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The antiporter-like subunit constituent of the universal adaptor of complex I, group 4 membrane-bound [NiFe]-hydrogenases and related complexes

Abstract: We have recently investigated the long-recognized relationship between complex I and group 4 [NiFe] hydrogenases and we have established the so-called Energy-converting hydrogenase related (Ehr) complex as a new member of the family. We have also observed that four subunits, homologues to NuoB, D, H and L, are common to the members of the family. We have designated this common group of subunits the universal adaptor. Taking into account the similarity of the Na^+/H^+ antiporter-like subunits of complex I (NuoL, NuoM and NuoN) and the unique structural characteristic of the long amphipathic α helix part of NuoL, the nature of the antiporter-like subunit of the universal adaptor was questioned. Thus, in this work we further explore the properties of the universal adaptor, investigating which antiporter-like subunit is part of the universal adaptor. We observe that the universal adaptor contains an antiporter-like subunit with a long amphipathic α helix, similar to NuoL. Consequently, the long helix is a common denominator that has been conserved in all members of the family. Such conservation surely reflects the key role of such helix in the energy transduction mechanism of this family of enzymes.

Keywords: complex I; hydrogenase-3; long amphipathic α-helix (LH); Mrp Na^+/H^+ antiporters; NADH:quinone oxidoreductase; NuoL.

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Introduction

Complex I (E.C.1.6.5.3) of respiratory chains transduces the energy released by NADH:quinone oxidoreduction to the difference of an electrochemical potential by translocating ions across the membrane. The dissipation of the potential is used for the synthesis of ATP, solute transport across the membrane or motility. Complex I is an L-shaped membrane protein with a membrane arm and a peripheral one facing the cytoplasm (Figure 1A-a). In bacteria, it is generally composed of 14 subunits, NADH:ubiquinone oxidoreductase (Nuo)A to N or NADH:quinone oxidoreductase (Nqo)1 to 14, whereas the mitochondrial enzyme consists of more than 40 subunits. The electron donor of several bacterial and mitochondrial complexes is NADH, while some archaeal complex I-like enzymes are $F_{420}H_2$ dehydrogenases. The oxidoreduction occurs at the peripheral arm, which consists of seven subunits. NADH is oxidized at the subunit containing an Flavin mononucleotide, which is localized at the top of the arm. Electrons then flow through an electrical wire constituted by seven iron-sulfur clusters, to the quinone-binding site located at the base of the peripheral arm, where this is in contact with the membrane arm. Ion translocation takes place at the membrane arm, which is also composed of seven subunits displayed in a linear arrangement along the membrane (Figure 1A-a) (Efremov and Sazanov, 2011). Three of such subunits are homologues of the so-called Mrp Na^+/H^+ antiporters (Mathiesen and Hagerhall, 2002). These subunits are most probably involved in ion translocation and are located at the opposite end of the arm in relation to its contact to the peripheral arm. For these reasons, a long-range communication between the base of the peripheral arm and the opposite end of the membrane arm has to be present. The crystal structure revealed the presence of a long amphipathic α helix (LH) that transverses the membrane arm over the three antiporter-like subunits, covering a distance of approximately 100 Å and which may accomplish such communication (Efremov and Sazanov, 2011). This long helix is part of the C-terminal of subunit NuoL (Efremov and Sazanov, 2011).

A close relationship between the so-called group 4 [NiFe] hydrogenases and complex I has long been recognized. Group 4 hydrogenases are membrane-bound enzymes

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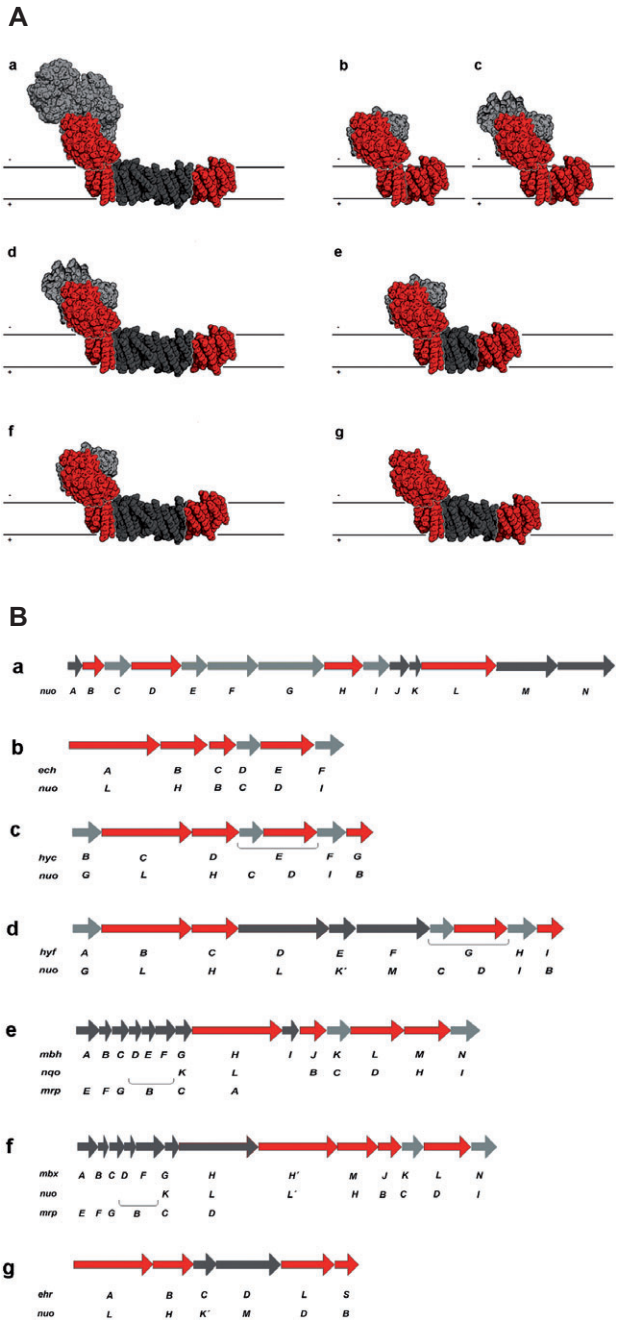

Figure 1 (A) Schematic representation of the protein structure of the chosen representatives of complex I, group 4 [NiFe] hydrogenases and energy-converting hydrogenase-related complexes (Ehr) family. The common subunits are colored in red. We termed this common denominator the Universal Adaptor. The representatives are: (a) complex I from *Escherichia coli*, (b) Ech from *Methanosarcina mazei*, (c) Hyc and (d) Hyf from *E. coli*, (e) Mbh and (f) Mbx from *Pyrococcus furiosus* and (g) Ehr from *M. mazei*. (B) Schematic representation of the gene cluster organizations coding for the chosen representatives of the complex I, group 4 [NiFe] hydrogenases and energy-converting hydrogenase related complexes (Ehr) family. Complex I from (a), *E. coli*, (b) Ech from *M. mazei*, (c) Hyc and (d) Hyf from *E. coli*, (e) Mbh and (f) Mbx from *P. furiosus* and (g) Ehr from *M. mazei*.

that receive electrons from cytoplasmic donors and reduce protons to hydrogen, thus being named hydrogen-evolving hydrogenases (Vignais and Billoud, 2007). The simplest known group 4 hydrogenases are the energy-converting hydrogenases (Ech), such as those purified from *Methanosarcina barkeri* and *Thermoanaerobacter tengcongensis* (Meuer et al., 1999; Soboh et al., 2004). They contain six subunits, EchA to F (Figure 1A-b), with the membrane subunits EchA and EchB being homologous to the antiporter-like subunits and NuoH, respectively, and the peripheral subunits EchC, D, E and F (also oriented toward the cytoplasm as in complex I) to subunits NuoB, C, D and I, respectively. The catalytic [NiFe] site is present in the subunit homologous to NuoD. These hydrogenases may receive electrons from a ferredoxin or be associated with other proteins, such as a formate dehydrogenase or a CO dehydrogenase, receiving electrons from the respective substrates (Vignais and Billoud, 2007; Marreiros et al., 2013).

We have recently made a thorough investigation of complex I and group 4 [NiFe] hydrogenases and unequivocally established a third member of this family of proteins: the energy-converting hydrogenase related complexes, Ehrs (Marreiros et al., 2013). These complexes are similar to the hydrogenases but lack the [NiFe]-binding site (Figure 1A-f and 1A-g) (Coppi, 2005; Marreiros et al., 2013). We have also observed that four subunits, homologous to NuoB, D, H and L, are common to the three types of complexes (Figure 1A). We have designated this common denominator the universal adaptor (Marreiros et al., 2013).

In this work we further explore the properties of the universal adaptor, investigating which antiporter-like subunit is part of it. As pointed out above, complex I has three homologous antiporter-like subunits, which are structurally similar (Efremov and Sazanov, 2011). Apart from the C-terminus of NuoL, the structures of those three subunits are superimposable, with 14 conserved helices containing two inverted repeats formed by transmembrane helices (TM) 4–8 and 9–13. Each repeat has a discontinuous helix where a lysine or a glutamate residue is present. NuoL has two additional transmembrane helices at the C-terminus that are linked by the long helix (Efremov and Sazanov, 2011). Accordingly, the amino acid sequence of NuoL is approximately 100 and 150 residues longer than those of NuoM and NuoN, respectively. As mentioned before, the long helix crosses the membrane arm over the three antiporter-like subunits. Not all members of the complex I, group 4 [NiFe] hydrogenases and Ehr family contain three antiporter subunits; for example, Ech contains only one such subunit. This may lead to us questioning the presence of the long helix and, as a consequence, the nature of the antiporter-like subunit present in the common denominator.

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Results

We have recently investigated the long-recognized relationship between complex I and group 4 [NiFe] hydrogenases and have established the so-called Ehr complexes as a new member of the family (Marreiros et al., 2013). The members of complex I, group 4 [NiFe] hydrogenases and the Ehr family contain what we have nominated the universal adaptor. This consists of four subunits, two of which are peripheral and homologous to NuoB and D, and the others are transmembrane proteins homologous to NuoH and to a Na^+/H^+ antiporter-like protein, which we considered to be NuoL. However, taking into account the similarity among the Na^+/H^+ antiporter-like subunits, NuoL, NuoM and NuoN and the unique structural characteristic of the long amphipathic α helix part of NuoL, the nature of the antiporter-like subunit (whether NuoL, NuoM or NuoN) present at the universal adaptor was questioned.

Sequence analyses reveal two types of Na^+/H^+ antiporter-like subunits

As representatives of the complex I, group 4 [NiFe] hydrogenases and Ehr family, we have chosen the complexes schematized in Figure 1A whose coding gene clusters are drawn in Figure 1B. To represent complex I, we chose that from *Escherichia coli* (Figure 1A-a and 1B-a), which is the best characterized and whose X-ray crystal structure has been determined (Efremov and Sazanov, 2011). The choice of the representatives of [NiFe] membrane-bound hydrogenases also took into account the available genetic and biochemical data. The simplest hydrogenase selected was that from *Methanosarcina mazei* (Figure 1A-b and 1B-b), which has already been isolated and functionally investigated (Welte et al., 2010). The so-called Hyc hydrogenase from *E. coli* (Figure 1A-c and 1B-c) has been characterized as a formate hydrogen lyase 1 (FHL-1). Besides the subunits composing Ech, this complex has an iron-sulfur subunit homologous to the N-terminal of NuoG and a formate dehydrogenase subunit (Fdh-F) that receives electrons from formate (Hedderich and Forzi, 2005). *Escherichia coli* contains another group 4 hydrogenase (Hyf) encoded by the *hyf* gene cluster (Figure 1A-d and 1B-d). This gene cluster is similar to *hyc*, but includes three additional genes (Andrews et al., 1997). 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 (Andrews et al., 1997; Efremov and Sazanov, 2012). The electron donor to this hydrogenase is still unknown. The hydrogenase from *Pyrococcus furiosus*, Mbh, has been intensively studied and is predicted to be composed of 13 subunits, having four to six subunits homologous to complex I (Bridger et al., 2011) (Figure 1A-e and 1B-e). Interestingly, we observed that in the *mbh* cluster, the gene following that encoding MbhH (*mbhI*) codes for a hypothetical protein that is homologous to the C-terminus of NuoL. In this way (and supported by the following analyses) we considered the antiporter-like subunit of Mbh the product of the *mbhH* plus *mbhI* genes. *P. furiosus* also contains a membrane complex similar to membrane-bound [NiFe] hydrogenases, but does not have the motifs for the binding of the [NiFe] center, like Ehrs. The complex, named Mbx (Figure 1A-f and 1B-f) has been genetically characterized and was suggested to function as ferredoxin:$NADP^+$ oxidoreductase (Bridger et al., 2011).

Although no biochemical data are available, in our list of representatives of the family we also included the Ehr complex from *M. mazei* (Figure 1A-g and 1B-g), because this is one of the simplest hydrogenase-like complexes, which does not contain the [NiFe] binding motifs (Marreiros et al., 2013).

Using clustalX we aligned the sequences of all Na^+/H^+ antiporter-like subunits of the chosen representatives of the complex I, group 4 [NiFe] hydrogenases and Ehr family (Figure 2). The alignment showed immediately that the sequences could establish two groups according to their average sizes, with one group of sequences having a C-terminus that was approximately 100 amino acid residues longer. The similarity and identity values obtained (see Supplementary Material, Table 1) do not allow us to unequivocally establish the identity of proteins such as the antiporter-like subunits from hydrogenases or Ehr as homologuess of NuoL, NuoM or NuoN. Nevertheless, we can consider that the antiporter-like subunit common to all representative complexes, and thus part of the universal adaptor, has a sequence as long as NuoL.

Moreover, we also aligned all known sequences from EchA, HycC, HyfB, MbhH plus MbhI, MbxH' and EhrA (not shown) and observed that all of them have a C-terminal extension. This observation indicated that the conclusion of the analyses performed for each representative may be generalized to the respective group of proteins.

Hydropathy profiles and secondary structure predictions of Na^+/H^+ antiporter-like subunits with C-terminal extension

In order to investigate whether the proteins with longer sequences have similar properties, specifically at their

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Figure 2 Alignment of the amino acid sequences from the Na^+/H^+ antiporter-like subunits present in the chosen representatives of complex I, group 4 membrane-bound [NiFe] hydrogenases and Ehr family.

The alignment was performed using clustalX. Glutamate residues corresponding to that at the middle of the TM helix 5 of NuoL (Glu^{144}–NuoL) and lysine residues corresponding to those at the middle of the discontinuous helices TM7 and TM12 of NuoL (Lys^{229}, Lys^{399}–NuoL), thought to be important in charge translocation, are highlighted in the black background. The transmembrane α helices observed in the structure of NuoL are shaded in light gray and the long amphipathic α helix is shaded in medium gray.

C-terminal extensions, we calculated their hydropathy profiles and predicted their secondary structures (Supplementary Figures 1 and 2). We used different algorithms for hydropathy profiling (Supplementary Figure 1). All proteins are predicted to have between 15 and 18 transmembrane regions, but most interesting is the observation that in all cases there is a significant non transmembrane stretch, composed of 46–102 amino acid residues (Table 1), between the two transmembrane regions closer to the C-terminus. This stretch in NuoL, containing 77 amino acid residues, corresponds to the long amphipathic α helix. The presence of such a helix in the other members of the family is supported by predictions of their secondary structure (Supplementary Figure 2). These indicate that the representative proteins may have several transmembrane helices, but most relevant is the prediction of an α helical structure for the non transmembrane stretch present between the two transmembrane regions closer to the C-terminus. Moreover, we specifically analyzed the hydropathy of these long α helices and we observed an amphipathic character for all representatives, such as that observed for NuoL (not shown). We can conclude that the antiporter-like subunits analyzed, with extended C-terminus, present similar hydropathy profiles and secondary structure predictions to that of NuoL.

A long amphipathic α helix is present in all Na^+/H^+ antiporter-like subunits with C-terminal extension

A possible existence of a LH in the common antiporter-like subunit present in the representatives of complex I, hydrogenases and Ehr is consistent with the respective sequence lengths, hydropathy profiles and secondary structure predictions. In order to further investigate this possibility,

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Table 1 Characteristics of the long amphipathic α helix predicted to be present in the Na^+/H^+ antiporter-like subunits with C-terminal extension.

	Long α-helix	
	Number of amino acid residues	Length (Å)[a]
NuoL	77	115.5
EchA	46	69
HycC	51	76.5
HyfB	101	151.5
MbhH+MbhI	66	99
MbxH′	72	108
EhrA	102	153

[a]The α-helix length was estimated based on the number of amino acid residues present between the two transmembrane regions at the C-terminus of each sequence and considering that one α helix turn contains 3.6 residues corresponding to an average length of 5.4 Å.

we have calculated homology structural models for all the representatives (Figure 3). Table 2 summarizes the properties of the models obtained. All models present the first 15 TMs of NuoL. EchA has two stretches of 36 and 15 amino acid residues, between NuoL's TM2 and TM3 and TM14 and TM15, respectively, which were not modeled. The presence of these extra amino acid segments seems not to alter the two inverted repeats arrangement present in all antiporter-like subunits. Although presenting different lengths, LH was also modeled in all cases. With the exception of EchA and HycC, all the other models showed the presence of a 16th transmembrane helix at the C-terminus. The last 41 amino acid residues from EchA could not be modeled, but its secondary structure prediction suggests that these constitute an α helix. Thus, the presence of a 16th transmembrane helix at the C-terminus of EchA is possible. A similar situation occurs for HycC, although in this case the presence of the C-terminal α helix is not so clear.

We have performed a rough estimation of the length of the LH, based on the number of amino acid residues present between the two transmembrane regions at the C-terminus of each sequence and considering that one α helix turn contains 3.6 residues corresponding to an average length of 5.4 Å (Table 1). The estimated length for LH of NuoL, 115.5 Å, is in accordance to that obtained by the crystal structure of 110 Å (Efremov and Sazanov, 2011). The representative complexes chosen contain different numbers of antiporter-like subunits. Ech, Hyc and Mbh have one antiporter-like subunit, Ehr and Mbx may

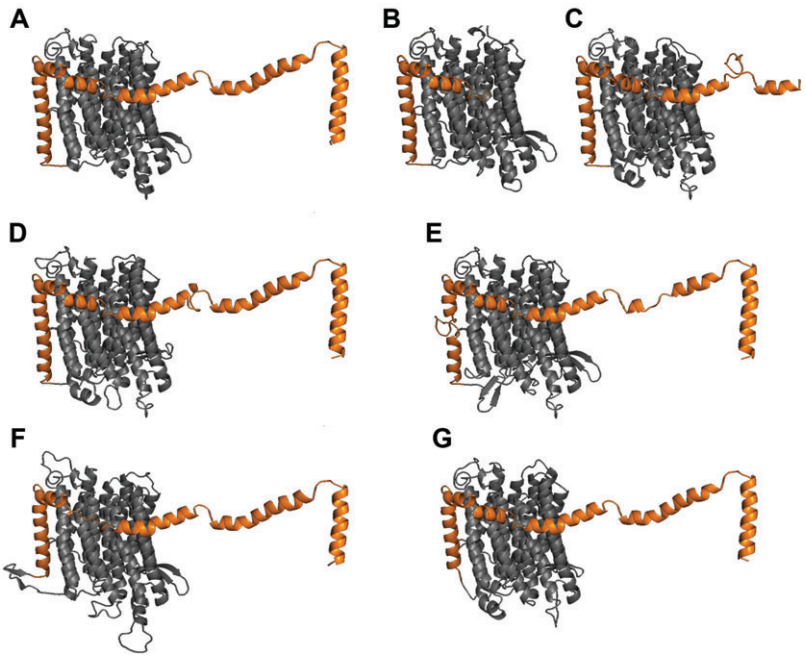

Figure 3 Structure of the NuoL Na^+/H^+ antiporter like subunit from *Escherichia coli* and homology models of the Na^+/H^+ antiporter-like subunits with extended C-terminus present in the chosen representatives of group 4 [NiFe] membrane-bound hydrogenases and Ehr family. The models represented were obtained using Phyre[2]. Side view, cytoplasmic side up: (a) NuoL from *E. coli,* (b) EchA from *Methanosarcina mazei*, (c) HycC and (d) HyfB from *E. coli*, (e) MbhH + MbhI and (f) MbxH′ from *Pyrococcus furiosus* and (g) EhrA from *M. mazei*. The proteins are colored in gray with their C-termini, including two transmembrene helices connected by a long amphipathic α helix highlighted in orange.

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Table 2 Properties of the structural models obtained of the Na^+/H^+ antiporter-like subunits with C-terminal extension.

	Protein			Homology model			Not modeled	
Name	Amino acid residues	Template	RMSD[a]	Amino acid residues	Transmembrane helices	(%)	Amino acid residues	Amino acid residues at C-terminus[b]
EchA	638	3RKO:L	1.05	534	15	84	104	41
HycC	608	3RKO:L	1.49	546	15	90	62	24
HyfB	672	3RKO:L	1.89	596	16	89	76	21
MbhH+MbhI	628[c]	3RKO:L	1.48	600	16	96	28	2
MbxH′	618	3RKO:L	1.29	581	16	94	37	3
EhrA	643	3RKO:L	1.90	587	16	91	56	24

[a]RMSD (root-mean-square deviation) of backbone. [b]Amino acid residues after TM15. [c]MbhH (510 aa) + MbhI (118 aa)

have two, and complex I and possibly Hyf contain three. According to the estimations of the length of LHs, EchA and HycC are expected to have the shortest LH and HyfB contains one of the longest. Nevertheless, a correlation between the estimated length of LH and the number of antiporter-like subunits present in each complex is not clear.

Additional conserved elements among all Na^+/H^+ antiporter-like subunits with C-terminal extension

The presence of additional elements connecting the antiporter-like subunits was shown by the crystal structure of complex I. These are β hairpins present between TM helices 2 and 3 and localized close to the opposite side of the membrane to that of LH. The β hairpin from one subunit is in contact with its neighbor via a stretch of a C-terminal amphipathic helix. These structural arrangements were suggested to contribute to the stability of the complex (Efremov and Sazanov, 2011). The crystal structure of the NhaA Na^+/H^+ antiporter from *E. coli* presents similar β hairpins (Hunte et al., 2005), which were suggested by cryo-electron microscopy to be at the dimer interface (Williams, 2000). The NhaA mutant devoid of the β hairpins is functional but the dimeric form is not observed (Rimon et al., 2007).

We also observed that all antiporter-like subunits contain a lysine or glutamate residue at the middle of each discontinuous helix (TM7 and TM12) and at TM helix 5 (Figure 2). These residues were suggested to be important for charge translocation (Efremov and Sazanov, 2011). Interestingly, HycC from *E. coli* seems to be an exception, since at the middle of its TM7 a leucine residue is present (Figure 2). Such substitution may lead us to question the role of lysine/glutamate residues in the middle of such helices or may simply reflect an adaptive event of the organisms that contain such proteins.

Discussion

We have investigated the nature of the antiporter-like subunit common to the representative members of the complex I, hydrogenases and Ehr family. We have observed that this antiporter-like subunit contains a long amphipathic α helix, similar to NuoL. Interestingly, Efremov et al have also suggested the presence of the LH in Hyf and Mrp complexes (Efremov et al., 2010). In this way we consider the antiporter-like subunit present in the universal adaptor to be a homologue of NuoL, even though this is not unequivocally supported by sequence similarities and identities (Supplementary Table 1). The presence of the LH in all complexes analyzed suggests that it is also a common denominator that has been conserved. The conservation in complex I, hydrogenases and Ehr family reflects the key role of LH in the energy transduction mechanism by these complexes.

The role of LH is one of the most frequently discussed topics in the current study of complex I. Based on the first crystallographic data, the LH was suggested to have a functional role in the energy coupling mechanism on complex I. It would work like a piston in order to allow transmission between the base of the peripheral arm, where the quinone is reduced, and the membrane antiporter-like subunits, in which ion translocation is suggested to occur (Efremov and Sazanov, 2011).

The available mutagenesis studies of LH have produced contradictory results (Belevich et al., 2011; Drose et al., 2011; Steimle et al., 2012). Some suggested that LH does not intervene in ion translocation and thus its function should be confined to a structural role that

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is necessary to stabilize the membrane arm (Belevich et al., 2011). However, *E. coli* complex I mutated in an aspartate residue of the LH, which to the NuoM subunit, presented approximately half of the H^+/2e stoichiometry. Thus, it was suggested that the transmission between LH and NuoL and NuoM is hampered (Steimle et al., 2012).

The LH was proposed to work as a long helical transmission element (Drose et al., 2011). Based on the observations that complex I from *Yarrowia lipolytica*, missing two of the antiporter-like subunits (and the LH), was still catalytically active and capable of proton translocation, although at half the stoichiometry of the complete enzyme, Drose et al suggested that complex I has two distinct ion pump modules connected in series by the LH, which would work as a transmission component (Drose et al., 2011).

Most interestingly, the only antiporter-like subunit present in the simplest [NiFe] hydrogenase, EchA, contains a LH, which transverses itself. In this case, a stabilizing role for LH, although not excludable, is unlikely. The compactness of the 14 core TM helices from EchA is predicted to be similar to those of other membrane proteins, including other antiporters, such as NhaA, that do not have a LH. In this way, we support the suggestion that LH should have a functional role, possibly as a transmission element, in agreement with what has been proposed by others (Drose et al., 2011; Steimle et al., 2012).

The presence of the β hairpins and the lysine/glutamate residues at the middle of the discontinuous TM7 and TM12 and at TM5 in addition to the presence of the LH, reinforces the idea that antiporter-like subunits present in the members of the complex I, hydrogenases and Ehr family operate in similar ways.

Conformational changes must occur in complex I to couple the catalytic reaction to charge translocation, whether NuoL, NuoM and NuoN work as Na^+/H^+ antiporters (Mathiesen and Hagerhall, 2002; Steuber, 2003; Stolpe and Friedrich, 2004; Batista et al., 2010, 2011; Batista and Pereira, 2011) or as H^+ channels. In this way, the element(s) responsible for such conformational changes, i.e. which may work as transmission devices, have to be present in the different complexes. The recognition of common structural and functional denominators in the universal adaptor is a step forward in the identification of such elements and consequently in the investigation of the mechanism of complex I.

Materials and methods

Sequence analyses

Amino acid sequences of the antiporter-like subunits were aligned using ClustalX v.2.0. (Larkin et al., 2007). The proteins selected were: NuoL, NuoM, NuoN, HycC, HyfB, HyfD and HyfF from *E. coli* K-12 MG1655 (P33607, P0AFE8, P0AFF0, P16429, P23482, P77416, P77437), EchA, EhrA and EhrD from *M. mazei* (Q8PUL4, Q8PY07, Q8PY04), MbhH plus MbhI, MbxH and MbxH' from *P. furiosus* DSM 3638 (Q8U100 plus Q8U0Z9, Q8U0Y4, Q8U0Y5). The default parameters of ClustalX 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 (Nicholas and Nicholas, 1997).

The secondary structure was predicted by Psi-pred 2.5 (Jones, 1999) using the ALI2D server (http://toolkit.tuebingen.mpg.de/ali2d). Transmembrane helices were predicted using the TMHMM server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). The TopPred 0.01 (Claros and von Heijne, 1994) and TMpred (Hofmann and Stoffel, 1993) programs were also used to make predictions about the transmembrane regions and orientations. The three different approaches gave similar results. Hydrophobic moments of amphipathic helices were predicted by MPex (Snider et al., 2009) and HeliQuest (Gautier et al., 2008). Both approaches yielded similar results.

Structural models

To generate the structural models of the antiporter-like proteins from *E. coli* (HyfB and HycC), *P. furiosus* (MbhH plus MbhI and MbxH) and *M. mazei* (EchA and EhrA), we used their amino acid sequences retrieved from Uniprot (see above). The structural models were predicted by the Protein Homology/analogy Recognition Engine V 2.0 (Phyre2) server (Kelley and Sternberg, 2009), which used the crystallographic structure of NuoL (PDB ID: 3RKO:L) as a template. After sequence alignment by HHpred (Soding, 2005) and using the structure of NuoL as a template, structural models were also predicted by MODELLER (Sali et al., 1995), providing the same result. Protein homology structure images were generated using PyMOL Molecular Graphics System, Version 1.4, Schrödinger, LLC.

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