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Biochimica et Biophysica Acta 1459 (2000) 61–68

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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The NADH oxidation domain of Complex I: do bacterial and mitochondrial enzymes catalyze ferricyanide reduction similarly?

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Received 4 January 2000; received in revised form 29 February 2000; accepted 13 March 2000

Abstract

The hexammineruthenium (HAR) and ferricyanide reductase activities of Complex I (H^+-translocating NADH:ubiquinone reductase) from *Paracoccus denitrificans* and bovine heart mitochondria were studied. The rates of HAR reduction are high, and its steady-state kinetics is similar in both *P. denitrificans* and bovine Complex I. The deamino-NADH:HAR reductase activity of Complex I from both sources is significantly higher than the respective activity in the presence of NADH. The HAR reductase activity of the bacterial and mitochondrial Complex I is similarly and strongly pH dependent. The pK_a of this activity could not be determined, however, due to low stability of the enzymes at pH values above 8.0. In contrast to the high similarity between bovine and *P. denitrificans* Complex I as far as HAR reduction is concerned, the ferricyanide reductase activity of the bacterial enzyme is much lower than in mitochondria. Moreover, ferricyanide reduction in *P. denitrificans*, but not bovine mitochondria, is partially sensitive to dicyclohexylcarbodiimide (T. Yagi, Biochemistry 26 (1987) 2822–2828). On the other hand, the inhibition of ferricyanide reduction by high concentration of NADH, a typical phenomenon in bovine Complex I, is much weaker in the bacterial enzyme. The functional differences between the two enzymes might be linked to the properties of their binuclear Fe–S clusters.

Keywords: Deamino-NADH; Fe–S cluster; FMN; NADH; NDH-1; Ubiquinone

1. Introduction

The H^+-translocating NADH:ubiquinone oxidoreductase (Complex I) is a very large enzyme that comprises the first segment of the respiratory chain in most eukaryotes and many bacteria. Bovine mitochondrial Complex I is composed of some 43 different protein subunits, seven of which are encoded and synthesized within the organelle. The redox centers of the enzyme include one FMN molecule, probably two binuclear Fe–S clusters, and five or six tetranuclear Fe–S clusters [1,2]. Bacterial Complex I (also called NDH-1) contains a similar set of redox centers, but 'only' 14 different protein subunits. Seven of these 14 subunits are homologues of the mitochondrially encoded subunits in eukaryotic Complex I,

Abbreviations: DCCD, dicyclohexylcarbodiimide; deamino-NADH, nicotinamide hypoxanthine dinucleotide, reduced form; HAR, hexammineruthenium(III); Q_1, ubiquinone-1

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0005-2728/00/$ – see front matter
PII: S0005-2728(00)00113-4

and the other seven are homologous to seven of the nuclear-encoded subunits that play central roles in the structure and function of the enzyme [3–5].

NDH-1 of the soil bacterium *Paracoccus denitrificans* is highly similar to bovine (and human) Complex I with respect to the primary structure of its 14 subunits [3,6], and the physicochemical properties of most of its Fe–S clusters [7–9]. However, the two binuclear Fe–S clusters of Complex I may represent exceptions to this rule. The midpoint redox potential of cluster N1-a in bovine heart Complex I is very low, −370 mV at pH 7.0 and −430 mV at pH 8.0 [9,10], or even lower [11]. Since the midpoint potential of the NADH/NAD^+ couple is higher, −320 mV, it is unclear whether or not cluster N1-a participates in electron transfer reactions within the enzyme. In *P. denitrificans*, on the other hand, the midpoint potential of cluster N1-a is higher than the potential of most of the tetranuclear Fe–S clusters, except cluster N-2 [8]. The main question regarding the other binuclear cluster, N1-b, is its stoichiometry in bovine Complex I. Albracht and co workers have calculated that cluster N1-b is present in bovine Complex I at only half the amount of the other redox centers [12]. Interestingly, work from the same laboratory suggest that the concentration of cluster 1 (presumably N1-b) in *P. denitrificans* Complex I is higher than in bovine Complex I, i.e. in the bacterial enzyme it is similar to the concentration of FMN and the tetranuclear Fe–S clusters [7].

Complex I can catalyze rotenone-insensitive reduction of ferricyanide, and this activity has been widely used as a convenient assay during purification of the enzyme or fragments thereof [13–16]. Ferricyanide reduction is inhibited by high concentrations of either NADH or ferricyanide, a phenomenon called double substrate inhibition [17,18]. In spite of this substrate inhibition, the ferricyanide reductase activity was often used in the past for kinetic studies on the NADH oxidation site of bovine Complex I [17,18].

Hexammineruthenium (HAR) is a water-soluble acceptor that supports high rate of NADH oxidation by Complex I, and in contrast to ferricyanide, its reduction is not inhibited by NADH [19]. Both ferricyanide and HAR reduction are also catalyzed by the flavoprotein fragment of Complex I (FP) that contains only the 51-, 24- and 10-kDa subunits [20]. The FP contains FMN and some iron and inorganic sulfur [14]. It is unclear, however, if intact Fe–S clusters are present in this fragment [11,21], and whether they are needed for either ferricyanide or HAR reduction.

Deamino-NADH is a NADH analogue in which the adenine moiety is replaced by hypoxanthine. The small NADH:ubiquinone reductase of *Escherichia coli*, NDH-2, does not oxidize deamino-NADH, but for NDH-1 this compound is a good electron donor [22]. Nonetheless, the effect of that difference in the donor structure on the kinetics of its binding and oxidation by NDH-1 has not been examined, yet.

The effects of dicyclohexylcarbodiimide (DCCD) on the activity of mitochondrial and bacterial Complex I have been studied [23–25]. In bovine mitochondrial and bacterial Complex I from *Escherichia coli* and *Thermus thermophilus*, DCCD inhibited ubiquinone reduction, but not ferricyanide reduction. In *P. denitrificans*, on the other hand, the ferricyanide reductase activity was also sensitive to DCCD [23]. The reasons for that unusual sensitivity have not been clarified.

We have studied the HAR and ferricyanide reductase activities of *P. denitrificans* and bovine Complex I. The kinetics of the reactions, their dependencies on pH, sensitivities to DCCD, and the effect of donor type on the activity have been carefully examined. Our findings indicate that in spite of the high similarity between *P. denitrificans* and bovine Complex I, there are clear differences between them at the electron input site of the enzyme.

2. Materials and methods

2.1. Materials

Ubiquinone-1 (Q_1) was a generous gift from Hofmann-La Roche, Switzerland. DCCD was purchased from Merck, rotenone and hexammineruthenium from Aldrich, NADH from Boehringer, and ferricyanide and deamino-NADH from Sigma.

2.2. Preparative procedures

P. denitrificans strain F2 was grown under high

aeration in a malate-containing medium. Membranes were prepared from mid-log phase cells as described previously [26]. Mitochondrial membranes from bovine heart were prepared as previously described [27].

2.3. Activity assays

Enzymatic activities were assayed at 30°C. The oxidation rate of NADH or deamino-NADH in the presence of HAR was monitored spectrophotometrically at 340 nm, or at 380 nm in cases of high NADH concentrations. The HAR reductase activity was carried out as described previously [26], and the reduction of ferricyanide was assayed according to Minakami et al. [17], but in the presence of 5 mM KCN (neutralized with HCl) and 15 μM rotenone. The pH dependency of the activities was assayed in the presence of 50 mM potassium phosphate of appropriate pH. The concentrations of the different substrates in these experiments (Fig. 3) were as follow: (1) HAR reductase activity, 2 mM HAR, 0.3 mM NADH or deamino-NADH; (2) Q_1 reductase activity, 60 μM Q_1, 0.2 mM NADH or deamino-NADH, rotenone and KCN as described above, 1 mg/ml sonicated phospholipid [26] and 2.5 μg/ml antimycin A; and (3) ferricyanide reductase activity, 0.45 mM ferricyanide and 0.1 mM NADH or deamino-NADH.

2.4. DCCD inhibition

Membrane samples of either *P. denitrificans* or bovine heart mitochondria were diluted to 1 mg/ml protein in a solution containing 1 mg/ml sonicated phospholipid, 250 mM sucrose and 50 mM potassium phosphate, pH 7.0, and placed on ice. DCCD was added from an ethanolic solution to a final concentration of 0.8 mM, and corresponding amounts of cold solvent were added to the control sample. The samples were exposed to the inhibitor for 16 h at 0°C, and special care was taken to maintain the temperature of the samples very close to 0°C for the entire incubation. The activity measurements involved a 1:50 dilution of the sample into the assay medium, and thus the DCCD concentration in the assay cuvette was too low to inhibit the activity during the few min at 30°C, as was shown in separate experiments.

Protein concentrations were determined according to Lowry et al. [28], in the presence of 0.2% SDS.

3. Results and discussion

Complex I is composed of many protein subunits and redox centers. The bacterial enzyme from *P. denitrificans* provides a good model system for mitochondrial Complex I, and it has recently been used in site-directed mutagenesis studies on the putative ubiquinone binding site of the enzyme [26]. Nonetheless, the kinetic properties of *P. denitrificans* Complex I, or any other such bacterial enzyme, have not yet been carefully examined. Characterization of enzymatic activities and their kinetics is important for a better understanding of the enzyme's function. In addition, this may help to determine which kinetic properties of Complex I are fundamental for its activity, and which mainly represent minor differences between species.

The tetranuclear, EPR-detectable, Fe–S clusters of *P. denitrificans* and bovine Complex I appeared to be highly similar with respect to their line shape and midpoint potential [7–9]. On the other hand, differences were observed between the two enzyme in the stoichiometry [7], or midpoint potential [8] of the binuclear Fe–S clusters. The two binuclear Fe–S clusters of Complex I, N1-a and N1-b, are most likely bound within the NADH dehydrogenase fragment of Complex I, i.e. one in the 24-kDa subunit (called NQO2 or NUOE in bacteria), and the other in the N-terminal segment of the 75-kDa subunit (NQO3 or NUOG) [3]. Hence, differences between *P. denitrificans* and bovine Complex I in activities that are catalyzed in the vicinity of the NADH oxidation domain could be the result of the different properties of their respective binuclear clusters.

In order to address this question, we have studied the rotenone-insensitive reduction of ferricyanide and HAR, using bacterial and mitochondrial Complex I in their native membranes. The concentration of Complex I in *P. denitrificans* membranes may depend on the growth conditions of the culture, and the degree of cell wall removal during membrane isolation. Due to this possible sample variability we took care to use the same membrane preparation whenever the different activities were analyzed on the basis

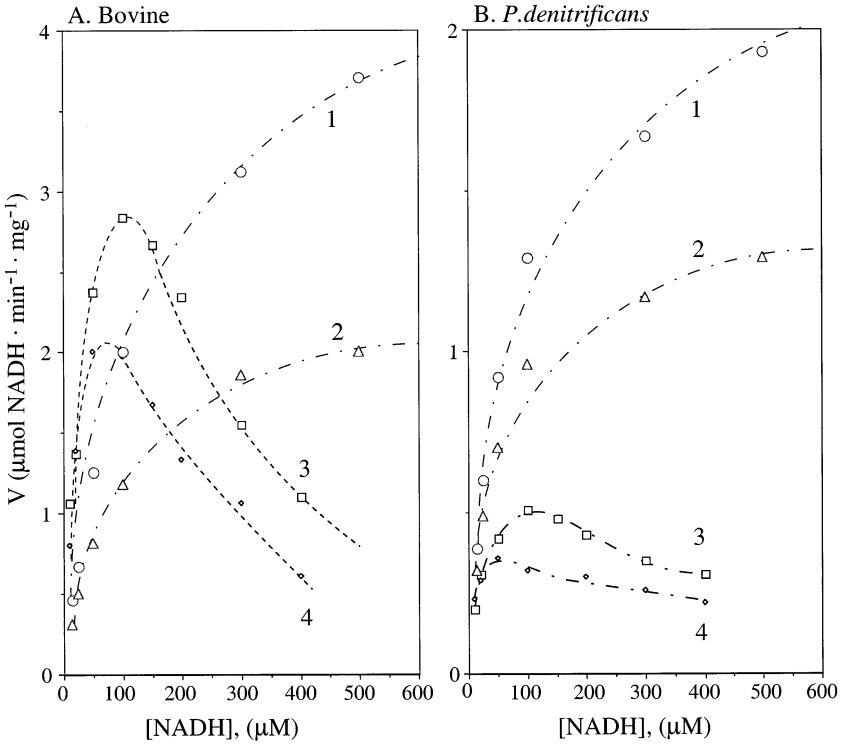

Fig. 1. Dependence of the ferricyanide and HAR reductase activities of Complex I on NADH concentration. Membrane-bound enzymes from bovine heart mitochondria (A) and *P. denitrificans* (B) were examined. The acceptor concentrations were 2 mM HAR (1), 0.5 mM HAR (2), 0.9 mM ferricyanide (3) or 0.45 mM ferricyanide (4). The activity is expressed as μmol NADH oxidized min^{-1} (mg membrane protein)$^{-1}$.

of protein concentration in the membrane. In other cases it was assumed that the HAR reductase activity is the best available estimate for the concentration of Complex I in the membrane, and it was used as a reference [26].

The specific HAR reductase activity of *P. denitrificans* Complex I in the membrane preparations was similar to the mitochondrial enzyme (Fig. 1). The specific ferricyanide reductase activity of *P. denitrificans* Complex I is, however, much lower than in mitochondria (Fig. 1). Furthermore, the typical substrate inhibition of the ferricyanide reductase activity of Complex I by high concentration of NADH (e.g. Fig. 1A, traces 3 and 4) is hardly detectable for *P. denitrificans* Complex I at the lower ferricyanide concentration (Fig. 1B, trace 4). Even at higher ferricyanide concentration, the substrate inhibition by NADH is clearly less than in bovine Complex I (trace 3 in Fig. 1A,B). These observations, namely the low ferricyanide reductase activity in comparison to the HAR reductase activity, and the absence of strong substrate inhibition by NADH, suggest that in functional terms the NADH dehydrogenase domain of *P. denitrificans* may be different from the bovine enzyme. This conclusion came as a surprise since *P. denitrificans* Complex I is highly homologous to the mitochondrial enzyme in many respects [3,6–9].

Sled and Vinogradov [19] have investigated the kinetics of the HAR reductase activity of bovine complex I, and found that it deviates from simple Michaelis–Menten kinetics [19]. They determined $K_{s(app)}$ values for NADH of 10 and 40 μM for isolated and membrane-bound Complex I, respectively, by extrapolation to zero acceptor concentration on a secondary plot of K_m vs. V_{max} [19]. We have analyzed kinetic data for *P. denitrificans* Complex I in a similar way and the results are presented in Fig. 2. The double reciprocal plots yield a series of straight lines that intercept in the third quadrant (Fig. 2A), as has been observed for bovine Complex I [19]. The $K_{s(app)}$ value for NADH, determined from the data

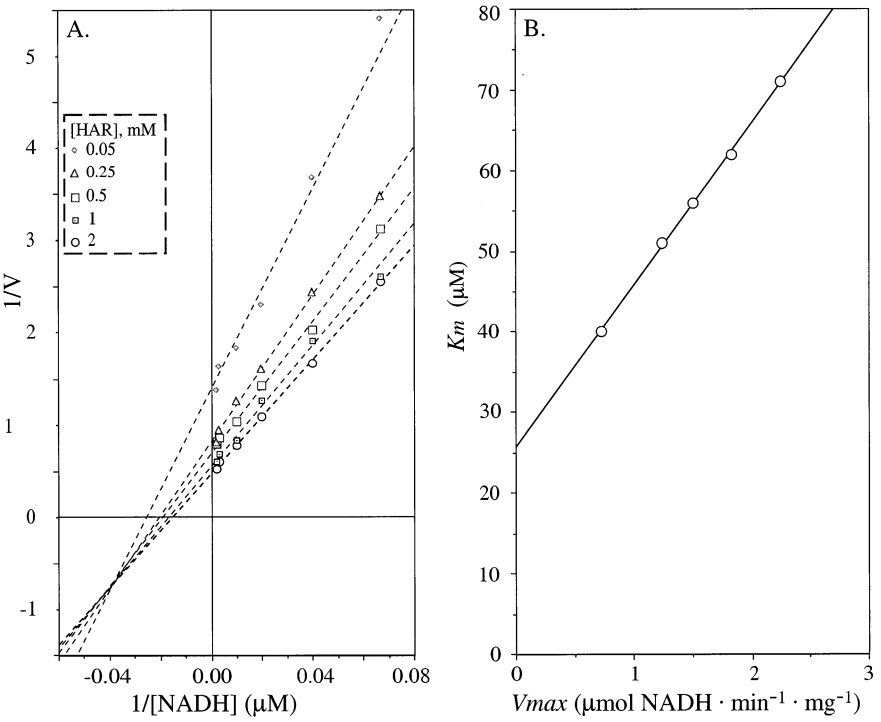

Fig. 2. (A) A double reciprocal plot of the NADH:HAR oxidoreductase activity of NDH-1 from *P. denitrificans*. NADH titrations were performed in the presence of the indicated HAR concentrations. (B), secondary plot of the K_m and V_{max} values, and extrapolation to zero acceptor concentration in order to determine $K_{s(app)}$ for NADH.

for *P. denitrificans* Complex I, is 26 μM (Fig. 2B), i.e. almost the same as for bovine Complex I. It may thus be concluded that as far as the rates and kinetics of the HAR reductase activity are concerned *P. denitrificans* Complex I is practically identical to the mitochondrial enzyme.

In our studies of *P. denitrificans* Complex I mutants it was often necessary to use deamino-NADH as the electron donor instead of NADH in order to eliminate NDH-2 activity from the assays [26]. We have now asked how similar deamino-NADH is to NADH with respect to the different activities of Complex I. In addition, we have examined the pH dependencies of these activities in the range of 6.5 to 8.0. These two assays were combined, and the results are presented in Fig. 3. The HAR reductase activity of Complex I of both *P. denitrificans* and bovine is pH dependent, regardless of whether the donor is NADH or deamino-NADH (Fig. 3A). However, the donor type had a marked effect on the rate of the reaction, and for both enzymes deamino-NADH was oxidized faster than NADH when the acceptor was HAR (Fig. 3A). The dependence of the HAR reductase activity on pH in both *P. denitrificans* and bovine Complex I (Fig. 3A) may suggest that deprotonation of a specific group close to the FMN is important for binding the positively charged acceptor, HAR. This is in agreement with previous work where positively charged ions were shown to inhibit the activity [20]. The observed difference in rate between NADH as compared to deamino-NADH (Fig. 3A) may also be interpreted according to the kinetics model that was suggested by Gavrikova and coworkers [20]. Thus, replacement of the amino group in NADH with a carbonyl oxygen in deamino-NADH changes the local charge in this area of the donor molecule, and may accelerate its binding to the pre-formed protein–HAR complex.

The pH dependence of the rotenone-sensitive Q_1 reductase activity is very different from HAR reduction, and a clear optimum was visible in each experiment (Fig. 3B). These reactions are slower and much more complex than HAR or ferricyanide reduction, hence the similarity between the two enzymes mainly reflects Q_1 binding or the rate-limiting step(s) of electron transfer reactions within the enzyme. The latter

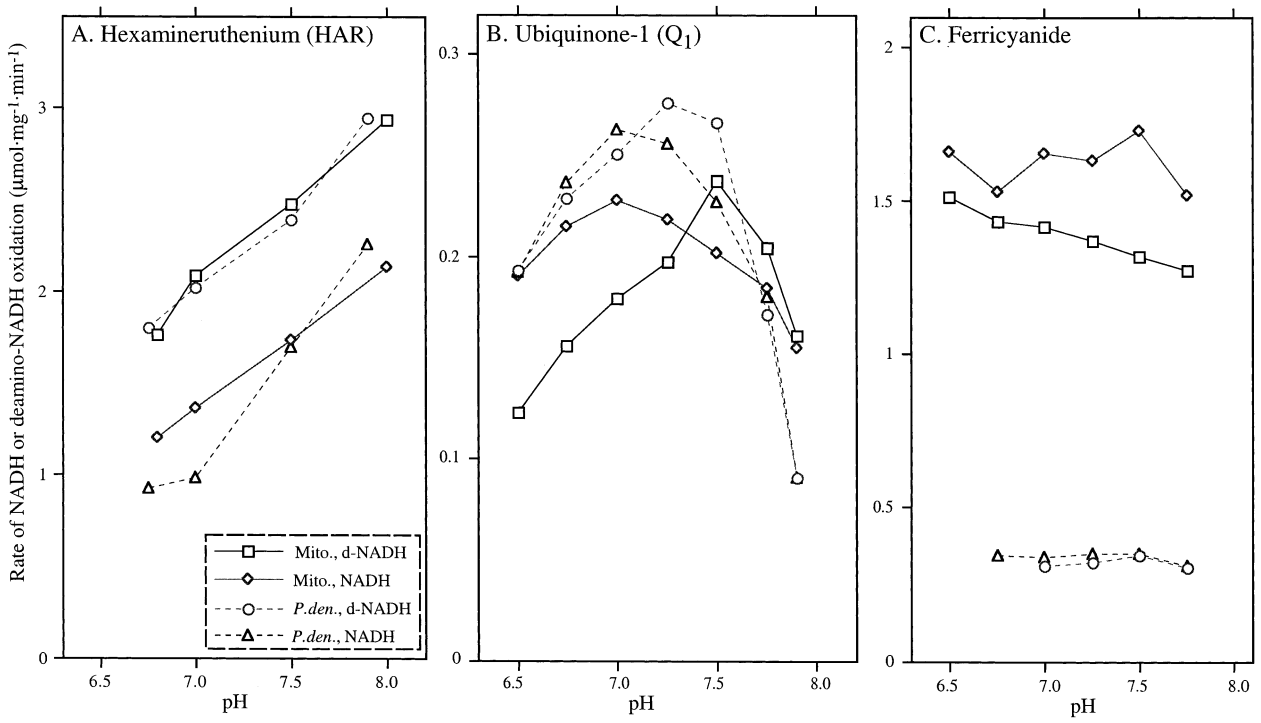

Fig. 3. The pH dependence of NADH and deamino-NADH oxidation in the presence of different acceptors. The HAR and ferricyanide activities are rotenone-insensitive (assayed in the presence of rotenone, see Section 2). In assays of the Q_1 reductase activity, the rotenone-insensitive activity (about 10%) was subtracted, and only the rotenone-sensitive activity is presented. Note that the scale of enzyme activity is different in each panel of the figure. Solid lines represent bovine Complex I in mitochondrial membranes, and dashed lines represent *P. denitrificans* Complex I in the bacterial membranes. NADH oxidation is marked by diamonds (bovine) or triangles (*P. denitrificans*), and deamino-NADH oxidation by squares (mitochondria) and circles (bacteria).

is not expected to be electron transfer from NADH to FMN since the rates of HAR reduction in both enzymes are about 10 times higher than the reduction of Q_1 (Fig. 3A). Thus, it is difficult to explain the small, but reproducible, lower pH optimum for NADH oxidation in comparison to deamino-NADH oxidation by Q_1 (Fig. 3B). This finding might be related to coordinated redox reactions that involve other redox centers within Complex I, and should be studied further.

The ferricyanide reductase activity, in contrast to HAR and Q_1 reductions, exhibits very little dependence on pH in the tested range (Fig. 3C). A dependence on donor type was detectable, however, and the rate of ferricyanide reduction was higher in the presence of NADH than with deamino-NADH (Fig. 3C). The same holds for the bacterial enzyme, even though the scale of the figure makes it difficult to observe the small differences between the NADH and deamino-NADH oxidation rates in *P. denitrificans*. Taken together with the HAR and Q_1 reductase activities, it may be suggested that the large rate difference in ferricyanide reduction between *P. denitrificans* and bovine Complex I is primarily due to lower affinity of ferricyanide for the bacterial enzyme, and not due to differences between the mammalian and prokaryotic Complex I in the primary events of NADH binding and oxidation.

The effect of DCCD inhibition on the activities of mitochondrial Complex I [23–25], and bacterial NDH-1 from *P. denitrificans*, *E. coli* and *T. thermophilus* have been previously studied [23]. It was observed that in *P. denitrificans* Complex I, the ferricyanide reductase activity is sensitive to DCCD, although to a lower extent than the Q_1 reductase activity [23]. It may be noted that in that work, the ferricyanide reductase activity present in *P. denitrificans* membranes was much higher than the Q_1 reduction, and that the ratio between these rates was similar to the ratio in bovine heart membranes [23]. This

result is contradictory to our findings (Figs. 1 and 3), and it is probably due to the absence of rotenone (or antimycin A) during the assays [23]. It is likely that under such conditions, ferricyanide was mainly reduced through the cytochrome bc_1 complex rather than Complex I. Due to this discrepancy, we have re-examined DCCD inhibition in bovine and *P. denitrificans* Complex I, and the results are summarized in Table 1.

The DCCD inhibition results reinforce the notion that the ferricyanide reductase activity of *P. denitrificans* Complex I is different from the activity of the mitochondrial enzyme (Table 1). This stands in sharp contrast to the HAR reductase activity that appears to be highly similar between the two enzymes (Figs. 1–3 and Table 1). The small percent inhibition of the HAR reductase activity in both systems, up to about 10%, appears to be within the variation of measurement method. However, the inhibition of the rotenone-insensitive ferricyanide reduction in *P. denitrificans* appears to be significant, if only partial. The DCCD inhibition may be related to the slow rate of ferricyanide reduction in *P. denitrificans* Complex I. The electron transfer to ferricyanide is not very efficient and hence a small conformational change that may be caused by DCCD binding somewhere else in the enzyme, might be sufficient to further reduce the rate of ferricyanide reduction. Another possibility is that there is a DCCD binding site inside the NADH oxidation domain in *P. denitrificans* Complex I. Such a possibility is currently being investigated in our laboratory.

The ferricyanide reductase activity of bovine Complex I has been extensively studied ([29] and references therein). A characteristic feature of this activity is its sensitivity to high concentrations of either the donor, NADH, or the acceptor, ferricyanide [17,18]. This inhibition by high concentrations of donor and acceptor was taken to indicate that the ferricyanide reduction is catalyzed by a ping-pong mechanism, whereby NADH and ferricyanide are competing with each other for access to the FMN, which is most likely buried inside the protein [18,29]. The results of the present study may suggest that in *P. denitrificans* Complex I, such a competition does not take place, except possibly, at very high ferricyanide concentrations (Fig. 1B). One possible explanation for the lack of substrate inhibition may be that while the access of ferricyanide to the FMN in this bacterial enzyme is restricted, the passage of NADH to its oxidation site is fast and similar to the situation in bovine Complex I. Alternatively, the access of ferricyanide to the flavin pocket in *P. denitrificans* may not be different from that in bovine Complex I, but its reduction rate in the bacterial enzyme may be much slower. In any case, the results of this work clearly show that the two enzymes differ in their ferricyanide reductase activity, but not in other activities that are catalyzed by the NADH oxidation domain of Complex I. This is a surprising finding when the high homologies between the two enzymes are considered, and it may point to the involvement of at least one of the binuclear Fe–S clusters in ferricyanide reduction. One may speculate that the presence of an oxidized Fe–S cluster in the vicinity of the flavin stabilizes the state of FMN that can reduce ferricyanide readily. The higher potential of this cluster in *P. denitrificans* may mean that it is reduced under the assay conditions and thus does not stimulates ferricyanide reduction. Fu-

Table 1
The effect of DCCD on Complex I activities in membranes from *P. denitrificans* and bovine heart mitochondria

Acceptor	NADH:acceptor reductase activities (% of initial activity ± S.D.) after incubation in the presence or absence of DCCD					
	P. denitrificans			Bovine mitochondria		
	−DCCD	+DCCD	% inhibition	−DCCD	+DCCD	% inhibition
Q_1	79.8 ± 3.4	13.2 ± 0.9	83.5	86.5 ± 2.3	31.9 ± 1.1	63.1
Ferricyanide	73.7 ± 4.1	50.9 ± 2.8	31.0	88.4 ± 1.4	81.0 ± 3.2	8.4
HAR	79.8 ± 3.0	74.3 ± 1.1	6.9	92.6 ± 2.2	84.0 ± 1.3	9.3

Membrane samples were incubated at 0°C and pH 7.0 for 16 h in the presence or absence of 800 μM DCCD. Three parallel tubes were used in each incubation. The percent activity values are in comparison to the respective activities at the onset of the incubations.

ture experiments using site directed mutagenesis of the bacterial subunits, NQO 1, 2 and 3 (NUO E, F and G), may shed light on the mechanism of this reaction.

One of the main findings of the present work is the high similarity of the HAR reductase activity between mitochondrial and bacterial Complex I (Figs. 1–3 and Table 1). In light of the above discussion, this result may suggest that the binuclear Fe–S clusters of Complex I do not play a role in HAR reduction. This observation is in agreement with the finding that the HAR reductase activity of the flavoprotein fragment of bovine Complex I (FP) is very similar to the activity of the intact enzyme [20]. It was earlier reported that although the FP fragment contains iron and inorganic sulfur, it lacks a clear EPR spectrum of intact Fe–S clusters [11,21,29]. Hence, if the Fe–S clusters in the vicinity of the bound FMN have been destroyed during the isolation of this fragment without significantly affecting HAR reduction [20], one can conclude that they are not involved in this activity. Interestingly, the FP fragment has a considerable ferricyanide reductase activity per mg protein. However, if the activity is calculated per bound FMN, it is about 10 times lower than in intact bovine Complex I [14,29].

Finally, the HAR reductase activity is a very useful tool in studies on both isolated and membrane-bound Complex I. This activity, at sufficiently high concentrations of HAR and NADH, and even more so deamino-NADH (Fig. 3), provides a sensitive tool for detection and determination of small amounts of Complex I, as we have recently demonstrated [26]. In the future, this activity could be beneficial for detailed studies on NADH binding and oxidation by Complex I, and for analysis of Complex I mutants in different organisms.

Acknowledgements

We would like to thank Dr. Pamela David for critical reading of the manuscript. This work was supported by the Academy of Finland, Biocentrum Helsinki, and the Sigrid Juselius Foundation. V.Z. is indebted to the Deutsche Forschungsgemeinschaft (DFG) for financial support.

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