Minireview

Proton pumping by NADH:ubiquinone oxidoreductase. A redox driven conformational change mechanism?

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Abstract **The modular evolutionary origin of NADH:ubiquinone oxidoreductase (complex I) provides useful insights into its functional organization. Iron–sulfur cluster N2 and the PSST and 49 kDa subunits were identified as key players in ubiquinone reduction and proton pumping. Structural studies indicate that this 'catalytic core' region of complex I is clearly separated from the membrane. Complex I from *Escherichia coli* and *Klebsiella pneumoniae* was shown to pump sodium ions rather than protons. These new insights into structure and function of complex I strongly suggest that proton or sodium pumping in complex I is achieved by conformational energy transfer rather than by a directly linked redox pump.**
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1. Introduction

Complex I (reduced nicotinamide adenine dinucleotide (NADH):ubiquinone oxidoreductase) is the last '*terra incognita*' among the respiratory chain complexes. Despite continuous efforts to understand its structure and function over the last five decades, even fundamental issues remain unsolved. This is in stark contrast to the growing interest in complex I due to its role in the generation of reactive oxygen species [1] and the increasing number of diseases that are caused by or related to complex I defects [2]. For a number of reasons, complex I is much more difficult to study than other respiratory chain complexes: Consisting of up to 45 different subunits and a total mass of almost 1000 kDa, mitochondrial complex I is one of the biggest and most complicated known membrane protein complexes. With 14 subunits and some 500 kDa, the prokaryotic counterpart is still rather big and complex. Moreover, the bacterial enzymes tend to be extremely unstable. So far complex I from *Escherichia coli* and the closely related *Klebsiella pneumoniae* are the only bacterial enzymes that could be purified in intact form [3,4].

Complex I is the only respiratory chain complex for which no X-ray structure is available so far. Iron–sulfur clusters, the prominent prosthetic groups of complex I, have no characteristic spectra in the visible region. Therefore, electron paramagnetic resonance (EPR) spectroscopy at very low temperatures has to be used, but this technique cannot be applied to rapid kinetics and requires large amounts of sample. Also structure/function studies based on the analysis of mutants are scarce for complex I: Even in *E. coli* mutagenesis of complex I is not a trivial task, as all structural genes are expressed and controlled by a single operon. For a long time mitochondrial complex I was studied primarily from bovine heart or from the filamentous fungus *Neurospora crassa*. While genetic manipulation of the mammalian enzyme is virtually impossible, even in *N. crassa* the introduction of site directed mutants is rather tedious. To overcome this limitation we recently introduced *Yarrowia lipolytica* as a model organism to study complex I [5]. For the first time, this strictly aerobic yeast allows efficient genetic manipulation of the nuclear coded subunits of mitochondrial complex I. Moreover a his-tagged version of complex I can be purified rapidly and with high yield from *Y. lipolytica* [6].

Numerous hypothetical mechanisms have been proposed over the years (see [7] for an overview). Here we compile the available evidence on structure and function of complex I and use the resulting constraints to narrow in on the components of the proton/sodium pumping machinery and the way they may operate.

2. Subunit composition and evolutionary origin

Eukaryotic complex I contains a total number of more than 35 subunits in fungi [8] and at least 45 subunits in mammals [9,10]. 14 of these subunits (Table 1) are also present in the minimal forms of complex I found in bacteria like *E. coli*, *Thermus thermophilus*, *Paracoccus denitrificans* and *Rhodobacter capsulatus* [11]. In eukaryotes, seven of the 14 'central' subunits are nuclear coded and contain all known redox prosthetic groups, namely one molecule of flavin adenine mononucleotide (FMN) and eight to nine iron–sulfur clusters (see below). The remaining seven 'ND' subunits are highly hydrophobic proteins with several putative transmembrane helices and are encoded by the mitochondrial genome in most eu-

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Table 1
Central subunits of complex I and homologies to subunits of other bacterial enzymes

Complex I subunit symbol			Redox prosthetic groups	Fragments or sub-complexes		Homologous subunits in related enzymes				
Bovine	*Y. lipolytica*	*E. coli*		*E. coli*	Bovine	NAD$^+$ reducing hydrogenase e.g. *A. eutrophus*/formate dehydrogenase *M. formicicum*	Water soluble [NiFe] hydrogenase e.g. *D. fructosovorans*	Membrane bound type-3 hydrogenase (*FHL-1*) *E. coli*/(Ech) *M. barkeri*	Membrane bound type-4 hydrogenase (*FHL-2*) *E. coli*	Antiporter e.g. *B. subtilis*
75 kDa	NUAM	NuoG	N1b, N1c, N4, N5	DF	Iλ	HoxU/FdhA	–	–	–	–
51 kDa	NUBM	NuoF	FMN, N3	DF	Iλ, FP	HoxF	–	–	–	–
49 kDa	NUCM	NuoD[a]	–	CF	Iλ	–	large subunit	EchE/HycE	HyfG	–
30 kDa	NUGM	NuoC[a]	–	CF	Iλ	–	–	EchD/HycE	HyfG	–
24 kDa	NUHM	NuoE	N1a	DF	Iλ, FP	HoxF	–	–	–	–
TYKY	NUIM	NuoI	N6a, N6b	HF	Iλ	–	–	EchF/HycF	HyfH	–
PSST	NUKM	NuoB	N2	HF	Iλ	–	small subunit	EchC/HycG	HyfI	–
ND1	ND1	NuoH	–	MF	Iγ	–	–	EchB/HycD	HyfC	–
ND2	ND2	NuoN	–	MF	Iγ	–	–	EchA[b]/HycC[b]	HyfB,D,F[b]	MrpD[b]
ND3	ND3	NuoA	–	MF	Iγ	–	–	–	–	–
ND4	ND4	NuoM	–	MF	Iβ	–	–	EchA[b]/HycC[b]	HyfB,D,F[b]	MrpD[b]
ND4L	ND4L	NuoK	–	MF	Iγ	–	–	–	HyfE?	–
ND5	ND5	NuoL	–	MF	Iβ	–	–	EchA[b]/HycC[b]	HyfB,D,F[b]	MrpA[b]
ND6	ND6	NuoJ	–	MF	–	–	–	–	–	–

Abbreviations: DF, dehydrogenase fragment; CF, connecting fragment; MF, membrane fragment; FP, flavoprotein.

[a] In *E. coli* both subunits are fused (NuoCD).

[b] The ND2, 4 and 5 subunits are weakly homologous to each other, an assignment of the individual subunits to other proteins is ambiguous.

karyotes (Table 1). Very little is known about the function of the remaining up to 31 'accessory' subunits.

It has been proposed that complex I was assembled from preexisting modules during evolution [12,13] and it can be expected that these modules form structural units in complex I (Table 1). The homology to hydrogenases has been especially useful for the understanding of complex I: The 49 kDa subunit and the PSST subunit are homologous to the large and small subunits of soluble [NiFe] hydrogenases. Membrane bound type-3 hydrogenases like the enzyme encoded by the *hyc* operon in *E. coli* or the *ech* operon in *Methanosarcina barkeri* contain additional proteins that are homologous to complex I subunits. In addition to the 49 kDa and PSST subunits, these are the 30 kDa, TYKY, ND1 subunits and one more hydrophobic subunit which could be homologous to either ND2, ND4 or ND5. The latter three proteins are weakly related to each other and show sequence similarities to Na^+/H^+ antiporters of the type encoded by the *mrp* operon in *Bacillus subtilis* and the corresponding *mnh* operon in *Staphylococcus aureus* [14,15].

In *E. coli* another type of hydrogenase was described that is related to complex I: Type-4 hydrogenase is encoded by the *hyf* operon [16] and contains the same homologs to complex I genes already found in type-3 hydrogenases. However, it comprises two more proteins of the Na^+/H^+ or K^+/H^+ antiporter, ND2/ND4/ND5 superfamily and a hydrophobic protein that exhibits some similarity to ND4L in its C-terminal half [13]. It is remarkable that only formate hydrogenlyase 2, the combination of formate dehydrogenase and type-4 hydrogenase is driven by an electrochemical proton gradient, while formate hydrogenlyase 1, the combination of formate dehydrogenase and type-3 hydrogenase is not [17].

The electron input domain of complex I is related to the oxidized nicotinamide adenine dinucleotide (NAD^+) reducing hydrogenase from *Alcaligenes eutrophus* [18]. The 24 and 51 kDa subunits are homologous to HoxF and the first 200 residues of the 75 kDa subunit are homologous to HoxU. HoxF and HoxU together form the NADH oxidoreductase part of the enzyme. It was suggested that the 75 kDa subunit is a fusion of two proteins of different origin because the C-terminal half shows sequence similarity to a formate dehydrogenase from *Methanobacterium formicicum* [13].

There are two more gene fusions in complex I and related enzymes that further support the view of a modular origin of complex I. In complex I from *E. coli* and in the corresponding subunits of the *hyc* and *hyf* operon the C-terminus of the 30 kDa subunit is fused with the N-terminus of the 49 kDa subunit. In the F_{420} reducing hydrogenase from *Archaeoglobus fulgidus* the subunits corresponding to the PSST and 30 kDa subunits are fused [19].

3. Redox groups

Complex I contains one non-covalently bound FMN and various iron–sulfur clusters as redox active groups [20,21] (Table 1). FMN (midpoint potential at pH 7.5 ($E_{m,7.5}$) = −336 mV) is the entry point for electrons from NADH. Due to the relative stability of the semiflavin ($K_{stab} = 3.4 \times 10^{-2}$ at pH 7) FMN functions as electron converter between the $n = 2$ electron donor NADH and the $n = 1$ electron transferring iron–sulfur clusters [22]. EPR spectroscopy of the flavin radical generated by reduction of complex I revealed an un-

usually broad line width of 2.4 mT and large spin relaxation enhancement. Both effects are explained by strong spin–spin interaction of the semiflavin with iron–sulfur cluster N3 which shows a concomitant broadening of its EPR spectrum [22]. In line with this interpretation, disruption of the gene encoding the NADH binding 51 kDa subunit in *N. crassa* resulted in the loss of FMN and iron–sulfur cluster N3 [23].

Depending on the origin of the enzyme, different numbers of iron–sulfur clusters have been identified. In the reduced form, these clusters possess paramagnetic $S = 1/2$ ground states. At very low temperatures, this property allows application of EPR spectroscopy, the main experimental approach to study the iron–sulfur clusters of complex I. The well-characterized enzyme of bovine heart mitochondria contains six EPR detectable iron–sulfur clusters designated N1a and N1b, N2, N3, N4, N5 according to their increasing spin relaxation rates [20]. In *E. coli* complex I eight clusters were identified and designated N1a, N1b, N1c, N2, N3, N4, N6a, and N6b [3,24,25]. In the yeast *Y. lipolytica* five clusters, N1, N2, N3, N4, N5 [8] and in *N. crassa* only four clusters, N1, N2, N3, N4 [26] could be identified by EPR spectroscopy so far. Binuclear (Fe_2S_2) and tetranuclear (Fe_4S_4) iron–sulfur clusters were found in complex I: Owing to their slower spin relaxation rates Fe_2S_2 clusters can be detected at somewhat higher temperatures (> 30 K) than Fe_4S_4 clusters (< 20 K).

3.1. Fe_2S_2 clusters

N1a is bound to the 24 kDa subunit [27,28]. In bovine complex I, this cluster has the lowest redox midpoint potential ($E_{m,7} = −370$ mV) and exhibits a pH dependence of −60 mV/pH [29,30]. Analysis of the isolated 24 kDa subunit from bovine mitochondria and different bacteria by protein-film voltammetry revealed that at low ionic strength the reduction potential changes only by ∼100 mV between pH 5 and 9 [31]. This pH dependence resulted from pH linked changes in protein charge, rather than from coupling to a specific ionizable residue. Although the 24 kDa subunit is present and the presumed liganding residues are conserved in *Y. lipolytica* and *N. crassa*, N1a is not detectable in complex I from these organisms by EPR spectroscopy. An extremely negative redox potential preventing reduction by NADH or an unusual spin state or magnetic interaction could render iron–sulfur cluster N1a EPR silent in these organisms. Cluster N1b could be assigned to the 75 kDa subunit [28]. According to its $E_{m,7}$ of about −250 mV it is, together with N3, N4, N5, one of the so called isopotential iron–sulfur clusters [20,30].

A third binuclear cluster, N1c, was first described for complex I from *E. coli* [3]. The 75 kDa subunit of *E. coli* contains an additional unique cysteine binding motif which seems to bind iron–sulfur cluster N1c [24]. This motif is also present in *T. thermophilus* [32]. Overexpression of this subunit, reconstitution and spectroscopic characterization revealed that this extra binding motif most likely harbors a Fe_4S_4 rather than a Fe_2S_2 cluster [33].

3.2. Fe_4S_4 clusters

Iron–sulfur cluster N2 has very distinct properties and therefore has been singled out from the other 'isopotential' clusters. There has been an intense controversy in recent years about the question which subunit ligates iron–sulfur cluster N2. While Albracht and colleagues still consider the TYKY subunit as the most likely candidate [34], there is now good

evidence suggesting that cluster N2 is bound to the PSST subunit [35–37] and resides at the interface between the PSST and the 49 kDa subunits [38] (see below). Because of its relatively high redox midpoint potential ($E_{m,7} = -150$ mV) and an EPR detectable magnetic interaction with semiquinone (SQ) radicals (see below) it is generally assumed that this redox center is the immediate electron donor for ubiquinone [20,39].

A redox midpoint potential dependence of -60 mV/pH unit around neutral pH values reported for bovine complex I [29] has been considered as an indication that cluster N2 may be directly involved in the proton translocation mechanism [7]. However, this attractive option seems unlikely now: For *Y. lipolytica* complex I we have determined a pK_{ox} of ~ 6 and a pK_{red} of ~ 7 for the protonable group associated with iron–sulfur cluster N2. Around neutral pH this results in a slope for the pH dependent redox midpoint potential change of less than 40 mV/pH (Zwicker et al., in preparation). As already mentioned, cluster N3 is located in the 51 kDa subunit [23,40] forming an electron input device together with FMN.

Cluster N4 resides in the 75 kDa subunit [28]. In addition to the binding motifs for clusters N1b and N4 there is a third motif in this subunit which has been proposed to ligate an additional tetranuclear cluster, N5 [20,30]. So far this cluster could only be detected in complex I from bovine heart mitochondria, *R. sphaeroides* [30] and *Y. lipolytica* [8]. Cluster N5 has a very high spin relaxation rate and a low spin concentration which makes EPR spectroscopic analysis rather difficult. A very low redox potential or magnetic interaction with another paramagnetic center may be the reason for the apparent sub-stoichiometric spin concentration of cluster N5.

Two conserved ferredoxin type binding motifs for iron–sulfur clusters could be identified in the sequence of the TYKY subunit. It was shown by ultraviolet visible (UV/Vis) spectroscopy that TYKY contains two additional Fe_4S_4 clusters that are not detectable by standard EPR spectroscopy. These clusters have been named N6a and N6b and seem to be arranged like in 8Fe-ferredoxins [35,41]. Although alternate stoichiometries have been proposed [42], binding motifs, protein chemical characterization, cofactor content and spectroscopic data overall strongly suggest that complex I contains one of each iron–sulfur cluster per FMN [3,5,20,26].

4. Semiquinones

During steady state NADH oxidation ubisemiquinone radicals could be identified in submitochondrial particles (SMP) from bovine heart by various groups using EPR spectroscopic approaches [20,43–46]. T. Ohnishi and coworkers identified three types of SQ species (SQ_{Nf}, SQ_{slow}, SQ_{Nx}) that contribute to the low temperature EPR signals (40 K, $g = 2.004$) in tightly coupled SMP. These differ in their spin relaxation properties, electrochemical potential difference for protons ($\Delta\mu_{H^+}$) dependence, temperature dependence and inhibitor sensitivity [47]. The fast relaxing SQ_{Nf} is only detectable in tightly coupled SMP (respiratory control ratio > 5) and is sensitive to piericidin A and rotenone. In contrast, SQ_{slow} is also detectable in uncoupled SMP and its rotenone sensitivity is less pronounced. However, its piericidin A sensitivity is the same as for SQ_{Nf}.

The unusual temperature dependence and the high spin relaxation rate of the SQ_{Nf} EPR signal indicate a magnetic interaction with a nearby paramagnetic center. A possible candidate is cluster N2. Its EPR signal shows splitting, or at least significant broadening, in the g_z region under conditions generating the SQ_{Nf} radical. Assuming a dipole–dipole interaction, a distance of 8–11 Å was calculated between cluster N2 and SQ_{Nf} [44]. Nearly the same distance (11 Å) was obtained by simulating the enhancement of the half saturation parameter of SQ_{Nf} based on the assumption that this effect results from an interaction with cluster N2 [47]. The EPR signal arising from SQ_{slow} follows the Curie law suggesting that this radical is at least 30 Å away from any other paramagnetic center. The third radical species, SQ_{Nx}, which also exhibits $\Delta\mu_{H^+}$ independence, features a very low spin relaxation rate and contributes to about 35% of the total free radical signal (SQ_{Nf}: 50%, SQ_{slow}: 15%) in coupled SMP [47]. This radical was not yet characterized in more detail. So far, complex I associated SQ were observed only in SMP from bovine heart and not in membrane preparations from bacteria or fungi.

5. Inhibitors

More than 60 different families of compounds (of natural and synthetic origin) are known to inhibit complex I [48,49]. Insect and fish mitochondria are particularly sensitive to complex I inhibition, which explains the traditional use of rotenone derivatives as fish and insect poison. Another important and highly specific group of natural complex I inhibitors are the piericidins. A number of synthetic insecticides/acaricides have complex I as their target [50] and can be grouped into two main classes: (i) pyrazoles and substituted pyrimidines, (ii) pyridines and quinazolines. Prominent examples of these two classes are fenpyroximate and DQA (2-decyl-4-quinazolinyl amine, formerly known as SAN 548A), respectively.

Most complex I inhibitors are hydrophobic or amphipathic compounds. Therefore, it was inferred that many of them may act as ubiquinone antagonists. Kinetic studies suggested that these inhibitors can be grouped into three classes represented by piericidin A and DQA (class I/A-type), rotenone (class II/B-type) and capsaicin (C-type) [51,52]. The demonstration of two independent binding sites for hydrophobic inhibitors had been a key experiment to demonstrate that a proton motive Q-cycle was operating in the cytochrome bc_1 complex [53]. Therefore, the three classes of complex I inhibitors stimulated discussions whether the pumping mechanism in complex I may be based on a reversed version of the Q-cycle and several hypothetical mechanistic schemes were proposed [7,54]. However, direct competition experiments with inhibitors from different classes revealed that they all share one common binding domain with partially overlapping sites [55]. In agreement with this observation, inhibitor resistant mutants of complex I in *R. capsulatus* [56,57] and *Y. lipolytica* [58] exhibit cross resistance between class I/A-type and class II/B-type inhibitors. The emerging picture is that one large amphipathic binding pocket accepts a plethora of chemically different compounds including even polyoxyethylene type detergents like Triton X-100 and Thesit [21]. Although this has not been demonstrated directly, it seems very likely that this pocket also comprises the ubiquinone binding site(s) of complex I.

Domains from several subunits seem to form this binding pocket. Inhibitor resistant mutants were found primarily in
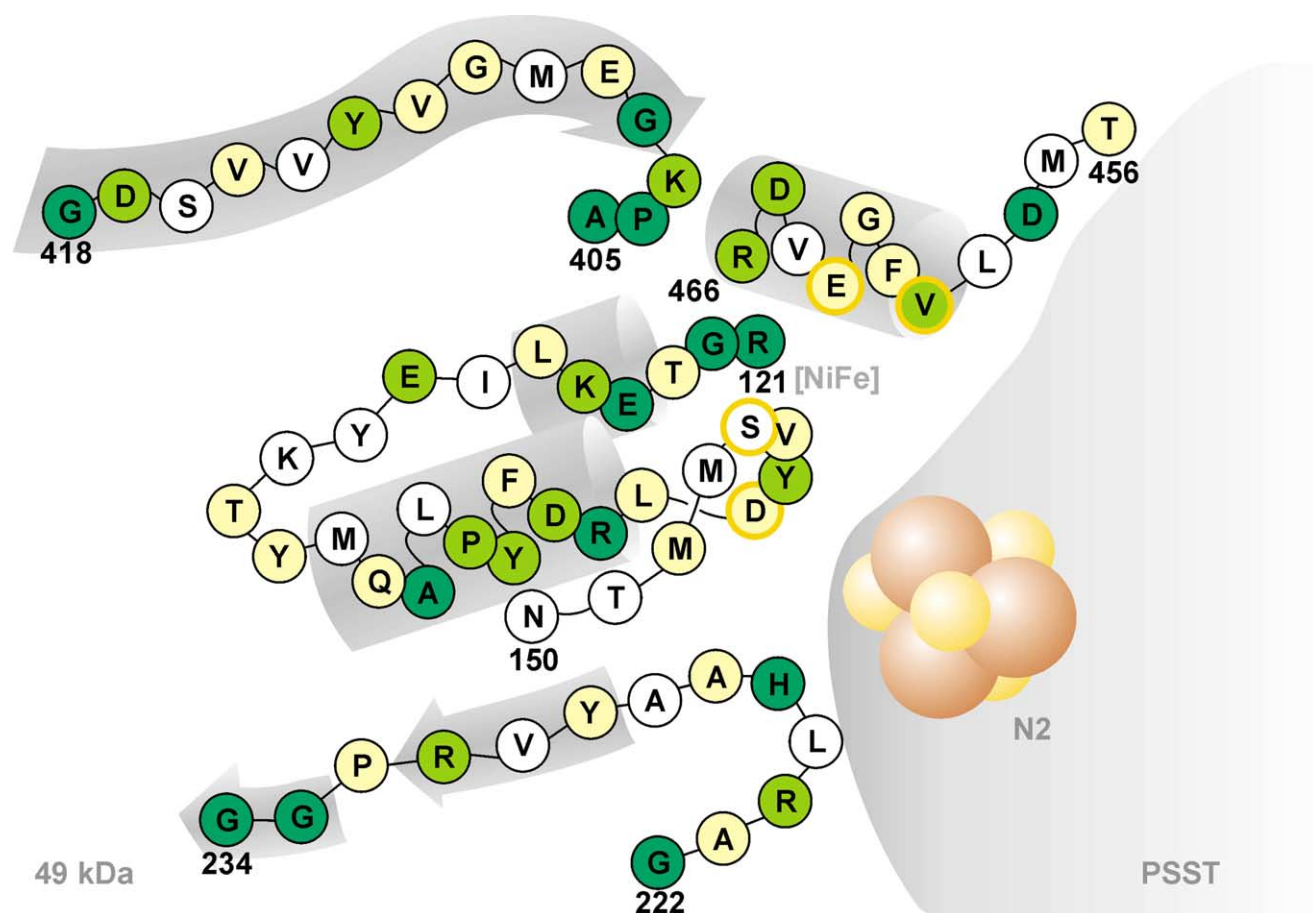
Fig. 1. Schematic view of conserved residues in the catalytic core region of complex I. The amino acid stretches from the 49 kDa subunit of *Y. lipolytica* complex I corresponding to the four loops that surround the [NiFe] site in water soluble hydrogenases are shown. Secondary structure elements and positions of metal ions were taken from the structure of the *D. fructosovorans* enzyme (PBD file 1FRF). Positions that are identical in complex I from *Y. lipolytica*, *N. crassa*, *B. taurus*, *R. capsulatus* are marked in light yellow, residues that are in addition conserved in *E. coli* are in light green, residues conserved between *Y. lipolytica* complex I and the *D. fructosovorans* hydrogenase are marked in dark green. Residues that correspond to the cysteine ligands in the hydrogenase are circled in orange. Iron–sulfur cluster N2 and the surface of the NUKM subunit (the PSST homolog) are indicated.

the 49 kDa subunit [56–58], but some point mutations in the PSST homologous subunit of *Y. lipolytica* exhibit altered inhibitor sensitivities as well [37]. Inhibitor binding to PSST was also demonstrated by photoaffinity labeling with a pyridaben derivative [59]. In this study rather unspecific labeling of the ND1 subunit was observed as well, like in earlier photoaffinity labeling studies with a rotenone derivative [60]. Very recently, a photoaffinity analog of fenpyroximate was reported to bind covalently to the ND5 subunit of complex I from bovine heart mitochondria [61]. Remarkably, pathogenic mutations were not only identified in mitochondrially coded subunits but also in the 49 kDa subunit [62] and the PSST subunit [63].

6. The 'catalytic core'

The essence of the functional studies reviewed so far is that in particular those subunits seem to be involved in ubiquinone reduction that was derived from the catalytic subunits of [NiFe] hydrogenases: The PSST subunit, the homolog of the small subunit of hydrogenase, was shown to carry iron–sulfur cluster N2, the probable immediate electron donor for ubiquinone; inhibitor resistant mutations were found in the 49 kDa

subunit, the homolog of the large subunit of hydrogenase. To further explore this evolutionary link and to get insight into complex I function, we reasoned that the X-ray structures of water soluble, two-subunit [NiFe] hydrogenases [64–66] may be useful as a model for the ubiquinone reactive 'catalytic core' of complex I. A first important clue in this direction came from the observation that three of the four cysteine ligands of the [NiFe] cluster in water soluble hydrogenases correspond to conserved residues in complex I: One cysteine has been replaced by the conserved valine that was identified as the target of the first randomly selected inhibitor resistant mutation of *R. capsulatus* [56]. Two other cysteine ligands have been replaced by conserved acidic residues in complex I. Site directed mutagenesis in *Y. lipolytica* of all three amino acids resulted in inhibitor resistance [58].

Inspection of the alignment of four loops of the *D. fructosovorans* [NiFe] hydrogenase that are in close contact to the [NiFe] site with the homologous regions of the 49 kDa subunit revealed a number of amino acids that are fully conserved even between these two rather distant enzyme families. Two of these loops contain a pair of cysteine ligands each (C72/C75 and C543/C546). One loop is bounded by conserved glycines and carries a highly conserved histidine (H228) at its tip. The

fourth loop contains a conserved proline (P475). Analysis of site directed mutants in these conserved regions (Fig. 1) resulted in specific changes of complex I activity, inhibitor sensitivity and the EPR signal of cluster N2 consistent with their position predicted from the structure of [NiFe] hydrogenase [58]: Mutations predicted to be closer to the former [NiFe] site tend to affect inhibitor binding. Mutations predicted to be closer to the former proximal iron–sulfur cluster in the small subunit of [NiFe] hydrogenases tend to affect the EPR line shape of iron–sulfur cluster N2. These findings support the following concept: (i) The structural fold of [NiFe] hydrogenases has been retained in complex I. (ii) Cluster N2 corresponds to the proximal iron–sulfur cluster of hydrogenases and is located at the interface between the 49 kDa and the PSST subunits. However, the identity of the fourth ligand of iron–sulfur cluster N2 remains unclear, because one cysteine ligand of the proximal cluster in hydrogenase is not conserved in the PSST subunit. It is tempting to speculate that this fourth ligand may reside on the 49 kDa subunit of complex I. (iii) A significant part of the quinone binding pocket of complex I is located within the 49 kDa subunit and has directly evolved from the [NiFe] site of hydrogenases.

7. Structural organization

To date there is no detailed structural information available for complex I. Electron microscopic analysis of single particles and two-dimensional (2D) crystals has been carried out with complex I from bovine heart [67,68], the filamentous fungus *N. crassa* [69–72], the aerobic yeast *Y. lipolytica* [8] and the

bacteria *E. coli* [72] and *Aquifex aeolicus* [73]. In all these studies an L shaped overall structure was observed with a membrane arm and a perpendicular peripheral arm protruding into the mitochondrial matrix or the bacterial cytoplasm. Recently, induction of a novel 'horse-shoe' conformation of the *E. coli* complex I was described under conditions of zero ionic strength [74]. However, the relevance of this observation is still unclear as induction of this alternate shape could not be reproduced in another laboratory working with the same organism [75].

A gross assignment of subunits to the two arms and their mutual structural interaction (see Table 1 and Fig. 2) can be based on (i) the dual genetic control of complex I by the mitochondrial and nuclear genome, (ii) the characterization of sub-complexes, and (iii) modules of common evolutionary origin: in higher eukaryotes seven hydrophobic subunits are encoded by the mitochondrial genome. In *N. crassa* grown in the presence of chloramphenicol, an inhibitor of mitochondrial protein synthesis, a small form of complex I is formed which consists of hydrophilic, nuclear encoded proteins only. The hydrophobic and hydrophilic parts of the enzyme could be analyzed separately by electron microscopy of 2D crystals allowing an assignment of the two different arms observed in the complete enzyme [70]. Different fragments and sub-complexes have been generated by dissociation of the purified complex I. From *E. coli* a fragment containing the 75, 51, and 24 kDa subunits can be generated [3]. Treatment of bovine complex I with chaotropes releases the so called flavoprotein (FP) [76] which contains the 51, 24 and 10 kDa subunits. This fragment represents the electron input part of the
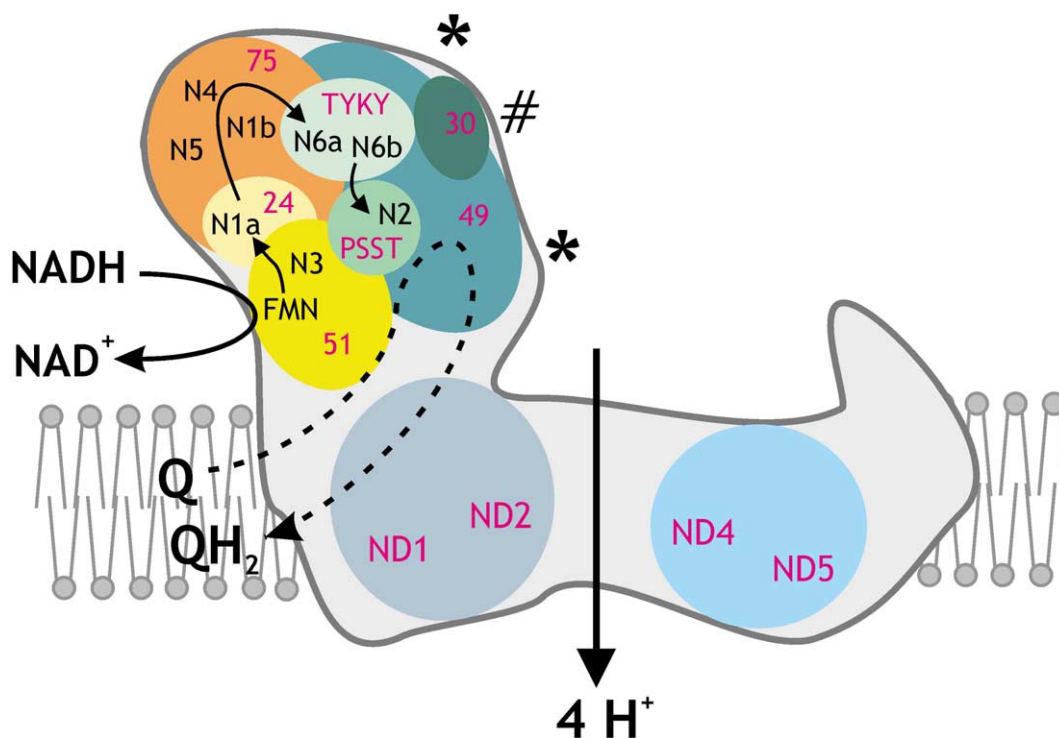

Fig. 2. Cartoon of the approximate positions of central subunits and iron–sulfur clusters within L shaped complex I. The binding sites of antibodies recognizing the 49 kDa subunit (*) and the 30 kDa subunit (#) are indicated. There is no evidence available for the arrangement of the other subunits in the peripheral arm which is oriented perpendicular to the membrane and protrudes into the mitochondrial matrix. The membrane arm consists of seven highly hydrophobic subunits (ND1–ND6 and ND4L). In eukaryotic complex I a substantial number of accessory subunits is found which are not indicated in the figure. The subunit symbol is given in red and redox groups in the complex are denoted in black. The hypothetical sequence of electron transfer steps from NADH to ubiquinone (Q) is indicated by small black arrows.

enzyme and transfers electrons from NADH to artificial acceptors like ferricyanide or hexaamineruthenium [77].

Complex I from bovine heart can be fractionated by sucrose gradient centrifugation [78] or ion exchange chromatography [79,80] in the presence of LDAO (lauryl-N,N-dimethylamine-N-oxide). A number of sub-complexes have been described and we focus here on Iλ, Iβ and Iγ (Table 1). Sub-complex Iλ as described in [78] contains hydrophilic subunits and presumably comprises the major part of the peripheral arm. Sub-complexes Iβ and Iγ together contain all of the hydrophobic ND subunits except ND6. It was inferred that in the membrane part of complex I the ND4 and ND5 subunits on one hand and the ND1 and ND2 subunits on the other hand are next to each other [80]. Electron microscopic analysis of 2D crystals suggested that subunit ND5 is localized at the distal end of the membrane arm [67]. Sub-complex Iγ contains subunits ND1 and ND2 and has been proposed to reside near the junction of the membrane and peripheral arms. Overall the next neighbor relationships for the central subunits of complex I obtained from biochemical and electron microscopic characterization fit with the evolutionary origin of the different parts of complex I discussed above.

A more precise localization of subunits has been achieved by electron microscopy of immunolabeled complex I (Zickermann et al., submitted): the position of the C-terminus of the 30 kDa subunit and two N-terminal epitopes of the 49 kDa subunit could be identified in 2D averages of *Y. lipolytica* complex I single particles decorated with monoclonal antibodies (Fig. 2). As one of the epitopes was found near the tip of the peripheral arm, it is obvious that the 49 kDa subunit and thus the 'catalytic core' of complex I must be clearly separated from the membrane arm of complex I. Remarkably, the hydrophobic ND2, ND4 and ND5 subunits seem to contain rather large hydrophilic domains that could connect the 49 kDa and PSST subunits to the membrane arm [14,81]. Fig. 2 summarizes the structural organization of complex I that can be deduced by combining all available evidence.

8. Proton/sodium pumping mechanism

Our knowledge about structure and function of complex I is still very limited. Therefore, any discussion about the mechanism how this respiratory chain complex uses redox energy to transport charges across the membrane is restricted. It can only analyze whether a given hypothetical concept is compatible with the constraints that are imposed by the available evidence. Thus hypothetical mechanisms are useful, if they make testable predictions. Over the years many proposals have been made regarding how mitochondrial complex I might pump protons. These can be subdivided into three basic types of mechanism: (i) directly redox linked proton pumps; (ii) redox linked ligand conduction mechanisms; (iii) conformational energy transfer. Examples for all three mechanisms can be found in oxidative phosphorylation: Cytochrome *c* oxidase is a directly linked proton pump [82], the proton motive Q-cycle of the cytochrome *bc₁* complex is a ligand conduction mechanism [83] and adenosine triphosphate (ATP) synthase makes ATP by conformational energy transfer [84]. In theory, the large number of redox prosthetic groups, some of which have a pH dependent midpoint potential, allows for a great variety of possible mechanistic scenarios for a directly linked proton pump (see [7] for an overview).

In essence all such mechanisms imply that electron transfer is directly translated into vectorial charge translocation. In most simple terms, this can be envisioned as a redox group within the membrane dielectric that takes up a proton from one side of the membrane upon reduction and releases it in a gated fashion to the other side upon reoxidation. However, as more and more information on the structural organization of complex I became available, it became clear that all known redox centers reside in the peripheral arm. Still, the observation that the stability of a SQ species near iron–sulfur cluster N2 was dependent on the membrane potential [47] seemed to suggest that this redox center was close to the membrane domain. Therefore, it seemed feasible that iron–sulfur cluster N2 was a component of a proton pump [20].

In a ligand conduction mechanism like the Q-cycle, charge is at least partly translocated across the membrane as electrons. This is translated into a proton gradient by electron transfer between two active sites and redox linked protonation and deprotonation of a suitable substrate like ubiquinone (the 'ligand') on opposite sides of the membrane. To account for a stoichiometry of 4 $H^+/2e^-$ mechanistic schemes were proposed in recent years that combined features of a direct pump with a reversed Q-cycle type mechanism [7,54]. However, recent evidence seems to exclude these hypothetical mechanisms like all other concepts involving directly linked pumps: Reverse Q-cycle schemes became unlikely, as the different classes of complex I inhibitors turned out to bind to the same large binding pocket [55].

It was shown by Steuber and colleagues that complex I from *K. pneumonia* and *E. coli* pumps sodium ions rather than protons [15]. As direct pumping mechanisms are essentially operating through redox linked pK_A changes, it is difficult though not impossible that the same charge compensation mechanisms would be possible with sodium ions. As the stoichiometry is only 2 $Na^+/2e^-$ it has been discussed that these complexes employ a completely different mechanism. However, as evident from the example of ATP synthase, conformational energy transfer mechanisms can be essentially the same for protons and sodium ions [85]. Finally, our recent finding that the 49 kDa subunit and thus the 'catalytic core' comprising the critical iron–sulfur cluster N2 is clearly separated from the membrane (Zickermann et al., submitted) places the site of ubiquinone reduction into the hydrophilic domain. As the SQ that can be detected at a distance of about 10 Å from iron–sulfur cluster N2 [47] strongly suggests that this redox center is in fact the immediate electron donor for ubiquinone, one has to assume that the ubiquinone headgroup can somehow reach up into the peripheral arm. This would fit with the concept of a large and rather unspecific inhibitor binding pocket that could provide an amphipathic 'ramp' guiding ubiquinone from the membrane domain into its catalytic site near the interface of the 49 kDa and PSST subunit.

Stimulated by the observation of redox dependent changes in cross linking patterns between subunits of the peripheral arm, a proton pumping mechanism reminiscent of the conformationally linked mechanism of ATP synthase has been proposed a long time ago [86]. However, only now one has to conclude mainly because evidence excludes other options that an indirect mechanism of proton and sodium pumping via long range conformational energy transfer is operating in complex I. At this point the most likely scenario is that the redox chemistry of ubiquinone reduction around iron–sulfur

cluster N2 induces specific conformational changes. These changes are then transmitted to the hydrophobic subunits in the membrane that have been derived from Na^+/H^+ or K^+/H^+ antiporters and act as ion pumps.

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