Polar:	No.	Percent
G	32	11.31	G	34	11.56	G	32	11.31
S	18	6.36	S	23	7.82	S	22	7.77
T	30	10.60	T	31	10.54	T	26	9.19
C	2	0.71	C	9	3.06	C	6	2.12
Y	11	3.89	Y	10	3.40	Y	12	4.24
N	19	6.71	N	15	5.10	N	19	6.71
Q	8	2.83	Q	8	2.72	Q	6	2.12
Acidic:	No.	Percent	Acidic:	No.	Percent	Acidic:	No.	Percent
D	14	4.95	D	17	5.78	D	14	4.95
E	15	5.30	E	12	4.08	E	11	3.89
Basic:	No.	Percent	Basic:	No.	Percent	Basic:	No.	Percent
K	25	8.83	K	23	7.82	K	27	9.54
R	7	2.47	R	7	2.38	R	4	1.41
H	3	1.06	H	4	1.36	H	4	1.41

The main activity of VDACs is to form pores in the mitochondrial outer membrane. The parameters of VDAC1 activity and the reconstitution methods were largely reported and we will not write about them (for reviews see [2,3,8]). We will focus this section on differences among the VDAC isoforms from mammals. Information about VDAC activity were obtained from purified and reconstituted protein and, in a cellular context, by complementation assays in yeast cell devoid of the endogenous porin [4] or by gene inactivation in mouse [45,46].

4.1. Pore-forming activity of VDAC

Functional experiments demonstrated that recombinant VDAC1 and VDAC2 isoforms are able to form pores in lipid bilayers [7,32,42], but recombinant VDAC3 has no evident pore-forming ability [42]. VDAC2 was obtained by solubilization of whole spermatozoa, containing the cellular mitochondria. Its channel-forming activity was characterized by a main single-channel conductance of about 3.5 nS in 1 M KCl. Very often small short-lived fluctuations were observed on top of the conductance steps. These fluctuations were absent for the purified VDAC1. VDAC2 was anion selective, as VDAC1, and showed to be similarly voltage-dependent [7]. Recombinant mouse VDAC2 expressed in yeast cells and purified from yeast mitochondria showed a similar broad distribution of channels' sizes, similar ion selectivity and voltage-dependence [42].

Recombinant VDAC3 (the native protein has never been purified) obtained from yeast mitochondria after its expression, was instead shown to be unable to form pores either after reconstitution in artificial bilayer or incorporation in liposomes loaded with low molecular weight molecules [42]. It is not yet clear whether recombinant VDAC3 is unable to form pores or instead unable to insert properly in the artificial membranes (Shoshan-Barmatz, unpublished observation). There are indications that VDAC3 is able to partially vicariate the pore-forming activity of the other isoforms (see below) but a definitive answer is still missing. This main difference between VDAC3 and the other two isoforms, despite the conserved sequence, is the most puzzling aspects of the VDAC isoforms in mammals.

Voltage dependence is one of the most intriguing aspects of VDAC. In simple terms, VDAC1 switches to closed states when the transmembrane voltage exceeds 20–30 mV. This observation is obtained in artificial systems. Transfer of it to the cell would produce a gating device that could control the bioenergetic metabolism, since, when in the closed state, VDAC does not allow the regular flux of ATP/ADP through the membrane. While VDAC1 sub-states (or closed states) have been extensively characterized [2,3], little is known about this feature in the other isoforms. VDAC2 showed to mantain voltage dependence features very similar to those of VDAC1 [7]. No relevant data are presently available about VDAC3. The ability to be voltage-dependent is a very important issue: it could explain the presence of three isoforms in the cell, linking them to specific metabolic relationships.

4.2. Complementation of yeast porin deficit

The complementation assay in yeast is a useful method that gives information about the effectiveness of VDAC isoforms to work in the cellular context. In a situation that force the yeast cell to be mitochondrial-dependent for its survival, the lack of the endogenous porin causes the yeast to stop its growth. Transformation of these Δporin yeast strains [4] with plasmids expressing the mammalian VDAC isoforms restores the normal growth phenotype upon mitochondrial-dependent carbon sources. In this condition, typically, VDAC1 and VDAC2 are able to restore the growth phenotype, while VDAC3 is not or it is at a very low level [14,47]. To obtain quantitative information about the complementation by eutherian VDACs, the growth curve of yeast was performed, showing that

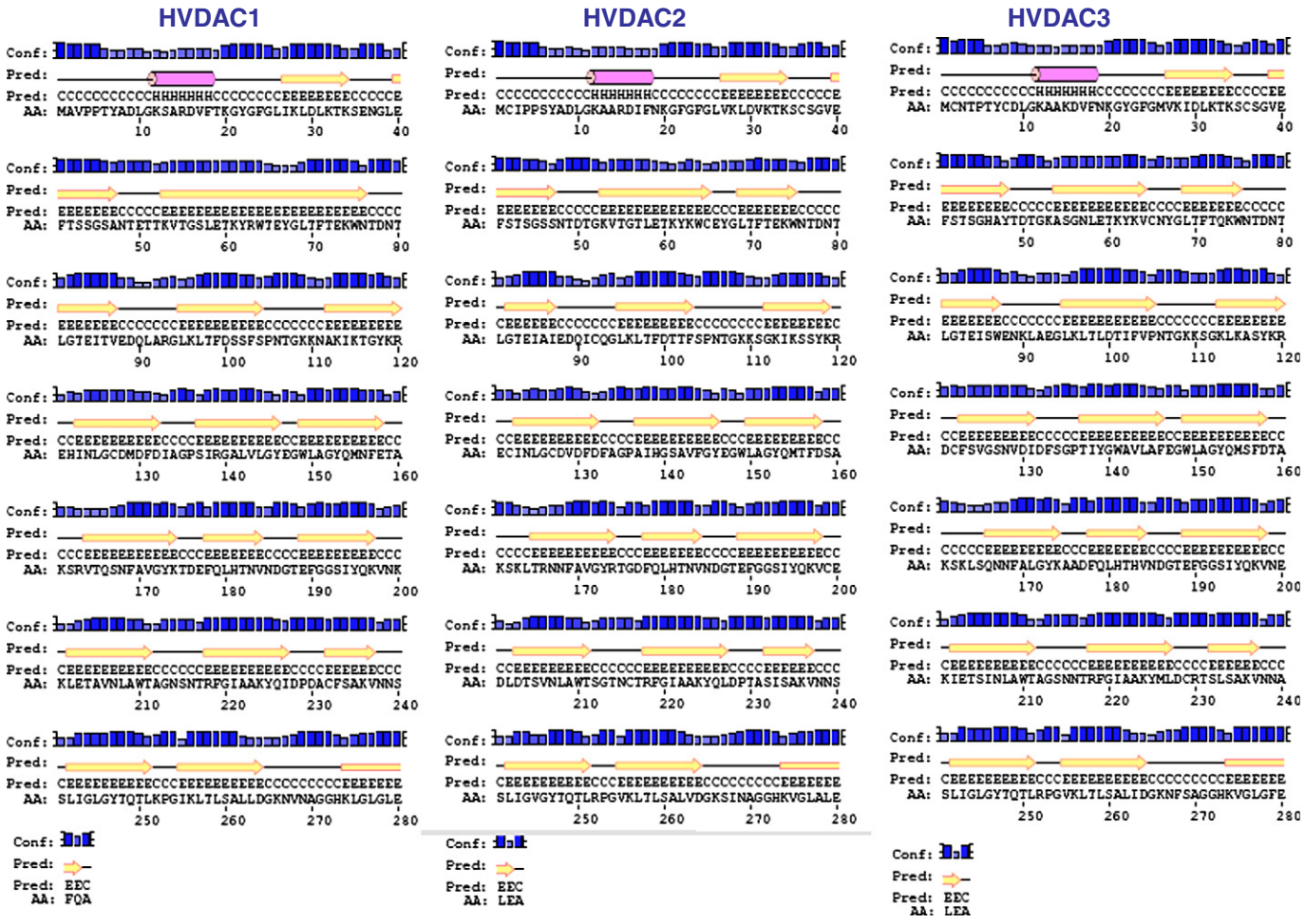

Fig. 4. Psipred secondary structure predictions of the human VDAC isoforms. The predictions were run at the Psipred server (http://bioinf.cs.ucl.ac.uk/psipred/). For the sake of an easier comparison, the additional 11-amino acids at the N-terminus of VDAC2 were deleted.

mouse VDAC1 and VDAC2 are able to replicate the growth conditions of the wild type [42]. VDAC3 has a very slow growth curve, in comparison with the other isoforms and even slower than Δporin yeast strains (C. Mazzoni, unpublished observation). These quantitative differences were confirmed by the NADH oxidation assay that exploits the presence of a NADH dehydrogenase on the outer surface of the inner membrane [48]. The import of NADH in the inter-membrane space is only possible when the Δporin yeast is complemented by VDAC1 and VDAC2 but not by VDAC3 [42].

Cronological life span experiments study the ability of single yeast cells to form colonies after days of growth. Striking difference was again noticed when the Δpor1 yeast was transformed with the human VDAC isoforms. These cells showed to live longer in the presence of VDAC1 and 2 but also in the presence of VDAC3 that showed a chronological life-span similar to the wild type. This outlines the importance of the presence of a pore-forming protein in the cell. On the other hand, VDAC3 showed to be less efficient than VDAC1 and 2 in restoring the energetic metabolism but anyway able to act as a porin, at a lower extent [47]. The indication that VDAC3 has the ability to recover the growth of yeast cells, even though at a lower level than the other isoforms, means that also VDAC3 is able to form pores in a cellular context [9,47]. For comparison, experiments of overexpression in the wild type yeast containing its own porin1 gene were performed, to investigate the effect of the over-expression of eutherian VDACs in addition to the physiological levels of the protein. Interestingly the over-expression of these genes generally reduced the viability of the yeast both in glucose and especially in glycerol. This result indicates that the dis-equilibrium of the VDAC levels is a sensible switch for the cell [9,49].

4.3. Gene knock out in mouse

Gene knock out of single or combination of VDAC genes has been performed in mouse [45,46,50]. Since this is the subject of another paper in this issue, we will just briefly summarize these results.

Mouse cell lines containing inactivated VDAC1, 2 or 3 gene were examined for loss of respiration and any reduction in respiratory chain activity [51]. ES cells lacking each isoform were viable but exhibited a ~30% reduction in oxygen consumption. VDAC1 and VDAC2 deficient cells showed a partial reduction in COX activity, whereas VDAC3 deficient cells had normal COX activity. These results indicate that each mouse VDAC isoform is not essential for the cell viability, since the lack of each of them could be compensated and thus gave just minor metabolite flux reductions [51]. The ES cells were used to generate mouse carrying VDAC genes K.O. [45,46]. Only K.O. mouse for VDAC1 and for VDAC3 have been produced. The VDAC2 K.O. mouse cannot be obtained since VDAC2 deficient embryos are not viable [52].

The VDAC1 KO mouse is relatively unaffected by the gene inactivation. Anflous et al discovered defects in skeletal muscles and modifications of kinetic parameters of some mitochondrial enzymes [45]. Ultrastructural changes in muscle mitochondria, where the cristae become very compact and the mitochondria enlarge many fold, and a certain degree of *in utero* lethality were also described [53] but

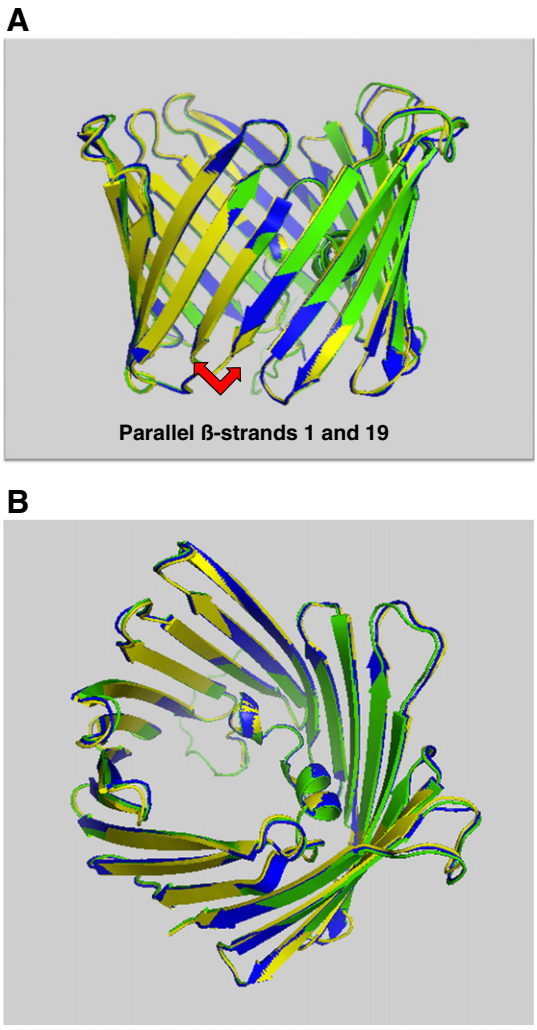

Fig. 5. Overposition of the predicted structures of VDAC2 and VDAC3 upon the PDB: 3EMN coordinates. VDAC1 is blue, VDAC2 is green and VDAC3 is yellow. A: lateral view of the proteins overposition. B: view from above. The caption in Panel A shows the two parallel β-strands, namely the strands 19 and 1, a peculiar feature of this structure. Panel B shows that the N-terminal sequences can be modeled in a similar way, but the extension of VDAC2, since there is no counterpart in VDAC1 structure, is just appended. The figure was obtained with PyMol.

the expected major deficits that one could associate to the role of VDAC1 in the cell were not found.

The VDAC3 KO mouse is again apparently not severely affected by the genetic deficiency [46]. In these mice, substantially healthy, the main problem is a high degree of male infertility. This problem has been associated with ultrastructural modifications in the sperm axoneme, leading to sperm immotility. Also, mitochondrial structure was found to be affected.

As said, the VDAC2−/− mice were never generated. The result was later explained with the anti-apoptotic function of VDAC2 isoform [52]. The proposed physiological role in vivo for the VDAC2 isoform is as a specific inhibitor of BAK-dependent mitochondrial apoptosis [52].

Baines et al.[50] produced the simultaneous ablation of the three VDAC isoforms by a combination of gene deletion and silencing to investigate the involvement of these proteins in the mitochondrial Permeability Transition. These authors proposed that the generalized inactivation of these three gene is simply uninfluent on the apoptotic mechanisms involving the Permeability Transition [50]. The absence of the three VDAC isoforms is a situation that should be lethal or otherwise supported by the opening of other channels in the outer membrane. This drastic conclusion is hardly compatible with living cell, and these data, in our opinion, should need further investigations and validations.

5. VDAC isoforms and Ca^{2+}

Ca^{2+} is an important cellular messenger. Since the extensive number of potential effects, its concentration is kept under strict control and the flow of the cation in the cell and to the various organelles is very well registered. Mitochondria are a storage site for Ca^{2+} but the cation, which is an effector for several intra-mitochondrial enzymes is not freely available. The flow of Ca^{2+} from outside the organelle instead is the signaling event that triggers other reactions and regulates the activity of several enzymes. Once more, VDAC is located in a critical position, at the entrance of mitochondria, towards the matrix-specific Ca^{2+} transporter systems. Investigations have been performed on two critical aspects of the VDAC permeability to Ca^{2+}: i) the influence of the cation upon the regulation of solute channeling by the pore; and ii) the involvement of VDAC in structures functionally devoted to the cation flow toward the organelle.

The first point was studied in [54–56]. For example, the work by Bathory showed that [Ca^{2+}] regulates the VDAC activity, showing that only at micromolar, but not at nanomolar, concentration of the cation, VDAC switches from a closed to an open conductance state [55]. This result gives a suggestive indication about the regulation of VDAC and, consequently, of the mitochondria by Ca^{2+}, proposing a novel mechanism for the control of OMM permeability to ions and small molecules. At low [Ca^{2+}] the VDAC would not show large conductance state but rather exhibit closed or subconducting states. Ca^{2+} addition would open to full conductance the pore [55]. The mechanism for this behaviour needs a direct interaction of the pore with the cation. A binding site for Ca^{2+} has been proposed in Ref. [57], following the competition between Ca^{2+} and Ruthenium Red and its derivative or other cations like La^{3+} and Tb^{3+}. Ca^{2+}-binding site(s) would involve peptides labelled by azido ruthenium (AzRu), a photoactivable compund with the same chemical reactivity of Ruthenium Red, an inhibitor of VDAC [58]. This picture has been questioned in Ref. [56]: the influence of Ca^{2+} on the pore-forming activity was not confirmed, asking for more investigation.

VDAC was considered as a molecular actor in the Ca^{2+} cellular traffic for the first time in [59], when the transient over-expression of VDAC in HeLa cells and skeletal myotubes increased the mitochondrial matrix accumulation of the cation. This has been the first indication of VDAC presence in the routes for Ca^{2+} transport across the mitochondrial membranes. Later, the finding that strict apposition of ER and mitochondria is mediated by a tethering structure visualized with several techniques like electron tomography [60] confirmed the proposal that such tethering would involve a component on the ER, the IP3 receptor, grp75 as tethering component and VDAC on the outer mitochondrial membrane [61].

The contribution of VDAC isoforms to Ca^{2+} traffic to mitochondria has been very recently considered. Di Stefano et al, performed both selective silencing or over-expression of each human VDAC isoforms, demonstrating that the mitochondrial Ca^{2+} transport may occur almost indifferently with any VDAC isoform [62]. Silencing of the various isoforms, in the presence of a pro-apoptotic stimulus, shows that they bear a different sensitivity to H_2O_2, confirming the notion that VDAC1 is pro-apoptotic and VDAC2 has a pro-survival role in HeLa cells. Finally, VDAC3 shows no significant influence on apoptosis [62].

During apoptotic events VDAC1 is preferentially involved in trasmission of Ca^{2+} dependent signals to mitochondria, while the other two isoforms do not show any special perturbation. The inactivation, by interference, of the IP_3R-3 impacts on the protective effect of VDAC1 silencing.

This work has discriminated, for the first time, the role of VDAC isoforms in Ca^{2+} traffic: VDAC1 selectively interacts with IP3-Rs and for this reason it is preferentially involved in the transmission of the

low amplitude apoptotic Ca^{2+} signals to mitochondria. At a variance from VDAC2 and VDAC3, both available for Ca^{2+} flow, VDAC1 is specifically involved in the signalling due to the specific flow of cations from the ER, with a regulatory meaning [62].

6. VDAC isoforms, VDAC chimeras and ROS

$O_2^{-\cdot}$ is mainly produced by Complex III as a subproduct of the Q cycle and it can be released both in the matrix and in the intermembrane space. The activity of SOD enzymes, that use $O_2^{-\cdot}$ as the substrate, produces the other ROS species, in particular H_2O_2. Since $O_2^{-\cdot}$ cannot freely permeate the membranes, it diffuses to the cytosol via the pores in the outer mitochondrial membranes [63]. The interaction of VDAC with $O_2^{-\cdot}$ is thus a continuous aspect of the physiology of this protein. Several investigators have already pointed out this and noticed, for example, that the closure of VDAC with the specific inhibitor dextran sulfate decreases the amount of ROS available in the cytosol [64]. The inhibition of VDAC by a specific antibody resulted in a block of the cytochrome C release [59], connecting in this way the control of ROS formation with the execution of apoptosis. It is thus not surprising that in systemic surveys reporting the list of protein modified due to overload of ROS generating species, VDAC is always among the most affected protein. In yeast mutants lacking MnSOD (SOD2) or CuZnSOD (SOD1) superoxide dismutase, it was found that few, specific mitochondrial proteins are modified (carbonylated) to a greater extent than in the wild type after exposure to paraquat, an oxidant-generating molecule [65]. Despite the action of superoxide dismutases, some proteins were not efficiently protected and thus were carbonylated. One of the proteins specifically carbonylated both in the *sod1*$^-$ and in *sod2*$^-$ mutant was the endogenous yeast porin 1 [65]. The reason for this might be the frequency of contacts with oxidative species, due to role of VDAC in their diffusion. In mammalian synaptosomes it was found that the exposure to acrolein, a chemical compound able to improve the carbonylation of proteins and suspected to cause Alzheimer's disease, enhances the ratio of carbonylation of VDAC1 13-fold. Only five proteins (among them VDAC1) were found exceedingly carbonylated [66]. The mechanism by which acrolein causes oxidative damage and neurotoxicity is presumed to involve the binding and depletion of cellular nucleophiles, such as reduced glutathione (GSH), lipoic acid and thioredoxin. The reaction of acrolein and GSH occurs spontaneously at neutral pH. VDAC was identified as a selectively oxidized target in Alzheimer's Disease brain [67], and it was proposed that oxidation of this protein could prevent the interaction of BcLxL with VDAC.

As seen for acrolein, another VDAC modification due to ROS action is the oxidation of the reduced SH group in cysteines. In a systematic study focused onto the oxidation of protein thiols in yeast, the oxidized proteome of unstressed cells was identified [68]. In this survey cysteines were reported to be oxidized at a various extent in 200, mostly cytoplasmatic, proteins. It was also found that porin 1 is predominantly present with its cysteines oxidized. ROS can oxidize protein thiols to thiyl radicals and then to sulfenic, sulfinic and sulfonic acid. Sulfenic acid can react with GSH to form PrSSG, thus preventing the irreversible oxidation to sulfonic acid. PrSSG is reduced back to protein thiol very effectively by Glutaredoxin. Saaed et al.[69] downregulated Glutaredoxin1 (Grx1) in cell and found that it was specifically associated with the oxidation of thiol groups of VDAC and mitochondrial dysfunction. Lefievre et al.[70] demonstrated the S-nitrosylation of VDAC3 in spermatozoa.

Thus VDAC(s) in the outer membrane are the highway of $O_2^{-\cdot}$ and can carry several modifications, like carbonylation or SH oxidation/derivatization, due to this role. In general, a drawback of the investigation about VDAC in ROS control, is the lack of consideration of the different VDAC isoforms. For example, in the work of Han et al.[64], the authors did not consider that in the outer mitochondrial membrane there are three different VDACs. Since dextran sulfate acts as a kind of clog upon the pore and since the VDAC isoforms are structurally very similar, it is very probable that the overall effect measured was not specific of VDAC1 but possibly the sum of the isoforms. Another striking difference among the VDAC isoforms is their cysteine content. Obviously human VDAC2 and VDAC3 with 9 and 6 cysteines, respectively (Table 1), are more subject to SH oxidation than VDAC1.

In the only work aimed to compare the ROS influence upon each VDAC isoform, Reina et al. transformed Δporin1 yeast with each human VDAC and looked, among the other, into the consequence of ROS producing agents on yeast containing single, distinct human VDAC isoforms [47]. Wild type and yeast strains transformed with human VDACs were compared following various parameters like life span and resistance to ROS generating agents. Starting point was the observation that yeast depleted of the endogenous porin is very sensitive to ROS. Complementation with VDACs restores the yeast surviving. VDAC3-transformed yeast was constantly the least resistant, while VDAC2 the most. Wild type yeast was also transformed to study the effect of an over-expression of VDACs. In this condition VDAC3 showed to be extremely toxic for the cell, resulting in a complete inhibition at 1.2 mM of H_2O_2. In apparent contradiction, the over-expression of VDAC in w.t. yeast showed high level of ROS. The over-expression of VDAC1 in addition to the yeast porin led to about 20% of ROS-positive cells, while the over-expression of VDAC3 led to about 8% of ROS-positive cells. This point was possibly explained by microscopy observation, showing that DHR123 fluorescence in VDAC1 over-expressing cells was mainly concentrated in cellular punctuate structures, probably mitochondria, while with VDAC3 the fluorescence was diffused [9]. Thus microscopy suggests that VDAC1 in some way forces the ROS to remain in the mitochondrion, while VDAC3 is not active in this respect. The ROS concentration could lead to the inactivation of mitochondrial function that in yeast causes the “petite” phenotype. Petite cells are unable to use respiratory carbon source. This could explain the longer lifespan observed in VDAC1 over-expressing cells grown on glucose [9,47]. On the contrary, cells grown on glycerol cannot accumulate petite and the VDAC over-expression toxicity is more evident [9].

VDAC(s) should have a key role in the control of ROS in the cell, as the many clues reported above seem to indicate. What can be the mechanism with which VDAC(s) can exert this function and what can be the difference among the various isoforms? In our opinion there are two possible mechanisms that could involve the pore-forming protein(s): i) to be part of the signalling route that from mitochondria, the source of ROS, switches on genes important for the cellular inactivation of these reactive species; ii) to be a sort of molecular buffer in the mitochondria when the overwhelming amount of ROS cannot be sufficiently inactivated by SODs and similar.

A mitochondria-to-nucleus signaling cascades must exist. This signaling pathway, most likely, can use proteins sensible to ROS increase and stimulate cellular detoxification [71]. It is completely unclear what are the mitochondrial sensors for oxidative stress and the way they can transmit signals from mitochondria to the nucleus. If VDAC(s) are involved in such cascade [72], it is tempting to speculate that structural modifications (like carbonylation or cysteine oxidation or derivatization) might cause conformational changes in mobile moieties of VDAC(s) (Fig. 6). Due to the expected rigidity of the pore walls, possible candidate to this role could be the N-terminal sequences of VDAC2 and VDAC3. Each of them contains two cysteines partially exposed to the water environment. Data from yeast complementation assay with human isoforms suggest that VDAC1 and VDAC2 are important for the ROS control, as it is for the endogenous yeast porin1. VDAC3 looks instead unable to exhert such a role. This has been confirmed by swapping experiments where the VDAC3 N-terminal sequence was substituted

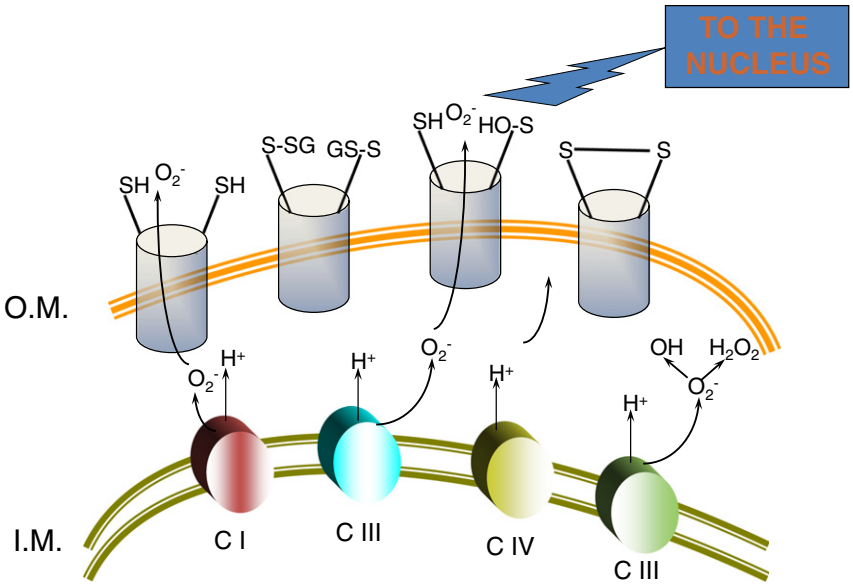

Fig. 6. A cartoon depicting potential role of VDAC(s) and their cysteines as mediator in the ROS signaling route mitochondria-to-nucleus. O.M.: outer membrane, I.M.: inner membrane, CI, CIII, CIV: cartoon of H^+ pumping mitochondrial OxPhos Complexes. Grey barrels represent VDAC(s) with cysteines carrying S in different oxidation states.

with the analogous moieties from VDAC1 or VDAC2: the chimeras VDACx-VDAC3 were able to restore the ROS control [47]. The conclusion is that the N-terminal sequence could be the important part of the protein for the ROS control. Another point is the different impact of VDAC3 and VDAC2 cysteines. Since VDAC3 is not able to control ROS, either its cysteines are not involved in this role or just act as chemical buffer for ROS modifications. In a last hypothesis VDAC3 cysteines cannot be involved in this function just because they already are in an oxidized state.

7. Conclusions

In this review we have tried to give a whole comparative report of the information available in the literature about the VDAC isoforms in mammals. A missing chapter is the involvement of VDAC isoforms in the germinal tissue. This intriguing point has been aroused with the discovery that VDAC3 KO mouse are male infertile because their sperm axoneme is not properly formed [46]. Next, other reports have been focused on mammalian spermatozoa (but similar interesting insights come from *Drosophila*, too [73,74]) showing that the localization of VDAC isoforms may not be canonical in this cell [41]. VDAC presence should be important in the development of germinal cells or in their ultrastructural organization. In particular VDAC2 and 3 are largely transcribed in the precursor of spermatozoa [75]. Recently the presence of VDAC in the plasmalemma of ovaries has also been shown [76]. Spermatozoa are very specialized and subcompartmentalized cells and they bear noteworthy differences among closely related species. Thus a comparative analysis of VDAC isoforms roles in these cells is still too early because not enough is known about the localization and function of VDACs in this context. There is no doubt, however, that this chapter is destined to become an active and surprising research field.

VDAC isoforms in mammals have differentiated roles, since the number of functional differences that is emerging in the last years justifies the evolution of three genes. VDAC isoforms show meaningful differences in their pore-forming function, Ca^{2+} sensitivity, ROS control. In the next years, the fine, molecular mechanisms of their coordination and function will be the frontier of research in the field.

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