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The superfamily of heme–copper oxygen reductases: Types and evolutionary considerations ☆

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ABSTRACT

Heme–copper oxygen reductases (HCO) reduce O_2 to water being the last enzymatic complexes of most aerobic respiratory chains. These enzymes promote energy conservation coupling the catalytic reaction to charge separation and charge translocation across the prokaryotic cytoplasmatic or mitochondrial membrane. In this way they contribute to the establishment and maintenance of the transmembrane difference of electrochemical potential, which is vital for solute/nutrient cell import, synthesis of ATP and motility. The HCO enzymes most probably share with the nitric oxide reductases, NORs, a common ancestor. We have proposed the classification of HCOs into three different types, A, B and C; based on the constituents of their proton channels (Pereira, Santana and Teixeira (2001) Biochim Biophys Acta, 1505, 185–208). This classification was recently challenged by the suggestion of other different types of HCOs. Using an enlarged sampling we performed an exhaustive bioinformatic reanalysis of HCOs family. Our results strengthened our previously proposed classification and showed no need for the existence of more divisions. Now, we analyze the taxonomic distribution of HCOs and NORs and the congruence of their sequence trees with the 16S rRNA tree. We observed that HCOs are widely distributed in the two prokaryotic domains and that the different types of enzymes are not confined to a specific taxonomic group or environmental niche. This article is part of a Special Issue entitled: Respiratory Oxidases.

1. Introduction

Heme–copper oxygen reductases (HCOs) are transmembrane enzymes characterized by the presence, in subunit I, of a low-spin heme and a binuclear center, which is the catalytic site. This center is formed by a high-spin heme and a copper ion (Cu_B), and a tyrosine residue (catalytic tyrosine Tyr-I), covalently linked to one of the histidine residues binding the copper ion. Only subunit I is common to all enzymes. A second subunit, generally named subunit II, can be present, which may have no cofactor (as in quinol oxidases) or a binuclear copper average valence center (Cu_A) (Fig. 1). In addition, the latter may also contain a C-terminal extension with one or two type C heme binding domains. The *cbb*$_3$ enzymes contain also extra subunits, having one and two C type hemes, respectively (Fig. 1C). Interestingly, these cytochrome domains are related to that present at the C-terminal part of Cu_A containing enzymes [1,2]. HCOs catalyze the four electron reduction of dioxygen to water in a process coupled to proton translocation across the membrane. Because electrons and protons needed for the reaction come from opposite sides of the membrane, the enzymes also promote the formation of a difference of electrochemical potential by charge separation. Thus, since protons are chemical and pumped substrates for all these enzymes, intramolecular proton conducting pathways should be present in subunit I. Based on amino acid sequence alignments, site directed mutations and on crystallographic structures, two proton channels (D- and K-channels) were identified for mitochondrial and mitochondrial-like enzymes, and named according to key residues of those channels. However, a detailed analysis of the amino acid sequences and structural information for other HCOs revealed important differences, in particular in the proton channels [3]: not only the residues lining those channels are not conserved but, most importantly, some enzymes do not have ionizable residues in between the cytoplasmatic surface of the enzyme and the binuclear site [4,5]. These observations imply an important role of water molecules in proton conduction as well as recommend a special care when proposing general catalytic mechanisms for proton pumping and its coupling to the chemical reaction. Based on the fingerprint of these proton conducting channels we proposed a division of HCOs into three types: type A (further divided into A1 and A2), B and C HCOs [3].

Nitric oxide reductases (NORs) catalyze the two electron reduction of NO to water and N_2O, do not pump protons across the membrane and do not promote charge separation (protons and electrons

Abbreviations: HCO, heme–copper oxygen reductase; NOR, nitric oxide reductase; LTG, lateral gene transfer; Gya, giga years ago

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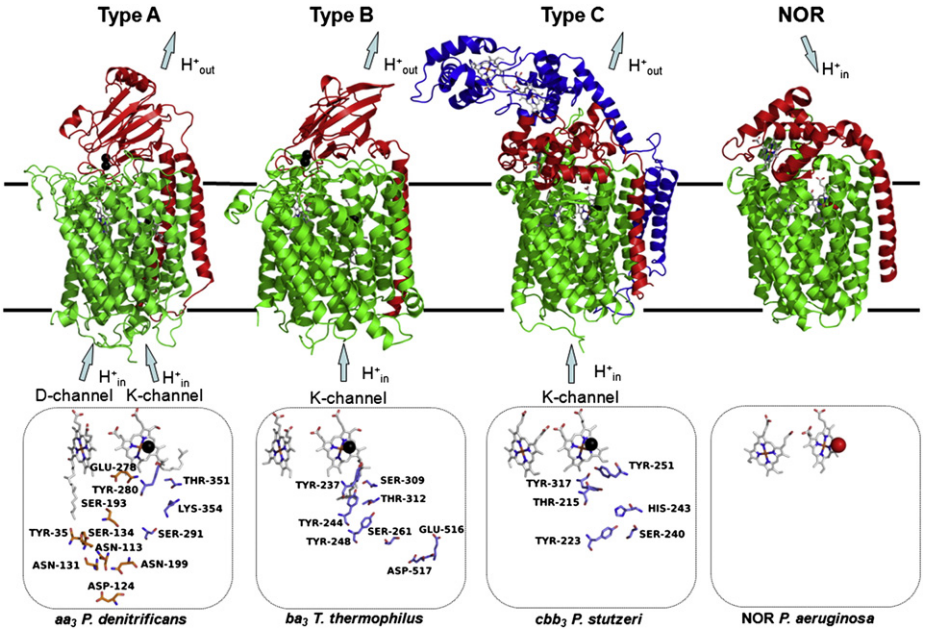

Fig. 1. Top—Crystallographic structures of heme–copper oxygen reductases and nitric oxide reductase. A representative structure is presented for each type of HCO. The catalytic subunit is shown in green, additional subunits are presented in red and blue. Copper ions are represented as black spheres and hemes are shown as sticks (orange). NORs are structurally similar to HCOs, but contain an iron atom (red sphere) in the catalytic subunit instead of a copper, and do not couple NO reduction to proton pumping. Bottom—Representation of the prosthetic groups and of the amino acid residues lining the proton channels from the different types of HCO and NORs. Structures were obtained in www.pdb.org.

come from the same side of the membrane); therefore they do not perform energy conservation [6,7]. Despite the functional and structural differences, NORs have been proposed to be evolutionary related to HCOs [7–10]. The catalytic subunit of NORs shares with that of HCOs the same general structural core, the presence of the low-spin heme, and of a binuclear center formed by a high-spin heme and an iron ion, instead of Cu_B [7]. The catalytic tyrosine residue is absent in NORs what may be considered to be a distinctive marker between HCOs and NORs. Also, no proton channels are observed in the cytoplasmatic side of the enzyme, in contrast to HCOs [7].

In this article we first review our recent expanded reanalysis of the HCO classification [9]. This comprehensive bioinformatics study strengthened our previously proposed classification [3,9]. Furthermore we analyze the taxonomic distribution of HCOs and NORs and the congruence of their sequence trees with the 16S rRNA tree. We observed that HCOs are widely distributed in the two prokaryotic domains and that the different types of enzymes are not confined to a specific taxonomic group or environmental niche. This classification of enzymes is important to establish in a systematic manner not only the natural diversity of HCOs but, most importantly, to probe for the general relevance of the so far proposed catalytic mechanisms. Moreover, the underlying analysis may allow identifying a higher number of "natural variants" which will be optimal candidates for future functional and structural studies.

2. Materials and methods

2.1. Taxonomic profile

We determined the phylogenetic profile of amino acid sequences from subunit I using completely sequenced genomes from 361 bacterial and 15 archaeal species present in the Superfamily database [9,11], and the HCO classification tool described before [9]. All retrieved sequences were mapped on NCBI Taxonomy using the BioSQL package from April 2009 available for download at ftp://ftp.ncbi.nih.gov/. The representativeness of a specific enzyme within a taxonomic group was determined based on a normalization in which the number of species with completely sequenced genomes having HCO or NOR sequences was divided by the total number of species of the same order with a completely sequenced genome available at Superfamily [12].

2.2. Phylogenetic analysis

Amino acid sequences from the HCO database (see [9] for details) were grouped according to each type of enzyme, A, B or C [9]. This analysis does not require completely sequenced genomes and the HCO database allows the most comprehensive taxonomic distribution. Multiple amino acid sequence alignments were performed using ClustalX 1.83 [13]. Default parameters were used, as no significant differences were observed with different parameter combinations. Protein weight matrix Gonnet, with Gap Opening 10 and Gap Extension 0.2 was used for multiple alignments that were manually refined in GeneDoc v.2.7.0 [14]. This manual adjustment is essential to assure an alignment that takes into consideration functional key amino acid residues, such as the metal ligands and the catalytic tyrosine residues, as well as parts of the proton channels already identified in the different enzymes. Neighbor joining (NJ) trees of each HCO type were constructed using the manually adjusted alignments with the following parameters: 10000 bootstraps, 1000 seeds and correction for multiple substitutions. One sequence of a different type of HCO or of a NOR enzyme was included to root each tree. Maximum likelihood (ML) trees were constructed using the PHYLIP package [15] with the following parameters: Jones–Taylor–Thornton probability model, 100 bootstraps and 13 seeds. In each tree, sequences of one HCO type or NOR were selected in order to have at least 3 representatives from each taxonomic class and one sequence of a different HCO or NOR type. The trees were visualized with Dendroscope v2.4 [16]. Groups were defined taking into account the first divergence of the rooted NJ tree.

3. Results

3.1. Classification

Based on the observation that all HCOs reduce oxygen to water and that those so far studied, pump protons and have the same general structural fold of the catalytic subunit (12–14 transmembrane

α-helices, which contain the same prosthetic groups) (Fig. 1), we previously proposed the existence of a common structural/functional denominator in HCOs. This common denominator should include specific elements, whether the prosthetic groups, amino acid residues, or water molecules indispensible for the operating mechanism. Thus, identifying the common elements will certainly be determinant for the recognition of the general mechanism of HCOs. The classification of HCOs can therefore be considered a step forward in the definition of the common denominator and may contribute to reset the so far proposed mechanisms, which have all in common being limited by the specific features of type A enzymes. We observed that all HCOs share the presence of proton channels, but the constituents and the number of the channels are not common. Remarkably, out of approximately 600 amino acid residues, only the six histidine residues, which are ligands of the prosthetic groups (hemes and Cu_B), are strictly conserved in the catalytic subunit.

Our previous classification separated HCOs into three major types: types A (subdivided into A1 and A2), B and C [3]. As this classification was based on a narrow number of amino acid sequences available then, we decided to reanalyze it with an enlarged data set combining bioinformatic and phylogenetic approaches. Furthermore, recently, Hemp and Gennis [17], proposed five additional types, D to H, some of them further subdivided, but comprising only 16 amino acid sequences from the Archaea domain. The comprehensive bioinformatics study performed [9] strengthened our previous classification and shows that there is no evidence for any further division, i.e., it does not support the existence of the extra types proposed by Hemp and Gennis.

The type A enzymes include the highest number of proteins known so far and are the best studied HCOs. This group contains the mitochondrial oxygen reductase and is characterized by the presence of the two first identified proton conducting channels (the D- and K-channel) and by the location of the catalytic tyrosine in helix VI (Tyr-I) [9]. In type B and C enzymes, only one type of channels, alternatives to the K-channel from type A enzymes, was identified. Moreover, type B HCOs contain the catalytic Tyr-I in helix VI, while in type C enzymes this tyrosine is present in helix VII (Tyr-II) [1,18]. We have also observed a correlation between the different types of HCOs and the properties of the catalytic site of the respective enzymes, such as ligand interaction, NO reduction and oxygen reducing kinetics [19].

3.1.1. Type A oxygen reductases

The mitochondrial enzyme and its close relatives are members of the type A, which is further divided in two types, A1 and A2. The type A1 is constituted by HCOs having the D- and K-channels first observed in the mitochondrial-like enzymes. Besides AspI-124 (D) (unless otherwise stated the numbering refers to the sequence of the aa_3 enzyme from *Paracoccus denitrificans*), located at the negative side of the membrane and after which the channel is named, the D-channel contains hydrophilic amino acid residues (AsnI-199, AsnI-113, AsnI-131, TyrI-35, SerI-134, SerI-193) ending at a glutamate residue, GluI-278, considered to be a key residue for the operating mechanism of the enzymes (Fig. 1). The residues LysI-354 (K), ThrI-351, SerI-291 and TyrI-280 are part of the K-channel (e.g., [3,20–23]) (Fig. 1). This last tyrosine residue (Tyr-I) is the one covalently bound to one of the histidine ligands of Cu_B (HisI-276).

Additional putative proton pathways such as the bovine aa_3 H-channel [24] or the *P. denitrificans* aa_3 E-channel [25] have been proposed but no experimental evidences supporting the functionality of these channels have been obtained. Furthermore, these putative channels were not observed in other members of this family.

In the members of the type A2, all the residues considered essential for the D- and K-channels are present, with the exception of the glutamate residue (GluI-278) present in helix VI. A homology based three dimensional (3D) model of *Rhodothermus (R.) marinus* caa_3 oxygen reductase suggested that a tyrosine residue, in a position equivalent to PheI-274, i.e., one helix turn below the glutamate residue in helix VI, and whose hydroxyl group was predicted to occupy the spatial place of the carboxyl group of the glutamate residue, may participate in proton transfer [26–28]. Furthermore, these enzymes also contain a conserved consecutive serine residue, which may be also relevant for proton transfer; this so-called YS motif is the fingerprint of the members of the type A2 [3]. In spite of these differences between A1 and A2 enzymes, both are proton pumps achieving, at neutral pH and reconstituted in liposomes, a stoichiometry of $H^+/e = 1$ [29,30].

3.1.2. Type B oxygen reductases

In the type B enzymes, the residues composing the D- and K-channels of type A enzymes are not conserved. However, a K-channel homologue was proposed with a threonine, a serine and a tyrosine residues replacing LysI-354, ThrI-351 and SerI-291, respectively, could be functional (Fig. 1). The tyrosine residue (Tyr-I) covalently bound to the histidine coordinating Cu_B is also present in these enzymes. The ba_3 oxygen reductase from *Thermus (T.) thermophilus* is the only member of this family whose crystallographic structure has been determined [31]. It was suggested that apart from the alternative K-channel, there are two other possible proton channels in this enzyme (a "D" and a "Q" channel). Inspection of the amino acid sequence alignment of the type B enzymes shows that none of the amino acid residues (or equivalent ones), constituent of those putative channels is common among type B members [3]. Furthermore, it was recently shown that mutations in these channels do not affect the proton pumping capability [32]. Interestingly in the structure from the ba_3 oxygen reductase it can be observed the absence of protonable amino acid residues between the cytoplasmatic surface and the plane in which the binuclear site is placed [4]. For the aa_3 oxygen reductase from *Acidianus* (*A.*) *ambivalens*, on the basis of a structural model [33], a pseudo D-channel was suggested but again, this putative channel was not observed in other members of this family. The ba_3, $b(o)a_3$ and aa_3 oxygen reductases from *T. thermophilus* [34], *Geobacillus stearothermophilus* [35] and *A. ambivalens* [36], respectively, were shown to pump protons but with lower stoichiometry (H^+/e between 0.50 and 0.75). If indeed these enzymes are less efficient pumps, it can be speculated that it is related to the existence of only one proton channel. This suggestion was recently reinforced by flow-flash and proton-uptake measurements on the ba_3 enzyme from *T. thermophilus* [37].

3.1.3. Type C oxygen reductases

The type C group only comprises cbb_3 oxygen reductases. These reductases apparently have only part of the alternative K-channel observed for the type B enzymes, with a serine and a tyrosine in the place of the ThrI-351 and SerI-291 [3,38] (Fig. 1). There is not a tyrosine residue in the same sequence position as TyrI-280, the tyrosine residue covalently bound to a copper histidine ligand. First, suggested based on homology models and later confirmed by both mass spectrometry [39,40] and the crystallographic structure [1], another tyrosine residue, from helix VII, was observed being also covalently bound to one of the copper histidine ligands [18,41] (Tyr-II). None of the canonical residues of the D-channel is present in these enzymes [1,3]. Up to now only for the cbb_3 oxygen reductase from *Bradyrhizobium japonicum* proton pumping has been observed in a liposome reconstituted system with a stoichiometry smaller than 0.5 [42]. Nevertheless, a H^+/e stoichiometry between 0.6 and 1 was obtained for proton pumping using whole cells from *P. denitrificans* and *Rhodobacter sphaeroides* [43,44].

3.1.4. Nitric oxide reductases (NORs)

NORs also constitute a diverse group of enzymes, being able to receive electrons from quinones (qNORs) and periplasmatic electron donor (cNORs). The available data concerns mostly the cNOR, including its crystallographic structure. cNORs do not translocate protons

across the membrane, and are not even electrogenic [6,10]. Accordingly, no proton channels or hydrogen-bonding network between the cytoplasmatic surface and its catalytic site is observed in the crystallographic structure of cNOR from *Pseudomonas aeruginosa* [7]. Two proton channels extending from the subunits' interface to the periplasmatic surface, involving three glutamate residues have been proposed [7].

3.2. Phylogenetic analyses

Our classification was also previously supported by phylogenetic analyses [3,9]. As an example, the Bayesian analysis is presented in Fig. 2. The construction of the tree, to reduce the computational cost of calculating it, included one NOR and one HCO of each type from each taxonomic order. From our analyses a clear separation between the type A, B and C enzymes as well as of the NOR enzymes can be observed. NORs and A type enzymes are evolutionary more separated, while the clades for the type B and C enzymes cluster in between NORs and type A enzymes (Fig. 2). Also, the Bayesian analysis does not support the existence of more than three types of HCO.

3.3. Bioinformatics classifier

Based on the established classification, we developed a bioinformatics classifier for HCOs and NORs, which is able to classify any given HCO and NOR sequence with a global recall and precision both of 99.8% [9]. This is available as a classification web tool and an integrated HCO and NOR protein database at www.evocell.org/hco.

3.4. Taxonomic distribution

The different types of HCOs are not confined to specific taxonomic groups or environmental niches. In fact the simultaneous presence of genes coding for different types of HCOs is observed in most prokaryotic organisms, which confers them an increased robustness, namely adaptability to the changing of the environment (e.g. changes in oxygen pressure).

We profiled the taxonomic distribution of each HCO type and NOR from Bacteria and Archaea. To determine the presence or absence of a protein within a taxonomic group, completely sequenced genomes were used and the sequences of HCOs and NORs from the different species were mapped against the NCBI taxonomic tree and organized according to the organism taxonomic order and HCO type/NOR. A total of 17 phyla (out of 23 present at Superfamily web-database) having at least one organism with a completely sequenced genome and containing at least one HCO or NOR enzyme were considered. The representativeness of a specific enzyme within a taxonomic group was determined after normalization (see Materials and methods). The resulting distribution of HCOs and NORs has different profiles (Fig. 3); while the occurrence of type A enzymes (including A1 and A2) is observed in most branches of the taxonomic tree, the other types have much narrower distributions.

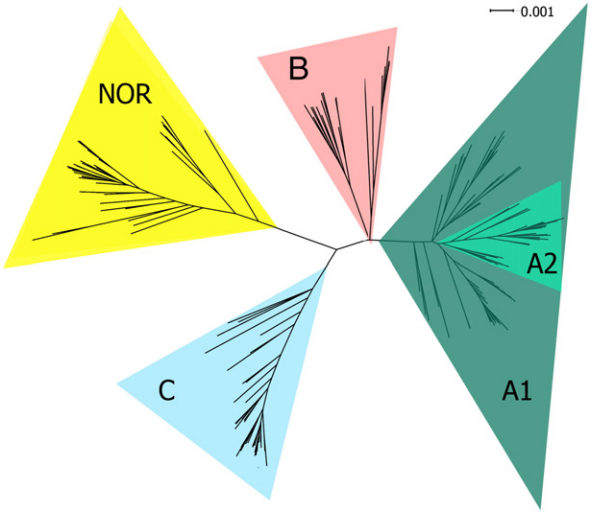

Fig. 2. Phylogenetic analysis of subunit I of HCO and NOR enzymes. Bayesian tree calculated with selected sequences of HCOs from each type and NORs. The sequences were chose in order to have a representative from each taxonomic class.

The type A enzymes are the most widespread oxygen reductases being present in all 17 phyla (Fig. 3) and in most cases, with dominant occurrence in relation to the other enzyme types. A predominance of type A1 versus A2 or vice-versa is always observed and therefore these enzymes are addressed separately.

Type A2 enzymes are only present in the Bacteria domain (Fig. 3), in 9 out of the 17 phyla. These enzymes are the predominant HCOs in several taxonomic groups, with a high representativeness in Cyanobacteria, Deinococcus-Thermus, and Planctomycetes phyla as well as in the δ-proteobacteria class.

Only enzymes belonging to the type A1 exist in all three domains of life, being present in 13 out of the 17 phyla (Fig. 3). Moreover, the presence of enzymes of this type is dominant in most orders. For example, in aerobic Archaea, 13 out of 24 species have type A1 enzymes and in γ, β and α-proteobacteria classes, these enzymes are present in 195 out of 218 species.

Although type B enzymes are the least represented HCOs (absent in 11 out of 17 phyla) they are present in both Archaea and Bacteria domains, being the predominant or highly represented HCOs in the Chloroflexi and Deinococcus-Thermus phyla, and equally represented as the type A1 enzymes in the Crenarchaeota phylum, Halobacteriales order and in two Proteobacteria orders, Nitrosomonadales (β-proteobacteria class) and Methylococcales (γ-proteobacteria class) (Fig. 3).

Type C enzymes are present in 11 out of the 17 phyla and confined to the Bacteria domain, mainly to the Proteobacteria phylum (Fig. 3), in which a wide distribution of type C enzymes is observed in four out of five classes. In the few cases in which the type C enzymes are highly representative, the presence of type A enzymes is usually also observed.

Nitric oxide reductases are present in both Archaea and Bacteria domains. From the 17 considered phyla, they are absent in 4, but are not representative in most of the phyla (Fig. 3). Moreover, although there is a considerable number of Proteobacteria containing NORs, they are not homogeneously distributed, i.e., within the same class, NORs are well represented in some orders and scarcely represented in others. For instance, in the γ-proteobacterial Alteromonadales order, 17 out of 24 species have NORs (71%) while in the entire γ-proteobacteria class, from the 99 species considered, only 29 have NORs (29%). A similar pattern is observed within other proteobacteria classes.

3.5. Taxonomic and protein sequence trees congruence

3.5.1. Type A oxygen reductases

Both ML and NJ trees of type A HCOs are congruent with each other. The topology of the NJ tree of type A enzymes (Fig. 4A) indicates the existence of six different groups. Type A2 enzymes are placed together in Group II in which the organization of the type A2 enzymes from Deinococcus-Thermus, Cyanobacteria and δ-proteobacteria is congruent with the taxonomic tree.

The five remaining groups only contain type A1 sequences. Interestingly, type A1 enzymes from Group I are more closely related to Group II (type A2 containing group) than to the remaining four A1 groups, a result corroborated both by a Bayesian analysis and the ML tree [9]. Group I comprises type A1 enzymes that are organized in subgroups corresponding to the Bacteroidetes (Flavobacteria class), Verrucomicrobia and Planctomycetes phyla. Group I also

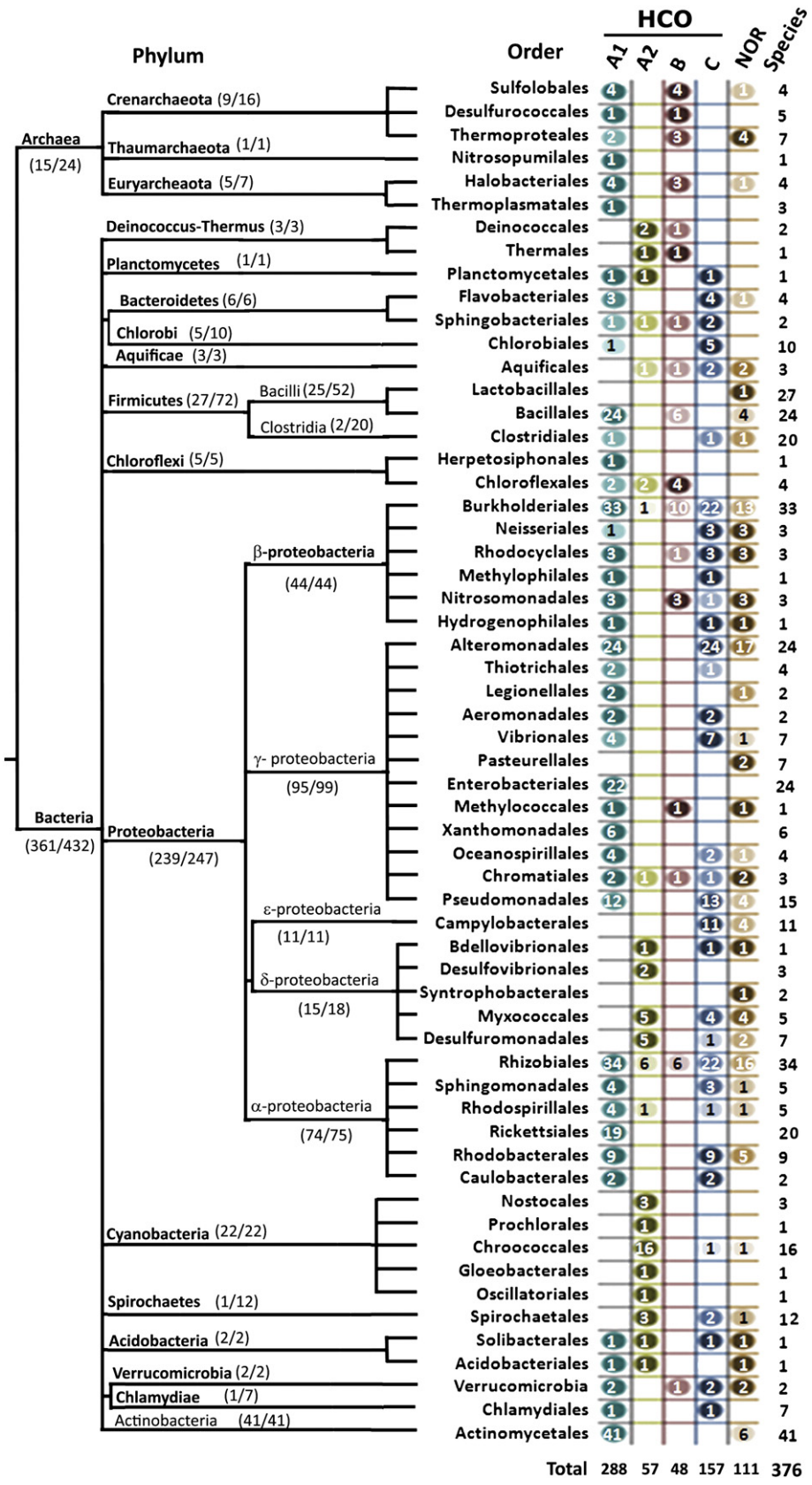

Fig. 3. Taxonomic distribution of different HCO types and NORs retrieved from completely sequenced genomes available from Superfamily database. Sequences are grouped by HCO type or NORs. The number of species containing each type is displayed as columns. A NCBI taxonomic tree is represented in the left in which each leaf (row) represents a different taxonomic order. The color gradient of each circle reflects the normalization of species within a certain taxon that has genes coding for that specific HCO type. The numbers inside the circles represent the number of species having genes coding for the respective HCO type. The last column refers to the total number of species within the respective taxon. Numbers in brackets represent the species within that taxon that contain either HCOs or NORs and the total number of species with completely sequenced genomes.

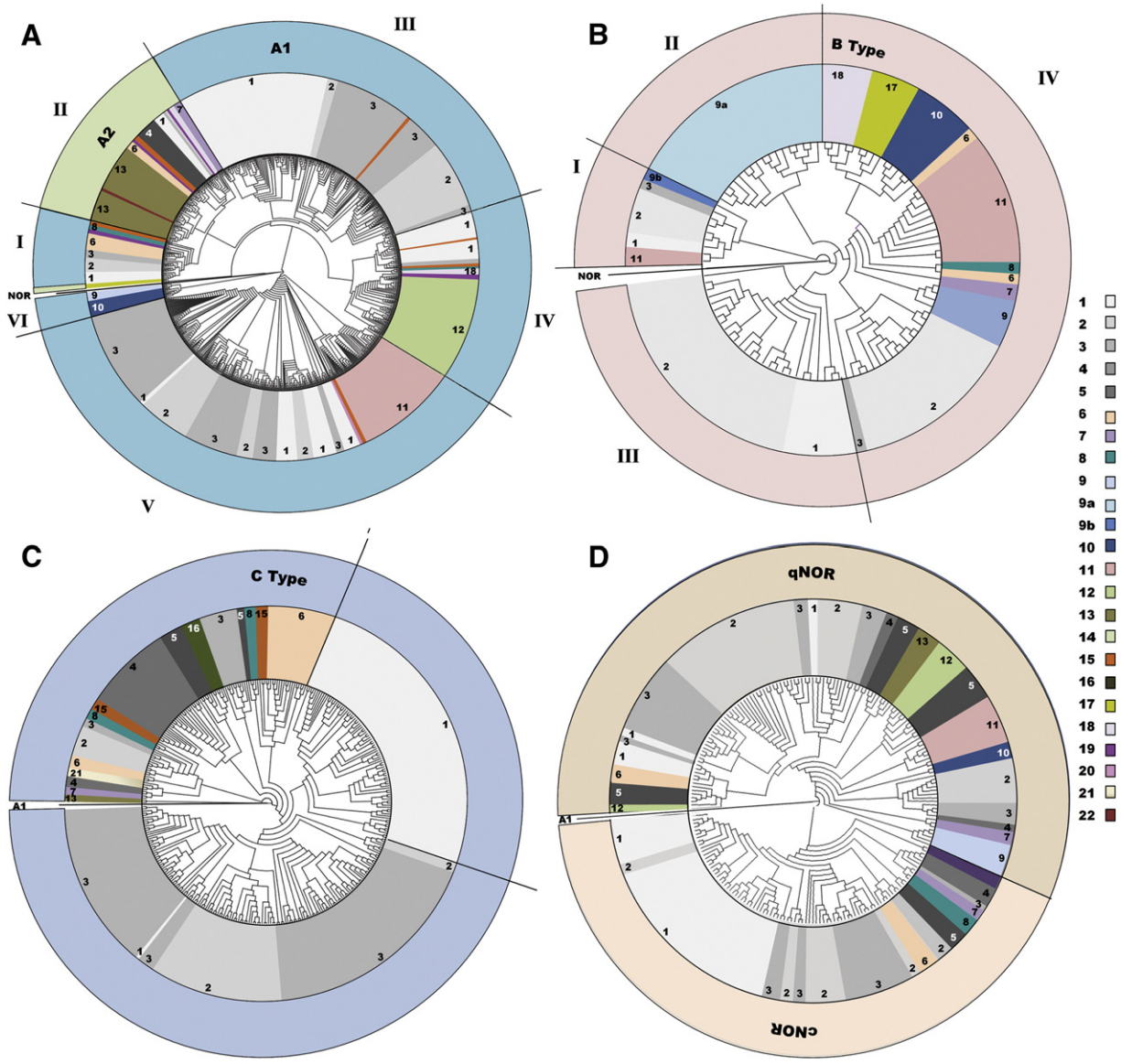

Fig. 4. Neighbor-joining (NJ) trees of subunit I from the different HCO types and NORs. A) type A HCOs, B) type B HCOs, C) type C HCOs, D) NORs. The NJ tree, the taxonomic groups (represented by a different color and number) and the enzyme type are indicated in the inner, medium and outer circle, respectively. 1—Alphaproteobacteria; 2—Betaproteobacteria; 3—Gammaproteobacteria; 4—Epsilonproteobacteria; 5—Deltaproteobacteria; 6—Flavobacteria, Sphingobacteria and Spirochaetes; 7—Aquificae; 8—Verrucomicrobiae; 9—Thermoprotei; (specific taxa, 9a—Sulfolobales order and 9b—*C. Maquilingensis* species); 10—Halobacteria; 11—Bacilli; 12—Actinobacteria; 13—Cyanobacteria; 14—Clostridia; 15—Planctomycetacia; 16—Chlorobia; 17—Deinococci; 18—Chloroflexi; 19—Chlamydiae; 20—Acidobacteria, Solibacteres and Spartobacteria; 21—Chlamydiae and Nitrospira; 22—Euglyphida.

contains some proteobacterial sequences belonging to different orders. The arrangement of proteobacterial type A1 enzymes in several groups (mainly in Group III and V) is incongruent with the taxonomic distribution within this phylum. On the contrary, the organizations of type A1 enzymes from Bacilli (Group V), Actinobacteria and Chloroflexi (Group IV) are congruent with the respective taxonomy. Group VI contains all archaeal type A1 enzymes evidencing the separation between archaeal and bacterial sequences of this type.

3.5.2. Type B oxygen reductases

The topology of the ML and NJ trees of type B enzymes (Fig. 4B) indicates the existence of several groups. One contains nine sequences from different taxa (Group I); a second one, comprises all sequences from the Sulfolobales order (Group II); a third group is dominated by sequences of organisms belonging to the Burkholderiales and Rhizobiales orders, in which 4 Burkholderiales sequences do not display a strict conservation of the residues of the K-channel of the type B enzymes (Group III); and a fourth group, in which the remaining bacterial and archaeal sequences are organized in agreement to their taxonomic order level (Group IV) but not to their respective domain. For instance, the archaeal Halobacteria sequences are present in the same subgroup containing Deinococci and Chloroflexi sequences and do not cluster with the remaining archaeal type B sequences from the Crenarchaeota phylum. The latter clusters with the majority of Bacilli sequences.

The existence of two separated groups of archaeal type B sequences within Group IV, and the existence of another group containing the Sulfolobales order sequences (Group II) are incongruent with the archaeal phylogeny.

It is also observed that in all type B enzymes from Group I, the catalytic tyrosine (Tyr-I) is absent. Recently, it was proposed based on homology structural models that in some of these enzymes two other tyrosine residues, located in helix VI or VIII, could replace Tyr-I [45]. However, this hypothesis was later questioned by the same type of models which revealed that the proposed tyrosine residues were too far from the catalytic center to be able to form a covalent bond

with histidine ligands of Cu_B [33]. If indeed the presence of the catalytic tyrosine residue is a fingerprint of HCOs, its absence could suggest a NO reductase activity for these enzymes.

Both the NJ and ML trees of type B enzymes are incongruent with the taxonomic tree at the domain level, as well as at the archaeal phyla level. Within Bacteria, with exception of sequences from organisms belonging to the Proteobacteria phylum, congruence between the protein sequence trees and the bacterial taxonomy is observed.

3.5.3. Type C oxygen reductases

Three main groups are identified in the ML and NJ trees of type C enzymes (Fig. 4C). The sequences from both ε and α-proteobacteria classes form well supported clades while those from the sister taxa, β and γ-proteobacteria, with a few exceptions, cluster together. The sequences from the Bacteroidetes and Chlorobi phyla are organized between proteobacterial sequences while the remaining non-proteobacterial type C enzymes are distributed among the ε and the α-proteobacteria classes.

3.6. Nitric oxide reductases

According to both their primary structure and nature of electron donors [10], NORs are divided into two groups, the cNORs (cytochrome oxidases) and the qNORs (quinol oxidases), which not only are differently distributed but also present distinct phylogenetic relationships. cNORs are mainly confined to the Proteobacteria lineages and are organized in well supported clades on the NJ and ML trees (Fig. 4D) that are congruent with the 16S rRNA tree. On the contrary, the qNORs show a wider taxonomic distribution and none of the several groups of sequences is congruent with the taxonomic tree.

4. Discussion

Subunit I, the catalytic one, is the only protein common to all HCO types and NORs. This structural similarity (Fig. 1) as well as their content in the same type of prosthetic groups is a strong indicative of their common ancestry. The origin and evolution of proteins may be inferred from molecular phylogenies; however, this single approach is not feasible for HCOs and NORs due to the indefinite of the root in a global tree comprising all NORs and the different HCO types (Fig. 2), i.e. the root cannot be indicated since the appropriate proteins to be used as an out-group were not identified yet. Without a proper root, it is not possible to order the appearance of each HCO type or NOR in relation to each other. Thus, it is not surprising that the available phylogenetic analyses of HCOs and NORs present contradictory results and that the order of appearance of the different enzymes has not been established, yet. From a very ancient origin, in the Last Universal Common Ancestor (LUCA) [46,47], or a later bacterial origin [3,45], many scenarios were considered. Furthermore, even the primordial function of the enzyme, i.e., whether a dioxygen reductase or instead, based on the assumption that dioxygen was inexistent in early Earth, a NO reductase, is still under debate [8,45,47].

The congruence of the type A1 enzymes and the 16S rRNA tree at the Archaea/Bacteria domain level may suggest their presence in the Last Universal Common Ancestor (LUCA). However, the absence of "true" eukaryotic HCO enzymes (these are present in the Eukarya domain resulting from an endosymbiotic process, being present in the mitochondrial membrane and not in the cellular membrane) may corroborate the proposal of a later appearance of these enzymes.

The existence of HCOs in LUCA was previously proposed [47–49], but without considering the different requirements for the existence of HCOs in a primitive organism and in the early environment. The catalytic subunit of HCOs and NORs is a transmembrane polypeptide chain having as cofactors hemes and a metal ion ($Cu^{2+/1+}$ or $Fe^{3+/2+}$). Thus, the appearance of these enzymes in LUCA or at any other time period cannot be dissociated from the existence of membranes and hemes, as well as from the availability of the different substrates (O_2 and NO) and metals.

Membrane proteins from Eukarya, Bacteria and Archaea are very similar, in spite of the different lipid composition of their membranes. The proposed (and widely accepted) ancestry of membrane enzymes in LUCA such as primordial V and F-ATPases [50] and the existence in Archaea and Bacteria of genes coding for SecE and SecY [51] (two essential subunits of a membrane-bound protein translocase) strongly suggest the existence of some kind of lipidic membrane in the first forms of life.

The appearance of HCOs and NORs also requires the existence of hemes. These could be a result of prebiotic organic synthesis [52] and thus could be available for biological incorporation or instead, a result from biotic synthesis. Moreover, the ubiquity of that cofactor and its role in many different biological reactions suggest that at least a (a)biotic protoheme precursor should have been present at the time of the first differentiation events.

The possible existence of NO in the early Earth is consensual: nitric oxide could be a product of lightning discharge reactions or of volcano emissions [53,54]. On the other hand, it is generally accepted that O_2 could not be accumulated before its photosynthetic production. However possible fluctuations on O_2 levels during the Archaean period are still under debate [55,56]. In any case, it is consensual that if O_2 was available prior to oxygenic photosynthesis, it would be confined to certain oases [57,58].

Before O_2 accumulation, the atmosphere and oceans of Earth are considered to have been reducing, and the metals needed for the catalytic centers of NORs and HCOs would be both in their lower redox state, Fe^{2+} and Cu^{+}, a case in which copper is considered to be hardly available for incorporation into proteins due to its disproportionation. In fact this argument has been invoked to support the ancestry of NORs over HCOs [59–61]. However, in biological systems, copper ions are transported in the reduced form, bound to thiolates, i.e., Cu(I) may become soluble and stable in the presence of suitable ligands. Moreover, the existence of copper containing enzymes in strictly anaerobic organisms [62], indicates that even in anaerobic environments, copper is available for biological incorporation. In the case of O_2 availability, copper would be in its oxidized state and thus, available for biological incorporation and iron would be incorporated into proteins by mechanisms similar to those used nowadays. In summary, the early bioavailability of copper or iron is possible, under either aerobic or anaerobic environments.

Both type C and NOR enzymes have been previously proposed to be at the origin of HCOs and NORs [49,63]. The first proposal, by Castresana and co-workers [49], based on the assumption that NO was present in the atmosphere prior to O_2, suggested that NORs were the first enzymes to appear and since type C enzymes are the closest HCOs to NORs, they were proposed to be the first O_2 reductases to evolve. The number of available sequences by Castresana and co-workers was too small to allow an enlarged phylogenetic and taxonomic study. More recently, Ducluzeau and co-workers proposed that type C enzymes were an ancient innovation of the Bacteria domain [63], based on the observation that these enzymes were present (regardless of their representativeness) in organisms from the lowest branches of the 16S rRNA tree, namely from the Aquificae phylum. However, due to the low representativeness (Fig. 3) it cannot be concluded that type C enzymes were present in the ancestor of this phylum.

Saraste and Castresana [49,60,64] also proposed the existence of at least NORs, A1 (SoxM) and B type (SoxB) HCOs in LUCA. The order of appearance of each enzyme suggested by Saraste and Castresana was based again on the possible existence of NO in the early atmosphere, being NORs the oldest enzymes. However, as also recognized by the authors, the function of primordial HCOs was the reduction of O_2 to water, implying that O_2 would have also to be present at LUCA's time.

The possible existence of HCOs before oxygenic photosynthesis does not require the presence of a global aerobic environment prior

to this advent, but certainly implies that oxygen was somehow available, possibly restricted to some niches. This idea may be supported by the recent study of Alm and David, who concluded on the existence of an Archaean expansion of gene births, containing a large number of genes coding for O_2 binding proteins, at a time prior to the appearance of oxygenic photosynthesis [65]. It is not implied that all organisms at that time respired O_2, but it is suggested that the species existing today are those whose ancestor already contained HCO enzymes and therefore, were better prepared to cope with the increase in the levels of atmospheric O_2 during the Great Oxidation Event [66].

In conclusion, the proposed classification of HCOs allowed organization of data and contributed to the recognition of the common denominator in HCOs and will certainly allow, in the future tracing the evolutionary scenarios of these enzymes. The existence of the common denominator has been previously proposed by us based on the rationale that if HCOs are structurally similar and perform the same function, the same operative mechanism should be present [29]. This mechanism will be executed by common elements, such as the prosthetic groups, amino acid residues or/and water molecules. Even without identifying the common denominator, the classification allowed to recognize uncommon elements and thus exclude these from being key players for the mechanism. For example, the first proposed mechanisms, which are still the most debated ones, invoke the fundamental role of the glutamate residue (GluI278) present in the D channel. Also, protonation and deprotonation events by this amino acid residue are suggested to occur. We have observed that there are enzymes, such as the ba_3 from *T. thermophilus*, that do not have protonable residues between the cytoplasmatic surface and the plane in which the catalytic center is [4]. We thus think that the discussion of the operating mechanism in HCOs should be reset in order to fit all the available structural data, without contemplating specific characteristics of some enzymes, which most probable are fine tunings of the mechanism in those cases, but are not essential for their operation. In particular, what can be concluded up to now is that HCOs need two hemes, one making a binuclear center with a Cu ion and close to a Tyrosine residue, that are needed for the chemical reaction; and a proton channel from the cytoplasmatic site to the binuclear center in order to promote charge separation and/or proton pumping. The available data does not lead to the identification of a protonatable amino acid common to all HCOs that could serve as a "controller" or gating element for proton conduction, making it difficult to consider only the thermodynamics (pkas) for the processes of the proton pumping mechanism and calling into play the role of water molecules.

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