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Mammalian NADH:ubiquinone oxidoreductase (Complex I) and nicotinamide nucleotide transhydrogenase (Nnt) together regulate the mitochondrial production of H₂O₂—Implications for their role in disease, especially cancer

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Abstract Mammalian NADH:ubiquinone oxidoreductase (Complex I) in the mitochondrial inner membrane catalyzes the oxidation of NADH in the matrix. Excess NADH reduces nine of the ten prosthetic groups of the enzyme in bovine-heart submitochondrial particles with a rate of at least 3,300 s⁻¹. This results in an overall NADH→O₂ rate of ca. 150 s⁻¹. It has long been known that the bovine enzyme also has a specific reaction site for NADPH. At neutral pH excess NADPH reduces only three to four of the prosthetic groups in Complex I with a rate of 40 s⁻¹ at 22 °C. The reducing equivalents remain essentially locked in the enzyme because the overall NADPH→O₂ rate (1.4 s⁻¹) is negligible. The physiological significance of the reaction with NADPH is still unclear. A number of recent developments has revived our thinking about this enigma. We hypothesize that Complex I and the Δp-driven nicotinamide nucleotide transhydrogenase (Nnt) co-operate in an energy-dependent attenuation of the hydrogen-peroxide generation by Complex I. This co-operation is thought to be mediated by the NADPH/NADP⁺ ratio in the vicinity of the NADPH site of Complex I. It is proposed that the specific H₂O₂ production by Complex I, and the attenuation of it, is of importance for apoptosis, autophagy and the survival mechanism of a number of cancers. Verification of this hypothesis may contribute to a better understanding of the regulation of these processes.

Keywords NADH:ubiquinone oxidoreductase · Transhydrogenase · Reactive oxygen species (ROS) · Cancer

Abbreviations

Ech Escherichia coli hydrogenase-3-type hydrogenase
from Methanosarcina barkeri
EM Electron microscopy
EPR Electron paramagnetic resonance
Fe-S Iron sulphur
HIF Hypoxia-inducible transcription factor
IRE Iron-response element
Nnt Δp-driven nicotinamide nucleotide transhydrogenase
PDH Pyruvate dehydrogenase
PDK Pyruvate dehydrogenase kinase
ROS Reactive oxygen species
SMP Submitochondrial particles
SOD Superoxide dismutase
TTF A 2-thenoyl-trifluoroacetone
UCP2 Uncoupling protein 2
Δp Protonmotive force
ΔΨₘ Mitochondrial membrane potential.
Introduction

NADH:ubiquinone oxidoreductase (EC 1.6.5.3; Complex I) from bovine heart is the largest complex of the mitochondrial respiratory chain. Its mechanism of action has been, and still is, extensively studied. There is strong evidence that the bovine enzyme contains two FMN groups and has a specific site for NADPH (for overview see (Albracht 2010a, b)). At physiological pH the reaction between Complex I in bovine-heart submitochondrial particles (SMP) and excess NADPH (rate 40 s⁻¹ at 22 °C) does not result in overall oxidation of NADPH by the respiratory chain. Likewise K₃Fe(CN)₆, the best artificial electron acceptor of the complex with NADH as electron donor, is not reduced when electrons are coming from NADPH. Hence, it has been proposed that Complex I contains two separate reactions sites for pyridine nucleotides each requiring a separate FMN group (Albracht and Hedderich 2000; Albracht 2010a, b). NADH reacts with the FMN group in the 51 kDa subunit (here referred to as FMN-b) resulting in oxidation of this substrate by the respiratory chain. NADPH reacts with the other FMN group (FMN-a) proposed to be bound to the conserved flavodoxin fold in the PSST subunit. Because this does not result in the overall oxidation of NADPH, the physiological relevance of this reaction has remained an enigma. Over the last 6 years certain developments in various, seemingly unrelated fields of research have inspired us to formulate a possible function.

Hypothesis in short

Our hypothesis is that the reaction of Complex I with NADPH can attenuate the levels of H₂O₂ generated by the enzyme during turnover with NADH under physiological conditions. When electrons come from NADH, FMN-a can be in the oxidized, semiquinone or reduced state, because electrons reach the flavin via Fe-S clusters, i.e. via n=1 redox groups (Fig. 1). With the NADPH/NADP⁺ couple the flavin shuttles between FMN and FMNH₂ in an n=2 redox reaction. Under physiological conditions the semiquinone concentration will decrease when the NADPH/NADP⁺ ratio increases. The membrane-bound Nnt has an apparent affinity for Complex I; it co-purifies with the complex in the classical purification procedure of Hatefi (Hatefi and Bearden 1976; Ragan and Widger 1975; Ragan 1976; Finel et al. 1992). Hence, it is proposed that the NADPH/NADP⁺ ratio local to the site where NADPH reacts with Complex I, is dominated by the ΔΨ-driven activity of Nnt. Reduced FMN-a cannot react with K₃Fe(CN)₆ and is not solvent accessible. Gaseous O₂ is considered to approach FMN-a through the hydrophobic tunnel in Complex I, which it has in common with [NiFe]-hydrogenases. Dioxygen can react fast with the flavosemiquinone radical to form superoxide (O₂⁻). Its rate of reaction with FMNH₂ is very much slower (spin forbidden). The charged superoxide anion cannot leave the site through the hydrophobic gas tunnel. A caging effect will stimulate its spontaneous dismutation into H₂O₂ which then leaves the enzyme.

The hypothesis predicts that the association and cooperation between the two membrane-bound enzymes enables the protonmotive force (Δp), reflecting the energy status of the mitochondrion, to rapidly and specifically regulate the H₂O₂ generation by Complex I. Thus, at a high Δp Nnt will contribute to a high local NADPH/NADP⁺ ratio, thereby attenuating the H₂O₂ production by Complex I, and vice versa. This specific H₂O₂ production, as well as the attenuation of it, is proposed to have a regulatory role in apoptosis and autophagy.

Dioxygen can also react with reduced FMN-b, as has been demonstrated with highly purified NADH dehydrogenase (Kussmaul and Hirst 2006). The redox state of FMN-b is controlled by the NADH/NAD⁺ ratio and generated O₂⁻ can rapidly diffuse from the solvent-accessible FMN-b into the bulk aqueous matrix medium.

Some general properties of the enzymes involved

Bovine-heart Complex I

Bovine Complex I (980 kDa) consists of 45 subunits (Fearnley and Walker 1992; Walker 1992; Hirst et al. 2003; Carroll et al. 2006). In its native form it contains two flavin molecules (FMN) and eight iron-sulphur (Fe-S) clusters, two of the [2Fe-2S] type and six of the [4Fe-4S] type (Fearnley and Walker 1992; Sazanov and Hinchliffe 2006; Albracht 2010a, b). It catalyzes the oxidation of NADH by Q₁₀ (ubiquinone, also called coenzyme Q) and translocates ca. two protons per electron from the mitochondrial matrix (via the intermembrane space) to the cytosol (Wikström 1984; Galkin et al. 1999). The resulting Δp is used for the production in the matrix of ATP from ADP and phosphate. The ATP is subsequently transported to the cytosol by the adenine-nucleotide translocase (ANT) in exchange for cytosolic ADP, a process driven by the mitochondrial membrane potential (ΔΨₘ).

In the present paper the term ‘Complex I’ will be used for the enzyme in membrane preparations and for purified preparations that catalyze an inhibitor-sensitive (rotenone or piericidin A) NADH→ubiquinone activity without any reconstitution efforts. Purified preparations without this activity will be called ‘NADH dehydrogenase’, even although inhibitor-sensitive quinone reductase activity can often be induced by treatment with phospholipids. As discussed elsewhere (Albracht 2010a) nearly all NADH dehydrogenases contain only a single FMN.
Recently the structures of the entire enzymes from *Thermus thermophilus* (bacterial enzyme, 4.5 Å resolution (Efremov et al. 2010)) and *Garnoria lipolytica* (mitochondrial enzyme, 6.3 Å (Hunte et al. 2010)) have been resolved by X-ray diffraction. The global structures resemble the shape of the capital character L. The base of the L shape is anchored in the membrane and contains the hydrophobic subunits of the complex. The stalk of the L sticks out into the inner (cellular or mitochondrial) aqueous space and is composed of the hydrophobic subunits. A similar global surface shape has been determined with cryo electron microscopy (EM) for the mitochondrial enzymes from *Neurospora crassa* (Leonard et al. 1987; Hofhaus et al. 1991; Guénebaut et al. 1997), *Escherichia coli* (Guénebaut et al. 1999; Morgan and Sazanov 2008), *Aquifex aeolicus* (Peng et al. 2003) and *Paracoccus denitrificans* (Yip et al. 2011). As the enzymes contain different numbers of subunits, the surface-structural details may differ (Zickermann et al. 2009). The X-ray ray structure showed that the hand of most EM-derived surface structures was a mirror image of the correct structure (Efremov et al. 2010), as was independently reported by investigators using the cryo-EM technique (Dudkina et al. 2010). The structure of the membrane domain of the *E. coli* enzyme has also been resolved (3.9 Å) and is similar to that of the *T. thermophilus* enzyme (Efremov et al. 2010).

The structure of the hydrophilic domain of Complex I from *T. thermophilus* is known in more detail (3.3 Å resolution) (Sazanov and Hinchliffe 2006). This major achievement disclosed the long-awaited information on the relative positions of the subunits and prosthetic groups involved in electron transfer. In contrast to the native bovine enzyme, which contains 2 FMN groups (Albracht et al. 2003; Van der Linden et al. 2004), the crystallized *T.
thermophilus enzyme has only one FMN and contains an extra [4Fe-4S] cluster in an extension of the largest subunit. The redox groups are approximately positioned along a straight line spanning a distance of ca. 90 Å. They form an ‘electric wire’ in the enzyme at an angle of ca. 120° (Efremov et al. 2010) to the plane of the membrane. The similarities between the global surface structures of Complex I from quite different organisms, as well as the high amino-acid sequence conservation between their prosthetic group-carrying subunits, has led to the consensus that the T. thermophilus structure may serve as a common template for the hydrophilic part of these enzymes. A schematic representation of the prosthetic groups in the bovine enzyme is shown in Fig. 1A and B.

Bovine-heart submitochondrial particles (SMP) can catalyze an NADPH→O2 reaction; its rate is highly dependent on pH and peaks at pH 5.7 (Fig. 1C). At pH 5.5 the oxidation of NADPH is as fast as that of NADH; both reactions are at least 99% inhibited by rotenone or KCN. The K_m values for both substrates differ widely, being 7 to 8 μM for NADH (Hatefi and Hanstein 1973) and 550 to 570 μM for NADPH (Hatefi et al. 1962; Rydström et al. 1978).

In the T. thermophilus enzyme one side of the isoalloxazine ring of FMN (FMN-b) is solvent accessible. This is the site where NADH reacts. When bound, its nicotinamide ring is positioned on top of the isoalloxazine ring, like in many other pyridine-nucleotide-binding flavoenzymes (Berrisford and Sazanov 2009). This is also the proposed site of reaction of the artificial electron acceptor K_3Fe(CN)_6 (Berrisford and Sazanov 2009). This is also the proposed site where NADH reacts. When bound, its nicotinamide ring of FMN (FMN-b) is solvent accessible. This is for NADPH (Hatefi et al. 1962; Rydström et al. 1978).

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The overall oxidation of NADPH by O2 at pH 8 occurs with a rate of at most 1.4 s^{-1} at 22 °C (Bakker and Albracht 1986; Van Belzen and Albracht 1989; Albracht 2010b). At this pH rotenone has no effect on the number of sites occupied (co-operative binding; for details see (Van Belzen et al. 1990; Albracht 2010b)). In the ‘steam-engine’ model of Sazanov and co-workers (Efremov et al. 2010) this might mean that structural changes in ND1 induced by reduction of Fe-S clusters not reducible by NADPH, and/or electron transfer to ubiquinone, is/are required for the simultaneous, tight binding of piericidin at both sites. Reduction with NADPH causes tight binding of only one inhibitor molecule (assumption: at site 1). DCCD binds to ND1 and blocks forward and reverse electrons transfer (Yagi 1987; Murai et al. 2007)
within 6 ms, even at 2 °C (Albracht 2010b); this is equivalent to a turnover number for NADH of at least 830 s$^{-1}$. Assuming a two- to three-fold change in rate for every 10 °C, the estimated turnover number at 22 °C would be at least 3,300 s$^{-1}$. The maximal rate of the NADH$\rightarrow$O$_2$ reaction in Fig. 1C equals a turnover number of at most 153 s$^{-1}$ at 22 °C. This demonstrates the well known property of Complex I in bovine-heart SMP that the rate of its reduction by NADH is much faster than its rate of reoxidation by ubiquinone. Thus, bovine Complex I comprises two separate FMN-containing dehydrogenase modules, one for NADH and the other for NADPH; both modules feed electrons to the TYKY subunit (Van Belzen and Albracht 1989; Albracht 2010a, b). Although rotenone and piericidin A have no effect on the rate or extent of reduction by NADH of FMN-b and the Fe-S clusters, the reduction by NADH is much faster than its rate of reoxidation by ubiquinone. Thus, bovine Complex I does so only in the absence of dioxygen (Warburg et al. 1924). They concluded that in tumours the oxidation rate of glucose (‘Zuckeroxidation’) cannot keep up with the rate of glycolysis (‘Zuckerspaltung’). They found that especially in malignant tumours the ratio Zuckerspaltung/Zuckeroxidation was very high. Often this finding is referred to as ‘the Warburg effect’. Strictly speaking it is not an ‘effect’ but a phenotype (the Warburg phenotype). Its use is also rather confusing because one of Warburg’s other discoveries, the inhibitory effect of O$_2$ on photosynthesis (Warburg and Krippahl 1960), became known as ‘the Warburg effect’ in photosynthesis research (Ellyard and San Pietro 1969; Robinson et al. 1980).

Experimental and theoretical basis for the hypothesis

An experimental link between the action of Complex I and cancer

Bioenergetics of cancer cells; the Warburg phenotype

In 1924, Warburg and collaborators first reported that tumour tissue produced considerable amounts of lactic acid under aerobic conditions, in contrast to normal tissue that did so only in the absence of dioxygen (Warburg et al. 1924). They concluded that in tumours the oxidation rate of glucose (‘Zuckeroxidation’) cannot keep up with the rate of glycolysis (‘Zuckerspaltung’). They found that especially in malignant tumours the ratio Zuckerspaltung/Zuckeroxidation was very high. Often this finding is referred to as ‘the Warburg effect’. Strictly speaking it is not an ‘effect’ but a phenotype (the Warburg phenotype). Its use is also rather confusing because one of Warburg’s other discoveries, the inhibitory effect of O$_2$ on photosynthesis (Warburg and Krippahl 1960), became known as ‘the Warburg effect’ in photosynthesis research (Ellyard and San Pietro 1969; Robinson et al. 1980).

Warburg hypothesized (‘Hypothese über die Entstehung der Tumoren’ (Warburg et al. 1924)) that tumours could be caused by chronic hypoxia of normal tissue. He assumed that some tissue cells survived because they had an enhanced capacity of (aerobic) glycolysis, whereas the majority of cells died due to shortage of O$_2$ because they did not have this property. This view (selective pressure) is still shared by present investigators (see e.g. (Borst and Rottenberg 2004; Mathupala et al. 2010)). A detailed insight into the precise causes for the metabolic reprogramming of cancer cells came from in depth genetic analyses of such cells (Parsons et al. 2008; Jones et al. 2008; Chin and Gray 2008; Stratton et al. 2009), but this will not be discussed here.

In vitro experiments. Effects of dichoro-acetate (DCA) on glucose metabolism in cancer cells

Michelakis and co-workers discovered that the abnormal glucose metabolism in cancer cells (Warburg phenotype)
can be normalized by a simple compound: dichloro-acetate (DCA) (Bonnet et al. 2007). It was demonstrated that the low level of glucose oxidation in cells of three human cancers (non-small-cell lung cancer, glioblastoma and breast cancer), having mitochondria with a hyperpolarized \( \Delta \Psi \text{m} \), is probably due to the constant inhibition of pyruvate dehydrogenase (PDH) by phosphorylation via one or more of its four kinases. Specific inhibition of the pyruvate dehydrogenase kinases (PDK) in these cancer cells by DCA, or by attenuation of the PDK2 activity by siRNA (Bonnet et al. 2007), caused (a/o): (i) A decrease of extracellular lactate; (ii) An increase of the intracellular pH; (iii) The decrease of the \( \Delta \Psi \text{m} \) in these cells to normal values (no effect in control cells); (iv) An increase of NADH in mitochondria isolated from the DCA-treated cancer cells; (v) A stimulation of electron transfer through the mitochondrial respiratory chain (also in control cells) with the concomitant increase (ca. 50%) in the cellular production of \( \text{H}_2\text{O}_2 \) (not in control cells). This increase was almost completely sensitive to rotenone whereas 2-thenoyl-trifluoroacetone (TTF-A), an inhibitor of succinate: ubiniquone oxidoreductase (Complex II), had no effect; (vi) An increase in the outward K\(^+\) current from the cells (no effect of DCA on control cells), leading to a hyperpolarization of the plasma membrane. This was largely inhibited by 4-aminopyridine, an inhibitor of voltage-dependent K\(^+\) channels. The increased K\(^+\) current was also blocked by rotenone or by micro-injected intracellular catalase, so it was apparently induced by an increased level of \( \text{H}_2\text{O}_2 \); (vii) An increase in the expression of the K\(^+\) channel \( \text{Kv}1.5 \). In cancer cells this expression is lower than in normal ones; and (viii) A rotenone-sensitive decrease of the cytosolic Ca\(^{2+}\) level.

Thus, rotenone inhibited the DCA-induced increase in (cytoplasmic) \( \text{H}_2\text{O}_2 \), the DCA-induced increase in the outward K\(^+\) current of the cancer cells and the DCA-induced decrease in the cytosolic Ca\(^{2+}\) concentration (Bonnet et al. 2007). The primary DCA effects on these cells were rather rapid, i.e. the normalization of the \( \Delta \Psi \text{m} \), as well as the induction of the K\(^+\) current and the decrease of cytoplasmic Ca\(^{2+}\), occurred within 5 min (and persisted after 48 h). We also note that the DCA-induced normalization of the hyperpolarized \( \Delta \Psi \text{m} \) was accompanied by an increase of the intracellular pH. This strongly suggests that the cancer-cell mitochondria had an abnormally high \( \Delta \rho \).

The (hyperpolarized) \( \Delta \Psi \text{m} \) in the cancer cell lines was hardly affected by KCN, but this inhibitor greatly decreased the (normal) \( \Delta \Psi \text{m} \) of DCA-treated cells. Hence, the authors concluded that the \( \Delta \Psi \text{m} \) hyperpolarization of non-treated cancer cells was apparently not caused by electron transfer. Uncoupler diminished the \( \Delta \Psi \text{m} \) of cancer cells, of DCA-treated cancer cells and of normal cells to the same low level (Bonnet et al. 2007). Moreover, it was shown that DCA induced apoptosis and decreased proliferation of the cancer cells, i.e. it induced an overall cell death. The experiments suggested that the DCA-induced apoptosis was achieved via two pathways, the mitochondria-dependent one (ca. 65%) and the plasmalemmal one (ca 35%). Noteworthy is that the DCA-induced changes in both processes were inhibited by rotenone. This specific effect of DCA has now been confirmed in several other studies, e.g. with two more human cancers, endometrical adenocarcinoma (uterin corpus) and prostate cancer (Wong et al. 2008; Cao et al. 2008; Michelakis et al. 2008), in rat metastatic breast cancer cells (Sun et al. 2010) and in colorectal cancer cells (Madhok et al. 2010) (for overview see e.g. (Papandreou et al. 2010)). Presently, DCA is being used in clinical trials (http://clinicaltrials.gov).

The crystal structure of PDK1 shows that DCA can bind to its N-terminal domain. The binding induces conformational changes relayed to the nucleotide and lipoyl-binding pockets of the protein (Kato et al. 2007), whereby a correct structure of a particular motif (DW-motif) in the C-terminus is essential (Li et al. 2009). Once the PDK is inhibited in the cell, the phosphorylated, inactive PDH can be rapidly activated again by the action of a specific phosphatase (Whitehouse et al. 1974). Especially PDK3, upregulated in cancer cells and the only enzyme of the four PDKs that is not inhibited by pyruvate, may play a dominant role in the suppression of the PDH activity (Lu et al. 2008).

Because many of the effects of DCA on cancer cells were largely inhibited by rotenone, Bonnet et al. proposed a ‘complex I-centered’ mechanism to explain them (Bonnet et al. 2007; Michelakis et al. 2010). Enhanced mitochondrial NADH levels would lead to enhanced production of reactive oxygen species (ROS) by Complex I and this would damage its Fe-S clusters, in fact a suicidal action. In turn, this would lead to a diminished \( \Delta \Psi \text{m} \). As discussed later on, we consider this explanation less likely.

**In vivo experiments with DCA**

In rat-model experiments DCA caused an immediate halt in tumour growth and a significant decrease in tumour size after 1 week of treatment, without apparent toxic effects on normal tissue (Bonnet et al. 2007). A recent study of the effects of DCA in patients with brain tumours (Michelakis et al. 2010) corroborated and extended the earlier in vitro and rat-model ones (Bonnet et al. 2007). The results were consistent with an inhibition of angiogenesis, induction of apoptosis and suppression of proliferation in the tumours by the DCA treatment. In accordance with earlier reports (reviewed by (Pastorino and Hoek 2008; Mathupala et al. 2009)) hexokinase-II was found to be bound to mitochondria in tumour tissue (but not in normal tissue). Hexokinase-II is upregulated in cancer cells and binds to
the outer-membrane voltage-dependent anion channel (VDAC), of which the ATP-ADP translocase (ANT) is a part. It has been proposed that this hexokinase taps off ATP right at the spot where it comes out of the mitochondrion (Pastorino and Hoek 2008; Mathupala et al. 2009). DCA removed this binding (Michelakis et al. 2010). In addition it was found that DCA induced activation of the pro-apoptotic tumour-suppressor transcription factor p53 and increased the level of downstream p21, an activator of many protein kinases (Van den Broeke et al. 2010). Recent evidence has shown that, in addition, p53 binds to, and inhibits the activity of, glucose 6-phosphate dehydrogenase so that the pentose phosphate pathway, the major producer of cytosolic NADPH, becomes compromised (Jiang et al. 2011). The study of Michelakis (Michelakis et al. 2010) demonstrated that the amounts of DCA required to inhibit PDK in cancer patients cause no or only very mild reversible neurotoxic side effects.

DCA also decreased the level of HIF-1 (hypoxia-inducible transcription factor) in the nucleus (Michelakis et al. 2010). HIF-1 is a most important transcription factor that safeguards cellular ATP production in healthy individuals under hypoxic conditions. It is a heterodimeric protein and its concentration in the cell is regulated via the level of one of the subunits, HIF-1α. Under normoxic conditions two specific proline residues in the HIF-1α subunit are readily hydroxylated by a specific enzyme (HIF-hydroxylase) containing a prolyl 4-hydroxylating domain as discovered in 2001 (Ivan et al. 2001; Jaakkola et al. 2001). Like the pro-collagen prolyl 4-hydroxylase, this enzyme requires 2-oxoglutarate and O2 as substrates and ascorbate (vitamin C) as an activator (Knowles et al. 2003; Vissers et al. 2007; Kaelin and Ratcliffe 2008) also in vivo (Kuiper et al. 2010). For the pro-collagen enzyme from chick embryos (reaction: 2-oxoglutarate+O2+Pro-R→succinate+CO2+4-OH-Pro-R) it has been demonstrated by EPR measurements that ascorbate is required to keep the iron ion in the active site in the ferrous (Fe2+) state (De Jong et al. 1982; De Jong and Kemp 1984). Ferrous iron can bind and activate O2, whereas ferric iron (Fe3+) cannot. In the absence of the target ‘Pro’ substrate a direct oxidation of ascorbate takes place (reaction: 2-oxoglutarate+Asc+O2→succinate+CO2+dehydroAsc) and catalysis (of this specific reaction) continues (De Jong and Kemp 1984). If ascorbate is absent as well, then catalysis stops due to oxidation of Fe2+.

Hydroxylation of either of the Pro residues in HIF-1α generates a binding site for the ‘von Hippel-Lindau’ tumour suppressor protein (pVHL), a component of the ubiquitin ligase complex. This results in poly-ubiquitination of HIF-1α, which is then subject to degradation by the 26S proteasome (Kaelin and Ratcliffe 2008). Under normoxic conditions (and in the presence of ascorbate) the active heterodimeric HIF-1 protein is hardly formed. In normal tissue the O2 concentration (10–30 μM) is well below the Km of the active HIF-hydroxylase for O2 (90–100 μM) (Kaelin and Ratcliffe 2008). Hence, the reaction rate is nearly directly proportional to the O2 concentration and so it slows down under hypoxic conditions. A decreased 2-oxoglutarate level will amplify this effect. Interestingly, a deficiency of ascorbate in neutrophils from vitamin-C-deficient mice leads to HIF-1α formation even under normoxic conditions. For these white blood cells ascorbate is essential for their spontaneous apoptosis (Vissers and Wilkie 2007). The active HIF-1 heterodimer regulates the expression of well over hundred nuclear genes, e.g. it causes the upregulation of genes encoding PDK1, PDK3 (Kato et al. 2007; Lu et al. 2008), and proteins and enzymes involved in glucose uptake, glycolysis, erythropoiesis, angiogenesis, and autophagy of mitochondria (Semenza 2008b). This is accompanied by downregulation of mitochondrial biogenesis (Kaelin and Ratcliffe 2008; Mole et al. 2009). As a result, the bioenergetic phenotype of the cell shifts from a glucose-oxidative type (bulk of ATP produced by mitochondria) to a glycolysis type (increased ATP production in the cytosol), in agreement with Warburg’s observations (Warburg 1927, 1954).

It is worthwhile to mention that in addition to the bioenergetic phenotype (Warburg phenotype), that may be normalized by DCA (Bonnet et al. 2007; Michelakis et al. 2010), many tumours also show an immunological phenotype, i.e. they manage to cripple the immune system. A simple re-activation appears to be an extremely efficient novel method to eradicate many cancers (see e.g. (Yamamoto et al. 2008, 2009) and reference therein).

Hyperpolarization of the mitochondrial inner membrane observed in cancer cells

Mitochondria in most types of cancers share an interesting bioenergetic property: they have a much higher ΔΨm of at least 60 mV, than mitochondria in normal cells. Because the outside of the inner membrane is positive, while the matrix side is negative, this has been measured by the accumulation of positive fluorescent dyes like Rhodamine 123 (Chen 1988). Although not discussed in (Chen 1988), where 200 different cancer cell lines were investigated, it is less likely that this increased ΔΨm is caused by respiration, because the rate of pyruvate oxidation in mitochondria in cancer cells is lower than that in normal cells (Warburg 1924; Warburg 1927; Bonnet et al. 2007). Also the observation that KCN and TTFA had no effect on the ΔΨm in cancer cells (Bonnet et al. 2007) is in agreement with the idea that the increased ΔΨm is not caused by respiration. It should be mentioned, however, that only a low dose (5 μM) KCN was used in these experiments. Cyanide inhibition of cytochrome c oxidase is easily reversed by pyruvate
(Cittadini et al. 1971) because pyruvate reacts non-

enzymically with cyanide to form cyanhydrin (Warburg 1948). Even although cyanide did decrease the $\Delta \Psi_m$ in DCA-treated cells, it could well be that the steady-state pyruvate concentrations in DCA-treated cells was lower than that in non-treated cells. Also the used concentration of TTFA (1 $\mu$M) was two orders of magnitude lower than that usually applied to inhibit succinate dehydrogenase (Ackrell et al. 1977). In contrast, and as discussed above, rotenone is a strong and specific inhibitor of Complex I and is effective in very low concentrations (Ernster et al. 1963; Gutman et al. 1970). Nevertheless, we support the view (Bonnet et al. 2007; Michelakis et al. 2010) that the hyperpolarization of $\Delta \Psi_m$ in cancer cells, representing an abnormally high $\Delta p$ as discussed above, is presumably not ruled by the mitochondrial respiration. Instead we will argue that conditions in the cytosol are responsible for that.

Pedersen (Pedersen 1978) has argued that hexokinase, which in cancer cells is also bound to the outside of the mitochondria, may channel large amounts of glucose-6-

phosphate into the upregulated glycolysis. The fast mitochondrial A TP synthesis and, in turn, this might lead to the assumption that the abnormally high $\Delta \Psi_m$ and an increase of the intracellular pH in agreement with (Cittadini et al. 1971) because pyruvate reacts non-

enzymically with cyanide to form cyanhydrin (Warburg 1948). Even although cyanide did decrease the $\Delta \Psi_m$ in DCA-treated cells, it could well be that the steady-state pyruvate concentrations in DCA-treated cells was lower than that in non-treated cells. Also the used concentration of TTFA (1 $\mu$M) was two orders of magnitude lower than that usually applied to inhibit succinate dehydrogenase (Ackrell et al. 1977). In contrast, and as discussed above, rotenone is a strong and specific inhibitor of Complex I and is effective in very low concentrations (Ernster et al. 1963; Gutman et al. 1970). Nevertheless, we support the view (Bonnet et al. 2007; Michelakis et al. 2010) that the hyperpolarization of $\Delta \Psi_m$ in cancer cells, representing an abnormally high $\Delta p$ as discussed above, is presumably not ruled by the mitochondrial respiration. Instead we will argue that conditions in the cytosol are responsible for that.

Pedersen (Pedersen 1978) has argued that hexokinase, which in cancer cells is also bound to the outside of the mitochondria, may channel large amounts of glucose-6-

phosphate into the upregulated glycolysis. The fast trapping of $\text{Pi}$, in glucose-6-phosphate might then limit mitochondrial ATP synthesis and, in turn, this might lead to an increased $\Delta \Psi_m$.

Another possibility, one that we favour, is that the cytosolic ATP/ADP ratio driven by the upregulated glycolysis in malignant tumour cells is considerably higher than that in normal cells. The release of inhibition of PDH by DCA in cancer cells increased the glucose-oxidation rate by ca. 23% (Bonnet et al. 2007) to 44% (Michelakis et al. 2010). This caused a decrease, not an increase, of the $\Delta \Psi_m$ and an increase of the intracellular pH in agreement with the assumption that the abnormally high $\Delta p$ was not caused by respiration. We note that the quickness of the DCA effect on the $\Delta \Psi_m$ (apparent within 5 min) nicely agrees with the onset of the activation of PDH by DCA in perfused rat heart (caused by DCA-induced inhibition of PDK) which was apparent after ca. 3 min (Whitehouse et al. 1974). Therefore we are inclined to think that the normalization of the $\Delta \Psi_m$ (from hyper- to normal polarization) by DCA in the experiments from Bonnet et al. (Bonnet et al. 2007) coincided with the stimulation and normalization of the PDH activity. DCA also increases respiration in normal cells (Whitehouse et al. 1974). However, this is not accompanied by a change in $\Delta \Psi_m$ (Bonnet et al. 2007).

We also note that the effects of DCA on cancer-cell metabolism are similar to those induced by knocking out lactate dehydrogenase in mammmary tumour cells. In that case pyruvate is forced to be oxidised by the mitochondria which resulted in increased respiration, a fall in $\Delta \Psi_m$ and a diminished tumourigenicity. Under hypoxic conditions (0.5% O$_2$), the growth rate of such cells was diminished by two orders of magnitude (Fantin et al. 2006). Similar effects could be induced by a specific inhibitor of lactate dehydrogenase (FX11, 3-dihydroxy-6-
methyl-7-(phenylmethyl)-4-propynaphthalene-1-carboxylic acid). This inhibitor also reduced the ATP levels in the cancer cells. In addition it inhibited the progression of human lymphoma and pancreatic cancer xenografts (Le et al. 2010).

An increase of the pyruvate-oxidation rate in cancer cells induced by DCA will cause a sharp decrease in the cytosolic lactate production. In turn, this leads to a sudden (large) increase in the cytosolic NADH/NAD$^+$ ratio. Normalization of this ratio then depends on the mitochondrial H-shuttles (glycerol-3-phosphate and malate-aspartate shuttles). Although the shuttles have not been considered as a rate-limiting factor in the control of glucose oxidation in tumour cells (Pedersen 1978) it must be stressed that flux through the shuttles is not only dependent on the enzyme activities but also on the steady state concentrations of the shuttle intermediates under these conditions. In the presence of DCA, a decreased capacity of these shuttles in tumour cells, a/o caused by a lower mitochondrial mass and respiration (which can be dramatic (Zhang et al. 2008)), will lead to a shortage of cytosolic NAD$^+$ and a diminished activity of the genetically upregulated aerobic glycolysis system. In turn, this will result in a decrease of the cytosolic ATP/ADP ratio under these conditions. A hyperpolarized $\Delta \Psi_m$ caused by a glycolysis-powered elevation of the cytosolic ATP/ADP ratio is also in agreement with the observation that the aggressiveness and the metastatic potential of colonic carcinoma cells with a high $\Delta \Psi_m$ are greater than those of carcinoma cells with a lower $\Delta \Psi_m$ (Heerd et al. 2006).

An abnormally high $\Delta p$, readily measured as a hyperpolarized $\Delta \Psi_m$, will diminish the activities of the proton-translocating Complexes I, III and IV of the respiratory chain. At the same time, it will stimulate the activity of Nnt, especially at higher NADPH concentration. Our hypothesis (discussed in detail later on) predicts that this would result in an (hyper)increase of the NADPH/NAD$^+$ ratio near the NADPH site of Complex I. Thus, the excessive $\Delta \Psi_m$ of most cancers may lead to a severe attenuation of the H$_2$O$_2$ production by Complex I, i. e. the sheer power of the aerobic glycolysis in the cancer cells forces the mitochondria to limit their Complex-I-produced H$_2$O$_2$ and this may enable the cells to, a/o, evade apoptosis. The hypothesis also predicts that the DCA-induced decrease of the $\Delta \Psi_m$ in cancer cells is the trigger for the increase in the H$_2$O$_2$ generation by Complex I.

It is conceivable that a high cytosolic ATP/ADP ratio in cancer cells may also lead to an increased ATP/ADP ratio in the mitochondrial matrix. An elevated ATP level in the matrix facilitates the phosphorylation of PDH by PDK, as convincingly shown by (Taylor et al. 1975), and so it will
add to the genetically increased suppression of the pyruvate-oxidation rate.

Reactions of dioxygen with bovine Complex I

**On the reactions of O₂ with biomolecules**

Dioxygen has an electronic ground state with two unpaired electrons in two antibonding π* orbitals and hence it is paramagnetic (S=1). The reactions of this triplet molecule with most organic molecules, which have a singlet (S=0) ground state, are kinetically very slow because they are spin forbidden. In addition, the one-electron reduction potential of O₂ (O₂+e⁻→O₂⁻) is unfavourably low, further restricting possible reactivity. Although biochemicals like ascorbate, dithiothreitol, cysteine and reduced flavin are said to be auto-oxidizable, the reactions are spin restricted and a catalyst, usually traces of a transition metal ion like Fe, Cu or Mn, are required for the reaction to proceed (Miller et al. 1990). In biochemical experiments extraneous iron (often called ‘dirty iron’, i.e. all iron except that inherent to proteins) is nearly always present and is complexed to buffers and virtually all proteins (causing the well known g=4.3 EPR signal). When working with tissue, mitochondria or SMP there is no lack of (complexed) iron ions and this is why auto-oxidation of thermodynamically-suited molecules may occur. Triplet dioxygen can readily react with radical forms (S=1/2) of biomolecules with a sufficiently low redox potential to form O₂⁻. Because superoxide is a doublet (S=1/2) species, this reaction is spin-allowed. Under physiological conditions the rate is often only limited by diffusion (Miller et al. 1990). Under special conditions, e.g. photosensitized reactions with triplet O₂, singlet dioxygen can be formed. This S=0 molecule readily react with many biomolecules. Thermal excitation of O₂ to singlet dioxygen, or of combustible material to the triplet state, also results in a fast reaction of mixtures of the two (fire).

Usually, the incompletely reduced oxygen species O₂⁻, H₂O₂ (hydrogen peroxide) and HO⁻ (hydroxyl radical), are collectively called ‘reactive oxygen species’ (ROS). Because of its rapid reaction with radical forms of suitable biomolecules, dioxygen itself is also a reactive oxygen species. The so-called ROS production in biological systems mainly concerns superoxide (O₂⁻) and/or hydrogen peroxide (H₂O₂) but, of course, not dioxygen. From the four oxygen species, only the HO⁻ radical can attack virtually all chemical bonds in biomolecules. The hydroxyl radical can only be produced from O₂ by three sequential one-electron reduction steps. The reduction of O₂(g) to O₂⁻ has a low oxidation-reduction potential at pH 7 (E₀' = -0.33 V). In aqueous solution this potential is ca. -0.17 V (Miller et al. 1990; Koppenol et al. 2010). For singlet O₂, both potentials are 1 V higher (Koppenol et al. 2010). The reduction of O₂⁻ to H₂O₂ proceeds with an E₀' of ca. +0.93 V, whereas the reaction H₂O₂+1e⁻+H⁺→H₂O+HO⁻ has an E₀' of ca. +0.36 V. The latter reaction easily proceeds in the presence of Fe²⁺ ions (Fenton chemistry). The one-electron reduction of HO⁻ to water occurs with an E₀' of +2.31 V, making it by far the strongest oxidant of all oxygen species (for a complete list of the redox potentials, see e.g. (Koppenol et al. 2010)).

The one-electron reduction of O₂⁻ (S=1/2) gives H₂O₂ (S=0); hence the superoxide anion can only react fast with electron-donating molecules in the doublet state. The reaction between two O₂⁻ anions is not occurring due to Coulomb repulsion. In fact, the rate of the spontaneous superoxide dismutation reaction is pH dependent (McCord and Fridovich 1978). Because the HO₂⁻ radical has a pKₐ of 4.8, the major dismutation reaction at physiological pH is

\[ \text{HO}_2^- + \text{O}_2^- + \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]

Although the rate constant for this reaction is large (ca. \(10^{9} \text{M}^{-1}\cdot\text{s}^{-1}\)) (McComb and Fridovich 1978), the low concentration of O₂⁻ and in particular that of HO₂⁻ under physiological conditions make that this spontaneous dismutase reaction would be insignificant in the absence of any catalysts, i.e. the half-life time of O₂⁻ could be considerable (up to minutes or hours). Superoxide dismutases react solely with O₂⁻ in two successive steps; sum reaction:

\[ \text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]

The rate constant of this reaction (ca. \(2 \times 10^9 \text{M}^{-1}\cdot\text{s}^{-1}\)) approaches the value set by diffusion. When present, nitric oxide (NO⁻, a radical formed from arginine by nitric oxide synthase), reacts with O₂⁻ in a diffusion-controlled reaction to form the highly reactive, toxic peroxinitrite (ONOO⁻). Most relevant for possible damage by ROS to the mitochondrial energy metabolism is the very fast reaction of O₂⁻ with the [4Fe–4S]³⁺ cluster in active aconitase in the matrix. Superoxide oxidizes the cluster to the [4Fe–4S]⁴⁺ state which is not stable and decomposes into a [3Fe–4S]⁺ cluster and Fe³⁺, resulting in an inactive enzyme. The second-order rate constant for this reaction is \(10^6\) to \(10^7 \text{M}^{-1}\cdot\text{s}^{-1}\) (Flint et al. 1993). The formed H₂O₂ reacts with Fe³⁺ to produce HO⁻ (Vásquez-Vivar et al. 2000), but this does not cause any irreversible damage in the enzyme (Flint et al. 1993; Vásquez-Vivar et al. 2000). Both H₂O₂ and O₂ can also oxidize the [4Fe–4S]⁺ cluster, but the rates are much smaller (\(10^3 \text{M}^{-1}\cdot\text{s}^{-1}\) and ca. \(10^2 \text{M}^{-1}\cdot\text{s}^{-1}\), respectively (Flint et al. 1993)). Other [4Fe–4S] cluster-containing hydrolysates are inactivated similarly (Flint et al. 1993), e.g. the cytosolic aconitase, which in fact is an iron-regulatory protein (also called iron-response element binding protein, IRE-BP). The cluster-containing protein has aconitase activity and cannot bind to IREs, stem-loops in mRNA for
a variety of Fe-S proteins. Oxidation of the cluster leads to a complete loss of it. The apo-enzyme has no aconitase activity, but can bind to IREs (Beinert et al. 1996). Thus, O₂, H₂O₂ and in particular O₂⁻ in the cytosol can have a prominent effect on the iron metabolism of the cell.

A hydrophobic gas tunnel common to both [NiFe]-hydrogenases and Complex I

It is now quite clear that the structure of the PSST subunit of Complex I is highly similar to that comprising the flavodoxin domain in the small subunit of standard [NiFe]-hydrogenases (Fig. 3). In addition, the structure of the 49 kDa subunit is similar to that of the large subunit of such hydrogenases (Sazanov and Hinchliffe 2006; Fontecilla-Camps et al. 2007). Also the relative positions of these subunits are the same. This was already predicted from extensive sequence comparisons (Albracht and Hedderich 2000). The interface surface between the two subunits in standard hydrogenases is very large (Völbeda et al. 1996) (see Fig. 3B). The PSST subunit misses the C-terminal domain, including two Fe-S clusters, of the related small subunit in standard hydrogenases (Fig. 3C). This is also the case for the small subunits of two particular [NiFe]-hydrogenases, namely for HoxY from the soluble NAD⁺-reducing enzyme from Ralstonia eutropha and for EchC in the proton-pumping hydrogenase Ech (E. coli hydrogenase-3-type hydrogenase) from Methanosarcina barkeri. As a result, part of the surface on the 49 kDa subunit in Complex I (Fig. 3C), and of the large hydrogenase subunits HoxH of the R. eutropha enzyme and EchC in the M. barkeri enzyme, would be exposed to the bulk medium. As predicted (Albracht and Hedderich 2000), the TYKY subunit occupies this surface area on the 49 kDa subunit in Complex I and at the same time ‘re-installs’ the two Fe-S clusters (Fig. 3C). In the R. eutropha soluble hydrogenase this surface area is predicted to be covered by the HoxU subunit and in the Ech by the EchF subunit (Albracht and Hedderich 2000; Albracht 2010a, b).

The structural comparison of Complex I and [NiFe]-hydrogenases (Sazanov and Hinchliffe 2006; Fontecilla-Camps et al. 2007) also disclosed that a hydrophobic gas tunnel formed by the small and the large subunits in [NiFe]-hydrogenases, a tunnel that leads to the Ni-Fe site, overlaps with a much wider hydrophobic tunnel in Complex I, now known to begin at the membrane surface (Eremov et al. 2010; Hunte et al. 2010). In Complex I it is a possible binding site for (part of) the long hydrophobic tail of Q₁₀ (Sazanov and Hinchliffe 2006). It may also allow the hydrophobic inhibitors rotenone and piericidin A to reach one of their specific binding sites close to the centre of the 49 kDa subunit (site 1.

Fig. 3  Comparison of the structures of flavodoxin from Desulfovibrio vulgaris Hildenborough (A), D. gigas [NiFe]-hydrogenase (B) and part of the hydrophilic domain of Complex I from T. thermophilus (C). For Complex I only the structures of the TYKY (Nqo9), PSST (Nqo6) and 49 kDa (Nqo4) subunits are shown as ribbons. Prosthetic groups are depicted in Van Der Waals radii. Flavodoxin contains one FMN group. Hydrogenase contains two [4Fe–4S] clusters, with a [3Fe–4S] cluster in between, in the small subunit, and the Ni–Fe(CO)(CN)₂ active site (Happe et al. 1997) in the large subunit. The prosthetic groups in Complex I are as in Fig. 1A. In (B) and (C) the images are positioned such that the Fe-S clusters in the TYKY and PSST subunits of Complex I are aligned with the three Fe-S clusters in hydrogenase. Co-ordinates were taken from the structure files (PDB entries: 3fx2 for flavodoxin (Watt et al. 1991), 2frv for hydrogenase (Völbeda et al. 1996) and 2fug for Complex I (Sazanov and Hinchliffe 2006)). The red circles indicate the position of the flavodoxin fold. The positions of the large interface between the hydrogenase subunits and of the hydrophobic tunnels in (B) and (C) are also indicated.
see Figs. 1 and 2). In the Desulfovibrio gigas hydrogenase (Volbeda et al. 2005) H₂ gas diffuses through the gas tunnel up to the Ni-Fe active site. Also O₂(g) is able to do this and can then be reduced to hydroxide or peroxide at the Ni-Fe site (George et al. 2004; Kurkin et al. 2004; Armstrong and Albracht 2005; Vincent et al. 2005) and van der Linden et al. 2006). In [NiFe]-hydrogenases this reaction is not merely a ‘leak reaction’; instead it enables these enzymes to quickly switch off their activity by occupying the H₂-reaction site at nickel with a hydroxide or peroxide group. In that way these enzymes avoid the highly-exothermic Knallgas reaction to take place at the Ni-Fe site and prevent wasting of precious H₂ for the bacterial host. For Complex I we propose that the diffusion of O₂(g) into the hydrophobic tunnel enables its reaction with the semiquinone and reduced forms of FMN-a (Figs. 3 and 4). We consider this to be a constitutive functional reaction of the enzyme and not simply an electron-leakage reaction.

It is conceivable that the wide hydrophobic tunnel formed by the PSST and 49 kDa subunits may also bind one or more phospholipids molecules. Removal of phospholipids from this tunnel may explain the loss of reaction with ubiquinone and the loss of the specific binding of the inhibitors rotenone and piericidin A. Apparently the correct structure of the electronic heart of Complex I, i.e. the module formed by the TYKY, PSST, 49 kDa and 30 kDa subunits (Albracht 2010a) (Fig. 2) stabilized by phospholipids and Q₁₀, is critically dependent on the presence and correct structure of each of its components. This delicate structural interdependence may also explain why the removal of phospholipids may result in loss of FMN-a (Albracht et al. 2003; Albracht 2010a) and affects the properties (e.g. the EPR linewidht and apparent midpoint potential) of the clusters f and g (1.92 line) in bovine Complex I (Ragan and Racker 1973; Ohnishi et al. 1974; Finel et al. 1992, 1994) and the E. coli enzyme (Sinegina et al. 2005). It may further explain e.g. why some point mutations in the 49 kDa subunit of the Y. lipolytica Complex I resulted in the disappearance of EPR signals from Fe-S clusters in other subunits (Kashani-Poor et al. 2001; Grgec et al. 2004; Zwicker et al. 2006; Tocilescu et al. 2010), whereas other mutations had no effect (Tocilescu et al. 2007). Similar observations have been made with the E. coli enzyme (Beleivich et al. 2007).

Reactions of O₂ with purified Complex I and NADH dehydrogenases

The properties of the classical intact Complex I from bovine heart were first described by Hatefi and co-workers in 1962 (Hatefi et al. 1962). Purification involved the use of the natural bile detergents deoxycholate and cholate in combination with inorganic salts. Hatefi’s Complex I contained ca. 22% lipids (w/w) and 2.8–3.2 molecules of Q₁₀ per FMN (Hatefi et al. 1962), i.e. 5.6–6.4 molecules Q₁₀ per Complex I. It catalyzed the rotenone-sensitive reduction of ubiquinone by NADH. Another classical preparation, containing much less phospholipids, was obtained from SMP by the action of the phospholipase A (from Naja naja venom) (Ringler et al. 1960, 1963; Cremona and Kearney 1964; Lusty et al. 1965). Its redox-linked properties, i.e. FMN, Fe and acid-labile sulphur content, reduction of K₃Fe(CN)₆, by NADH, and the EPR properties were highly similar to those of Hatefi’s Complex I. However, it did not contain Q₁₀, nor did it react with quinones in a rotenone- or piericidin-A-sensitive way. This preparation was usually called the high-molecular weight type NADH dehydrogenase, but we will refer to it as Singer’s NADH dehydrogenase. These classical preparations contained two FMN groups per enzyme molecule (Albracht et al. 2003).

When Hatefi’s Complex I (16–18 Fe atoms per FMN; see below) was further purified, using dodecyl maltoside, glycerol, a sucrose gradient and column chromatography, a fairly active preparation (NADH → Q₁ activity 0.516 μmol.min⁻¹.mg⁻¹, i.e. 9 s⁻¹ at 20 °C, pH 8, 85% rotenone sensitive) was obtained with 28 Fe atoms (and 3 Q₁₀ molecules) per FMN (Nakashima et al. 2002), indicating the loss of FMN-a. More recently the same laboratory, using a different preparation procedure, purified a more active enzyme (NADH → Q₁ activity 1.27 μmol.min⁻¹.mg⁻¹, i.e. 21 s⁻¹ at 20 °C, pH 8, 90% rotenone sensitive; the mentioned activity, 1.27 μmol.min⁻¹.mL⁻¹, must have been a printing error) with 30 Fe atoms (and 1 Q₁₀ molecule) per FMN (Shinzawa-Itôh et al. 2010). This preparation will be referred to as Yoshikawa’s Complex I.

A monodisperse preparation of the bovine enzyme has been obtained by the group of Hirst using synthetic
detergents, salts and column chromatography (Sharpley et al. 2006). It contained 3% phospholipids (w/w) and 0.3 molecules Q₁₀ per enzyme molecule. The enzyme did not react with quinones (decyubiquinone) unless phospholipids were present; this reaction was completely rotenone sensitive. In contrast to the classical preparations, it contained only 1 to 1.1 molecules of FMN per enzyme molecule. Its non-heme Fe content was 22–26 Fe atoms per FMN (Sharpley et al. 2006) against 16–18 Fe atoms per FMN for the classical preparations (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). We will refer to this enzyme as Hirst’s NADH dehydrogenase.

The prosthetic groups in Complex I have a redox potential in the range −380 to 0 mV. In contrast to Fe-S proteins from most anaerobic micro-organisms, the Fe-S clusters in Complex I are not destroyed by O₂ because the surrounding protein prevents its access. The most likely places where dioxygen can react are the substrate sites. Hence, FMN-b, FMN-a, the Q-reaction site, as well as ubiquinone itself may be possible targets.

The reactions of dioxygen with Hirst’s NADH dehydrogenase have been studied in detail (Kussmaul and Hirst 2006). The enzyme generates superoxide with a rate of ca. 45 nmol.min⁻¹.mg⁻¹ in the presence of NADH in aerobic buffer (pH 7.5, 32 °C, measured by the reduction of cytochrome c). This rate (ca. 1 s⁻¹ as the apparent molecular mass is close to 980 kDa) was not affected by rotenone. The direct generation of H₂O₂ was estimated to be less than 10% of the superoxide production. The redox-potential dependence of the superoxide production in an NADH/NAD⁺ titration, showed a clear n=2 behaviour and coincided with the expected amount of FMNH₂ (see Fig. 5 in (Kussmaul and Hirst 2006)). Because the Fe-S clusters remained reduced in the relevant redox-potential window (−0.3 to −0.4 V), the reduced form of flavin was assigned as the prime target of O₂. Note that the used preparation contained only one flavin, the one reacting with NADH, i.e. FMN-b; hence this is a superoxide-generating site (Fig. 4).

The reaction with O₂ has also been studied with Yoshikawa’s Complex I (Ohnishi et al. 2010). Based on EPR studies it was hypothesized that the semiquinone forms of both FMN (i.e. FMN-b) and ubiquinone, induced by reduction with NADH, contribute to the overall formation of O₂⁻. We note that the actual measured rates (1.3 to 1.76 nmol.min⁻¹.mg⁻¹ at pH 7.5 and 32 °C, i.e. ca. 0.022 to 0.029 s⁻¹) were at least one order of magnitude lower than the NADH→O₂ reaction reported for Hirst’s NADH dehydrogenase or Hatefi’s Complex I (discussed hereafter). Hatefi reported that Complex I preparations, in which the Nnt was inactivated, showed low rotenone-sensitive NAD(P)H→O₂ activities (Hatefi and Bearden 1976). For NADH this activity was 20 nmol.min⁻¹.mg⁻¹ in the absence of inhibitor and 7 nmol.min⁻¹.mg⁻¹ in its presence. For NADPH the values were 5 and 1 nmol.min⁻¹.mg⁻¹, respectively (pH 7.4 and 22 °C). The rotenone-sensitive activity with NADH (13 nmol.min⁻¹.mg⁻¹) cannot be caused by reduced FMN-b or any of the Fe-S clusters because their rate of reduction is not inhibited by rotenone. Instead, this inhibitor prevents reduction of both FMN-a and Q. Ubiquinol is an unlikely candidate for the reaction with O₂. Its redox potential is thermodynamically unfavourable and QH₂ will not readily react with O₂ (spin forbidden). A reaction with ubisemiquinone is also less likely because no such radical is observed in Hatefi’s Complex I reduced with NADH. Instead, it was shown (Sled et al. 1994) that a flavin radical, ascribed by us to FMN-a (see details later on), is always present under these conditions. Hence, we consider the semiquinone of FMN-a as the best candidate for the reaction with O₂ (spin-allowed reaction; thermodynamically favourable).

NADPH does not reduce FMN-b or Q. Instead, it reduces FMN-a to FMNH₂. Reaction of FMNH₂ with O₂
will be very slow (spin forbidden). Because the reaction of Complex I with NADPH is not sensitive to rotenone (Albracht 2010b), the inhibition of the NADPH→O₂ reaction of the enzyme (Hatefi and Bearden 1976) suggests that it cannot be excluded that rotenone has a second inhibitory effect, e.g. it could interfere with the passage of O₂(g) through the hydrophobic tunnel.

One significant side effect of the reactions between dioxygen and isolated Complex I or NADH dehydrogenases in the presence of NADH is that one of the Fe-S clusters is modified by superoxide. Unlike the enzyme in SMP, Hatefi’s Complex I is not stable when reduced with NADH in air. Dramatic changes in the EPR signal of cluster c (main EPR line at g=1.94, see (Beinert and Albracht 1982; Albracht 2010b)) can be observed. Within a minute after addition of NADH at room temperature the g=1.94 line starts to decrease while a new signal with g₂=1.97 appears (Hatefi et al. 1962; Kawakita and Ogura 1969; Orme-Johnson et al. 1974). The signals of the other clusters are not affected. This instability is introduced in Hatefi’s isolation procedure (Hatefi et al. 1962) at the step where Complex I-III is prepared from Complex I-II-III (Albracht et al. 1977). The presence of dithionite, which effectively consumes all O₂, prevents these NADH-induced changes (Albracht et al. 1977). A g=1.97 line, a marker for the NADH-induced instability, is also present in Hirst’s NADH dehydrogenase upon reduction with NADH (Sharpley et al. 2006).

Fragmentation of Singer’s NADH dehydrogenase by exposure to NADH in air has been observed long ago (Beinert et al. 1963; Rossi et al. 1965). At 0 °C this resulted in a drastic decrease (60% in 1 h) in the NADH→O₂ reaction of the enzyme (Hatefi and Bearden 1976) suggests that it cannot be excluded that rotenone has a second inhibitory effect, e.g. it could interfere with the passage of O₂(g) through the hydrophobic tunnel.

As first reported in 1967 (Hinkle et al. 1967) bovine submitochondrial particles (SMP) can generate H₂O₂ (1.1 nmol.min⁻¹.mg⁻¹) in the presence of dioxygen, NADH and KCN. A similar production (1.2 nmol.min⁻¹.mg⁻¹) was obtained via ATP-induced reverse electron transfer in the presence of succinate and KCN. Rotenone stimulated the first reaction by ca. 30%, but inhibited the second one and so the aspecific reaction with dioxygen was assigned to Complex I.

The difficulty with O₂⁻⁻/H₂O₂ generation by mitochondria and SMP is that the net productions are dependent on reactions that generate these species and reactions that can remove them. Submitochondrial particles, generally considered as a suspension of inside-out vesicles of inner membranes, are usually obtained by ultrasound. Depending on the power of the ultrasound, the duration of the treatment and the composition of the medium, variable amounts of residual proteins from the matrix and the intermembrane space, as well as membrane-associated, loosely-bound proteins may be present. Hence, SMP suspensions can contain multiple non-protein redox compounds as well as protein-bound redox groups that may be directly or indirectly reducible with NADH and then react with O₂. In addition, the presence of contaminants like...
Cu,Zn-SOD (from the intermembrane space (Okado-Matsumoto and Fridovich 2001), cyanide sensitive) as well as Mn-SOD (soluble in the matrix as well as bound to the inner membrane (Okado-Matsumoto and Fridovich 2001), cyanide insensitive) make the interpretation of \( \mathrm{O}_2^- \) and \( \mathrm{H}_2\mathrm{O}_2 \) generation by SMP not straightforward. It should also be kept in mind, that the relative content of mitochondrial outer membranes of SMP obtained by ultrasound is the same as that in mitochondrial preparations (Albracht and Heidrich 1975). For a proper separation of inner and outer membranes a special procedure is required (see, e.g. (Heidrich et al. 1978)). The variable quality of the SMP preparations is presumably one of the main reasons that reports on \( \mathrm{O}_2^-/\mathrm{H}_2\mathrm{O}_2 \) production and the effect of inhibitors are confusing and often contradictory (summarized e.g. in (Andreyev et al. 2005; Lambert et al. 2008a; Murphy 2009; Brand 2010; Murphy et al. 2011)). The recent study of Hirst and co-workers (Pryde and Hirst 2011) on the \( \mathrm{O}_2^-/\mathrm{H}_2\mathrm{O}_2 \) production of bovine SMP is no exception. Although not mentioned, the results confirmed and extended data from 1988 (Krishnamoorthy and Hinkle 1988). Reduced FMN-b was proposed as the only reaction site for \( \mathrm{O}_2 \) and the rate of \( \mathrm{O}_2^-/\mathrm{H}_2\mathrm{O}_2 \) production with succinate was found to be considerably smaller than the NADH-induced one (Pryde and Hirst 2011). These results with SMP sharply contradict data obtained with rat-muscle mitochondria (Lambert and Brand 2004a, b; Lambert et al. 2008a, b). We note that two steps in the preparation procedure of the SMP (Pryde and Hirst 2011), i.e. the treatment of mitochondria at pH 9 for ca. 30 min, and an incubation of the SMP with 1 mM NADH for 60 min, may lead to partial (reversible) release of FMN from Complex I as demonstrated by Vinogradov and co-workers (Gostimskaya et al. 2007). Reduction of the \textit{R. eutropha} soluble NAD\(^+\)-reducing [NiFe]-hydrogenase by NADH at pH 8 results in the specific rapid (reversible) release of FMN-a, whereas FMN-b remains firmly bound (Van der Linden et al. 2004).

It cannot be ruled out that ultrasound itself may cause subtle mechanically- and/or thermally-induced changes in a large membrane-bound enzyme like Complex I, especially when being part of “respiratory strings” (Dudkina et al. 2010). Acoustic cavitation produced by ultrasound can lead to local hot spots with very high temperatures (5,000 °C) and pressures (500 bar) resulting in the decomposition of water into highly reactive hydrogen atoms and hydroxyl radicals (Suslick 1990; Suslick and Flannigan 2008). In the biochemical context, the reduction of ferricytochrome \( c \) in water by radicals, notably superoxide, created by ultrasound was already reported in 1972 (Lippitt et al. 1972). The structure of the inner-mitochondrial membrane is certainly affected by ultrasound: the membrane becomes more leaky to protons the more powerful the ultrasonic treatment.

Even during the routine isolation of bovine mitochondria considerable mechanical forces, e.g. the sheering forces in a Potter-Elvehjem homogenizer or those in a Waring Blender, are applied to free the mitochondria from tissue cells. In addition, the use of mitochondria adds a number of additional complications for the study of \( \mathrm{O}_2^-/\mathrm{H}_2\mathrm{O}_2 \) generation (Andreyev et al. 2005; Lambert et al. 2008a; Murphy 2009; Brand 2010; Murphy et al. 2011). The study of cell cultures may best approach the in vivo situation, but the techniques for \( \mathrm{O}_2^- \) and \( \mathrm{H}_2\mathrm{O}_2 \) detection are more demanding (Forkink et al. 2010; Murphy et al. 2011).

In contrast to Complex I, the other respiratory complexes are thought to play a minor role in \( \mathrm{O}_2^-/\mathrm{H}_2\mathrm{O}_2 \) production. Succinate:ubiquinone oxidoreductase (Complex II) does not noticeably react with \( \mathrm{O}_2 \). For Complex III it has been demonstrated that in the presence of the specific inhibitor antimycin A, but not in its absence, it can produce \( \mathrm{H}_2\mathrm{O}_2 \) and possibly also \( \mathrm{O}_2^- \) (Jensen 1966; Andreyev et al. 2005; Murphy 2009). Complex IV reduces \( \mathrm{O}_2 \) to two molecules of \( \mathrm{H}_2\mathrm{O} \) in a reaction involving four electrons. This complex produces no apparent oxygen by-products. Hence, Complex I is considered to be the main producer of \( \mathrm{O}_2^-/\mathrm{H}_2\mathrm{O}_2 \) in mammalian mitochondrial inner membranes.

Influence of reactive oxygen species, in particular \( \mathrm{H}_2\mathrm{O}_2 \), on the cell

It is generally accepted that the \( \mathrm{H}_2\mathrm{O}_2 \) concentration in mammalian cells must be carefully kept within certain limits. A high concentration (>3 \( \mu \)M) will lead to direct destruction of cells (necrosis). Intermediate \( \mathrm{H}_2\mathrm{O}_2 \) concentrations (1 to 3 \( \mu \)M) will trigger the mitochondria-dependent process of natural cell death (apoptosis) (Cadenas 2004), while still lower concentrations may just trigger non-specific (“bulk”) autophagy or the specific autophagy of damaged cell organelles (e.g. of mitochondria: mitophagy) (Scherz-Shouval et al. 2007; Kim et al. 2007; Semenza 2008a, b; Chen and Gibson 2008; Meijer and Codogno 2009; Chen et al. 2009; Terman et al. 2010). Numerous studies indicate that the mitochondrial \( \mathrm{H}_2\mathrm{O}_2 \) generation is the trigger for these processes (reviewed by (Murphy 2009)). Some recent results suggest that mitochondrial superoxide could be the major trigger of autophagy during starvation (Chen et al. 2009). We note that the mentioned \( \mathrm{H}_2\mathrm{O}_2 \) concentrations (up to 3 \( \mu \)M) are two orders of magnitude lower than those required to affect the NADH\(\rightarrow\mathrm{O}_2 \) activity of SMP by 10% (Zhang et al. 1990).

A low \( \mathrm{H}_2\mathrm{O}_2 \) production may enable the cell to evade the normal \( \mathrm{H}_2\mathrm{O}_2 \)-regulated turnover cycles of cells, but to keep autophagy going. In healthy tissue this may lead to long-lived cells (Terman et al. 2010). As apparent from the study of Bonnet et al. (Bonnet et al. 2007) also cancer cells...
apparently suppress mitochondria-generated \( \text{H}_2\text{O}_2 \) levels to achieve a long life time. In this context, and as indicated above, it is important to keep in mind that the elevated level of HIF-1 in (hypoxic) tumours upregulates proteins involved in mitophagy and downregulates mitochondrial biogenesis. On a longer time scale this can result in a (greatly) decreased mitochondrial density (Pedersen 1978; Luciaková and Kuzela 1992; Zhang et al. 2008) and thus adds to the decrease of their contribution to the cellular \( \text{H}_2\text{O}_2 \) production and mitochondria-induced apoptosis. It is instructive to recall that rat liver contains ca. 2500 mitochondria per cell (Estabrook and Holowinsky 1961), presumably as part of mitochondrial reticula (Westermann 2010).

Role of Nnt in oxidative stress

NADPH produced by Nnt was generally thought to contribute to the NADPH pool in the matrix (Rydström 2006a, b). This pool can be used for the reduction of mitochondrial GSSG to GSH (by glutathione reductase) and for reduction of several mitochondrial redoxins, e.g. thioredoxin (by NADPH-reduced thioredoxin reductase), peroxiredoxin (by thioredoxin) and glutaredoxin (by GSH-reduced thioredoxin reductase). GSH is the main electron donor for the reduction of mitochondrial \( \text{H}_2\text{O}_2 \) (by glutathione peroxidase); for overviews see (Cadenas 2004; Andreyev et al. 2005; Rydström 2006a; Muller et al. 2007; Murphy 2009). In mammalian heart and skeletal muscle the NADPH-producing capacity of the mitochondrial IDH2 is apparently greater than that of Nnt; therefore, the comparison between the activities, determined as maximal initial activities, did not take into account that Nnt, because of its linkage to the mitochondrial NADPH/NADP\(^+\) ratio to much higher values than IDH2 can. Of course, mitochondrial NADPH is also essential for a number of biosynthetic reactions (Hoek and Rydström 1988; Hatefi and Yamaguchi 1996; Bizouarn et al. 2000).

In vivo effects of Nnt deficiency

Experiments with *Caenorhabditis elegans* with a knockout of Nnt revealed for the first time that Nnt is important in the defence against mitochondrial oxidative stress (Arkblad et al. 2005). It was found that there was an increased sensitivity towards \( \text{O}_2^- \) (created in mitochondria by addition of methyl viologen). In addition the GSH/GSSG ratio (in lysates) was drastically decreased from 56 in the wild type to 12 in the mutants, a decrease of 80%. If the mitochondrial and cytosolic pools of glutathione were entirely separate, an oxidation of the mitochondrial GSH would not be expected to decrease cellular GSH by more than 10–15%, being the mitochondrial GSH+GSSG content in eukaryotic cells, e.g., hepatocytes. Hence, it was concluded that there might be some kind of communication between the redox states of the glutathione pools in the mitochondrial and cytosolic compartments. The isocitrate-2-oxoglutarate-NADPH shuttle may be responsible for such communication (Papa 1969). An additional possibility, inspired by our hypothesis, is that the specific \( \text{H}_2\text{O}_2 \) production by FMN-a in Complex I is no longer attenuated by Nnt-generated NADPH. The \( \text{H}_2\text{O}_2 \) can escape into the cytosol and contributes to the decrease of the cytosolic GSH/GSSH ratio.

Additional experimental evidence for a specific role of Nnt in the suppression of \( \text{H}_2\text{O}_2 \) came from experiments with mice deficient in Nnt. In 2005 a direct link between Nnt and a metabolic defect (glucose intolerance reminiscent of human type 2 diabetes (Jitrapakdee et al. 2010)) was discovered in C57BL/6J mice with an inactive Nnt (Toye et al. 2005). It was found that the release of insulin induced by high glucose concentrations was strongly diminished when compared to insulin release in other mouse strains. The C57BL/6J mice have a mutation in the NNT gene, resulting in an inactive Nnt. Apparently, this prevented glucose from closing the \( K^+\text{ATP} \) channel in the plasma membrane and so from opening the voltage-gated \( \text{Ca}^{2+} \) channel in the pancreatic \( \beta \)-cells, resulting in impaired insulin release. Silencing of the NNT gene in an insulin-secreting cell line by siRNA confirmed this. In addition it was found that the \( \text{O}_2^-/\text{H}_2\text{O}_2 \) production increased three-fold relative to the control (Freeman et al. 2006a, b, c). The defects in the C57BL/6J mice could be rescued by transgenic expression of the entire NNT gene (Freeman et al. 2006c), whereas increased levels of Nnt in DBA/2 mice (a strain with a normal Nnt activity) led to hypersecretion of insulin in response to glucose challenge (Aston-Mourney et al. 2007).

It was hypothesized that the increased \( \text{O}_2^-/\text{H}_2\text{O}_2 \) levels in Nnt-deficient mice, induced by a glucose-induced \( \Delta p \), would stimulate the action of UCP2 (uncoupling protein 2) in the mitochondrial inner membrane. In turn, this would lead to a dissipation of \( \Delta p \) (whereby the \( \text{O}_2^-/\text{H}_2\text{O}_2 \) production would decrease again), to decreasing ATP levels and hence to a decrease in insulin secretion. (Freeman et al. 2006a, b). However, a knockout of the UCP2 gene had essentially no effect (Parker et al. 2009), suggesting that the glucose intolerance in the C57BL/6J mice does not involve UCP2 but some type of oxidative stress.

Our hypothesis would predict that inactivation of the Nnt will lead to an increased level of \( \text{H}_2\text{O}_2 \) generated by FMN-a in Complex I. In addition, the redox power of the mitochondrial NADPH pool will decrease. The effects will
weaken the power of the cytosolic as well as of the mitochondrial \( \text{H}_2\text{O}_2 \)-detoxifying systems. This contributes to the oxidative stress of the cell, followed by oxidative damage, diminished ATP synthesis and so a lower insulin secretion.

C57BL/6J mice show an increased sensitivity to \( \text{O}_2^-/\text{H}_2\text{O}_2 \), especially in the absence of mitochondrial Mn-SOD (SOD2). A deficiency of both Nnt and Mn-SOD resulted in mice barely surviving 1–2 days (Huang et al. 1999, 2006). In the light of our hypothesis, this would mean that (i) \( \text{O}_2^- \) generated at FMN-b will have a much longer life time and will cripple the citric-acid cycle by inactivation of aconitase; (ii) \( \text{H}_2\text{O}_2 \) generation at FMN-a will no longer be attenuated resulting in the effects discussed above. C57BL/6J mice where one copy of the \( \text{NNT} \) gene had been re-introduced showed improved cardiovascular functions, i.e. they were more ‘normal’ than other Nnt-containing mice. However, postnatal survival was not supported. (Kim et al. 2010).

**An experimental link between the action of Nnt and cancer**

It has been noticed, that the occurrence of spontaneous as well as some types of chemically induced cancers is much lower in C57BL/6J mice than in other mouse strains. Pre-neoplastic lesions and tumours are considerably less common in C57BL/6J than in e.g. C3H/He mice. This was explained by assuming that C3H/He, in contrast to C57BL/6J mice, are genetically predisposed to a high susceptibility to, especially, hepatocarcinogenesis (Bursch et al. 2005). This discrepancy was apparently not dependent on differences in apoptotic activities, but rather on rates of cell division; thus, C57BL/6J mice show a lower rate of cancer cell growth (Bursch et al. 2005). Colorectal tumourigenesis is also less frequent in C57BL/6J mice than in C57BL/6N mice (Diwan and Blackman 1980). Our hypothesis would predict that the absence of Ntt activity will lead to higher \( \text{H}_2\text{O}_2 \) levels. This is analogous to the situation in DCA-treated cancer cells; also in that case a decrease in cell proliferation was observed (Bonnet et al. 2007; Michelakis et al. 2010). In rat liver, apoptosis seems to play a more dominant role in the prevention of tumour formation (Bursch et al. 2005).

**Increased \( \text{O}_2^-/\text{H}_2\text{O}_2 \) generation in heart cells with inhibited Nnt**

Inhibition of Nnt with NBD-chloride (4-chloro-7-nitrobenzo-2-oxa-1,3-diazole) in isolated heart cells resulted in an increase of mitochondrial \( \text{O}_2^-/\text{H}_2\text{O}_2 \) production. The inhibition also contributed to the opening of the innermembrane anion channel (IMAC) and the mitochondrial permeability transition pore (MPT) through a decreased cellular GSH/GSSG ratio (Aon et al. 2007). We note that the phenomena could also be caused by the lack of attenuation of the \( \text{H}_2\text{O}_2 \) production at FMN-a.

**Hypothesis in detail**

A potential regulatory role for NADPH in the \( \text{H}_2\text{O}_2 \) production by bovine Complex I

The solvent-accessible FMN-b is the site where dissolved dioxygen reacts with FNMH\(_2\) resulting in superoxide formation (turnover rate with excess NADH ca. 1 s\(^{-1}\)) (Kussmaul and Hirst 2006; Berrisford and Sazanov 2009; Hirst 2010). The \( \text{O}_2^- \) anion can rapidly escape to the bulk (matrix) aqueous phase. Its detoxification is the task of the Mn-SOD. This enzyme occurs in two forms, a soluble form in the matrix and a innermembrane-bound form (Okado-Matsumoto and Fridovich 2001). The precise binding site in the membrane is not known. It cannot be excluded that it would bind to Complex I close to the site where superoxide escapes after being formed at reduced FMN-b. If not taken care off, \( \text{O}_2^- \) will rapidly bring the citric-acid cycle to a halt by inactivating aconitase.

FMN-a can be reduced in two ways, i.e. indirectly by electrons from NADH or directly by hydride transfer from NADPH (Fig. 4). Electrons from NADH reach FMN-a via the chain of Fe-S clusters; hence, the initial reduction via this route is a one-electron step. The effective redox power of the electrons reaching FMN-a is considerably lower than that of the NADH/NAD\(^+\) couple. Three redox states of FMN-a may be expected: FMN, FNMH\(^+\) and FNMH\(_2\). In redox titrations performed with Hatefi’s Complex I (Sled et al. 1994) in the presence of mediating redox dyes, a bell-shaped curve composed of two \( n=1 \) reactions was observed for a flavosemiquinone radical EPR signal (linewidth 2.4 mT, midpoint potentials –336 mV and –415 mV at pH 7.5). A maximal semiquinone concentration of 0.1 spin per FMN was obtained at –368 mV. Piericidin A or rotenone did not affect the titration. The radical showed a strong spin-spin interaction with a nearby Fe-S cluster having a \( g_3 \) EPR line at 1.86. As a result its spin relaxation was greatly enhanced and it was not saturated by a microwave power of 100 mW at 169 K. A titration with NADH/NAD\(^+\) and AcPyADH/AcPyAD\(^+\) (AcPyADH, reduced 3-acetylpyridine adenine dinucleotide), both obligatory \( n=2 \) redox couples, at pH 8.2 in the absence of redox mediators gave very different results (see Fig. 8 in (Sled et al. 1994)). A maximal semiquinone signal (0.035 spins per FMN, linewidth 1.7 mT) was now observed at –310 mV. With mediators the signal peaked at –390 mV (0.11 spins per FMN) at the same pH. In contrast to the titrations with mediators, the signal did not disappear completely at the
lowest potentials (−440 mV and below) obtained with NADH/NAD⁺; ca. 0.02 spins per FMN persisted. The radical’s relaxation properties and its spin-spin interaction with the \( g_3 = 1.86 \) signal were the same as observed in the titration in the presence of mediators.

At the time, the authors (Sled et al. 1994) assumed that Complex I contained only one FMN group, and hence explanations for the observed phenomena were based on that. We suggest that both flavins may have equally contributed to the flavosemiquinone signal in potentiometric titrations in the presence of mediators. However, in the pyridine-nucleotide titrations the contributions are expected to differ. It is questionable whether FMN-b will contribute because the nucleotides are obligatory \( n=2 \) redox agents. In contrast, FMN-a receives electrons via the Fe-S clusters (\( n=1 \) redox groups). Hence, we assume that in that case the observed flavosemiquinone radical signal was mainly due to FMN-a. This explains why even the lowest redox potentials, obtained with NADH/NAD⁺, could not eliminate the radical signal. The expected redox behaviour of FMN-b in the presence of NADH/NAD⁺, i.e. an FMNH₂/FMN equilibrium without the flavosemiquinone form, is in agreement with the observed superoxide generation of Hirst’s NADH dehydrogenase (which contains only FMN-b) with NADH/NAD⁺ (Kussmaul and Hirst 2006). We also note that in contrast to Hatefi’s Complex I (Sled et al. 1994), there was no hint of a flavosemiquinone EPR signal in Hirst’s NADH dehydrogenase at either −0.3 V or −0.4 V, even in the 40 K spectra (Kussmaul and Hirst 2006). This is in agreement with our explanation that the NADH-induced flavosemiquinone radical EPR signal in Hatefi’s Complex I is caused by FMN-a. Our interpretation also suggests that the spin-spin interaction between the flavosemiquinone radical and the reduced Fe-S cluster with \( g_3 = 1.86 \) observed by Ohnishi and co-workers (Salerno et al. 1977; Ingledew and Ohnishi 1980; Sled et al. 1994) is one between FMN-a and cluster \( h \). This is consistent with the observed rapid reduction of cluster \( h \) (\( g_3 = 1.86 \)) by NADPH (Albracht 2010b).

The direct redox reaction of FMN-a with the NADPH/NAD⁺ couple will result in an FMNH₂/FMN equilibrium. However, in the in vivo situation FMN-a will receive reducing equivalents both from NADH, via the chain of Fe-S clusters, and from NADPH. Hence, there will be three different reactions. Two are induced by the NADH/NAD⁺ couple:

\[
\text{FMN} + e^- + H^+ \rightleftharpoons \text{FMNH}^-
\]

\[
\text{FMNH}^- + e^- + H^+ \rightleftharpoons \text{FMNH}_2
\]

The kinetics of the two forward reactions are rotenone sensitive. Also electrons coming in via energy-induced reverse electron transfer from the Q-pool can contribute here (rotenone sensitive). The third reaction is ruled by the NADPH/NAD⁺ couple (kinetics insensitive to rotenone):

\[
\text{FMN} + 2e^- + 2H^+ \rightleftharpoons \text{FMNH}_2
\]

The concentration of the flavosemiquinone, the form that can rapidly react with dioxygen to form \( O_2^- \) (a spin-forbidden reaction), is thus dependent on both the NADH/NAD⁺ and the NADPH/NAD⁺ couples. At high NADPH/NAD⁺ ratios, the level of FMNH₂ will be maximized. The reaction of FMNH₂ with dioxygen is spin forbidden. For free flavin in aqueous solution the rate of reaction of FMNH₂ with \( O_2 \) (second order rate constant ca. \( 10^4 \text{M}^{-1} \text{s}^{-1} \)) is three orders of magnitude faster than that of the reaction with FMNH₂ (Bruce 1982; Anderson 1982; Lind and Merenyi 1990). The superoxide anion formed at FMN-a cannot escape through the hydrophobic tunnel (caging) and so \( H_2O_2 \) is formed by spontaneous dismutation (second order rate constant ca. \( 10^5 \text{M}^{-1} \text{s}^{-1} \)), as well as by further reduction via regenerated FMNH₂. The hydrogen peroxide then leaves the enzyme at a site close to the inner membrane. Thus, increasing NADPH/NAD⁺ ratio’s will attenuate the rate of \( H_2O_2 \) generation at FMN-a independently of the NADH/NAD⁺ ratio. Under turnover conditions rotenone will inhibit the NADH-induced flavosemiquinone formation at FMN-a, and so it will minimize the \( H_2O_2 \) production. The observed inhibition in cancer cells of the DCA-induced \( H_2O_2 \) production by micro-injected intracellular catalase (Bonnet et al. 2007) suggests that most of the hydrogen peroxide can escape into the intermembrane space and from there to the cytoplasm. This suggests that potential target sites for this signal molecule may be situated (also) outside the matrix. Although it is generally assumed that \( H_2O_2 \) can pass freely through biological membranes, recent studies indicate that specific aquaporins may be involved (Miller et al. 2010).

Co-operation between Complex I and transhydrogenase in the control of \( H_2O_2 \)

The major generators of NADPH in mammalian mitochondria are Nnt, the mitochondrial NADP⁺-linked isocitrate dehydrogenase (iso-enzyme 2, IDH2 (Pollard and Ratcliffe 2009)), malic enzyme (see e.g. (Rydström 2006a)) and perhaps glutamate dehydrogenase (Meijer and Codogno 2009). The NADH site of Complex I sticks out well (ca. 130 Å) into the matrix space (Efremov et al. 2010). The proposed reaction site for Complex I with NADPH is, however, very close to the membrane. It has consistently been reported that the Nnt in bovine-heart SMP partly co-purifies with Complex I in the Hatefi procedure (Ragan and Widger 1975; Hatefi and Bearden 1976; Ragan 1976; Finel et al. 1992). Hence it is conceivable that the Nnt, a membrane-bound enzyme, is weakly bound to Complex I.
in the mitochondrial inner membrane. The Complex I content of bovine-heart SMP is 0.06 nmol mg\(^{-1}\) (Albracht 2010b). From the reverse (NADPH generation) Nnt activity of SMP (ca. 0.2 \(\mu\)mol/min/mg), the activity of purified Nnt (ca. 15 \(\mu\)mol/min/mg) and the molecular mass (218 kDa) (Persson et al. 1984), it follows that SMP contain ca. 0.06 nmol Nnt per mg of protein. Thus, the concentrations of the two enzymes in bovine-heart SMP are about the same.

The structural information on Nnt (Bizouarn et al. 2000; Cotton et al. 2001; Jackson 2003; Pedersen et al. 2008) shows that its NADPH-producing site is close to the membrane surface, as was already predicted from inhibitor studies in 1972 (Rydström 1972). An association between the two enzymes could rule the NADPH/NADP\(^+\) ratio in the vicinity of the NADPH site of Complex I (Fig. 5). This would enable a rapid and specific control mechanism of Complex I-dependent \(\text{H}_2\text{O}_2\) generation steered by the mitochondrial energy status, the protonmotive force. Excess NADPH will diffuse into the bulk matrix space. Because Nnt is a key NADPH generator in cancer cells, using Nnt inhibitors as pharmaceuticals may help to improve future cancer therapy.

The expression of several of the enzymes involved mitochondrial \(\text{O}_2^-/\text{H}_2\text{O}_2\) detoxification, e.g. Mn-SOD and glutathione peroxidase, is stimulated by \(\text{H}_2\text{O}_2\) (Jo et al. 2001). These biosynthetic processes are much slower than the proposed attenuation by NADPH/NADP\(^+\) of \(\text{H}_2\text{O}_2\) generation by Complex I. We therefore consider these scavenging systems as more general (global) ones. They may even be unable to sufficiently cope with \(\text{H}_2\text{O}_2\) produced by Complex I close to the inner membrane, especially when, as indicated by experiments of Michelakis and co-workers (Bonnet et al. 2007), the rotenone-sensitive \(\text{H}_2\text{O}_2\) produced in DCA-treated cancer cells can easily escape into the cytoplasm. Rather, they will deal with \(\text{H}_2\text{O}_2\) produced elsewhere in matrix. We note that the \(\text{H}_2\text{O}_2\)-detoxifying systems are all involved in the indiscriminate removal of this oxygen product. In contrast, our hypothesis predicts that the reaction of the NADPH/ NADP\(^+\) couple with Complex I may result in an increase or decrease of the \(\text{H}_2\text{O}_2\) levels specific for signalling in apoptosis and autophagy, depending on the mitochondrial energetic conditions.

The hypothesis is in agreement with the results and conclusions of Lambert and Brand (Lambert and Brand 2004a, b; Lambert et al. 2008a, b) that Complex I in rat-muscle mitochondria produces \(\text{O}_2^-/\text{H}_2\text{O}_2\) at two sites. The minor site was proposed to be the FMN reacting with NADH (i.e. FMN-b). The major site was thought to be in the region of the Q-binding site, possibly ubisemiquinone itself (Lambert and Brand 2004b). Our hypothesis predicts that this second site is FMN-a.

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