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# The reaction of NADPH with bovine mitochondrial NADH: ubiquinone oxidoreductase revisited

## II. Comparison of the proposed working hypothesis with literature data

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**Abstract** The first purification of bovine NADH:ubiquinone oxidoreductase (Complex I) was reported nearly half a century ago (Hatefi et al. J Biol Chem 237:1676–1680, 1962). The pathway of electron-transfer through the enzyme is still under debate. A major obstacle is the assignment of EPR signals to the individual iron-sulfur clusters in the subunits. The preceding paper described a working model based on the kinetics with NADPH. This model is at variance with current views in the field. The present paper provides a critical overview on the possible causes for the discrepancies. It is concluded that the stability of all purified preparations described thus far, including Hatefi's Complex I, is compromised due to removal of the enzyme from the protective membrane environment. In addition, most preparations described during the last two decades are purified by methods involving synthetic detergents and column chromatography. This results in delipidation, loss of endogenous quinones and loss of reactions with (artificial) quinones in a rotenone-sensitive way. The Fe:FMN ratio's indicate that FMN-a is absent, but that all Fe-S clusters may be present. In contrast to the situation in bovine SMP and Hatefi's Complex I, three of the six expected [4Fe-4S] clusters are not detected in EPR spectra. Qualitatively, the overall EPR lineshape of the remaining three cubane signals may seem similar to that of Hatefi's Complex I, but quantitatively it is not. It is further proposed that point mutations in any of the TYKY, PSST, 49-kDa or 30-kDa subunits, considered to make up the delicate structural heart of Complex I, may have unpredictable effects on any of the other subunits of this quartet. The fact that most point mutations led to inactive enzymes makes a correct interpretation of such mutations even more ambiguous. In none of the Complex-I-containing membrane preparations from non-bovine origin, the pH dependencies of the NAD(P)H→$O_2$ reactions and the pH-dependent reduction kinetics of the Fe-S clusters with NADPH have been determined. This excludes a proper discussion on the absence or presence of FMN-a in native Complex I from other organisms.

**Keywords** NADH:ubiquinone oxidoreductase · Complex I · Prosthetic groups · EPR

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## Introduction

There is a large body of data on NADH-dehydrogenase preparations purified from Complex-I-containing membranes, mainly from non-mammalian origin, that seems to be at variance with the proposed model for electron transfer in bovine NADH:ubiquinone oxidoreductase (EC 1.6.5.3; Complex I) in the preceding paper (Albracht 2010). Hence, an overview focussing on possible reasons for these discrepancies is in place.

Like in the preceding paper the term 'Complex I' is used here for the enzyme in membrane preparations and for purified enzymes that catalyze an inhibitor-sensitive (rotenone or piericidin A) NADH→quinone activity without any reconstitution efforts. Preparations without this activity are called 'NADH dehydrogenase', even although inhibitor-sensitive quinone reductase activity can often be induced by treatment with phospholipids.

## Bovine Complex I contains two FMN groups

The general idea that bovine Complex I holds only one FMN per enzyme molecule stems from the time that its molecular mass was not yet known. Taking the FMN content as the basis for the enzyme concentration was a reasonable minimum hypothesis. However, when more detailed information on the number, masses and sequences of the subunits, and on the potential binding sites for prosthetic groups became available (Walker et al. 1992; Fearnley and Walker 1992; Walker 1992) the ideas in my group in Amsterdam about the FMN content changed. Our freeze-quench kinetic data on the reaction of NADPH with bovine submitochondrial particles (SMP) did not fit a model of the enzyme with only one FMN. Also the FMN content of the best preparations of Hatefi's Complex I (up to 1.5 nmol per mg of protein (Hatefi et al. 1962)) exceeded the expected FMN content of a complex with a molecular mass of nearly 1,000 kDa by 50%. When we investigated the least accurate of the methods to determine the FMN content, i.e. the protein determination, we discovered large errors in these methods when applied to bovine Complex I. It was established that the colorimetric methods (biuret (Gornall et al. 1949; Cleland and Slater 1953), Lowry (Lowry et al. 1951), Bradford (Bradford 1976) and bicinchoninic acid (BCA) (Smith et al. 1985)) greatly overestimate its protein content (Albracht et al. 2003). Hence, taking its molecular mass as 900 kDa, the real FMN content appeared to approach ca. two FMN per Complex I (Albracht et al. 2003). An example where this presumably also applies is a bovine enzyme preparation (Okun et al. 2000) that was reported to contain 1.1 nmol FMN.mg$^{-1}$. As the protein content was determined by the Lowry method, and because this method overestimates the protein content of Hatefi's Complex I two-fold (Albracht et al. 2003), the real FMN content is twice as high, i.e. this preparation also contained 2 FMN groups per enzyme molecule. This error in the protein content led to a two-fold overestimation of the content of Complex I in SMP (Schägger and Pfeiffer 2001).

## Differences between NADH:ubiquinone oxidoreductases from different organisms

Some general differences between the enzymes involve a/o optimum temperature of action (20–85 °C), number of subunits (ranging from 13 to 45), number of prosthetic groups, detergents used for solubilization (bile salts or synthetic detergents), stability upon solubilization, purification methods and application of column chromatography, contents of lipids and quinones of the purified enzymes and the instability of purified preparations upon reduction with NADH in air.

*Subunits, iron-sulfur clusters, lipids and quinones* In prokaryotes the number of subunits in Complex I is mostly 14 (Friedrich and Scheide 2000) (13 in *Escherichia coli* where the 30-kDa and 49-kDa subunits are fused (Friedrich 1998)). Eukaryotic enzymes contain more subunits, e.g. 32 in *Neurospora crassa* (Tuschen et al. 1990; Nehls et al. 1992), 40 in the yeast *Yarrowia lipolytica* (Abdrakhmanova et al. 2004; Brandt et al. 2005; Morgner et al. 2008), and at least 45 in the bovine enzyme (Hirst et al. 2003; Carroll et al. 2006). The number of Fe-S clusters also differs. Cluster N5 in the 75-kDa subunit of the *T. thermophilus* enzyme is absent in the mitochondrial enzymes.

Differences in growth temperature of the various organisms (20–85 °C) are reflected in the lipid composition (melting temperature) of the membranes. In addition the inner membranes of mitochondria contain high levels of cardiolipin (phosphatidyl-glycerol), a phospholipid nearly exclusively present in these cell organelles. Cardiolipin is not present in prokaryotes. Eukaryotic membranes contain cholesterol essential for their fluidity. Cholesterol is absent in prokaryotes. Complex-I-containing membranes from various species also contain different quinones, like $Q_{10}$ (*Bos taurus*, *Homo sapiens*, *Rhodobacter capsulatus*), $Q_9$ (*Y. lipolytica*), $Q_8$ (*Paracoccus denitrificans*), both $Q_8$ and menaquinone-8 (*E. coli*; ratio controlled by the oxygen tension during growth), or just menaquinone-8 (*Thermus thermophilus*). Also *Rhodothermus marinus* contains menaquinone.

This implies that the vital interaction of ion-translocating Complex I with the lipid membrane and quinones may require specific adaptations of the protein on the periphery of the enzyme and in its large hydrophobic cleft in the hydrophilic domain (Fig. 2 of the preceding paper).

*Purification methods* The method of purification is highly important. In a review of 1985 Hatefi (Hatefi 1985) compared his Complex I with other preparations and remarked that "the isolation of a complex I-like enzyme with retention of the above physiological properties has not yet been achieved from another source, or by an alternative procedure from the same source." This still holds today for nearly all reported preparations.

In contrast to Hatefi's Complex I, most of the purified preparations described during the last two decades do not contain bound native quinones, contain very few lipids and have lost the ability to react with (artificial) quinones in an inhibitor-sensitive (rotenone or piericidin) way. Usually some activity can be restored with the help of phospholipids. A good example is the highly-purified bovine enzyme prepared by the group of Hirst (Sharpley et al. 2006), hereafter referred to as Hirst's NADH dehydrogenase. This contained 3% (w/w) lipids against 22% in Hatefi's Complex I. It did only contain 0.3 molecules of $Q_{10}$ per

enzyme molecule against 4.2–4.5 $Q_{10}$ for Hatef's complex and it hardly reacted with n-decylubiquinone, unless asolectin was added.

For the solubilization and purification of nearly all of these preparations artificial detergents were used, whereas for Hatefi's Complex I only bile salts were applied. In addition, to obtain highly pure monodisperse preparations for crystallisation purposes, the purifications often involved anion-exchange and size-exclusion column chromatography. This author suspects that removal of phospholipids and the native quinones from Complex I induces a destabilisation of the binding of FMN-a in enzymes that contain such a group. Together this may result in changes of the properties of the cubane clusters in e.g. the PSST (cluster *h*), TYKY (clusters *f* and *g*) and 75-kDa (in particular cluster *e*) subunits. Experience with the evolutionary-related soluble hydrogenase from *Ralstonia eutropha* (Van der Linden et al. 2004) learns that loss of FMN-a in that enzyme starts within seconds after the addition of NADH.

*Stability* The stability of the various solubilized enzymes can differ greatly. All attempts, including those in Amsterdam (Bakker 1988), to purify an intact complex from *P. denitrificans* have failed. Once solubilized, the complex breaks up in at least two subcomplexes (Bakker 1988). For the only enzyme preparation described (Yagi 1986), no EPR spectra have ever been published. Dissociation of the peripheral arm from the membrane arm also occurs upon solubilization of the *T. thermophilus* enzyme (Hinchliffe et al. 2006).

Unlike the native bovine enzyme in SMP, nearly all purified preparations, including Hatefi's Complex I (Hatefi et al. 1962; Kawakita and Ogura 1969; Orme-Johnson et al. 1974) and Singer's NADH dehydrogenase (Beinert et al. 1963; Rossi et al. 1965), are degraded upon reduction with NADH in air. Dramatic changes in the EPR signal of cluster N1b in Hatefi's Complex I are observed within a minute after addition of this substrate at room temperature (Hatefi et al. 1962; Kawakita and Ogura 1969; Orme-Johnson et al. 1974). A convenient marker of this change is the appearance of a $g_2$=1.97 line, detectable up to 150 K. This instability is introduced in Hatefi's isolation procedure (Hatefi et al. 1962) at the point where Complex I–III is prepared from Complex I–II–III (Albracht et al. 1977). The presence of dithionite, which effectively consumes all $O_2$, prevents these NADH-induced changes (Albracht et al. 1977). A $g_2$=1.97 line is also present in EPR spectra from Hirst's NADH dehydrogenase reduced with NADH (Sharpley et al. 2006).

Also the purified bacterial NADH dehydrogenases show this instability. In the *E. coli* enzyme NADH (in air at 4 °C) induced covalent links between the subunits 30 kDa, 49 kDa and PSST. At the same time a degradation of the PSST subunit took place (Berrisford et al. 2008). Similarly, with the isolated *T. thermophilus* enzyme, exposure to NADH induced a covalent link between the 49-kDa and PSST subunits, as well as between the PSST and TYKY subunits. In addition a degradation of these three subunits, as well as of the 75-kDa subunit, was observed. These processes did not occur under anaerobic conditions (Berrisford et al. 2008).

It has been reported early on that removal of 50% lipids from the bovine enzyme resulted in a considerable decrease (75 mV) of the midpoint potential reflected by the N2 EPR signal, while that of all other signals did not change significantly. Also the rotenone-sensitive NADH→Q reductase activity was decreased (Ohnishi et al. 1974; Ohnishi 1998). Both phenomena could be partly restored by addition of phospholipids. Removal of 50% lipids of Hatefi's Complex I also considerably increased the sensitivity of the enzyme to trypsin (Ragan 1976). This could not be repaired by addition of phospholipids. Hence, Ragan concluded that removal of lipids leads to irreversible structural changes in Complex I. Even the presence of $Q_{10}$, an amphipatic molecule just like phospholipids, is required for the tight binding of piericidin A to Complex I in SMP (Van Belzen et al. 1990). This underlines the idea that $Q_{10}$ is required for a correct environment for the binding of that inhibitor.

The lesson is that the stability and the protection against the damaging attack by $O_2$ of NADH-reduced Complex I in bovine mitochondrial inner membranes apparently relies on components that are not present, or that are considerably changed, upon purification by classical (Hatefi's Complex I and Singer's NADH dehydrogenase) or modern means (Hirst's NADH dehydrogenase). Apparently, this also hold for the enzymes from other sources.

*Enzymatic reactions with NADPH* The pH-dependent oxidation of NADPH by bovine SMP as shown in Fig. 3A of the preceding paper has never been published for mitochondrial inner membranes from lower organisms like *N. crassa* or *Y. lipolytica* or for membrane preparations of Complex-I-containing prokaryotes. Because the presence of FMN-a in the bovine enzyme is considered by the author to be essential for this reaction, a discussion on the absence or presence of FMN-a in Complex I from other organisms is only relevant once this behaviour has been investigated. Freeze-quench kinetic experiments with NADPH have only been reported for bovine-heart SMP (Amsterdam group) and for Singer's NADH dehydrogenase (Beinert et al. 1965).

For Hirst's NADH dehydrogenase (Sharpley et al. 2006) no information is available about possible reduction of Fe-S clusters with NADPH. This preparation is reported to contain only one operational reaction site for pyridine

nucleotides, which is in agreement with the presence of only one flavin group (FMN-b). At pH 7.5 and 32 °C the enzyme catalyzed the reduction of $Ru(NH_3)_6^{3+}$ by NADPH ($K_M^{NADPH} = 3.7mM$) with a rate of 18 $s^{-1}$, which is 0.7% of the rate with NADH (Yakovlev and Hirst 2007).

Purified *Y. lipolytica* NADH dehydrogenase oxidizes NADPH with $Ru(NH_3)_6^{3+}$ as acceptor with a rate of 2.2 μmol.$min^{-1}$.$mg^{-1}$ at pH 5.5, which is 3.8% of the NADH→$Ru(NH_3)^{3+}$ reaction rate at neutral pH (Zickermann et al. 2007). Likewise, the oxidation rate of NADPH with n-decylubiquinone as acceptor at pH 6.0 was 3.8% of the NADH-quinone activity measured at neutral pH. It was stated that in this enzyme both NADH and NADPH can completely evolve all EPR signals, but no rates were given. These data are consistent with the finding that the purified *Y. lipolytica* enzyme contains only a single FMN group and with the conclusion that both pyridine nucleotides react at the NADH site, be it with quite different rates (Zickermann et al. 2007). Mitochondrial membranes of *Y. lipolytica* show a piericidin-sensitive oxidation of NADH by 5-nonylubiquinone of 0.4–0.5 μmol.$min^{-1}$.$min^{-1}$ (at pH 7.4 and 30 °C). However, no activity could be observed with NADPH (Kerscher et al. 1999).

## Quantitative aspects

The structure of the *T. thermophilus* enzyme dictates some important quantitative properties like the number of Fe atoms per flavin and the spin concentrations of Fe-S EPR signals (assuming $S$=1/2 systems) relative to the FMN concentration.

*Number of Fe atoms per FMN* The structure of the *T. thermophilus* enzyme shows the presence of 32 Fe atoms per FMN. The mitochondrial enzymes, lacking cluster N5, should contain 28 Fe atoms per FMN. Of course, these numbers are 16 and 14, respectively, in case of two FMN groups.

The best classical preparations of the bovine-heart enzyme, Hatefi's Complex I and Singer's NADH dehydrogenase, contained maximally 16–18 non-heme Fe atoms per FMN (Hatefi et al. 1962; Ringler et al. 1963; Cremona and Kearney 1964; Lusty et al. 1965; Orme-Johnson et al. 1974; Ohnishi et al. 1981; Paech et al. 1981; Kowal et al. 1986). The real values are somewhat lower due to contamination with the Complexes II and III, which also contain non-heme Fe. Hirst's NADH-dehydrogenase (Sharpley et al. 2006) hold 22–26 Fe atoms per FMN, indicating loss of FMN.

For some purified eukaryotic, non-mammalian enzymes the Fe:FMN ratio's are 27.5 (*Torulopsis utilis* (Tottmar and Ragan 1971)), 28–38 (*N. crassa* (Schulte et al. 1998)), 33.2 (*Pichia pastoris* (Bridges et al. 2009)) and 30.4 (*Pichia angusta* (Bridges et al. 2009)). The values for the purified *Y. lipolytica* enzyme have not been reported (Djafarzadeh et al. 2000; Kashani-Poor et al. 2001a).

The Fe:FMN ratio's for some purified prokaryotic enzymes are 21–27 (*E. coli*, wild-type enzyme (Leif et al. 1995)), 37–42 (*E. coli*, His-tagged enzyme (Pohl et al. 2007)) and 42 (*T. thermophilus* (Hinchliffe et al. 2006)). Remarkably, the enzyme purified from *R. marinus* contains 13.5 Fe atoms per FMN (Fernandes et al. 2002, 2006). It also contains 3.7 molecules of menaquinone and has a rotenone-sensitive quinone reductase activity. Hence this preparation seems to have properties similar to those of Hatefi's Complex I.

The conclusion is that the purified NADH-dehydrogenase preparations, except Singer's NADH dehydrogenase, show an Fe:FMN ratio indicative for the presence of a single FMN group. Only the purified enzyme from *R. marinus* has an Fe:FMN ratio consistent with the presence of 2 FMN groups. The iron content of all preparations is consistent with the presence of the expected amount of Fe-S clusters.

*Spin concentrations of the individual EPR signals* The structure of the *T. thermophilus* enzyme also dictates the EPR spin concentrations of the Fe-S clusters relative to the FMN concentration. Therefore, a most useful exercise is the comparison between the spin concentrations represented by the N2 signal and the signal(s) from the binuclear cluster(s), and the FMN concentration in a sample. This circumvents errors in the protein determination which can be large for Hatefi's Complex I (Albracht et al. 2003). The N1b signal (and that of cluster N1a if reduced) can be easily monitored at 45 K. A simulated lineshape should be used for quantification (Van Belzen et al. 1992). Direct double integration of the N1b signal detected at 45 K or higher, as often used by other research groups, leads to a large overestimation of its spin concentration due to contributions of the underlying relaxation-broadened signals of the cubane clusters (Van Belzen et al. 1992). In the mitochondrial enzymes the axial N2 signal is the sharpest of the signals from the cubane clusters and hence dominates the spectrum at 15–17 K. The area of the left half of its $g_1$ line at 2.05 can be conveniently used to calculate the N2 spin concentration (Albracht et al. 1979; Van Belzen et al. 1992), but a simulated lineshape of the complete signal also suffices. Determination of the acid-labile FMN content is straightforward.

In the Amsterdam group it has been consistently found with the classical preparations of the bovine enzyme and with SMP, that the spin concentration represented by the signal assigned to cluster N1b is 50% of that of the N2

signal (and 50% of the concentration of FMN) (Albracht et al. 1977, 1979; Albracht and Bakker 1986; Bakker and Albracht 1986; Van Belzen et al. 1992). This is in agreement with the presence of two cubane clusters responsible for the N2 signal and two FMN groups per Complex I in these preparations.

An independent observation underpinning the notion that the N2 signal represents two spins per Complex I is that the initial velocity of the NADH→$O_2$ reaction of bovine SMP activated by NADPH can be completely inhibited by a concentration of piericidin A equal to 50% of the spin concentration represented by the N2 signal ((Van Belzen et al. 1990) and the preceding paper).

As discussed above, most NADH-dehydrogenase preparations contain a single FMN group and sufficient iron for the expected amount of Fe-S clusters. For the NADH dehydrogenase from *Y. lipolytica* it was assumed (but not shown) that cluster N1a is not reduced by NADH (Zickermann et al. 2007). The individual spin concentrations represented by the N1b, N2, N3 and N4 signals were reported to be the same and equal to the FMN concentration (Kerscher et al. 2002). This means that three of the six cubane clusters are not detected in EPR spectra of the NADH-reduced enzyme.

Also the four main EPR signals of NADH dehydrogenase from *N. crassa* represent spin concentrations equal to that of the FMN concentration (Ohnishi et al. 1981). Cluster N1a cannot be reduced with NADH (Wang et al. 1991). This means that also in this preparation three of the cubane clusters are not detected in EPR spectra of the NADH-reduced enzyme.

In the *E. coli* enzyme both cluster N1a (formerly called N1c (Uhlmann and Friedrich 2005; Nakamaru-Ogiso et al. 2005)) and cluster N1b are reduced by NADH. The concentration ratio's were determined as FMN:N1a:N1b:N2:N3:N4=1.0:1.2:1.1:1.0:0.9:1.1 (Leif et al. 1995; Ohnishi 1998). Hence, at least three cubane clusters do not show up in EPR spectra of the NADH-reduced enzyme.

For the highly-purified monodisperse bovine NADH dehydrogenase of the Hirst group no spin concentrations were reported (Sharpley et al. 2006). Although the spectra of Hirst's NADH dehydrogenase reduced with NADH qualitatively resemble those of Hatefi's Complex I and SMP (Sharpley et al. 2006), a more detailed analysis (Reda et al. 2008) shows some serious quantitative deficiencies. It appeared that the N1b signal at 40 K, induced with NADH (ca. −0.4 V), increased 3.3 times when a more powerful reductant was used (ca. −1 V). From the areas of the $g_1$ lines of the N2 and N1b signals at 2.05 and 2.02, respectively, in spectra at 12 K (Fig. 5 in (Reda et al. 2008)), one can easily estimate that at ca. −1 V the double integrated intensity (i.e. the spin concentration) of the N1b signal is ca. 5 times that of the N2 signal. Together this implies that at most 30% of the enzyme molecules contribute to the EPR spectra of the NADH-reduced enzyme. This is in sharp contrast with all previous quantitative studies on Hatefi's Complex I, where it was established that the spin concentration of the N2 signal equals the FMN concentration (Orme-Johnson et al. 1974; Beinert and Albracht 1982; Ohnishi 1998). Surprisingly, the authors did not provide any spin concentrations of the N2 and N1b signals, although this is only a matter of a few minutes (e.g. using the simulated spectrum in the inset in Fig. 3 and simulations of the spectra in Fig. 6B in (Reda et al. 2008)). Another flaw was that the comparison of spectra at different temperatures was highly confusing, because a fixed microwave power of 0.5 mW was used. This led to serious saturation of the N1b and N2 signals at temperatures of 12 K and below. Such comparisons should always be carried out at non-saturating powers, e.g. like in (Finel et al. 1994). As the EPR instrument was not connected to a microwave-frequency meter, small *g*-value shifts in the spectra will go unnoticed. Needless to say that it of utmost importance that quantitative EPR data of Hirst's NADH dehydrogenase should be published. In addition, the spin concentration of the N2 signal relative that of the N1b signal, and the FMN concentration should be followed during the purification procedure. Until then, conclusions from previous and future experiments obtained with this enzyme preparation, concerning the mechanism of action of native Complex I, cannot be regarded as trustworthy.

It can be concluded that, in contrast to the situation in bovine SMP, Hatefi's Complex I and Singer's NADH dehydrogenase, three of the six cubane clusters are not detected in EPR spectra of the NADH-reduced enzymes from *E. coli*, *N. crassa* and *Y. lipolytica*. Since the relative intensities of the N2 (1.92 line), N3 (1.88 line) and N4 (1.86 line) signals were equal, the overall EPR lineshape of the combined cubane signals may be very similar (*N. crassa* and *Y. lipolytica*) to that of Hatefi's Complex I. Thus, qualitatively the EPR spectra would seem to be alright, but quantitatively they are not. Three cubane clusters, one contributing to the 1.92 line, one to the 1.88 line and one to the 1.86 line, do apparently not show up in these NADH-reduced enzymes. The Fe:FMN ratios indicate that the clusters may be present, but that FMN-a is absent. Hence, I suspect that the absence of FMN-a (and lipids) may change the properties of the clusters *h* (1.86 line), *e* (1.88 line) and one of the clusters *f* or *g* (1.92 line) such, that they are not detected anymore in EPR spectra. Either they are not reduced or their magnetic properties have changed.

*Total number of spins* An additional and most useful control measurement is to determine the total spin concentration by direct double integration of an EPR spectrum of

NADH-reduced purified enzyme recorded at 4.5 K and an incident microwave power that is non-saturating for any of the clusters. If all clusters, except N1a, are reduced and EPR detectable as $S=1/2$ clusters, then seven spins per N1b (or per FMN) should be detected in case of one FMN group. With two FMN groups, seven spins per N1b but only 3.5 spins per FMN should show up.

The actually determined spin concentration of the combined Fe-S EPR signals from the classical enzyme preparations reduced with NADH is ca. 3.5 times that of the signal of N2 or the FMN concentration (2.5–4.0 spins/FMN (Orme-Johnson et al. 1974); 3.1–3.25 spins/FMN (Albracht et al. 1977); 3.8 spins/FMN (Finel et al. 1994); 3.4–3.7 times the N2-signal intensity (Albracht and De Jong 1997); 4 spins/FMN (Ohnishi 1998)). Because, as discussed above, the N1b:N2 signal ratio is 0.5, this means that the number of spins represented by the combined Fe-S EPR signals amounts to seven per cluster N1b. Thus, in the classical enzyme preparations all clusters but one (cluster N1a) are observable by EPR upon reduction with NADH. Unless the author has overlooked it, the current literature provides no such quantitative data for any of the other enzymes preparations.

Efforts to isolate the minimal catalytic unit of the bovine enzyme have resulted in the description of several subcomplexes. One of them, containing 22 mostly hydrophilic subunits (subcomplex Iα (Finel et al. 1992)) and separated from bovine Complex I, showed qualitatively all EPR signals of the parent enzyme. The 2.05 and 1.92 lines of the N2 signal were slightly broadened and the 2.05 line had shifted somewhat. A shift was also noticed for the 2.10 line. Subcomplexes prepared in slightly different ways (Iλ, 15 subunits; IS, 22 subunits; IλS, 13 subunits (Finel et al. 1994)) likewise showed qualitatively all EPR signals, but the $g_1$ line of the N2 signal was shifted from 2.05 to 2.04. Important was the loss of spin concentration of all signals by 40–60%. In subcomplex Iλ the N2 signal could no longer be induced with dithionite, only with NADH (Finel et al. 1994). In another version of subcomplex Iλ (King et al. 2009) the N2 EPR signal could not even be evoked by NADH. All of these subcomplexes lacked the capacity to react with quinones in a rotenone-sensitive way whether or not phospholipids were present.

## The presumable driving unit involved in proton translocation has a delicate structure

The core subunits making up the driving unit for the proton-translocating activity of Complex I are quite likely the TYKY, PSST, 30-kDa and 49-kDa subunits. In the hydrophobic domain the ND1 and ND5 subunits are essential. This is hypothesised from the fact that homologues of these six subunits constitute the proton-translocating membrane-bound [NiFe]-hydrogenase, called Ech, of *Methanosarcina barkeri* (Meuer et al. 1999; Albracht and Hedderich 2000; Hedderich and Forzi 2005). This is visualised in Fig. 1. The hydrogenase requires protons for the production of $H_2$. These protons (green colour) enter via one or more proton-transfer pathways in the EchE subunit (homologue of the 49-kDa subunit), just like in standard [NiFe]-hydrogenases (Volbeda et al. 1996; Matias et al. 2001; Fontecilla-Camps et al. 2007). The extra subunit EchF (TYKY homologue), which makes Ech a non-standard [NiFe]-hydrogenase (Albracht 2001), is thought to provide the enzyme with the driving unit to power proton translocation (red colour). It is proposed that Complex I uses similar proton-transfer pathways. However, in that case the 'green' protons are presumably required for the reduction of Q to $QH_2$ (Fig. 1).

The *g* values of the broad axial EPR signal of one of the cubane clusters in Ech ($g_{1,2,3}$=2.05, 1.94, 1.92 (Kurkin et al. 2002)) are closely similar to those of the N2 signal in bovine Complex I. Like the N2 signal, the cluster has a pH-dependent midpoint potential (−53 mV/pH). Cys to Ser mutations of seven of the eight conserved Cys residues in the EchF (TYKY) subunit indicated that the 2.05, 1.94, 1.92 EPR signal comes from one of the two clusters in this subunit (Forzi et al. 2005). This is reminiscent to the results from Cys to Ser mutations in the TYKY subunit of *R. capsulatus* Complex I, showing that each of the two cubane clusters in the TYKY subunit contribute equally to the N2 signal (Chevallet et al. 2003).

As discussed above, bovine Complex I in SMP is dependent on the presence of phospholipids, $Q_{10}$, and unknown factors involved in the defence against $O_2$, for a correct functioning and stability. The *g*-strain (Fritz et al. 1971; Hagen 2009) in the EPR signals of bovine Complex I is surprisingly small because the linewidths in units of *g* decrease as the microwave frequency increases (determined at 1.1, 3.4, 9.2 and 34.7 GHz) (Albracht 1974, 1984). This shows that, unlike the situation in ordinary ferredoxins, the protein structure is quite rigid and does not allow for much flexibility in the immediate co-ordination of the clusters (Hagen and Albracht 1982). Hence, any disturbance of the delicate structure in or around the protein core consisting of the TYKY, PSST, 49-kDa and 30-kDa subunits may affect the properties of the clusters in the TYKY and PSST subunits as well as the binding of FMN-a. The most noticeable changes in EPR spectra are expected to be those that affect the dominant sharp axial N2 signal from the clusters in the TYKY subunit. From a quick glance at the primary structure, this subunit looks just like an ordinary 2 [4Fe-4S] ferredoxin. However, a closer inspection showed that it must have a unique structure among Fe-S proteins.

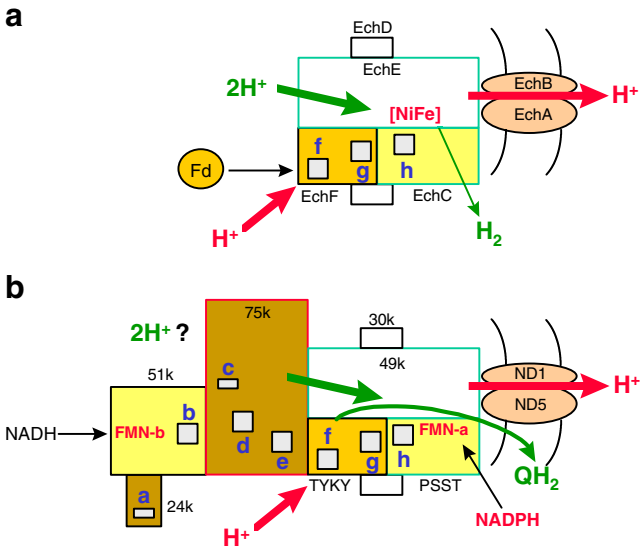


**Fig. 1** *Structural and mechanistic similarities between Complex I and a proton-pumping [NiFe]-hydrogenase.* (**a**) Modular representation of the membrane-bound proton-pumping Ech from *Methanosarcina barkeri* (adapted from (Albracht and Hedderich 2000)). The protons required for reduction to $H_2$ in the centre of the enzyme enter from the medium through one or more proton-transfer pathways in the EchE subunit (thick green arrow) (Fontecilla-Camps et al. 2007). Electrons from ferredoxin (Fd) are transferred to the Ni-Fe site via three Fe-S clusters, two in the EchF subunit and one in the EchC subunit. The thick red arrows indicate the possible entry and exit sites of an additional proton-transfer path solely involved in quinone-independent proton translocation over the membrane (curved structure). (**b**) Modular representation of seven of the hydrophilic subunits of bovine Complex I. Two of the hydrophobic subunits, i.e. ND1 and ND5 which are homologous to the EchB and EchA subunits in (**a**), respectively, are added as well. Ubiquinone ($Q_{10}$), which measures ca. 50 Å in the stretched conformation, is proposed to react deep inside the hydrophilic domain of Complex I close to cluster *f*. Homologous subunits in the enzymes have the same colour and shape and homologous Fe-S clusters have the same name (*a* to *h*). Note that the 30-kDa subunit in Complex I, a subunit only found in proton-pumping hydrogenases, and in Complex I and related enzymes, clamps the 49-kDa and TYKY subunits in Complex I together. The analogous EchD subunit in Ech is thought to strengthen the association of the EchE and EchF subunits. Squares stand for [4Fe-4S] clusters and rectangles for [2Fe-2S] clusters (clusters *a* and *c*)

The profile $Tx_3Px_{11,12}Gx_{8,14}CxxCxxCxxxC$ is exclusively found in TYKY and related subunits of ion-pumping Complex I and proton-pumping [NiFe]-hydrogenases (Albracht and Hedderich 2000).

*Point mutations in the 49-kDa subunit affect the N2 EPR signal* The following example demonstrates that the properties of the Fe-S clusters (*f* and *g*) in the TYKY subunit are strictly dependent on a correct structure of the 49-kDa subunit. A number of point mutations in the 49-kDa subunit of *Y. lipolytica* Complex I resulted in a decrease or even the loss of the N2 signal, while others had no effect. The assembly of the complex was usually quite normal (Kashani-Poor et al. 2001b; Grgic et al. 2004). One mutation (H226M) led to a 80 mV decrease of the midpoint potential of the N2 signal and an apparent loss of the pH dependence of its midpoint potential (Zwicker et al. 2006). Other mutations in the 49-kDa subunit and a series mutations in the PSST subunit, some very close to its Fe-S cluster assumed by these authors to be cluster N2 (Zickermann et al. 2009), did not induce any qualitative changes in the EPR signal of N2 (or any of the other signals) of the mutant enzyme (Tocilescu et al. 2007). Tale telling is the observation that mutations of a conserved Tyr residue in the 49-kDa subunit (Y144) did either not affect the N2 signal (Y144F), considerably affected (broadened and shifted) this signal (Y144W, Y144H, Y144S) or completely abolished it (Y144I, Y144R) in NADH-reduced mitochondrial membrane preparations (Tocilescu et al. 2010). The deamino-NADH (dNADH) oxidizing capacity of membrane preparations of all mutants, i.e. the activity of Complex I, declined to below 5%, while the rotenone sensitivity decreased noticeably. The EPR properties of enzyme with yet another set of mutations in the 49-kDa and PSST subunits (Fendel et al. 2008) were not disclosed. Unless this author has overlooked it, results of possible mutations of any of the conserved Cys residues in the PSST or TYKY subunits have not been reported.

In view of all these considerations, this author holds the opinion that point mutations in any of the TYKY, PSST, 49-kDa or 30-kDa subunits, which form the delicate structural heart of Complex I, may have unpredictable effects on any of the other subunits of this quartet. The facts that in most purified NADH-dehydrogenase preparations three of the cubane clusters go unnoticed in EPR spectra and that many point mutations lead to inactive enzymes, makes a correct interpretation of such mutations even more ambiguous. A thorough demonstration of the intactness of the mutant complex, like e.g. in (Chevallet et al. 2003), is required to minimise incorrect conclusions.

## Assignment of the EPR signals to Fe-S clusters in specific subunits

Such assignments have been based on results from four main approaches: (a) Resolution of Complex I into subcomplexes by chaotropic agents (Galante and Hatefi 1979; Ohnishi et al. 1985) or by special detergents (Finel et al. 1992, 1994); (b) Overexpression of individual subunits, often followed by chemical reconstitution (with iron and sulfide) of the Fe-S clusters; (c) Disruption of genes encoding a prosthetic-group-containing subunit; (d) Point mutations of Cys (or other) residues and EPR studies of membrane- and/or purified preparations of the enzyme.

The N2 EPR signal ($g_1$=2.05, $g_{2,3}$=1.92) is the most eye-catching signal in spectra at 15–17 K of the mitochondrial and most prokaryotic enzymes. Because the assignment of that signal to Fe-S cluster(s) is a main point of disagreement between the results presented here and the studies from other groups, I will focus this part of the overview on that point.

The Amsterdam group and initially also the Cambridge group assumed that the two clusters N2 in the bovine enzyme were located in the TYKY subunit (Fearnley and Walker 1992; Finel et al. 1992; Albracht and Hedderich 2000; Chevallet et al. 2003). Other groups believed that there was only one cluster N2 and initially thought that this cluster was bound to ND5 (Wang et al. 1991; Weiss et al. 1991). Later it was believed that three of the Cys ligands of cluster N2 were in the PSST subunit, while ligand number four was provided by the 49-kDa subunit (Kashani-Poor et al. 2001b). Presently the Complex I community, except the author of this paper, believes that there is only one cluster N2 and that this cluster has its four Cys ligands in the PSST subunit. This view is mainly based on point-mutation studies combined with EPR characterization of the mutant Complex I in membranes and/or in the purified enzyme.

Point mutation of three conserved Cys residues in the TYKY subunit have been reported for the *N. crassa* Complex I (Duarte and Videira 2000). They all resulted in a lack of assembly of the complex. Mutations of each of the tandem Cys residues in the cluster-binding motif of the PSST subunit have also been published (Duarte et al. 2002). In this case a normal assembly of the complex was observed although the purified enzyme hardly showed any NADH-decylubiquinone activity, not even in the presence of added phospholipids. None of the enzymes showed an N2 signal; qualitatively, the signals of the other clusters did not change. It was concluded that the N2 signal was caused by a cubane cluster in the PSST subunit.

Point mutations of conserved Cys residues in the PSST and TYKY subunits of the *E. coli* Complex I were reported by (Flemming et al. 2003). None of the mutants had any dNADH→$O_2$ activity as measured with membranes. Purification of NADH dehydrogenase from most of the PSST mutants was possible and EPR spectra of the enzyme reduced with excess dithionite in the presence of redox mediators were shown. Purification of the enzyme from the TYKY mutants was nearly impossible. Only one spectrum of a partly purified enzyme of the C102A mutant was shown. It was concluded that the signal of N2 was absent in the five PSST mutant enzymes, but still present in the TYKY mutant.

However, a close comparison of the spectrum of the wild-type *E. coli* enzyme (reduced with dithionite plus redox mediators) in Fig. 5 of (Flemming et al. 2003) and spectra of the wild-type enzyme (reduced with NADH) in Fig. 5 of (Sinegina et al. 2005) learns the following: (a) The conditions used for the EPR spectra in (Flemming et al. 2003), 13 K and 10 mW, were quite saturating for some of the signals, resulting in a relative loss of these signals; (b) Under non-saturating conditions and 10 K the areas of the $g_1$ peaks ascribed to the clusters N2 (at 2.05) and N3 (at 2.04) are much smaller than that of the $g_1$ peak of cluster N4 (at 2.10) (Sinegina et al. 2005). This means that the spin concentrations represented by the signals of N2 and N3 in these NADH-generated spectra are well below that represented by the signal of N4. Only at higher microwave powers the lines in the 2.05–2.04 region show up well (due to saturation of the other signals).

With that in mind, the spectra of the mutant enzymes published in (Flemming et al. 2003) can also be interpreted as follows. In NADH dehydrogenase of the TYKY/C102A mutant the remaining $g_1$ line at 2.04 is mainly due to signal N3, consistent with its position at a higher field than the $g_1$ line at 2.05 of the N2 signal in the wild-type signal and consistent with the loss of this line in the mutant enzyme. In the complexes from the PSST mutants there is virtually no peak left in the 2.05–2.04 region, pointing to loss of both the signals N2 and N3. Thus, the TYKY mutant reports the loss of the N2 signal, while the N3 signal is still present. Mutations in the PSST subunit remove the N3 signal and destabilise the TYKY subunit such that its clusters are no longer detectable. Such a destabilisation is reminiscent to the one induced by a number of point mutations in the 49-kDa subunit of the *Y. lipolytica* enzyme discussed above.

With *R. capsulatus* two of the Cys-to-Ser point mutations of conserved Cys residues in the TYKY subunit (C67S and C106S, each residue involved in the binding of one, but not the same, of the two clusters) resulted in fully active Complex I and a normal growth rate of the cells under anaerobic, photosynthetic conditions (Chevallet et al. 2003). Under these conditions, Complex I in this purple photosynthetic bacterium is essential for the production of NADH via reduction of $NAD^+$ driven by reversed electron transfer. Unlike in other mutant studies, the $g_{2,3}$ line at 1.92 in 17 K EPR spectra of membranes of the mutants was measured against an internal standard, namely the $g_{2,3}$ line at 1.94 of the [2Fe-2S] signal (cluster *c*) at 36 K (Chevallet et al. 2003). In both mutants the 1.92 line halved; the EPR signature of the Ser-bound cluster could not be established. The N3 and N4 signals did not change, therefore the two cubanes in the TYKY subunit were equally contributing to the 1.92 line.

In all the mutation studies using purified NADH dehydrogenases there is the uncertainty created by the absence of EPR signatures of three of the cubane clusters. Hence, some of the point mutations may induce changes

going unnoticed in EPR spectra, while others (e.g. some of those in the 49-kDa subunit discussed above) apparently destabilise the structure of the nearby TYKY subunit. In addition nearly all of the mutated preparations were inactive in the inhibitor-sensitive reaction with quinones. This author therefore feels that the effects of point mutations of the conserved Cys residues in the PSST subunit of Complex I from fungi or bacteria on the EPR signature of the 2.05 and 1.92 lines, i.e. the N2 signal, is not a solid basis for assigning these lines to the cluster in this subunit.

## Are the unusual properties of the cubane cluster in the PSST subunit of the *T. thermophilus* enzyme reflected in the N2 EPR signal?

Recent investigations on the crystal structure of the *T. thermophilus* enzyme in the reduced state showed unusual changes in the co-ordination of cluster *h* in the PSST subunit (Nqo6) (Berrisford and Sazanov 2009). The changes were dependent on the reductant. With NADH or NADH plus dithionite the second Cys residue (C46) of the tandem C-C ligands of the cluster, seems to be removed from one of the Fe atoms. With dithionite alone the first Cys residue (C45) becomes disconnected (from another Fe atom), whereas C46 remains connected. It was proposed that in both cases a water molecule or a hydroxide ion replaced the Cys ligand. The loss of a Cys ligand is expected to have a profound impact on the magnetic properties of the cluster. In addition the results imply that this impact should be different for dithionite or NADH as reductant. As it was assumed that the reduced cluster in the PSST subunit produces the N2 EPR signal (Hinchliffe et al. 2006), one should expect a signal shape that varies with the reductant. No such spectra are available for the *T. thermophilus* enzyme (Hinchliffe et al. 2006), so this remains to be established. For a similar cluster in adenosine 5′-phosphosulfate (APS) reductase, likewise having a tandem Cys co-ordination (Chartron et al. 2006) no EPR signal of the reduced cluster could be detected (Kim et al. 2004). Addition of $K_3Fe(CN)_6$ converted the $[4Fe\text{-}4S]^{2+}$ cluster ($S=0$) into a $[3Fe\text{-}4S]^{+}$ cluster with a normal $S=1/2$ EPR signal (Kim et al. 2004).

A signal from a $[3Fe\text{-}4S]^{+}$ cluster has never been observed with Hatefi's Complex I. In addition it is well established that the lineshape of the N2 signal of Hatefi's Complex I induced by dithionite (Albracht et al. 1977) is precisely the same as that induced by NADH (Orme-Johnson et al. 1974; Albracht et al. 1977). This implies that the properties of cluster *h* in the purified hydrophilic domain of the *T. thermophilus* enzyme are not those of the clusters that induce the N2 EPR signal in bovine Complex I.

## Significance of double electron-electron resonance (DEER) spectroscopy of Hirst's NADH dehydrogenase

Roessler et al. (Roessler et al. 2010) have attempted to assign cluster-cluster interactions observed by double electron-electron resonance (DEER) spectroscopy of the reduced enzyme prepared in their laboratory (Sharpley et al. 2006). As a basic reference frame the *T. thermophilus* structure was used. Following Ohnishi's assignment of three of the EPR signals (Ohnishi 1998), the N3 signal was assigned to cluster *b* , the N1b signal to cluster *c* and the N2 signal to cluster *h*. Unlike Ohnishi, they assumed that the N4 signal could be due to either of the clusters *d*, *f* or *g* and that at the measuring temperature (10 K) the clusters *d*, *e*, *f* and *g* can produce only one detectable EPR signal, i.e. three of these clusters were assumed not to contribute to the EPR spectrum at 10 K. As discussed above, this situation does not apply to Hatefi's Complex I where all clusters but one (N1a) are detectable. In the DEER experiments the major detection-pulse position was chosen at $g=1.927$–$1.929$ ($g_{2,3}$ line of the N2 signal) at the top of the total integrated spectrum. Pump-pulse positions were at *g* values of 2.0580, 2.0410, 1.9835 and 1.8904.

It was assumed that the oscillations in the DEER spectra only contained contributions from dipolar interactions between clusters at distances of 20–35 Å. Hence, cluster *b* (assumed by the authors to evoke the N3 signal) was dismissed, because that cluster was too far away from cluster *h* (assigned by the authors to evoke the N2 signal with $g_{2,3}=1.92$). Cluster *c* (N1b signal) was dismissed for other reasons. Simulations showed that the only possible interaction that cluster *h* could have was with cluster *f*. Hence, the latter cluster was assigned to evoke the N4 EPR signal.

A few remarks and questions to this approach are in place.

(a) As discussed above, the reported redox experiments (Reda et al. 2008) strongly suggest that 70% of the enzyme molecules in the preparation do not contribute to EPR spectra of the NADH-reduced enzyme. Spectra of the remaining 30% were not quantitatively analysed, i.e. the relative stoichiometries of the spin concentrations represented by the signals were not reported. This is not a good basis for the application of an advanced technique as DEER spectroscopy.

(b) The assignment of the N3 signal to cluster *b* has originally been based on the observation that in redox titrations with Hatefi's Complex I and pigeon-heart SMP, the 1.86 line showed a dip in the titration curve corresponding with the maximum appearance of a flavin-radical signal (Salerno et al. 1977; Ingledew and Ohnishi 1980; Sled et al. 1994). In addition, a half-

field signal accompanied this effect, suggesting formation of a $S=1$ system from a $S=1/2$ flavin radical and a S=1/2 Fe-S cluster. This is the most straightforward demonstration of a spin-spin interaction between a flavin radical and a [4Fe-4S] cluster in Complex I. However, because the 1.86 line represents two clusters (this and the preceding paper (Albracht 2010)), and since Complex I holds two FMN groups, the minimal hypothesis should be that one of these clusters interacts with one of the flavins, i.e. it could be an interaction between cluster *b* and FMN-b or one between cluster *h* and FMN-a. The second possibility would be in agreement with the cluster assignment proposed in the preceding paper. In that case, the basis for the interpretation of the DEER data (Roessler et al. 2010) would collapse. Also relevant to this point is that spin-spin interaction of a [4Fe-4S] cluster with a flavin radical has not yet been confirmed for Hirst's NADH dehydrogenase which lacks FMN-a.

An indirect assignment of the N3 signal to cluster *b* was based on the following observation. The soluble low-molecular weight type bovine NADH dehydrogenase, consisting of the 9-kDa, 24-kDa and 51-kDa subunits (Galante and Hatefi 1979), showed an EPR signal with $g_{1,2,3}=2.045$, 1.95, 1.87 (Ohnishi et al. 1981). This signal was taken as the N3 signal and assigned to the cubane cluster in the 51-kDa subunit. However, extrapolation of this interpretation to Complex I is ambiguous because it is not clear whether the $g_3$ line at 1.87 of this isolated subcomplex corresponds to the 1.88 or to the 1.86 line of Complex I in Hatefi's preparation and SMP. Hence, it cannot be excluded that in the intact enzyme cluster *b* might have an EPR signal with $g_{1,2,3}=2.04$, 1.95, 1.88. Again, that would be in line with the proposed cluster designation in Fig. 2 of the preceding paper.

(c) Lastly, in the study of Roessler et al. it was assumed that the N2 signal represents only one cluster (cluster *h*). This makes that the authors rule out the possibility that within the N2 signal a pump pulse around $g=2.05$ (exciting a particular set of clusters in the randomly-oriented frozen ensemble of clusters) could give rise to a contribution in the DEER spectrum observed at $g=1.928$ (representing another set of clusters with quite a different orientation). As discussed above and in the preceding paper (Albracht 2010), the N2 signal receives equal contributions from the clusters *f* and *g*. Hence, it should be considered whether a pump pulse at 2.05 exciting one of the clusters in the TYKY subunit could give a contribution to the detection pulse in the DEER spectrum at 1.928 of the other cluster. After all, the maximum responses in the DEER spectra were precisely those from the 2.0580/1.93 and 2.0410/1.93 pulse/detection combinations (Roessler et al. 2010). It has always been surprising that the two $S=1/2$ cubanes in the TYKY subunit do not give a spin-spin interaction as observed in X-band EPR spectra from many 2[4Fe-4S] ferredoxins. This is presumably due to a special relative orientation of the total magnetic moments of the clusters *f* and *g*. Only under energy-coupled conditions in bovine SMP, such an interaction was observed, although the true nature of the interaction, also including a $Q_{10}$ radical, is not yet completely clear (Van Belzen et al. 1997; Yano et al. 2005).

## Concluding remarks

Summarizing it can be said that the best estimates of the true properties of Complex I, as an ion-translocating membrane-bound enzyme molecule, can at present only come from studies with membrane preparations. Purification will inevitably lead to changes, e.g. loss of the defence against the destructive attack by $O_2$, or even loss of bound quinones and lipids, loss of Q reactivity, loss of the EPR signature of some or all of the Fe-S clusters and, as indicated by the Fe:FMN ratio, loss of FMN-a assuming that it was initially present. It is most important to establish whether Complex-I-containing membrane preparations from non-bovine origin posses the same pH dependencies of the NAD(P)H→$O_2$ reactions and the same pH-dependent reduction kinetics of the Fe-S clusters with NADPH as determined in the preceding paper with bovine SMP.

Recently, magnificent work of the group of Sazanov (Efremov, R.G., Baradaran, R. and Sazanov, L.A. (2010). Nature 465, 441–445) revealed the X-ray structure of the entire *T.thermophilus* enzyme. An ingenious 'steam-engine' model for its redox-driven proton translocation was proposed. Reduction of the clusters *f* and *g* in the TYKY (Nqo9) subunit and of cluster *h* in the PSST (Nqo6) subunit would induce the observed conformational changes in the 49-kDa (Nqo4) and PSST (Nqo6) subunits (Berrisford and Sazanov 2009). These structural changes would be relayed via ND1 (Nqo8) and the (non-resolved) subunits ND3, ND6 and ND4L (Nqo7, 10, 11) all the way down to ND5 (Nqo12), the most distal subunit in the membrane domain. It was proposed that each of the ND5, ND4 and ND2 subunits would be involved in the direct translocation of one proton from the cytoplasm to the periplasm in the bacterium. An additional proton might originate from changes involving the unique tandem Cys ligands around cluster *h* in the PSST subunit during redox cycling of this cluster. As outlined in the present and preceding papers the N2 EPR signal is caused by the clusters *f* and *g*. Since this

signal shows clear uncoupler-sensitive changes during tightly-coupled electron transfer in bovine SMP, I consider the model of Efremov et al. as highly attractive. It would also largely apply to Ech of *M. barkeri* (Fig. 1); however, cluster *h* in that enzyme has no tandem Cys residues. In the model of Efremov et al. the input site for the 'red' protons in Fig. 1 would be on the ND5/EchA subunits (and on ND4 and ND2, which are not shown). Binding of inhibitors like piericidin and DCCD would obstruct conformational changes, resulting in inhibition of electron transfer.



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