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A missing link between complex I and group 4 membrane-bound [NiFe] hydrogenases ☆

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ABSTRACT

Complex I of respiratory chains is an energy transducing enzyme present in most bacteria, mitochondria and chloroplasts. It catalyzes the oxidation of NADH and the reduction of quinones, coupled to cation translocation across the membrane. The complex has a modular structure composed of several proteins most of which are identified in other complexes. Close relations between complex I and group 4 membrane-bound [NiFe] hydrogenases and some subunits of multiple resistance to pH (Mrp) Na^+/H^+ antiporters have been observed before and the suggestion that complex I arose from the association of a soluble nicotinamide adenine dinucleotide (NAD^+) reducing hydrogenase with a Mrp-like antiporter has been put forward. In this article we performed a thorough taxonomic profile of prokaryotic group 4 membrane-bound [NiFe] hydrogenases, complexes I and complex I-like enzymes. In addition we have investigated the different gene clustering organizations of such complexes. Our data show the presence of complexes related to hydrogenases but which do not contain the binding site of the catalytic centre. These complexes, named before as Ehr (energy-converting hydrogenases related complexes) are a missing link between complex I and group 4 membrane-bound [NiFe] hydrogenases. Based on our observations we put forward a different perspective for the relation between complex I and related complexes. In addition we discuss the evolutionary, functional and mechanistic implications of this new perspective. This article is part of a Special Issue entitled: The evolutionary aspects of bioenergetic systems.

1. Introduction

Complex I (E.C.1.6.5.3) is an energy transducing enzyme that couples the electron transfer from NADH to quinone to charge translocation across the membrane. It is an L-shaped membrane protein, consisting of a peripheral and a membrane arm. The peripheral arm contains a series of iron–sulphur centres and a FMN at the catalytic site, where NADH is oxidized [1]. The membrane arm includes the cation translocating machinery [2,3].

Homologues of complex I exist in bacteria, archaea and eukarya. In the latter domain, the complex is present both in the mitochondria and chloroplasts. Bacterial complex I is generally composed of 14 subunits designated NuoA to N (from NADH:ubiquinone oxidoreductase) or as Nqo1 to 14 (from NADH:quinone oxidoreductase) (Supplementary Table 1), whereas the mitochondrial enzyme is constituted by more than 40 subunits [4]. The electron donor of several bacterial and mitochondrial complexes is NADH, whilst some archaeal complex I-like enzymes work as $F_{420}H_2$ dehydrogenases [5]. In general, 11 subunits (NuoA to D and NuoH to N), suggested to be responsible for charge translocation and quinone binding, are common to the complexes from the three domains of life.

The research on complex I has been boosted in the last two years by the wealth of crystallographic structural data that have been obtained [3,6]. These data may corroborate the predictions based on amino acid sequences that the subunits composing complex I have homologies to the subunits of so-called Mrp Na^+/H^+ antiporters, to soluble NAD^+ reducing hydrogenases and to group 4 membrane-bound [NiFe] hydrogenases [3,7,8].

1.1. Complex I is constituted by modular units

Complex I is an example of the modular structure observed in several energy transducing enzymes [7–12]. The prototype complex I from prokaryotes may be viewed as a combination of 14 subunits most of which have been identified in other complexes. Fig. 1 schematically represents the L-shape structure of bacterial complex I, constituted by a peripheral and a membrane arm [1,3]. The peripheral arm has a Y shape with a length of 130 Å and is composed of seven subunits (NuoB to G and NuoI). One of the Y arms is formed by the

Abbreviations: DUF, domain of unknown function; Ech, energy-converting hydrogenase; Eha, energy-converting hydrogenase A; Ehr, energy-converting hydrogenase related complex; FHL-1, formate hydrogen lyase 1; Fpo, $F_{420}H_2$:phenazine oxidoreductase; Mbh, membrane-bound hydrogenase; Mbx, membrane-bound hydrogenase related complex; Mrp, multiple resistance to pH; Nuo, NADH:quinone oxidoreductase

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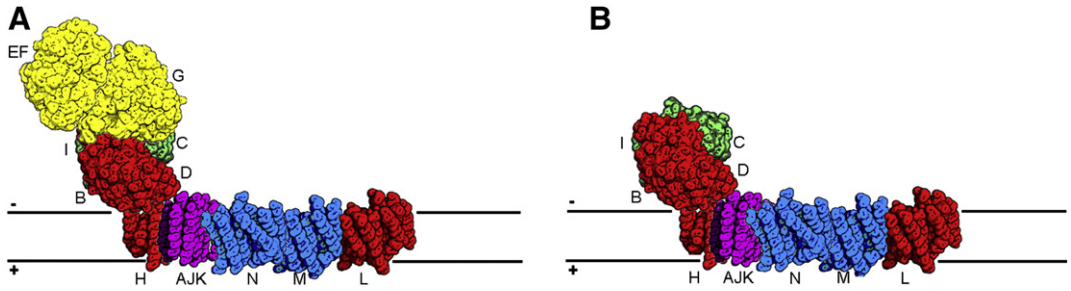

Fig. 1. Schematic representation of respiratory complex I and complex I-like enzymes. A) Canonical complex I composed of 14 subunits. B) Complex I-like enzymes with 11 subunits (unknown electron input module). The colour code is: red — subunits NuoB, D, H, and L (universal adaptor); green — subunits NuoC and NuoI; blue — subunits NuoM and NuoN (antiporter-like subunits); purple — subunits NuoA, J and K and yellow — subunits NuoE, F and G (the electron input module).

subunits NuoE and NuoF and the other by the C-terminal domain of NuoG. The central part is formed by the N-terminal domain of NuoG and by the subunits NuoC and NuoI. At the end of the Y are the subunits NuoD and NuoB, forming an interface with the membrane domain. The membrane arm has a curved shape with a total length of 180 Å and is also constituted by seven subunits (NuoA, H, J to N). NuoH is located at the basis of the peripheral arm and the membrane arm is then extended in an almost linear arrangement by subunits NuoA, NuoJ, NuoK, NuoN, NuoM and NuoL [3,13].

Subunits NuoE, F and G constitute the electron input modules and to the exception of the C-terminal domain of NuoG, are homologous to the subunits of soluble NAD^+ reducing hydrogenases. The C-terminal domain of NuoG is homologous to molybdopterin containing enzymes, such as formate dehydrogenase [1]. NuoB, C, D, I and H are homologous to subunits of the membrane-bound [NiFe] hydrogenases (see Section 1.2), whereas the subunits NuoK to N are homologous to subunits of Mrp Na^+/H^+ antiporters (see Section 1.3) [7]. Several organisms have genes coding for all subunits of complex I with the exception of those composing the input module (NuoEFG). In some archaea, for those complexes with $F_{420}H_2$:phenazine oxidoreductase activity, a flavoprotein (FpoF) has been shown to constitute the input module [5]. In the case of several other prokaryotic complex I-like enzymes, such as those of cyanobacteria, bacilli and other archaea, the input module is still unknown [14] (Fig. 1B).

Complex I couples the redox reaction to charge translocation. H^+ has been identified as the coupling ion of the system [15–18], although in the case of some bacterial complexes I Na^+ has been proposed to have that role [19,20]. It was also shown that some bacterial complexes I are capable of H^+ and Na^+ translocation, but in opposite directions, with H^+ being the coupling ion. A model for the functional mechanism of complex I was proposed suggesting the presence of two different energy coupling sites, both operating by indirect coupling mechanisms. One coupling site may work as a proton pump and the other as a Na^+/H^+ antiporter [21–23].

1.2. Relationship between group 4 membrane-bound [NiFe] hydrogenases and complex I

A close relationship between the so-called group 4 hydrogenases and complex I has long been recognized [8,10,24–27]. Group 4 hydrogenases are membrane-bound enzymes, which receive electrons from cytoplasmatic donors and reduce protons to hydrogen, thus being named hydrogen evolving hydrogenases [26]. The simplest known functional group 4 hydrogenases are the energy-converting hydrogenase (Ech), such as those purified from *Methanosarcina barkeri* and *Thermoanaerobacter tengcongensis* [28–30]. They are constituted by six subunits EchA to F (Fig. 2A). The membrane subunits EchA and EchB are homologous to NuoL and NuoH respectively, whilst the peripheral subunits EchC, D, E and F (also oriented toward the cytoplasm as in complex I) are homologous to subunits NuoB, C, D and I, respectively. The catalytic centre is located in subunit EchE and is constituted by a [NiFe] centre, which is bound to the protein by specific amino acid residues composing two CxxC motifs, one located close to the N-terminus and the other at the C-terminus. Ech receives electrons from a cytoplasmatic ferredoxin [24].

Formate hydrogen lyase 1 (FHL-1) from *Escherichia coli* includes a group 4 hydrogenase, with the same composition as Ech, that receives electrons from formate *via* a formate dehydrogenase subunit (Fdh-F) [24] (Fig. 2B). Interestingly, the latter protein is homologous to the C-terminus of subunit NuoG from complex I. In addition, a gene coding for a protein homologous to the N-terminus of NuoG, and thus possibly involved in electron transfer, is also present in the gene cluster (*hyc*) coding for this hydrogenase [10].

E. coli contains another group 4 hydrogenase (Hyf) encoded by the *hyf* gene cluster (Fig. 2C). This gene cluster is similar to *hyc*, but includes three additional genes [31]. Two of these encode homologues of the antiporter-like subunits from complex I and another (*hyfE*) encodes a protein, whose C-terminus is homologous to NuoK [10]. The electron donor to this hydrogenase is still unknown.

Rhodospirillum rubrum and *Carboxydothermus hydrogenoformans* contain a group 4 hydrogenase, encoded by genes *CooMKLXUH*, whose electron donor is CO *via* a CO dehydrogenase and a ferredoxin [29]. These hydrogenases were suggested to conserve energy, allowing *R. rubrum* to grow on CO as the sole energy source [32].

More complex membrane-bound [NiFe] hydrogenases were also identified, such as the membrane-bound hydrogenase (Mbh) from *Pyrococcus furiosus* [33] (Fig. 2D) and energy-converting hydrogenase A (Eha) from *Methanobacterium thermoautotrophicum* [34]. These hydrogenases are predicted to be composed of 13 or more subunits, having 4 to 6 subunits homologous to complex I [33,34].

The presence of group 4 hydrogenases has been reported for several other prokaryotic organisms [26,35]. Importantly, experiments with inverted membrane vesicles showed that the hydrogenases from *M. mazei* (Ech) and *P. furiosus* (Mbh) coupled H_2 production to the establishment of a transmembrane difference of electrochemical potential [33,36], *i.e.* the results showed that these enzymes are capable of energy transduction.

1.3. Relationship between Mrp Na^+/H^+ antiporters and complex I

Mrp (Multiple resistance to pH) Na^+/H^+ antiporters are considered as secondary active transporters energized by the transmembrane difference of electrochemical potential [37,38], for which biochemical and biophysical characterization at the protein level is still missing. This type of antiporters, generally multimeric complexes, was first identified in *Bacillus halodurans* C-125 where it is crucial for cytoplasmatic pH homeostasis in this alkaliphilic bacterium [39].

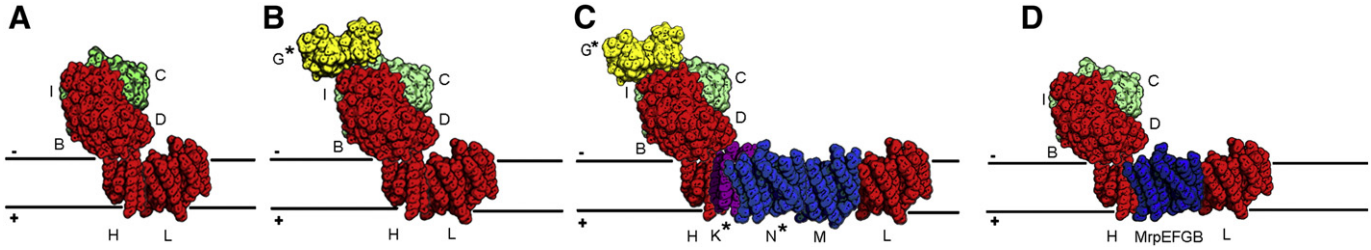

Fig. 2. Schematic representation of group 4 membrane-bound [NiFe] hydrogenases. A) Energy-converting hydrogenase (Ech). B) Membrane-bound hydrogenase Hyc. C) Membrane-bound hydrogenase Hyf. D) Membrane-bound hydrogenase Mbh. The colour code is: red — homologous subunits to NuoB, D, H, and L (Ech: EchC, EchE, EchB and EchA; Hyc: HycG, HycE, HycD and HycC; Hyf: HyfI, HyfG, HyfC and HyfB; Mbh: MbhJ, MbhL, MbhM and MbhH); green — homologous subunits to NuoC and NuoI (Ech: EchD and EchF; Hyc: HycE and HycF; Hyf: HyfG and HyfH; Mbh: MbhK and MbhN); blue — homologous subunits to NuoM/N and MrpE, F, G, B (Hyf: HyfD and HyfF; Mbh: MbhA to MbhF); purple — homologous subunit to NuoK (Hyf: HyfE; Mbh: MbhG) and yellow — homologous subunit to NuoG (HycB and HyfA). (G*: protein is homologous to the C-terminus of NuoG; K*: protein is homologous to the C-terminus of NuoK; N*: protein is homologous to antiporter-like).

The *mrp* gene cluster has typically seven genes encoding different integral membrane proteins, named MrpA to G, that were suggested to be coordinately expressed as an operon [40–43]. This entity is called group 1 *mrp* gene cluster [38]. Group 2 *mrp* gene clusters are similar to group 1 with *mrpB* fused to *mrpA*. Group 3 is more heterogenous, the gene clusters do not have *mrpA*, but may contain multiple copies of *mrpD* [37,38].

The products of *mrpA* and *mrpD* are homologous to subunits NuoL/M/N of complex I and that of *mrpC* has homology to NuoK [7,44]. Interestingly, the functional similarity of MrpA and MrpD to NuoL/M/N was recently corroborated by complementation studies using *Bacillus subtilis* MrpA and MrpD deletions strains, in which the subunits of complex I could replace those of the Mrp antiporter [45].

Evolutionary scenarios for complex I have been suggested over the past years, most of which propose that it resulted from the assembly of a soluble NAD^+ reducing hydrogenase with an Mrp-like antiporter [7,14,27]. Based on our observations described here we put forward a different perspective and discuss its implications also in functional and mechanistic terms.

2. Material and methods

2.1. Search of KEGG's database

Genes coding for proteins homologous to the different subunits of complex I were searched amongst the prokaryotic domains using protein BLAST (pBLAST) analysis tool running at KEGG's (Kyoto Encyclopedia of Genes and Genomes) database platform [46–48]. This database was selected because it contains data only on fully sequenced organisms, which is a requirement when searching for the presence or absence of genes in specific organisms.

Amino acid sequences and respective information were retrieved using a homemade script [49]. The following protein sequences were used as queries for pBLAST: NuoA to N from *Rhodothermus marinus* DSM 4252 (YP_003291419.1, YP_003291420.1, YP_003291421.1, YP_003291422.1, YP_003291423.1, YP_003291427.1, YP_003291429.1, YP_003291319.1, YP_003291891.1, YP_003290523.1, YP_003290524.1, YP_003290525.1, YP_003290526.1) and *Paracoccus denitrificans* (YP_916038.1, YP_916022.1, YP_916023.1), FpoF (NP_632651.1) from *M. mazei* Goe1, MrpA to G and the hypothetical protein with DUF2309 domain (Pfam) from *B. subtillis* (NP_391038.2, NP_391039.1, ZP_03592952.1, ZP_03592953.1, ZP_03592954.1, ZP_03592955.1, NP_391044.1, NP_388065.2). The selected *e-value* was ≤ 1 to guarantee a robust and representative sampling. 31 phyla, containing 580 classes and 1223 species (1117 species from bacteria and 106 from archaea) were investigated. Only one strain of each species was considered. The information used was that available by April 2012.

This search gave rise to different datasets containing the amino acid sequences, the species and gene identifications of each NuoA to N, FpoF, MrpA to G and DUF2309 related proteins.

2.2. Taxonomic profile of the universal adaptor

Each dataset obtained as described in Section 2.1 was processed on Microsoft Office Excel using Visual Basic for Applications (VBA) programming language. We were able to organize the data in a profile of presence and absence of each gene (*nuoA* to *N*, *fpoF*, *mrpA* to *G* and *duf2309*) by species. We consider that the universal adaptor is constituted by four subunits, NuoB, D, H and L. The number of universal adaptors was quantified by the presence of groups of the four genes that code for their constituting subunits, which implies that one repeat of such a group of four counts as one universal adaptor, two repeated group count as two universal adaptors, and so forth.

2.3. Taxonomic profile of hydrogenases, complex I and related complexes

Two groups of universal adaptors were considered: those belonging to hydrogenases and those present in non-hydrogenases. These groups can be distinguished based on the presence or absence of the binding site for the [NiFe] centre (Supplementary Fig. 1). Thus, a homemade script on VBA allowed us to separate the amino acid sequences of NuoD dataset by the presence or absence of CxxC binding motifs of the [NiFe] centre. Matching the NuoD dataset without CxxC motifs against the profile of the universal adaptor resulted in the profile of non-hydrogenases and matching the profile of NuoD with CxxC motifs against the remaining universal adaptors resulted in the profile of hydrogenases (Supplementary Fig. 1). Afterwards, the profile of complex I-related enzymes (module NuoBDHL IC MNAJK) and that of energy-converting hydrogenase related complexes (Ehr) were obtained by matching the profiles of NuoA, C, I, J, K, M and N against non-hydrogenases profile.

Finally, matching the profiles of NuoE, F, G (in bacteria) and FpoF (in archaea) against complex I-related enzymes (module NuoBDHL IC MNAJK) profile resulted in the profiles of complex I and of complex I-like enzymes (Supplementary Fig. 1).

2.4. Gene cluster organization of hydrogenases, complex I and related complexes

For each of the four final datasets (hydrogenases, complex I, complex I-like enzymes and Ehr complexes) obtained, the gene clustering organization was analyzed. The genes coding for complex I and complex I-like enzymes, when in a cluster have a well established organization. In this work these genes correspond to 73% of the data and thus their organization was automatically analyzed using a homemade script running on VBA, which allowed grouping genes by their identification number. Two combinations were considered, namely the 14 genes cluster (*nuoA-N*) and the 11 genes cluster (*nuoA-N* without *nuoE, F* and *G*). The cases in which genes *nuoB*, *nuoC* and *nuoD* are fused were also taken into account. The other datasets (hydrogenases and Ehr complexes) corresponding to 27% of the total data were

analyzed manually on KEGG's platform, since general information on their gene clustering organization is not available.

2.5. Structural modelling

To generate the structural model of the standalone Mpr-like protein from *Natranaerobius thermophilus* we used its amino acid sequence from Uniprot (B2A3T0). This sequence was aligned with that of NuoL from *E. coli* (via MobWeb) and the structural model was calculated with MODELLER using as template the crystallographic structure of NuoL (PDB ID: 3RKO:L).

The sequence of NuoH from *E. coli* was obtained from Uniprot (C6E9S0). Sequence alignments were made using both HHPRED [50] and GenTHREADER [51]. Both methods provided the same result, which was used as input in MODELLER [52] to calculate the homology model of the NuoH from *E. coli*. As structure templates we used the crystal structures of the homologue proteins identified by the sequence alignment algorithms: NuoL (PDB ID: 3RKO:L), NuoM (PDB ID: 3RKO:M), NuoN (PDB ID: 3RKO:N) and CysZ (PDB ID: 3tx3:A). To test the robustness of the structural model, we used the protein sequence of NuoH from *E. coli* as input in the ITASSER server [53] and a second model of NuoH was generated.

Protein structure images and cartoons were generated using PyMOL Molecular Graphics System, Version 1.4, Schrödinger, LLC.

3. Results

3.1. Taxonomic profile of hydrogenases, complex I and related complexes.

Complex I can be considered as being composed of three main parts. These have been named differently [4,7,10,54], but essentially they intend to define the peripheral arm in which the electron entry occurs, the membrane ion translocating machinery and a central membrane adaptor. We considered that this central membrane adaptor is composed of the soluble subunits NuoD and NuoB and the membrane subunits NuoH and NuoL, and since it is common to complex I and group 4 hydrogenases we will refer to it as the universal adaptor.

Using the sequences of NuoB, D, H and L from *R. marinus* as templates we looked for the presence of genes coding for universal adaptors in all prokaryotes with fully sequenced genome deposited in KEGG's database (Supplementary Fig. 1). We observed that from a total of 1223 species, 831 contain at least one group of the four genes coding for the universal adaptor (Table 1, column a). In total, we found 1145 groups of the four genes coding for the universal adaptor. With three exceptions these groups of genes are presented in all prokaryotic phyla, represented in the database (Table 1).

In the sequence of NuoD homologues, we searched for two CxxC motifs in which the cysteine residues provide the ligands for the [NiFe] centre of hydrogenases. This allowed us to distinguish possible complexes I from possible hydrogenases (Supplementary Fig. 1). We observed that 777 species contain 959 groups of genes coding for a universal adaptor without the [NiFe] centre binding site, whilst 146 species contain 186 groups of genes with this binding site (Table 1, columns b and c). The latter, most probably encode membrane-bound hydrogenases.

Amongst the species that contain genes coding for the universal adaptor without the [NiFe] centre binding site we searched for the presence of genes encoding the other subunits of complex I: NuoA, C, I, J, K, M and N. We found that 724 species have 833 groups of those genes *plus* the genes coding for the universal adaptor subunits (Table 1, column d). From these species, 579 have 645 groups of genes coding for a complex I with the NuoE, F and G or FpoF input modules (Table 1, column e). Most interestingly we observed the presence of 126 groups of genes, in 114 species, coding for universal adaptors without the [NiFe] centre binding site, which do not have genes coding for the other subunits of complex I (Table 1, column f).

Table 1 clearly indicates the existence of three types of complex: group 4 hydrogenase, complex I (and related enzymes) and a membrane-bound complex related to hydrogenases, but which seems to lack the [NiFe] binding site. This type of complex is known as energy-converting hydrogenase related complex (Ehr) [26,55].

3.2. Gene clusters of hydrogenases, complex I and related complexes.

Table 1 gives us information on the number of groups of genes coding for hydrogenases, complex I and related complexes existing in a certain genome, but does not provide any information about their possible organization into clusters. This prompted us to investigate the different organizations of gene clusters from the three types of complexes identified in Table 1. Fig. 3 contains schematic representations of the different gene clusters we have identified present in organisms belonging to the 31 considered phyla. We could observe that from 626 groups of genes coding for complex I with NuoE, F, G as the input module, 247 are organized in gene clusters similar to that present in *E. coli* genome, either with NuoCD fused or not (Fig. 3A). A similar gene cluster organization, but without the three genes encoding the input module proteins, is observed in 63 cases. Epsilonproteobacteria contain 38 groups of genes coding for all subunits of complex I, with the exception of genes encoding homologues of NuoE and F. We found two types of gene clusters in this phylum; one cluster containing genes coding for two hypothetical proteins, which are located between genes *nuoD* and *nuoG* and a second cluster containing an additional NuoG coding gene, a gene encoding a protein homologous to NuoE (but with a smaller size — Pfam: PF11390) and another one encoding a homologue to a protein annotated as FAD-dependent pyridine nucleotide-disulfide oxidoreductase (Pfam: PF07992). A gene coding for FpoF, the input module of some archaeal complex I-like enzymes, is present in 19 genomes (Fig. 3A and Table 1).

We observed 56 groups of genes coding for group 4 membrane-bound hydrogenases organized as *EchABCDEF* (Fig. 3B). Similar gene organizations to those observed in *E. coli* genome, *hyc* and *hyf* gene clusters, which code for its hydrogenases are present in other Gammaproteobacteria and in Epsilonprotobacteria (Fig. 3B). Most interesting is the observation that the gene clusters of some hydrogenases (mbh type) contain genes coding for proteins homologous to the small subunits of Mrp Na^+/H^+ antiporters, *mrpE*, *F* and *G*, which are not present in complex I (Fig. 3A and B).

Additionally, we observed the presence of genes coding for NuoD like subunits without the binding motifs for the [NiFe] centre, which are not part of gene cluster encoding complex I or complex I-like enzymes. The genes, which code for Ehr complexes are also organized in clusters (Fig. 3C). The simplest of these clusters contains a gene coding for an additional antiporter-like subunit and another one encoding a protein homologous to HyfE/NuoK, besides the genes coding for the universal adaptor proteins (Fig. 3C). We also observed another gene cluster with an organization similar to that encoding for the Mbh hydrogenase, but in which the gene coding for the NuoD homologous subunit does not contain the motifs for the [NiFe] centre binding. This observation has been reported before and the product of those clusters was named Mbx [56] (Fig. 3C).

In some cases we identified a gene cluster constituted by two genes: one encoding a NuoL homologue and the second a soluble/periplasmic protein containing a domain of unknown function (DUF2309). Isolated genes coding for NuoL homologous have also been observed and were called standalone Mrps [37]. In order to clarify the discussion we included in Fig. 3 the representation of the gene clusters coding for Mrp-like Na^+/H^+ antiporters (Fig. 3D). Initial studies of Mrp antiporters considered their gene clusters to be composed of six to seven genes, but later different gene arrangements

Table 1
Taxonomic profile of the genes coding for the subunits of the universal adaptor (a), type 4 membrane-bound [NiFe] hydrogenases (c), complex I-related enzymes (d), complex I (e), energy-converting hydrogenases related complexes (f).

				(a) Universal Adaptor			(b) Universal Adaptor (NuoD without CxxC)			(c) Universal Adaptor (NuoD with CxxC)			(d) Universal Adaptor (NuoD without CxxC) With IC MN AJK			(e) Universal Adaptor (NuoD without CxxC) With IC MN AJK and EFG/FpoF			(f) Universal Adaptor (NuoD without CxxC) Absence of IC MN AJK		
	Phylum	Class	Species	Species (+)	Gene (+)	Species (−)	Species (+)	Gene (+)	Species (−)	Species (+)	Gene (+)	Species (−)	Species (+)	Gene (+)	Species (−)	Species (+)	Gene (+)	Species (−)	Species (+)	Gene (+)	Species (−)
Bacteria	1. Gammaproteobacteria	88	175	115	159	60	111	121	4	35	38	80	110	115	5	110	115	5	6	6	109
	2. Betaproteobacteria	44	78	77	84	1	77	84	0	0	0	77	77	78	0	77	78	0	5	6	72
	3. Epsilonproteobacteria	11	37	36	50	1	35	41	1	8	9	28	35	38	1	3	3	33	3	3	33
	4. Deltaproteobacteria	22	45	37	74	8	35	60	2	9	14	28	34	52	3	26	40	11	8	8	29
	5. Alphaproteobacteria	66	171	166	212	5	166	203	0	4	9	162	166	188	0	166	188	0	13	15	153
	6. Other proteobacteria	1	1	1	2	0	1	2	0	0	0	1	1	1	0	1	1	0	1	1	0
	7. Chrysiogenetes	1	1	1	2	0	1	2	0	0	0	1	1	2	0	1	2	0	0	0	1
	8. Firmicutes	74	249	80	101	169	59	64	21	35	37	45	55	59	25	17	20	63	5	5	75
	9. Tenericutes	6	30	0	0	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	10. Actinobacteria	67	123	81	116	42	78	112	3	3	4	78	78	100	3	77	84	4	12	12	69
	11. Chlamydiae	5	12	2	2	10	2	2	0	0	0	2	2	2	0	2	2	0	0	0	2
	12. Spirochaetes	6	27	3	3	24	3	3	0	0	0	3	3	3	0	3	3	0	0	0	3
	13. Acidobacteria	6	7	7	11	0	7	11	0	0	0	7	7	11	0	7	8	0	0	0	7
	14. Bacteroidetes	43	58	35	45	23	35	45	0	0	0	35	35	45	0	23	25	12	0	0	35
	15. Fibrobacteres	1	1	1	2	0	1	2	0	0	0	1	1	1	0	1	1	0	1	1	0
	16. Fusobacteria	5	5	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	17. Verrucomicrobia	4	4	4	8	0	4	8	0	0	0	4	4	5	0	4	5	0	2	3	2
	18. Gemmatimonadetes	1	1	1	2	0	1	2	0	0	0	1	1	2	0	1	2	0	0	0	1
	19. Planctomycetes	4	5	3	5	2	3	5	0	0	0	3	3	5	0	3	3	0	0	0	3
	20. Elusimicrobia	2	2	1	1	1	0	0	1	1	1	0	0	0	1	0	0	1	0	0	1
	21. Synergistetes	3	3	2	2	1	0	0	2	2	2	0	0	0	2	0	0	2	0	0	2
	22. Cyanobacteria	12	15	15	15	0	15	15	0	0	0	15	15	15	0	0	0	15	0	0	15
	23. Green sulfur bacteria	5	10	10	11	0	10	11	0	0	0	10	10	11	0	1	1	9	0	0	10
	24. Green nonsulfur bacteria	7	12	12	22	0	12	21	0	1	1	11	12	19	0	12	18	0	2	2	10
	25. Deinococcus-Thermus	6	12	10	11	2	10	11	0	0	0	10	10	11	0	10	10	0	0	0	10
	26. Hyperthermophilic bacteria	24	33	27	40	6	27	36	0	4	4	23	18	24	9	15	17	12	12	12	15
Archaea	27. Euryarchaeota	44	70	69	111	1	50	55	19	38	56	31	31	31	38	19	19	50	23	24	46
	28. Crenarchaeota	17	31	31	50	0	30	39	1	6	11	25	11	11	20	0	0	31	21	28	10
	29. Thaumarchaeota	2	2	2	2	0	2	2	0	0	0	2	2	2	0	0	0	2	0	0	2
	30. Nanoarchaeota	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	31. Korarchaeota	2	2	2	2	0	2	2	0	0	0	2	2	2	0	0	0	2	0	0	0
TOTAL 31		**580**	**1223**	**831**	**1145**	**392**	**777**	**959**	**54**	**146**	**186**	**685**	**724**	**833**	**107**	**579**	**645**	**252**	**114**	**126**	**715**

were observed, including the association of some of these genes with genes coding for group 4 hydrogenases [37,38].

Curiously, some genomes contain gene clusters coding for the membrane arm subunits of complex I (Fig. 3D) which seem not to have the peripheral partners. This may suggest the existence of an unknown peripheral arm, however we cannot exclude the possibility that some peripheral arm may associate with different membrane arms.

3.3. Standalone Mrp Na^+/H^+ antiporters show structural homology to the NuoL subunit

To further investigate the relation of standalone Mrp Na^+/H^+ antiporters with the NuoL subunit from complex I we performed a structural homology model of the standalone Mrp from *N. thermophilus*. The sequence of the standalone Mrp was used as query in Modweb and an alignment of this sequence with that of NuoL from *E. coli* with 25% similarity was obtained. The homology model was calculated using the structure from NuoL (3rko:L) as template. The final model presents 13 transmembrane α-helices organized in a similar arrangement as the NuoL subunit but does not contain a region equivalent to the 14th transmembrane helix and to helix HL. Superimposition of the standalone Mrp model with the structure of NuoL highlights the sequence and structural conservation between the two proteins, especially on amino acid residues: E144, K229 and K399 in NuoL to E144, K223 and K399 in the standalone Mrp-like protein from *N. thermophilus* (Fig. 4). These amino acid residues were suggested to have a key role in charge translocation by complex I [2].

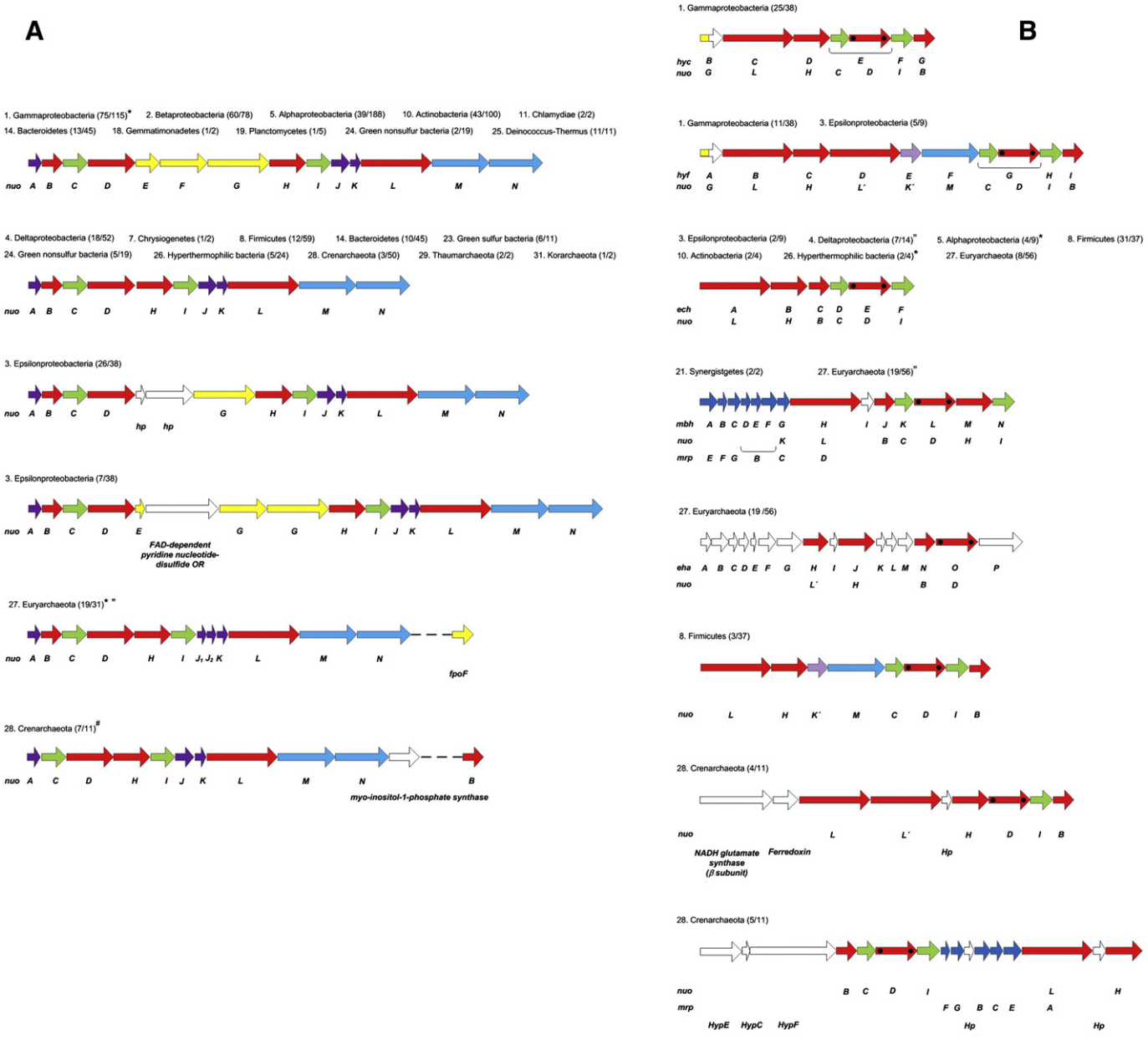

Fig. 3. Schematic representation of different gene cluster organizations present in the different prokaryotic phyla. A) Complex I and complex I-like enzymes. B) Group 4 membrane-bound [NiFe] hydrogenases. C) Energy converting hydrogenases related complexes (Ehr). D) Mrp, hypothetical protein containing Duf2309 and isolated membrane module (NuoA, H, J, K, L, M and N). The spheres represent the ligands of the NiFe centre (motif: CxxC). The colour code is: red — genes coding for homologues of subunits NuoB, D, H, and L (universal adaptor); green — genes coding for homologues of subunits NuoC and NuoI; blue — genes coding for homologues of subunits NuoM and NuoN (antiporter-like subunits); purple — genes coding for homologues of subunits NuoA, J and K and yellow — genes coding for homologues of subunits NuoE, F and G (the electron input module). (Hp: hypothetical protein; *genes may be fused; #NuoJ may be divided in two; "genes may be in a different order).

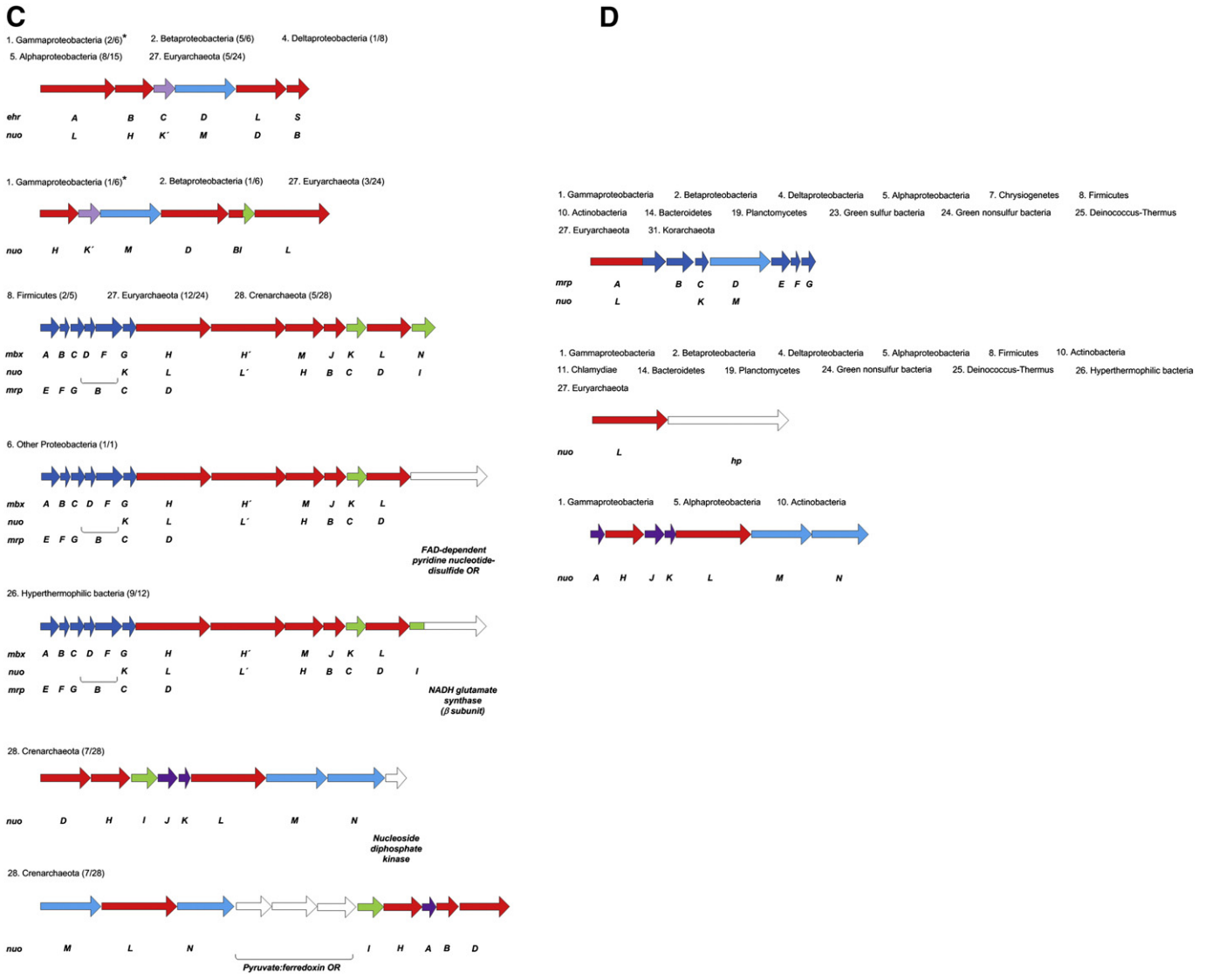

Fig. 3 (*continued*).

3.4. NuoH subunit is structurally related to the antiporter-like subunits of complex I

Besides the 1179 genes coding for NuoH subunits integrated in the universal adaptor, we observed the existence of 34 other genes coding for this protein. These genes are never observed isolated and are part of a group of genes coding for the membrane subunits homologous to those of complex I, which do not have the known peripheral counterpart (Fig. 3D).

NuoH seems to be unique to group 4 hydrogenases, Ehr complexes and complex I like proteins and is considered functionally unrelated to other known proteins [10,27]. Curiously, blast searches for homologues only indicated other NuoH, but if we looked for paralogues within a certain genome the result was frequently one of the complex I antiporter-like subunits NuoL, NuoM or NuoN. This led us to hypothesize a relation between NuoH and these subunits. In order to investigate this possibility we have performed a structural homology model of NuoH.

We used HHPRED and GenTHREADER to look for proteins homologous to NuoH. This strategy allowed us to identify distant sequence–structure relationships. Both algorithms showed a homology of NuoH to NuoL, NuoM and NuoN from *E. coli*, generating the same amino acid sequence alignment. This alignment was used to model the structure of NuoH from *E. coli*, using both MODELLER and ITASSER. The resulting models were similar. The model obtained by MODELLER, which is based on the combination of several high resolution structures, shows a RMSD of 3.1 Å in the homologous regions to NuoL (Fig. 5). This RMSD value is within the range of values acceptable when comparing a meaningful model with its template [57]. The NuoH structural model includes 7 transmembrane helices. Helices 3 to 6 are structurally homologous to helices 5 to 8 of NuoL and include conserved residues, D178, T174, Y151, Y119, suggested to be part of the putative ion channel of the antiporter-like subunits [2]. Helix 3 from NuoH does not contain E144 located in homologous helix 5 from NuoL. Most interestingly helix 4 from NuoH includes E157, conserved in NuoH subuntis [10] spatially close to the location where the E144 is in NuoL (blue sticks in Fig. 5). In helix 6 from NuoH we can also identify a helix kink structurally homologous to the kink observed in helix 7 from NuoL and where a highly conserved aspartate residue of NuoH [10] is located (D213).

Not aiming at providing a structure for NuoH the model supports our hypothesis for a structural relation between NuoH and the antiporter-like subunits of complex I (NuoL/M/N).

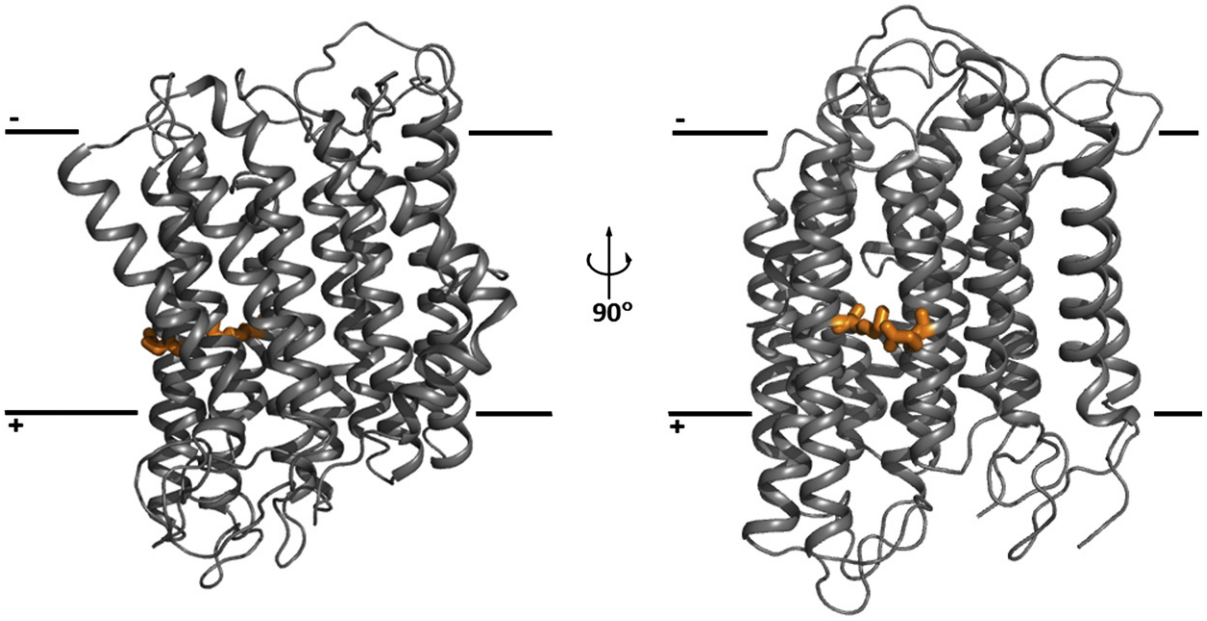

Fig. 4. Homology model of the standalone Mrp model from *N. thermophilus*. Side view, cytoplasmic side up. Cartoon representation of the homology model of standalone Mrp model from *N. thermophilus*. E144 and K223 in orange stick representation.

4. Discussion

4.1. Ehr complexes, a new piece for the puzzle of hydrogenases and complex I

Based on the data presented in Table 1 and on the organizations of the gene clusters (Fig. 3), we identified membrane complexes similar to membrane-bound [NiFe] hydrogenases, which do not have the motifs for the binding of the [NiFe] centre. The possible existence of such complexes had already been reported [26,55,56], and were named Ehr (Energy-converting hydrogenases related complexes) [26,55].

As described on Section 3.2, the simplest gene clusters coding for Ehr complexes, such as those from Proteobacteria and Euryarchaeota, should code for two peripheral subunits homologous to NuoD and NuoB and four membrane proteins, one homologous to NuoH, two equivalent to NuoL and one homologous to HyfE (whose C-terminus is similar to NuoK) of Hyf hydrogenase from *E. coli* (Figs. 3C and 6A).

In Firmicutes, hyperthermophilic bacteria and archaea the Ehr complex may be composed of additional small membrane subunits homologous to MprB, E, F and G. In this case the presence of subunits homologous to NuoC and NuoI is also expected (Figs. 3C and 6B). Interestingly, the NuoL-like subunit from hyperthermophilic bacteria seems to be fused to a domain similar to the small subunit of the glutamate synthase (Pfam: PF07992; PF12798). This is a flavoprotein that interacts with NADH [58,59] and is thus most probably the input module of these systems (Fig. 3C).

The so-called Mbx from the archaean order Thermococcales, which includes *P. furiosus* and *Thermococcus (T.) kodakarensis* has been genetically characterized and was suggested to function as ferredoxin: $NADP^+$ oxidoreductases [60,61]. It can be hypothesized that these

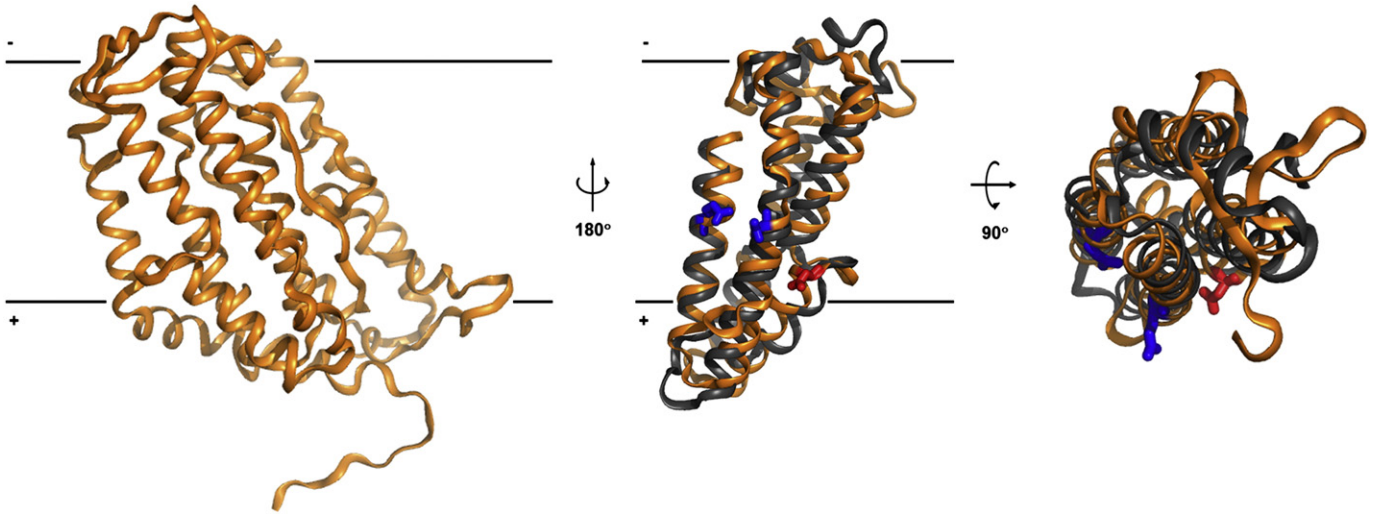

Fig. 5. Homology model of the NuoH from *E. coli*. Side view, cytoplasmic side up. Left: homology model of the NuoH from *E. coli* in cartoon representation. Middle: superimposition of the homologous region of NuoH (in gold) to the NuoL (from 3rko:L) from *E. coli* (in dark grey), in blue sticks the E144 (from NuoL) and the E157 (from NuoH). In red sticks is represented the D213 from NuoH. Right: top view from the homologous region of NuoH (colouring as before).

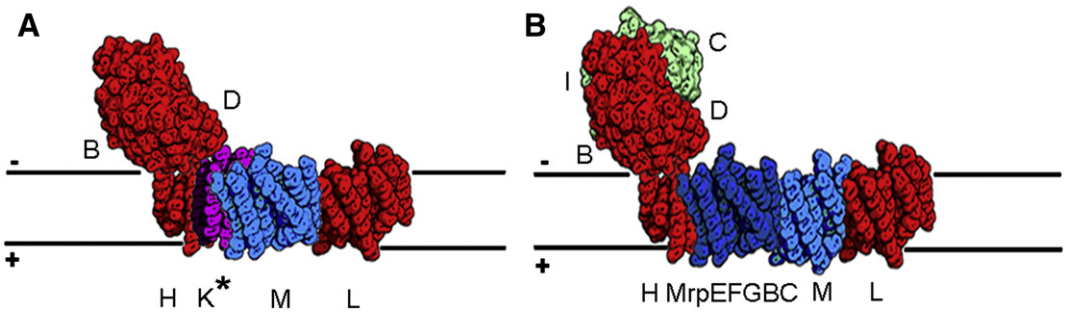

Fig. 6. Schematic representation of energy-converting hydrogenase related complexes (Ehr). A) energy-converting hydrogenase related. B) Membrane-bound hydrogenase related (Mbx). The colour code is: red — homologous subunits to NuoB, D, H, and L (Ehr: EhrS, EhrL, EhrB and EhrA; Mbx: MbxJ, MbxL, MbxM and MbxH); green — homologous subunits to NuoC and NuoI (Mbx: MbxK and MbxN); blue — homologous subunit to NuoM/N and MrpE, F, G, B (Ehr: EhrD; Mbx: MbxH′, MbxA to D and MbxF) and purple homologous subunit to NuoK (Ehr: EhrC; Mbx: MbxG) (K*: protein is homologous to the C-terminus of NuoK).

complexes couple peripheral catalytic reactions to charge translocation, without involving hydrogen as substrate or product. Since the genes coding for Mbx complexes are very similar to those coding for the Mbh hydrogenase, which was shown to perform ion translocation, Mbx complexes most probably also do so [60]. Curiously, in these complexes the subunit homologous to NuoB and NuoI also contains the binding motifs for the iron–sulphur centres. The presence of such centres allows electron conduction up to the membrane surface and thus we hypothesize that the catalytic reaction of at least some of these new complexes may involve quinones.

Ehr complexes are here recognized as members of the large family of proteins including hydrogenases, complexes I and complex I-like enzymes.

4.2. Evolution of group 4 membrane-bound [NiFe] hydrogenases and complex I

As described above (Section 1) a close evolutionary relationship between group 4 membrane-bound [NiFe] hydrogenases and complex I has long been assumed [8,10,24–27]. Common to the two complexes are four subunits (NuoB, D, H and L) that we nominated as the universal adaptor. Two of the subunits are peripheral (NuoB and D), facing the cytoplasm whereas the other two are integral membrane proteins (NuoH and L). Performing a taxonomic profile of the universal adaptor we observed that, with the exceptions of three phyla, the genes that code for the four proteins are present in all prokaryotic phyla (Table 1). Furthermore, besides complex I and group 4 membrane-bound [NiFe] hydrogenases, Ehr complexes also contain that same universal adaptor (Table 1). The genes coding for all these complexes are mainly organized in clusters (Fig. 3).

4.2.1. Origin of the universal adaptor

The subunits NuoB and NuoD are homologous to the small and large subunits of soluble [NiFe] hydrogenases [8,10]. These soluble hydrogenases are capable of performing the reduction of protons to hydrogen without the involvement of any other protein. So why did a soluble enzyme become membrane-bound and associated with proteins homologous to NuoH and NuoL, as it happens in the case of Ech? In order to answer this question we may analyze which evolutionary constrains or advantages existed for a soluble enzyme to become membrane-bound. We anticipate three possibilities: 1 — the catalytic reaction is not thermodynamically favourable and needs energy, such as the dissipation of the transmembrane difference of electrochemical potential, 2 — the catalytic reaction is thermodynamically favourable and the free energy may be transduced and conserved in the form of the transmembrane difference of electrochemical potential or 3 — the catalytic reaction evolves to have a membrane soluble substrate.

Membrane-bound hydrogenases kept their catalytic activities and the enzymes from the archaea *M. mazei* (Ech) and *P. furiosus* (Mbh) were shown to perform energy conservation [33,36]. Thus, we hypothesize that the initial advantage for the membrane association of hydrogenases was to benefit from or contribute to the membrane potential.

Since the catalytic activity occurs at the peripheral subunits the contribution to the membrane electrochemical potential cannot occur by a charge separation mechanism, thus charge translocation across the membrane must be involved. In this way it is not surprising that hydrogenases have recruited NuoL, an antiporter-like protein.

The origin of NuoH (and the reason for being recruited) is not so clear. This protein is considered functionally unrelated to any other known protein [10,27] and seems to be unique to complex I, Ehr complexes and group 4 membrane-bound [NiFe] hydrogenases. However, our incident search for paralogues of NuoH and its structural homology model (Fig. 5) leads us to propose a relation between NuoH and the antiporter-like subunits (NuoL, NuoM and NuoN), which may reflect an evolutionary link between NuoL and NuoH, the two membrane subunits of the universal adaptor. It can be hypothesized that NuoH arose by a gene duplication of NuoL, which then became structurally and functionally specialized in order to allow efficient coupling between the cytoplasmatic reaction and the membrane charge translocation.

4.2.2. Origin of the additional subunits

4.2.2.1. Peripheral subunits. The peripheral arm of complex I and of most membrane-bound [NiFe] hydrogenases is constituted, besides the two subunits NuoD and NuoB, by two other subunits, NuoC and NuoI. The latter is similar to soluble ferredoxins, whilst the role of the former is less clear; the protein does not contain any cofactor and in several cases it is fused to NuoD.

The classical complex I receives electrons from NADH through subunits NuoE, F and G. The need for all these subunits is not known and especially intriguing is the conservation of the C-terminus of NuoG. As mentioned in the Introduction (Section 1) this domain is homologous to formate dehydrogenase, although the binding ligands of the catalytic centre, a molybdopterin, are not present. The C-terminus of NuoG is not involved in electron transfer and is a large domain. The NuoE, F and G composition is similar to the soluble NAD^+ reducing formate dehydrogenase from *Ralstonia eutropha* [62]. The same composition, but without the domain equivalent to that of the C-terminus of NuoG is observed in the so-called bidirectional NAD^+ reducing hydrogenases (Hox) from cyanobacteria [63].

It can be speculated that NuoF could directly interact with NuoB, thus the need for the long electron conducting wire composed of the iron–sulphur centres is again intriguing. The constitution of the peripheral arm of complex I could have been the result of a random evolutionary assembling of pre-existing modules. However, the synthesis of the peripheral subunits is such an energetically expensive

process which most probably exerts evolutional pressure to change complex I composition. In this way, we hypothesize that the composition of the peripheral arm of complex I is mechanistically relevant (see below).

4.2.2.2. Membrane subunits. Complex I and more complex membrane-bound [NiFe] hydrogenases evolved by one or several duplications of the gene coding for NuoL and recruitment of other small membrane proteins, such as those homologous to HyfE/NuoK (these are in turn homologuos to MrpC, Fig. 3B). The replication of NuoL would confer additional ion translocating sites and would possibly allow a higher efficiency in energy conservation. The function of the small subunits is still enigmatic. Most interestingly is the observation that the small subunits in membrane-bound hydrogenases, are homologous to the Mrp small subunits, MrpE, F and G (Fig. 3). The association of some of the genes coding for Mrp subunits to hydrogenase or hydrogenase-like genes has been indicated before [64].

4.3. Complex I is not the evolutionary result of the association of a soluble NAD^+ reducing hydrogenase with a Mrp antiporter

Contrary to what has been proposed [27] complex I does not seem to be the evolutionary result of the association of a soluble NAD^+ reducing hydrogenase with a multisubunit Mrp antiporter complex. Complex I seems to result from evolutionary pressures on soluble hydrogenases to become membrane associated: soluble hydrogenases associated with one or two antiporter-like subunits. One of these antiporter subunits evolved as NuoH. Later the genes coding for the other antiporter duplicated possibly to increase energy transduction efficiency. Eventually this new membrane arm dissociated from the complex giving rise to the multisubunit Mrp Na^+/H^+ antiporter complexes. In this way we suggest that multisubunit Mrp complexes are the results of the evolution of the membrane arm of hydrogenases or related complexes.

Ehr complexes, most probably arose from hydrogenases by the loss of their [NiFe] centre binding site. Two types of Ehr complexes may be considered; one close to Ech and the other related to Mbh hydrogenases. In fact it has been suggested that hydrogenases and this type of complexes were acquired by different bacteria by three gene transfer events [65]. Two of these involved the transfer of the *ech* gene cluster independently from the *Methanosarcina* genus to some species from the Deltaproteobacteria and Firmicutes phyla. The other was the transfer of the *mbx* gene cluster from the order Thermococcales to species of hyperthermophilic bacteria [65]. These observations suggest that the loss of the [NiFe] binding site occurred more than once, which means that evolution of these complexes was not linear, but comprised multiple and parallel event.

The catalytic reaction of Ehr complexes is not known, however it is tempting to speculate that some may have acquired a quinone interacting site. Complex I-related enzymes may have evolved from these quinone interacting Ehr complexes or directly from hydrogenases. Most interesting is the observation, in the genome of *Pelobacter propionicus*, of a complex (YP_900275.1–YP_900287.1) composed by the same subunits as those present in complex I-like enzymes, but whose NuoD homologous subunit contains the binding motif of the [NiFe] centre.

4.4. Functional implications

Our analysis led us to several observations that allow a new discussion on the mechanism of not only complex I and complex I-like enzymes, but also of group 4 membrane-bound [NiFe] hydrogenases and Ehr complexes. All these complexes have in common the subunits composing the universal adaptor module. This module is most probably structural and functionally involved in the coupling of the two activities performed by the complexes, *i.e.* the coupling between the peripheral catalytic activity and the membrane charge translocation.

It is proposed that Mbx complexes have ferredoxin:$NADP^+$ oxidoreductase activity [60,61]. This reaction does not involve the presence of a catalytic site close to the membrane surface, therefore suggesting the action of long range conformational changes for the coupling of the catalytic reaction to charge translocation. Furthermore, we have mentioned that the length of the peripheral arm of complex I is intriguing, since in theory a NuoF-like protein could directly interact with NuoB. We then hypothesize that, considering its size, shape and its relative position to the membrane arm the peripheral arm total assembly may be functionally relevant to the coupling mechanism and not merely be a scaffold for the electron transfer wire (which should not be present in Ehr complexes). We suggest that the peripheral arm may work as a *mechanical lever* and as such a small effect at its top may have a large repercussion at its base.

The discussion presented here has also repercussions in terms of the ion translocation machinery including that of the multisubunit Mrp antiporter complexes. The homology between the antiporter-like subunits (NuoL, M and N) of complex I and subunits of the Mrp Na^+/H^+ antiporter complexes, besides the amino acid sequence similarity is now also supported by structural analyses [2,3]. Until recently Mrps were thought to be functional only within a complex [38]. However the standalone Mrp-like protein from *N. thermophilus* was shown to have Na^+/H^+ antiporter activity [66]. The homology model of this Mrp (Fig. 4) highlights the similarity of this protein to NuoL, especially in the case of amino acid residues considered key elements for charge translocation. Furthermore, membrane vesicles containing over-expressed C-terminally truncated NuoL subunit were shown to transport Na^+, which was prevented by an inhibitor of Na^+/H^+ antiporters [67] and complementation studies using *B. subtilis* Mrps deletions strains demonstrated that subunits of complex I could replace those of the Mrp antiporter [45]. These observations strongly suggest that NuoL homologues alone may have Na^+/H^+ antiporter activity. Thus in multisubunit Mrp antiporter complexes the Na^+ transport occurs most probably through MprA and/or D subunits and not at the interface of the subunits as proposed before [2].

5. Conclusions

The understanding of the modularity of complex I, group 4 membrane-bound [NiFe] hydrogenases and Ehr complexes will help to unravel the coupling mechanism between catalytic reactions and ion translocation by this family of proteins. We hypothesize, based on their close relationship that the three types of complexes may have a similar coupling mechanism. Membrane-bound hydrogenases, complex I and complex I-like enzymes have a catalytic centre ([NiFe] or quinone binding site) close to the membrane surface. By contrast some Ehr complexes may perform catalytic reactions distant from the membrane surface. In this case the coupling mechanism should be based on long range conformational changes, and not on possible conformational changes at the catalytic sites close to the membrane surface. This could also provide hints for an explanation for the need of a long peripheral arm in complex I.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2012.09.012.

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