Hypothesis

Learning from hydrogenases: location of a proton pump and of a second FMN in bovine NADH–ubiquinone oxidoreductase (Complex I)

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Abstract Hydrogenases have clear evolutionary links to the much more complex NADH–ubiquinone oxidoreductases (Complex I). Certain membrane-bound [NiFe]-hydrogenases presumably pump protons. From a detailed comparison of hydrogenases and Complex I, it is concluded here that the TYKY subunit in these enzymes is a special 2[4Fe–4S] ferredoxin, which functions as the electrical driving unit for a proton pump. The comparison further revealed that the flavodoxin fold from [NiFe]-hydrogenases is presumably conserved in the PSST subunit of Complex I. It is proposed that bovine Complex I and the soluble NAD^+-reducing hydrogenase from *Ralstonia eutropha* each contain a second FMN group.

Key words: [NiFe]-hydrogenase; NADH–ubiquinone oxidoreductase; Complex I; Proton pump; FMN

1. Introduction

NADH–ubiquinone oxidoreductase (Complex I) catalyzes electron transfer from NADH to ubiquinone and couples this to the translocation of protons across a mitochondrial or bacterial membrane. Closely related enzymes, NADH–plastoquinone oxidoreductases [1], are found in chloroplasts and cyanobacteria. Electron-microscopy studies with 2D membrane crystals of Complex I from three different sources [2–4] showed that the enzyme extends across the membrane and protrudes extensively from the surface into the internal space. Enzymes related to Complex I have also been identified in the archaeal kingdom. The $F_{420}H_2$–quinone oxidoreductase from *Archaeoglobus fulgidus*, which uses coenzyme $F_{420}H_2$ instead of NADH as electron donor [5,6], and the $F_{420}H_2$–methanophenazine oxidoreductase from *Methanosarcina mazei*, which uses methanophenazine instead of quinones as electron acceptor [7], are the best characterized examples.

Hydrogenases catalyze the reversible reaction $H_2 \Leftrightarrow 2H^+ + 2e^-$ and are found in a wide variety of microorganisms. Based on their metal content, two classes can be distinguished [8]: those that contain both Ni and Fe (termed: [NiFe]-hydrogenases) and those that contain only Fe ([Fe]-hydrogenases). The active site of [NiFe]-hydrogenases is a $NiFe(CN)_2(CO)$ group [9–12]. The [Fe]-hydrogenases contain a Fe(CO)(CN)-Fe(CO)(CN) center, sometimes with a bridging CO [13–16].

In the last decade it was recognized that some subunits of Complex I resemble (parts of) subunits from hydrogenases [8,17–19]. This resemblance became even more pronounced when a group of membrane-bound, multi-subunit [NiFe]-hydrogenases with significant sequence similarities to subunits of Complex I were identified. They contain a core of six subunits which look like the TYKY, PSST, 49 kDa, 30 kDa, ND1 and ND5 subunits of bovine Complex I (the bovine nomenclature is used here also for similar subunits in other enzymes). The list includes *Escherichia coli* hydrogenases 3 and 4 [20,21], postulated to be part of two formate hydrogenlyase complexes, a CO-induced hydrogenase (Coo) from *Rhodospirillum rubrum* [22] and a similar enzyme (Ech) from *Methanosarcina barkeri* [23]. From growth characteristics of *R. rubrum* and from cell-suspension experiments with *M. barkeri*, it can be inferred that the [NiFe]-hydrogenases in these organisms probably pump protons [24,25].

This report describes new insights into the functioning of Complex I and some hydrogenases, which emerged from of a detailed re-examination of sequence similarities between the subunits of these enzymes.

2. Resemblance of the 49 kDa–PSST subunit couple in Complex I with the basic [NiFe]-hydrogenase dimer module

2.1. Comparison of the large [NiFe]-hydrogenase subunit with the 49 kDa subunit of Complex I

Five common sequence motifs were detected in 50 sequences from the 49 kDa subunit and in 50 sequences of the small subunit of [NiFe]-hydrogenases. The position of the motifs is visualized in the 3D structure of the *Desulfovibrio gigas* enzyme (Fig. 1). The motifs A and B in the large subunit form the major part of the interface region with the small subunit. Motif C, a clear α-helix, runs from the center of the molecule to the outside of the protein. Motifs D and E are inside the protein and make close contact with the motifs A, B and C. It is worthwhile to mention that motif D forms part of the capping structure around the CN/CO ligands in [NiFe]-hydrogenases.

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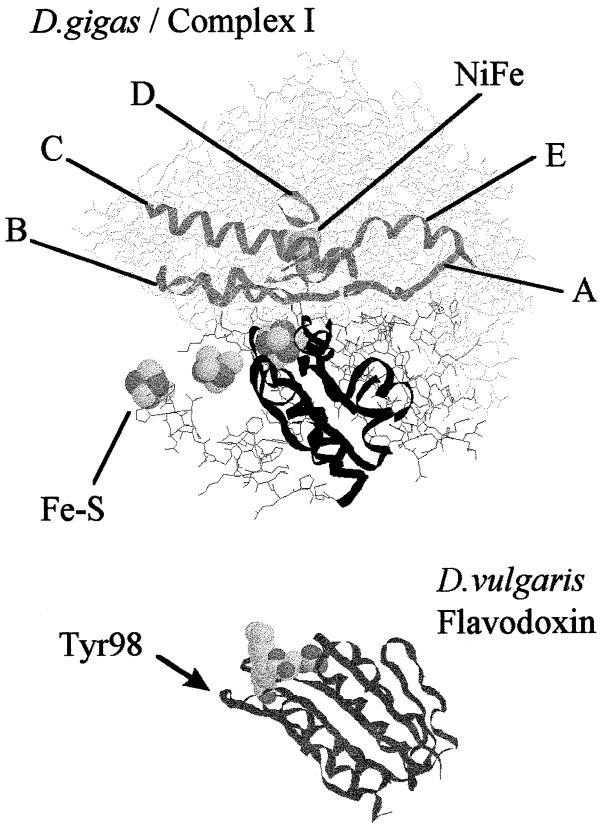

Fig. 1. Common sequence motifs in Complex I and [NiFe]-hydrogenases visualized in the structure of *D. gigas* hydrogenase (2frv [10]). Upper picture: five common motifs (A–E) in the 49 kDa subunits of Complex I and the large subunits of [NiFe]-hydrogenases are shown as gray ribbons in the large subunit (upper part of molecule; light gray) of *D. gigas* hydrogenase. They comprise the amino acid stretches: 9–20 (A), 41–69 (B), 88–100 (C), 460–465 (D) and 517–536 (E). Four common motifs in the PSST-like subunits of Complex I and the small subunits of [NiFe]-hydrogenases are shown as black ribbons in the (shortened) small subunit (lower part of molecule; dark gray) of the *D. gigas* enzyme (stretches 15–39, 71–83, 95–119 and 134–165). The hydrogenases of interest and Complexes I have only the N-terminal part of the small subunit of standard [NiFe]-hydrogenases (holding the proximal Fe–S cluster) in common. Hence, only this part of the small subunit is shown. The other two Fe–S clusters have been included as reference points only. Lower picture: flavodoxin from *D. vulgaris* Hildenborough (3fx2 [60]). FMN is shown in van der Waals radii. From this figure the possible position of a second FMN in Complex I (in the PSST subunit) and the soluble, NAD-reducing [NiFe]-hydrogenase from *R. eutropha* (in the HoxY subunit) can be predicted.

2.2. Comparison of the small [NiFe]-hydrogenase subunit with the PSST subunit of Complex I

Four clear, common motifs were detected in 46 PSST sequences and in 43 sequences of the small hydrogenase subunit (visualized in Fig. 1). In the *D. gigas* enzyme, the four motifs form a distinct structure of several α-helices and β-sheets. Fontecilla-Camps and coworkers [9,26] noted that residues 7–170 in the small subunit of the *D. gigas* enzyme form a specific structure (called the I_s domain) with a striking similarity to the structure formed by the first 136 residues of *Clostridium* MP flavodoxin, an FMN-binding redox protein. They reported that 89 out of the 136 amino acids of the *Clostridium* flavodoxin can be superimposed on the I_s domain and that even the site for binding of the phosphate from FMN seems to be present. The four motifs found in the present report form the major part of this flavodoxin fold. As Volbeda et al. [9] did not actually show the comparison, it is included in Fig. 1. The aromatic amino acid, which partly shields the isoalloxazine ring from the bulk medium in many flavodoxins (Tyr-98 in the *Desulfovibrio vulgaris* Hildenborough flavodoxin in Fig. 1), was not detected in the present comparison.

These comparisons suggest that the 49 kDa subunit forms a complex with the PSST subunit just like the large and small subunits from [NiFe]-hydrogenases.

2.3. FMN attachment to the flavodoxin fold

Common [NiFe]-hydrogenases, like the one from *D. gigas*, do not contain FMN [9,10,26]. One reason may be that there is simply no space for it in the tightly packed protein structure. We felt that if the C-terminal part of the small subunit, holding two more Fe–S clusters, is absent (like in some special [NiFe]-hydrogenases and in the PSST subunit), then the space limitation might be less strict and this might allow for the binding of FMN. The small hydrogenase subunit (HoxY) from the soluble, NAD^+-reducing hydrogenase (SH) from *Ralstonia eutropha* [27] is such a case. The HoxF subunit in the SH binds one FMN group (Fig. 2B), which is considered to react with the NAD^+–NADH couple. The H_2-activating site in the HoxH subunit, a $Ni(CN)Fe(CN)_3(CO)$ center, lacks any redox activity [28]. Yet, after the heterolytic cleavage of H_2, the hydride has to pass on its two electrons to a suitable redox group. This group is proposed here to be a second FMN bound to the flavodoxin fold in the HoxY subunit. This can explain the forgotten observations by Schneider and Schlegel [29], that maximal activity of this enzyme is only obtained when two FMN groups are bound. These findings were recently confirmed and it could be shown that the enzyme can specifically loose the second FMN (H.P. van der Linden, B. Faber, B. Bleijlevens, T. Burgdorf, M. Bernhard, B. Friedrich and S.P.J. Albracht, manuscript in preparation). The second FMN has been included in Fig. 2.

The rapid reduction of part of bovine Complex I with NADPH at pH 8 and higher, at a site different from the NADH binding site [30,31], can be explained by invoking a second FMN. For unknown reasons, only a limited number of Fe–S clusters (2a, 3a and 4a) and no electron transfer to ubiquinone takes place (for overview see [19]). A second FMN reducible by NADPH is in line with the observations [30] that the bleaching of Complex I (observed at 475–510 nm) induced by NADPH is only one half of that induced by NADH. The reduction with NADPH also suggests that the EPR signal ascribed to cluster 4a might be due to a [4Fe–4S] cluster in the PSST subunit, comparable to the proximal cluster in [NiFe]-hydrogenases. As the CxxC stretch, providing two thiol ligands for this cluster in hydrogenases, reads like L/TxCC in the PSST subunits, the ligand structure of such a cluster in the PSST subunit must be slightly different.

From studies on bovine Complex I with the inhibitor ADP-ribose [32], it has been concluded, that the site for NAD^+ reduction in the energy-induced reversed electron transfer reaction (virtually no inhibition) is not the same as that used for NADH oxidation (inhibition). It is proposed here that the FMN group in the PSST subunit is involved in this reversed electron transfer (Fig. 2).

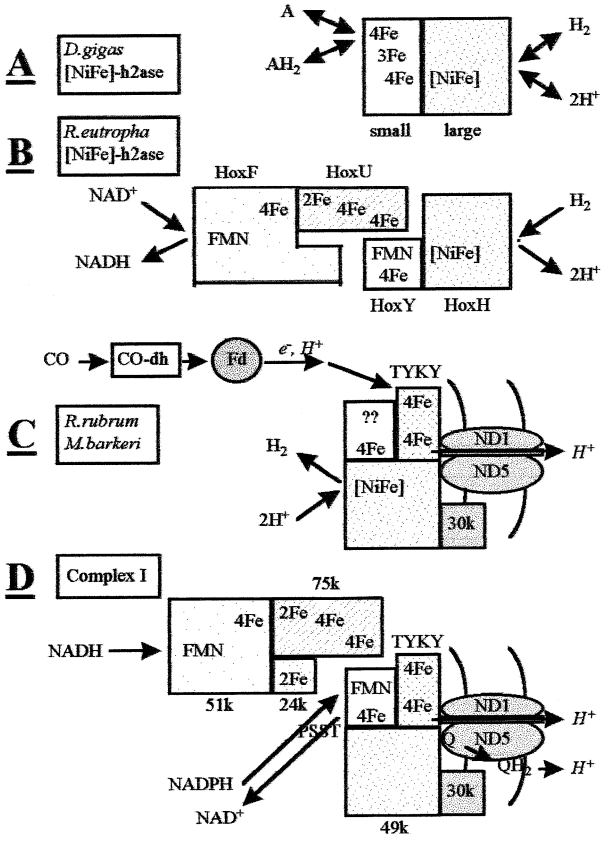

Fig. 2. Schematic representation of the common protein modules in several [NiFe]-hydrogenases and Complex I. Only the subunits expected to be involved in electron transfer, and the most essential ones involved in proton translocation, are represented. A: The [NiFe]-hydrogenase from *D. gigas* with its extensive interface surface between the large and small subunits. B: The soluble [NiFe]-hydrogenase (SH) from *R. eutropha*. C: The membrane-bound [NiFe]-hydrogenases from *R. rubrum* and *M. barkeri*, receiving electrons from a ferredoxin (Fd) [23]. The four subunits, in excess of the two hydrogenase subunits, have been labelled with the names from their bovine Complex I counterparts. D: Bovine Complex I. Sequence similarities between the subunits in the several enzymes are indicated by similar shapes and filling patterns (see [19] for details). The curved lines depict a membrane.

2.4. A second FMN in bovine Complex I explains most if not all of the available data

Already in 1977, one of us has shown [33] that in purified bovine Complex I the EPR signal later ascribed to the NADH-reducible [2Fe–2S] cluster (1b or N1-b [34]) represents a (spin) concentration equal to half the concentrations of cluster 2 and FMN. This was the first indication that a functional Complex I molecule must contain two FMN groups, one cluster 1b and two clusters 2. Subsequently, extensive experimental evidence corroborating this hypothesis has been published by the Amsterdam group (for overviews see [19,35]). In order to accommodate two FMN molecules, initially a dimeric enzyme model was proposed [36]. Information on the amino acid sequence of the bovine enzyme [37] made the dimeric model redundant [19]. It did, however, not resolve the problem of where to put the second FMN molecule. A monomeric enzyme with two 51 kDa subunits was a possible, but rather uncomfortable way out [19], since this contradicted experiments on the stoichiometry of the subunits of Complex I [38,39]. Binding of the second FMN to the flavodoxin fold in the PSST subunit, however, would agree with the experimental findings.

From the amino acid sequences of the subunits of bovine Complex I, Fearnley and Walker [37] predicted the binding sites for eight Fe–S clusters (two 2Fe and six 4Fe clusters) and one FMN, indicating that Complex I should contain at least 28 Fe atoms per FMN. Such a prediction has also been made for Complex I from *Paracoccus denitrificans* [39]. Extensive analyses of FMN and Fe of the bovine enzyme purified in the laboratories of Hatefi [40,41] and Singer [42–44] showed the presence of only 16–18 non-heme Fe per FMN. As Complex I preparations are often contaminated with some Complex II and III, these numbers represent upper values. This would mean that the bovine enzyme would have lost about 40% of its Fe during purification, or that many Fe–S binding motifs are not occupied. The EPR spectra were indistinguishable from those of the enzyme in sub-mitochondrial particles, however (for overview see [35]).

The full reduction of all eight Fe–S clusters predicted on basis of the sequence information would result in EPR spectra with eight unpaired spins per FMN. There is a general agreement in the field, however, that only 3.5–4 spins per FMN can be detected [19,35,45] (one of the [2Fe–2S] clusters, cluster 1a or N1-a, in bovine Complex I is not reducible by NADH [46]). Since MCD spectra eliminated the possibility of additional, paramagnetic, EPR-silent Fe–S clusters in the enzyme [47], this would mean that 50% of the expected Fe–S clusters cannot be detected by EPR or MCD. The presence of two internal FMN groups in Complex I would resolve these discrepancies.

The basis for the viewpoint, that Complex I contains only one FMN, is the amount of FMN per mg of protein, being 1.2–1.5 nmol in the best preparations [40–44]. From the standard methods to determine flavin, Fe or protein, the latter one is the least reliable. Protein is routinely determined by the Bradford, Lowry, biuret or BCA method. Also here the hydrogenase field presents a warning example: the protein content of [Fe]-hydrogenases has been overestimated (up to two-fold) when using standard methods [48,49]. This has long obstructed a correct characterization of the active site [50,51]. A systematic overestimation of the protein content of Complex I preparations by some 50% would explain all available data. We are currently investigating this highly important issue.

3. The TYKY subunit enables membrane-bound [NiFe]-hydrogenases and Complex I to pump protons

In the enzymes Ech and Coo a [NiFe]-hydrogenase unit, like the one in the *R. eutropha* SH, is supplemented with a TYKY-like subunit predicted to contain two [4Fe–4S] clusters, and three subunits (resembling the ND1, ND5 and 30 kDa subunits of bovine Complex I) without any obvious redox centers (Fig. 2C). The sequence similarities of the ND1-, ND5- and 30 kDa-like subunits have already been described in literature [21,52,53]. The ND1 subunit in bovine Complex I can bind the proton-translocation inhibitor DCCD (*N*,*N*′-dicyclohexylcarbodiimide) [54,55]. The 30 kDa-like subunits are only found in proton-pumping hydrogenases, Complex I and related enzymes. In some enzymes this subunit is fused to the N-terminus of the 49 kDa subunit. *R. rubrum* can grow on CO as the only energy source, suggesting that its membrane-

Table 1
Compilation of TYKY-like subunits

A: Formate-hydrogenlyases		
P16432	*E. coli* (HycF)	|CIGCAACVNACP| – 23 – |CIFCGRC**EE**VCP|
	K. pneumoniae (from contig937)	|CIGCAACVNACP| – 23 – |CIFCGRC**EE**VCP|
P77423	*E. coli* (HyfH)	|CIACGACACACP| – 23 – |CIYCGRC**EE**VCP|
O59116	*Pyrococcus horikoshii* (206 aa)	|CIGCNFCGQICP| – 27 – |CTFCQFCV**D**VCP|
Q9V0S4	*Pyrococcus abyssi* (NADH dh)	|CIGCNFCGQICP| – 27 – |CTFCQFCV**D**VCP|
O59110	*P. horikoshii* (136 aa)	|CVGCRMCVTVCP| – 19 – |CVFCKQCV**D**VCP|
Q9V0S0	*P. abyssi* (CO-induced H2ase)	|CVGCRMCVTVCP| – 19 – |CVFCKQCV**D**VCP|
B: NADH–plastoquinone oxidoreductases		
P56755	*Arabidopsis thaliana* (mouse-ear cress)	|CIAC**E**VCVRVCP| – 28 – |CIFCGNCV**E**YCP|
P06252	*Nicotiana tabacum* (common tobacco)	|CIAC**E**VCVRVCP| – 28 – |CIFCGNCV**E**YCP|
P46722	*Zea mays* (maize)	|CIAC**E**VCVRVCP| – 28 – |CIFCGNCV**E**YCP|
P12099	*Oryza sativa* (rice)	|CIAC**E**VCVRVCP| – 28 – |CIFCGNCV**E**YCP|
P05312	*Triticum aestivum* (wheat)	|CIAC**E**VCVGVCP| – 28 – |CIFCGNCV**E**YCP|
O98692	*Hordeum vulgare* (barley)	|CIAC**E**VCVRVCP| – 28 – |CIFCGNCV**E**YCP|
P06253	*Marchantia polymorpha* (liverwort)	|CIAC**E**VCVRVCP| – 28 – |CIFCGNCV**E**YCP|
Q00236	*Plectonema boryanum*	|CIAC**E**VCVRVCP| – 28 – |CIFCGNCV**E**YCP|
Q9WWM6	*Anabaena* sp.	|CIAC**E**VCVRVCP| – 28 – |CIFCGNCV**E**YCP|
P26525	*Synechocystis* sp.	|CIAC**E**VCVRVCP| – 28 – |CIFCGNCV**E**YCP|
Q9TKV4	*Nephroselmis olivacea*	|CIAC**E**VCVRVCP| – 28 – |CIFCGNCV**E**YCP|
C: NADH–ubiquinone oxidoreductases		
P29921	*P. denitrificans*	|CIACKLC**E**AVCP| – 27 – |CIYCGFCQ**E**ACP|
P42031	*Rhodobacter capsulatus*	|CIACKLC**E**AICP| – 27 – |CIYCGYCQ**E**ACP|
P42028	*Bos taurus* (bovine)	|CIACKLC**E**AVCP| – 27 – |CIYCGFCQ**E**ACP|
O00217	*Homo sapiens* (human)	|CIACKLC**E**AICP| – 27 – |CIYCGFCQ**E**ACP|
P80269	*Solanum tuberosum* (potato)	|CIACKLC**E**AICP| – 27 – |CIYCGFCQ**E**ACP|
O24143	*N. tabacum* (common tobacco)	|CIACKLC**E**AICP| – 27 – |CIYCGFCQ**E**ACP|
Q42599	*A. thaliana* (mouse-ear cress)	|CIACKLC**E**AVCP| – 27 – |CIYCGFCQ**E**ACP|
O21233	*Reclinomonas americana*	|CIACKLC**E**AICP| – 27 – |CIYCGFCQ**E**ACP|
Q9VF27	*Drosophila melanogaster (CG3944)*	|CIACKLC**E**AICP| – 27 – |CIYCGFCQ**E**ACP|
Q9ZCF8	*Rickettsia prowazekii*	|CIACKLC**E**AICP| – 27 – |CIYCGLCQ**E**ACP|
Q22619	*Caenorhabditis elegans*	|CIACKLC**E**AICP| – 27 – |CIYCGLCQ**E**ACP|
Q12644	*Neurospora crassa*	|CIACKLC**E**AVCP| – 27 – |CIYCGFCQ**E**SCP|
Q9UUT8	*Yarrowia lipolytica* (NuiM)	|CIACKLC**E**AICP| – 27 – |CIYCGYCQ**E**SCP|
D: Others		
P30826	*Trypanosoma brucei brucei*	|CIACRLC**D**LICP| – 27 – |CIYCGFCMHVCP|
O79959	*Crithidia oncopelti*	|CIACRLC**D**FICP| – 27 – |CIYCGFCMHVCP|
Q56224	*Thermus aquaticus*	|CIGCSLCAAACP| – 33 – |CIFCGLC**EE**ACP|
Q9RU95	*Deinococcus radiodurans*	|CIGCSLCAAACP| – 33 – |CIFCGLC**EE**ACP|
P95173	*Mycobacterium tuberculosis*	|CIGCELCAWACP| – 33 – |CIGCGLCI**E**ACP|
Q9XAR2	*Streptomyces coelicolor*	|CVGCELCAWACP| – 33 – |CILCGLCI**E**ACP|
P33604	*E. coli* (NuoI)	|CVACNLCAVACP| – 27 – |CIFCGLC**EE**ACP|
	M. mazei Gö1 (FpoI)	|CIGCGICANTCP| – 28 – |CLFCGLCI**D**QCP|
Q9UYN5	*P. abyssi* (H2ase-4)	|CIGCGACVNACP| – 23 – |CIRCYRCV**E**VCP|
O59657	*M. barkeri* (EchF)	|CILCGLCQKKCP| – 19 – |CIMCT**E**CVNGCP|
	Archeoglobus fulgidus (FqoI)	|CISCFRCAQICP| – 20 – |CIFCHFCV**D**SCP|
P72318	*R. rubrum* (CooX)	|CVGCKMC**E**HVCP| – 21 – |CVNCGLCSHYCL|
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Three sequences, fitting the search profile $Tx_3Px_{11,12}Gx_{8,14}CxxCxxCxxxC$ were not from the SwissProt+TrEMBL banks (no identification number). The alignment order was obtained with Clustal W. Only the two 4xCys motifs are shown, together with the number of spacer amino acids. Acidic residues are in bold.

bound hydrogenase can build up a proton-motive force [22,25]. Like Complex I, this hydrogenase is inhibited by DCCD. This implies the presence of a similar, redox-linked proton pump in both. In turn, this suggests that the TYKY subunit is essential for this extra function (the proton pump) and that it might have some special properties.

3.1. The TYKY subunit: a special ferredoxin

The amino-acid sequences from TYKY-like subunits were inspected and a simple, highly specific, common motif was uncovered in the N-terminal part of all sequences: $Tx_3Px_{11,12}Gx_{8,14}CxxCxxCxxxC$. With this search pattern, only 42 sequences were retrieved from a total of 384566 sequences in the SwissProt and TrEMBL databases. Two of these (a subunit of glutamate synthase from *Thermotoga maritima* and a polyferredoxin from the methyl viologen-reducing hydrogenase from *Methanococcus jannaschii*) were discarded, since the sequences were much larger than the TYKY subunits. The remaining 40 sequences all belonged to NADH–quinone oxidoreductases or membrane-bound hydrogenases. It thus appears that the structure formed by this motif is unique among Fe–S proteins. It indicates, that the TYKY subunits in NADH–quinone oxidoreductases have evolved from the TYKY-like subunits in membrane-bound hydrogenases, or that both have a common ancestor.

The two 4xCys motifs in the sequences are shown in Table 1 in the order as obtained by an alignment with Clustal W. NADH–plastoquinone and NADH–ubiquinone oxidoreductases form the two main groups. They both have one conserved E residue in each of the two 4xCys motifs and a strictly conserved spacing between the two motifs. All other sequences have only one or two acidic residues in one of the 4xCys

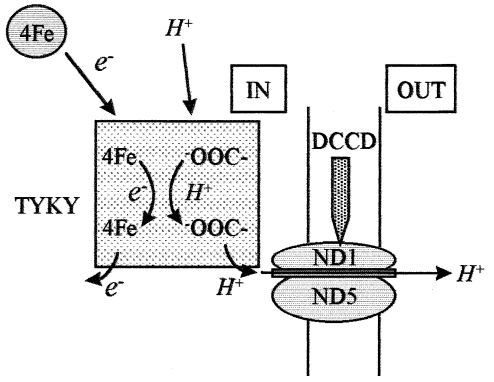

Fig. 3. Schematic representation of the TYKY subunit as the driving device for a proton pump. Reduction of the first 4Fe cluster in the TYKY subunit requires a proton from the inside space to compensate the negative charge from the nearby carboxylic acid group. The electron and proton are subsequently transferred to the second 4Fe cluster in the TYKY subunit. The many conserved acidic residues are expected to play a critical role here. Upon oxidation of the second 4Fe cluster, the electron and the proton are separated. In hydrogenases, the electron goes to the Ni–Fe site where protons from the inside space are reduced to H_2. In Complex I the electron is used to reduce ubiquinone. The proton is conveyed to the ND1/ND5 subunits which function as membrane anchors and also provide a proton channel through the membrane to lead the proton to the external medium. DCCD (which binds to the ND1 subunit in Complex I) obstructs the proton channel and so inhibits electron transfer. It is expected that a precise folding and embedding of the unique TYKY-subunit structure in the intact enzyme is essential for the properties of its [4Fe–4S] clusters (e.g. the clusters in the overexpressed TYKY subunit have a redox potential of −600 mV [61]).

motifs. Also the total number of conserved polar residues within each group is different. Group A has only one conserved R residue; group B has 4 D, 8 E, 6 R and 4 K residues in conserved positions. Group C has 4 D, 9 E, 7 R and 4 K conserved residues, while the fourth group has no polar residues in conserved positions at all. Generally, 2[4Fe–4S] proteins are rather acidic. We found that the acidic residues have no conserved sequence position relative to the two 4*Cys motifs, however; also the spacing between the two motifs is highly variable (11–33 residues).

On basis of the foregoing, we propose that the TYKY subunit forms the electrical driving unit of a proton pump in Complex I and related enzymes. Oxidation–reduction of one or both of the [4Fe–4S] clusters in the TYKY subunit is proposed to be dependent on charge compensation when acidic residues are close to the Fe–S clusters (Fig. 3). The clusters are expected to have a pH-dependent redox potential. Ohnishi and coworkers [56] have discovered that this holds for the behavior of the EPR signal ascribed to cluster 2. Reduction kinetics of bovine Complex I with NADPH demonstrate that this EPR signals represents two [4Fe–4S] clusters [31] and hence we propose that these clusters are bound to the TYKY subunit.

The redox-potential difference between the donor redox protein and the accepting couple ($2H^+$–H_2 or Q–QH_2) provides the driving force for the proton pump. If this driving force is rather small, like in the *E. coli* formate-hydrogenlyase (Hyc) [20,21], an enzyme which is very active during fermentation, the proton pump must only be loosely coupled to electron transfer, otherwise the enzyme could not be used for fast electron transfer. This requirement is expected to be dependent on the number and the distance of acidic residues in the vicinity of the Fe–S clusters, i.e. the coupling can be tight or virtually absent. The ancestor pump units in pumping [NiFe]-hydrogenases apparently do not require quinones for their action. Quinone-reducing Complexes I may therefore have a second (Q-cycle) device to translocate protons, in addition to the hydrogenase-based pump unit. This would enable them to translocate more protons (maximally $2H^+/e^-$ [57]).

Recently it has been shown that Complex I in *Klebsiella pneumoniae* is primarily a Na^+ pump [58] and that *E. coli* Complex I can pump such ions as well [59]. It may well be that the TYKY subunit is one of the players determining this property. As remarked in [59] a Q-cycle type of mechanism can be excluded for Na^+ ion translocation. Note that the NuoI subunit from the *E. coli* Complex I does not belong to the main class of TYKY-subunits from NADH–ubiquinone oxidoreductases (Table 1).

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