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Superoxide Radical Formation by Pure Complex I (NADH:Ubiquinone Oxidoreductase) from *Yarrowia lipolytica**

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Generation of reactive oxygen species (ROS) is increasingly recognized as an important cellular process involved in numerous physiological and pathophysiological processes. Complex I (NADH:ubiquinone oxidoreductase) is considered as one of the major sources of ROS within mitochondria. Yet, the exact site and mechanism of superoxide production by this large membrane-bound multiprotein complex has remained controversial. Here we show that isolated complex I from *Yarrowia lipolytica* forms superoxide at a rate of 0.15% of the rate measured for catalytic turnover. Superoxide production is not inhibited by ubiquinone analogous inhibitors. Because mutant complex I lacking a detectable iron-sulfur cluster N2 exhibited the same rate of ROS production, this terminal redox center could be excluded as a source of electrons. From the effect of different ubiquinone derivatives and pH on this side reaction of complex I we concluded that oxygen accepts electrons from $FMNH_2$ or FMN semiquinone either directly or via more hydrophilic ubiquinone derivatives.

Over the last decade the processes leading to the production of superoxide and other reactive oxygen species (ROS)[1] have gained much attention. ROS seem to be involved in apoptosis, the development of various pathological states, aging, and the regulation of cell metabolism. It is generally accepted that production of reactive oxygen species is an inherent property of the mitochondrial respiratory chain of eucaryotic cells. Oxidation of certain redox centers in complex I and III by molecular oxygen results in the production of superoxide anion radical $O_2^{\cdot-}$ (see Ref. 1 for a review). Superoxide can then convert into hydrogen peroxide, the highly active hydroxyl radical ($OH^{\cdot}$), and other ROS. It has been shown that $O_2^{\cdot-}$ production by complex I occurs in the mitochondrial matrix, whereas the cytochrome bc_1 complex reduces oxygen primarily on the intermembrane side (2, 3) (see, however, Ref. 4). It has been demonstrated for the cytochrome bc_1 complex that oxygen reduction occurs at the Q_P site and is increased markedly under conditions of "oxidant-induced reduction" (5, 6). However, much less is known about the site and mechanism of $O_2^{\cdot-}$ generation in complex I. Thermodynamically, any of the complex I redox centers in the reduced state is capable of donating an electron to molecular oxygen to form a superoxide anion.

Eucaryotic NADH:ubiquinone oxidoreductase (complex I or type I NADH dehydrogenase) is the largest and most complex enzyme of the respiratory chain, residing in the inner membrane of mitochondria. In mammals, the enzyme is composed of 46 different subunits (7) and contains non-covalently bound FMN and up to eight iron-sulfur clusters as redox cofactors. Two complex I-associated, electron paramagnetic resonance-detectable semiquinone species with different spin relaxation times have been characterized (8, 9). Complex I catalyzes the transfer of electrons from matrix NADH to membrane ubiquinone coupled to the translocation of four protons across the membrane (10, 11). Numerous hypothetical schemes for the coupling mechanism of complex I can be found in the literature; the most recent ones involve long range conformational changes in the enzyme complex rather than variations of a classical redox loop or pump (12–15). In the presence of $\Delta\mu H^+$ across the membrane, the enzyme is also able to catalyze the reverse reaction and reduce NAD^+ by the quinol pool (16, 17). Besides its "primary" reaction, complex I is capable of one electron reduction of "artificial" acceptors, including molecular oxygen, during both reverse and forward electron transfer.

Almost 40 years ago it was shown in the laboratory of E. Racker and colleagues that submitochondrial particles produce hydrogen peroxide during direct and reverse electron transfer at "coupling site one" (18). Later it was found that $O_2^{\cdot-}$ originating from respiring mitochondria is a stoichiometric precursor of mitochondrial H_2O_2 (19). In a pioneering study of Boveris and Cadenas and co-workers (20), ubiquinol molecules were identified as sources of superoxide radicals; however, other possibilities have also been discussed in the literature. Later, the same group proposed flavine mononucleotide as a source of $O_2^{\cdot-}$ based on its negative redox potential and, by analogy, to other flavoproteins (2). In recent studies the involvement of other cofactors has been discussed, *i.e.* the most negative iron-sulfur cluster, N1a of complex I (21), or tightly bound semiquinone molecules (22, 23). However, FMN has not been excluded (24). Even an enzyme-bound NAD radical has been considered as a possible source of electrons by some authors (25).

Because of the elusive nature of $O_2^{\cdot-}$ and the high variability of ROS production in mitochondria from different tissues and species (24, 26), it is still not possible to pinpoint the precise site(s) of superoxide generation in complex I. Almost all studies over the last two decades were performed on either intact mitochondria or submitochondrial particles or on cell cultures. This makes it very hard to unambiguously identify the site of $O_2^{\cdot-}$ generation in complex I. The only studies on superoxide

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[1] The abbreviations used are: ROS, reactive oxygen species; $C_{12}E_8$, octaethyleneglycol mono-*n*-dodecyl ether; DBQ, *n*-decylubiquinone; DPI, diphenyleneiodonium; DQA, 2-decyl-4-quinazolinyl amine; EPR, electron paramagnetic resonance; HAR, hexaammineruthenium (III)-chloride; $O_2^{\cdot-}$, superoxide radical; Mops, 4-morpholinepropanesulfonic acid; Q_1, 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone.

TABLE I
dNADH-dependent activities of mitochondrial membranes

DNADH oxidation			O_2^- generation[a]				
HAR[a]	O_2	DBQ[b]	No additions	Antimycin	DQA	Stigma	Azide
μmol dNADH·min^{-1}·mg^{-1}			*nmol AcCyt c·min^{-1}·mg^{-1}*				
1.2 ± 0.2	0.17 ± 0.20	0.42 ± 0.13	0.25 ± 0.09	1.74 ± 0.33	0.70 ± 0.21	0.65 ± 0.26	0.57 ± 0.21

[a] AcCyt *c* is acetylated cytochrome *c*.
[b] In the presence of azide and stigmatellin.

formation that were performed with the isolated enzyme (20, 25, 27) used the original purification protocol for complex I by Hatefi and Rieske (28), which suffers from low inhibitor sensitivity and is contaminated by significant amounts of other respiratory chain enzymes.

Here we have examined the generation of superoxide radical by an affinity-purified, homogenous preparation of complex I from the aerobic yeast *Yarrowia lipolytica* (29). In addition to the wild-type enzyme, a variant carrying a point mutation in the 49-kDa subunit containing no detectable iron-sulfur cluster N2 but retaining significant specific activity (30) was examined. We show that the terminal iron-sulfur cluster N2 is not involved in superoxide production by complex I and propose FMN as the reductant for molecular oxygen.

MATERIALS AND METHODS

Mitochondrial membranes were prepared according to published protocols (31). Complex I from both mutant and wild-type was affinity-purified from isolated mitochondrial membranes that were solubilized with *n*-dodecyl-β-D-maltoside essentially as described previously (29). Construction of the mutant and its characterization have been reported elsewhere (30). Protein concentrations were determined according to a modified Lowry protocol (32).

Purified complex I (0.5 mg/ml) was activated with 10 mg/ml lipids (76% phosphatidylcholine, 19% phosphatidylethanolamine, and 5% cardiolipin) in 2.3% octylglucoside as described previously (33) and used after extensive dialysis (24 h) against the measuring buffer. For preparation of complex I-containing proteoliposomes, 0.3–0.5 mg/ml enzyme were mixed with 10 mg/ml asolectin solubilized in 1.6% octylglucoside and dialyzed for 24 h against the measuring buffer. The liposomes were collected by centrifugation at 90,000 × *g* for 1 h, and the pellet was gently resuspended in a small volume of the same buffer. Only uncoupled proteoliposomes were used for standard measurement. For the preparation of proteoliposomes with a higher degree of respiratory control, the procedure essentially as described in Ref. 34 was used. The aliquot of the enzyme (final concentration 0.8 mg/ml) was mixed with 30 mg/ml asolectin and 60 mM cholate in 40 mM Na^+/Mops, pH 7.6, and 50 mM KCl. The mixture was dialyzed against 200 volumes of the same buffer for 4 h followed by a change of buffer and dialyzed overnight at 4 °C.

NADH oxidation was measured spectrophotometrically at 340–400 nm in 40 mM Na^+/Mops, pH 7.0, 0.2 mM EDTA, and 20 mM NaCl by using either a diode array spectrophotometer (MultiSpec 1501, Shimadzu) or a SpectraMax plate reader spectrophotometer (Molecular Devices). The concentrations of the additions were 100 μM NADH, 2 mM HAR, and 60 μM DBQ or Q_1. For inhibition of the individual complexes of the mitochondrial respiratory chain, 1 μM stigmatellin, 0.9 μM antimycin A, 2.2 μM DQA, or 10mM sodium azide was used. Routinely, 0.5–1.5 μg of isolated enzyme per milliliter were used for measuring NADH oxidation, and 5–15 μg of protein per milliliter were used for measuring O_2^- generation. All activities were assayed at 28 °C. For measurements of pH dependence, a buffer containing 20 mM Tris/Cl^-, 20 mM Na^+/Mops, 20 mM NaCl, and 0.2 mM EDTA was used.

The formation of superoxide radicals was monitored as the reduction of acetylated cytochrome *c* ($\epsilon_{550-539\ nm}$ = 21.5 mM^{-1}·cm^{-1}) (35, 36) in 40 mM Na^+/Mops, pH 7.0, 0.2 mM EDTA, 20 mM NaCl, and 27 μM acetylated cytochrome *c* using a SpectraMax plate reader spectrophotometer (Molecular Devices). After the addition of all components, the mixture was distributed into the wells of the plate and the reaction was started by the addition of 100 μM NADH. Ubiquinone was added only where indicated. In this assay the rate of superoxide formation is determined as the superoxide dismutase-sensitive rate of acetylated cytochrome *c* reduction measured in quadruplicate pairs (with or without 15 units/ml CuZn-superoxide dismutase). The rates of the superoxide dismutase-insensitive reaction were ~50% or 5–7% of the total rate when the activity of the mitochondrial fragments or of the isolated enzyme, respectively, was measured. The calculated rates were proportional to the amount of enzyme used. Data were analyzed statistically and are given as mean ± S.E in Figs. 1–3 and Tables I–IV.

Suitable concentrations of the components (acetylated cytochrome *c* and superoxide dismutase) and the kinetic parameters of the measuring system were established using the xanthine/xanthine oxidase reaction as a reference system (37). The addition of catalase did not affect the rate of cytochrome reduction in the presence of superoxide dismutase. Acetylated cytochrome *c* was prepared as described (38).

Asolectin (total soy bean extract with 20% lecithin), phosphatidylethanolamine, phosphatidylcholine, and sodium cholate were purchased from Avanti Polar Lipids (Alabaster, AL). *n*-Dodecyl-β-D-maltoside was obtained from Glycon (Luckenwalde, Germany), and octyl-β-D-glucopyranoside was from Biomol. Superoxide dismutase, cytochrome *c* (from horse heart), diphenyleneiodonium (DPI), and cardiolipin were from Sigma.

RESULTS

Superoxide Radical Generation by Mitochondrial Membranes of Y. lipolytica—dNADH-dependent activities of mitochondrial membranes from *Y. lipolytica* are shown in Table I. As was typically observed for the membrane preparation used that largely consists of mitochondrial fragments, rates of dNADH oxidation were 2–3 times lower than those of dNADH:ubiquinone oxidoreductase activities. This was largely due to the loss of endogenous cytochrome *c* during isolation.

Superoxide dismutase-sensitive rates of acetylated cytochrome *c* reduction were measured during oxidation of dNADH. The superoxide formation rate in the absence of inhibitors was 0.15% of the dNADH-oxidation rate and increased 7-fold upon the inhibition of center N (Q_i) of complex III by antimycin A. Much of this increase reflects the well characterized high rate of superoxide formation at center P (Q_o) of complex III under these specific conditions (5, 6). If the center P inhibitor stigmatellin was present, the rate of superoxide formation increased only 2–3-fold. Remarkably, virtually the same increase was observed when the complex I inhibitor DQA or the complex IV inhibitor azide was added. This finding indicated that the observed moderate increase was not due to a specific effect on the chemistry occurring in any of the inhibited complexes but was rather due to an overall increase of the reduction level of upstream redox centers by preventing electrons from passing onto oxygen. Still, as the effect was approximately the same no matter which complex was inhibited, it seems likely that a higher reduction level of the redox centers in complex I was responsible for the increase in superoxide production as has been shown previously for bovine heart submitochondrial particles (39).

Superoxide Radical Generation by Complex I Proteoliposomes in the Presence and Absence of Ubiquinones—The rates of NADH-dependent electron transfer and superoxide formation by a typical proteoliposome preparation containing the wild-type enzyme were measured under different conditions (Table II). The specific NADH:ubiquinone oxidoreductase activities for DBQ and Q_1 depended somewhat on the batch of protein and varied between 4–6 μmol·min^{-1}·mg^{-1}. However, it always correlated with the rate of superoxide radical genera-

TABLE II
NADH-dependent activities of complex I containing proteoliposomes

	NADH oxidation			O_2^- generation		
	HAR	DBQ	Q_1	No quinone	DBQ	Q_1
	μmol NADH min^{-1}·mg^{-1}			*nmol AcCyt c·min^{-1}·mg^{-1}*		
Proteoliposomes	21.0 ± 0.3	6.3 ± 0.2	4.3 ± 0.3	16 ± 3	22 ± 2	125 ± 10
Proteoliposomes + Q_9	22.1 ± 1.0	6.5 ± 0.3	4.1 ± 0.2	16 ± 2	20 ± 3	113 ± 12

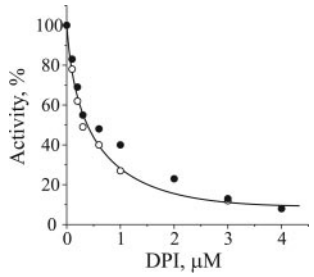

FIG. 1. **Effect of DPI on superoxide production (○) and NADH: DBQ reductase activity (●) by complex I.** Proteoliposomes (0.2 mg/ml) were preincubated on ice for 70 min with different concentrations of inhibitor in the presence of 50 μM NADH. It should be noted that even in the absence of DPI, NADH:DBQ oxidoreductase decreased somewhat during the necessarily long incubation period, most likely due to damage of complex I by the formed superoxide. Therefore, it was essential to keep the incubation time the same for all measurements.

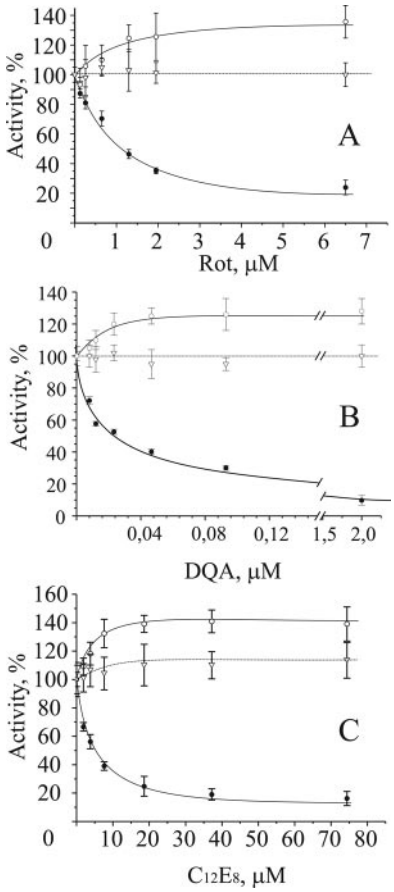

FIG. 2. **Complex I inhibitors do not inhibit superoxide production.** The effect of increasing concentrations of complex I inhibitors on NADH:DBQ oxidoreductase activity (●) and O_2^- generation by complex I proteoliposomes with (○) or without (▽) 60 μM DBQ was tested using rotenone (*Rot*) (*A*), DQA (*B*), and the detergent $C_{12}E_8$ (*C*). Proteoliposomes (0.7 mg/ml) were preincubated with different concentrations of inhibitor on ice. After 30 min, small aliquots of vesicles were taken for activity determination.

tion. Superoxide generation in the absence of excess ubiquinone as an electron acceptor was rather small, and the electron transfer rate amounted to ~0.15% of the rate measured for catalytic turnover in the presence of saturating amounts of NADH and DBQ. A slight increase in superoxide formation was observed when the more hydrophobic ubiquinone derivative DBQ was added as substrate for catalytic turnover. However, if hydrophilic Q_1 was added as a substrate instead, a dramatic 7–8-fold increase in the rate of superoxide formation was observed (Table II). This high rate decreased over time, most likely due to the consumption of ubiquinone (not shown). It has been shown previously (20) that in the case of Q_1 the rates of the H_2O_2 production depend on the concentration of oxidized acceptor. In contrast, NADH:ubiquinone reductase activity with Q_1 was only two-thirds of that with DBQ. None of the determined specific activities was affected when 100 moles of ubiquinone Q_9, the endogenous electron acceptor of the *Y. lipolytica* complex I, were added per mole of enzyme to the phospholipid/detergent mixture used for reconstitution. The incorporation of added Q_9 was checked by the redox spectra of the proteoliposomes in the presence of detergent (at 275 nm, reduced by borohydride), revealing that at least 60% of added Q_9 was retained in the vesicles.

Effect of Inhibitors on Superoxide Radical Generation—DPI is a flavoprotein inhibitor that reacts specifically with the FMN of complex I (40, 41). The preincubation of the proteoliposomes with a small amount of NADH resulted in a reduction of the redox centers of the enzyme and allowed DPI to bind to the reduced flavine. As shown in Fig. 1, both NADH:DBQ reductase activity and superoxide production were fully sensitive to the inhibitor. With oxidized complex I, no inhibition was observed up to 5 μM DPI (not shown). In fact, all NADH-dependent activities listed in Table II and the NADPH:HAR oxidoreductase activity were fully sensitive to DPI in the micromolar range (not shown).

Conflicting results are found in the literature on the effect of classic complex I inhibitors on O_2^- production by complex I. Therefore, we tested rotenone (class B), DQA (class A), and the detergent $C_{12}E_8$ (class C), which, according to our previous results (42), bind to complex I at different sites but share a common binding pocket. It should be noted that the inhibitors inhibited complex I activity at concentrations very similar to those reported previously for mitochondrial membranes (43). As shown in Fig. 2*A*, rotenone had no effect on the rates of O_2^- production in the absence of the substrate, quinone. If superoxide production was monitored during steady-state turnover using DBQ as a substrate, a small but significant increase in radical production was observed with progressive inhibition of complex I by rotenone. A similar relative increase by 20–30% was found when Q_1 was used as a substrate instead (not shown). Virtually identical results were observed if complex I was inhibited by DQA (Fig. 2*B*). In the case of $C_{12}E_8$, a slight stimulation of superoxide production occurred already in the absence of DBQ, and the increase was somewhat more pronounced than that for the other two inhibitors in the

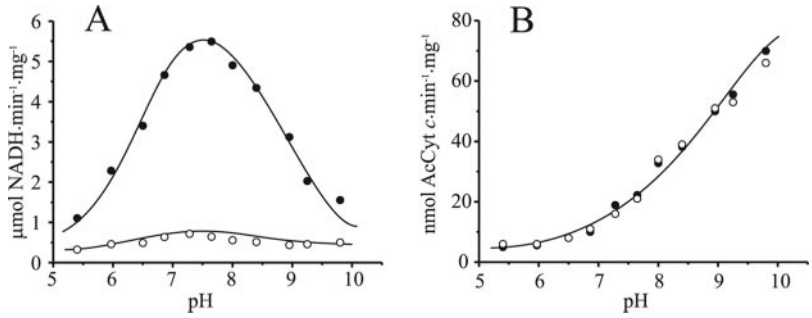

FIG. 3. **The pH profiles for ubiquinone reduction and superoxide formation of complex I are different.** Shown are the pH dependence of the NADH:DBQ oxidoreductase activity (*A*) and the pH dependence O_2^- generation by complex I (*B*) in proteoliposomes with (○) and without (●) the addition of 1 μM DQA.

TABLE III
NADH-dependent activities of complex I containing proteoliposomes from parental strain and a cluster N2 deficient mutant

	NADH:HAR oxidoreductase	NADH:DBQ oxidoreductase	O_2^- generation	
			Absolute	Normalized[a]
	μmol NADH·min⁻¹·mg⁻¹	*μmol NADH·min⁻¹·mg⁻¹*	*nmol AcCyt c·min⁻¹·mg⁻¹*	*%*
Parental	23.0 ± 0.3	5.5 ± 0.2	18 ± 3	100 ± 17
Mutant R141M	16.1 ± 1.0	2.3 ± 0.5	12 ± 4	96 ± 39

[a] O_2^- generation activities were normalized to complex I content as estimated from NADH:HAR oxidoreductase reductase activities.

presence of quinone as the substrate. The rather high concentrations needed for maximal inhibition by $C_{12}E_8$ came close to the critical micellar concentration of 90 μM for this detergent. Therefore, the gradually enhanced effects by $C_{12}E_8$ can be explained by alterations in the lipid environment of complex I and the improved accessibility of the interacting agents. Also, in the presence of Q_9 only, no significant effect of the inhibitors was seen (data not shown). Our results suggest that the ubiquinone reduction reaction itself was not directly involved in superoxide formation. Rather, as observed with mitochondrial membranes, this side reaction seemed to be affected by the state of other redox centers of the complex that was modulated by titrating down the steady-state turnover of the enzyme.

pH Dependence of Superoxide Radical Generation—Confirming earlier findings (43), we found an optimum for NADH:DBQ oxidoreductase activity with phospholipid-activated complex I at around pH 7.5 (Fig. 3*A*). If most of the turnover was blocked by 1 μM DQA, the residual catalytic turnover still exhibited a very similar pH profile. However, the observed pH dependence was quite different for O_2^- production in the absence of substrate ubiquinone. The rate of radical generation was ~10-fold faster at pH 10 than at pH 6 (Fig. 3*B*). Up to a pH value of 7.5, an excess of the specific complex I inhibitor DQA had no significant effect on superoxide radical generation. Above pH 7.5, some reduction of the rates by up to ~20% at pH 10 was observed.

Superoxide Radical Generation by Complex I Lacking Detectable Cluster N2—As described recently (30), mutation of arginine 141 to methionine in the 49-kDa subunit of *Y. lipolytica* complex I results in the loss of all electron paramagnetic resonance-detectable cluster N2 (in both intact membranes and the isolated enzyme), whereas complex I specific activity remained significant. To further explore a possible involvement of iron-sulfur cluster N2 in superoxide formation by complex I, we included mutant R141M of the 49-kDa subunit in this study. Confirming our previous result, NADH:DBQ oxidoreductase activity of proteoliposomes with complex I from mutant R141M was found to be ~40% of that of complex I from the parental strain value (Table III). Normalized to HAR:DBQ oxidoreductase to account for differences in complex I purity, ubiquinone activity amounted to 50% of the parental strain. In absolute terms, the specific rate of O_2^- generation was somewhat reduced for complex I from mutant R141M. However, normalizing the rates for complex I content based on NADH:HAR oxidoreductase activities revealed that the mutation abolishing electron paramagnetic resonance-detectable cluster N2 had virtually no influence on superoxide formation. Basically the same results were found in preparations of the enzymes activated with mixture of pure phospholipids (not shown).

Effect of Potential across the Membrane on Superoxide Radical Generation by Complex I Proteoliposomes—Reconstitution of complex I into proteoliposomes supports the formation of an electric potential across the membrane during NADH:DBQ reaction, and preparations show a various degree of respiratory control.[2] To obtain proteoliposomes with higher degree of respiratory control, a reconstitution procedure with cholate was used in addition to the standard octyl-glucoside protocol. In these preparations a 4-fold increase of NADH:DBQ reductase activity was observed upon the addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Table IV). Despite different values of respiratory control in *n*-octyl-β-D-glucopyranoside and cholate-reconstituted enzyme, the observed rates of O_2^- production were not affected by the addition of uncoupler (Table IV). It should be noted that *n*-octyl-β-D-glucopyranoside and cholate-reconstituted liposomes were measured at pH 7.0 and 7.6, respectively, explaining the ~2-fold difference in the specific rates of superoxide formation.

DISCUSSION

Complex I from *Y. lipolytica* reactivated by lipids or by reconstitution into proteoliposomes has full NADH:ubiquinone oxidoreductase activity (33). Here we show that these preparations of pure complex I also exhibit NADH-dependent formation of superoxide radicals at a rate of ~0.15% of the maximal catalytic electron transfer rate (calculated for two-electron reduction). The reported rates for O_2^- production are in the same range as the previously published values (10–20 milliunits/mg or ~0.1–0.2% of NADH:Q_1 oxidoreductase activity) (20, 27). One should keep in mind, however, that these earlier studies used Hatefi and Rieske (28) preparations of complex I contaminated by complexes III and IV, as well as rather different measuring conditions.

In contrast to the Hatefi and Rieske (28) preparation, affinity-purified complex I from *Y. lipolytica* contains little tightly bound coenzyme Q_9 (~0.2 nmol/nmol enzyme) (33). Still, introducing excess Q_9 during the reconstitution procedure by adding

[2] S. Dröse, A. Galkin, and U. Brandt, submitted for publication.

TABLE IV
Effect of uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) on superoxide production in NADH:DBQ reductase reaction of complex I containing proteoliposomes (PL)

	NADH:DBQ		O_2^- generation	
	PL-cholate	PL-OG[a]	PL-cholate	PL-OG[a]
	μmol NADH min^{-1}·mg^{-1}		*nmol AcCyt c·min^{-1}·mg^{-1}*	
−FCCP	1.4 ± 0.2	2.2 ± 0.1	60 ± 5	33 ± 3
+FCCP	5.4 ± 0.3	4.3 ± 0.3	59 ± 6	35 ± 6

[a] OG, *n*-octyl-β-D-glucopyranoside.

it to the lipid/detergent/protein mixture did not affect any of the activities of the proteoliposomes, including O_2^- production in the presence or absence of short chain ubiquinone derivatives (Table I). It has been demonstrated previously that endogenous ubiquinone is not required for NADH:ubiquinone oxidoreductase activity (33). Our present findings suggest that this finding also holds true for superoxide formation by isolated complex I from *Y. lipolytica*. This is in line with a previous study (39) in which pentane extraction of endogenous Q_{10} from bovine SMP was shown to have no effect on superoxide radical formation by complex I.

Our finding that the addition of exogenous Q_1 quinones markedly increased O_2^- generation by an isolated enzyme is in agreement with the pioneering studies of Boveris and co-workers (20). Also, in bovine submitochondrial particles blocked by the center Q_p inhibitor strobilurin, Q_1 has been shown to increase superoxide production during oxidation of NADH to a greater extent than the more hydrophobic DBQ (39). As such, the observed high rates of O_2^- generation in the presence of the rather hydrophilic Q_1 may have been interpreted as supporting a direct involvement of substrate ubiquinone in this side reaction of complex I. However, the much less hydrophilic *n*-decyl derivative of ubiquinone, DBQ, also increased superoxide production but to a much lesser extent. The most straightforward explanation for this difference is that Q_1 acts as redox mediator between molecular oxygen and the enzyme mostly at a non-physiological site in the hydrophilic domain of complex I, whereas DBQ accepts electrons almost exclusively from the physiological ubiquinone binding site of complex I. Thus, our results obtained with ubiquinone derivatives suggest that there is another site in complex I where ROS formation can be mediated by hydrophilic quinones. However, these results do neither support nor exclude an involvement of the physiological ubiquinone site in the production of superoxide by complex I. The binding pocket for ubiquinone could be ruled out as source of oxygen radicals based on the effect of quinone-analogous hydrophobic inhibitors of complex I. Rotenone, DQA, and $C_{12}E_8$, *i.e.* representatives covering all three subsites within the inhibitor binding pocket of complex I (42), had no effect on O_2^- production by complex I in the absence of substrate quinones. The 20–30% increase in the presence of Q_1 or DBQ and any of the three inhibitors does not argue against this conclusion. To understand this moderate effect, one has to consider that titrating the enzyme under steady-state conditions with an inhibitor leads to an increased reduction of the redox centers of complex I promoting oxygen reduction. In fact, the same explanation can be applied to the inhibitor-induced increase of superoxide formation with mitochondrial membranes that was the same no matter at which complex of the respiratory chain the electron flow was blocked. The residual turnover of complex I even in the presence of large excess of inhibitor certainly was still fast enough to not limit the even lower rate of superoxide production. Thus, different and independent mechanisms are responsible for the increase in the rates of superoxide formation by the inhibition of electron transfer and the addition of more hydrophilic ubiquinone derivatives.

All previous studies showing that rotenone increases ROS production have been performed on preparations of mitochondria, and H_2O_2 rather than O_2^- production was measured. Moreover, in most cases the effect of the inhibitor has been reported to be very weak and tissue-specific (24, 26, 44). On the other hand, rotenone did not increase ROS production in crude preparations of isolated enzyme (27) or even inhibited it (20). In fact, results from our own laboratory on ROS formation by other complexes suggest that complex interactions have to be taken into account to understand mitochondrial superoxide production if several respiratory chain enzymes connected by the same donor/acceptor couple (*e.g.* quinone/quinol for complexes I and III) are present.[3] Thus, although it is essential to study the isolated enzyme to understand the basic mechanisms involved in superoxide formation, one should keep in mind that it may behave differently under certain conditions in intact mitochondria, where complex I physically and functionally interacts with other respiratory chain enzymes. In this context it is worth noting, however, that we were not able to detect respiratory chain supercomplexes in *Y. lipolytica* under conditions where they are observed in mammalian mitochondria.[4]

As shown in Fig. 1, DPI inhibits both NADH:DBQ oxidoreduction and superoxide production by the enzyme. The concentration of DPI sufficient to give maximum inhibition of both activities was equivalent to 20 mol of DPI per mole of complex I. No inhibition was observed if DPI was incubated with the oxidized enzyme, confirming previous findings (41) that the inhibitor reacts with reduced flavine to yield a non-reoxidizable product (40, 41). DPI inhibits oxidation of pyridine nucleotides by the artificial acceptor HAR, showing the same titration curve as NADH:DBQ reductase (not shown). Recently, moderate increase of H_2O_2 release by heart mitochondria during the oxidation of endogenous substrates was observed after the addition of DPI (23); however, this effect could have been mediated by the effect on other NAD(P)H-dependent enzymes such as mitochondrial nitric oxide synthase (45, 46), NAD(P)H dehydrogenase (47), or dehydrogenases of α-keto acids (48) rather than by inhibition of complex I. At the same time, DPI strongly inhibits mitochondrial H_2O_2 production during the oxidation of succinate by heart (23) or brain mitochondria (24). Another difficulty in interpreting the direct effect of DPI on respiring intact mitochondria is the issue of permeability of the inner membrane for the positively charged inhibitor molecule.

Our results showing that even the uncoupling of rather tight proteoliposomes had no effect on superoxide generation during the direct reaction of complex I (Table IV) suggest that membrane potential has no influence on this reaction. It has been shown, however, that there is a threshold ΔΨ value (near ΔΨ in state III respiration) in mitochondria respiring on succinate above which there is a very steep dependence on H_2O_2 production (49). Below this level, the potential has no effect. It seems likely that this ΔΨ dependence in whole mitochondria is due to superoxide formation of complex III, because its heme *b* centers

[3] S. Dröse and U. Brandt, manuscript in preparation.
[4] U. Brandt, unpublished observation.

are located within the membrane and arranged perpendicular to it. In contrast, none of the known redox centers of complex I is located in the membrane domain. However, we cannot fully exclude the possibility that a membrane potential higher than the one we could reach with our liposome preparation would have affected superoxide formation by indirect mechanisms.

The results discussed up to this point indicate that the site of O_2^- production within complex I is located upstream of the inhibitor binding site. It seems likely that the very negative redox potential of the electrons passing through complex I has provided selective pressure to optimize the insulation of internal redox cofactors to reduce the potentially dangerous leaking to molecular oxygen. In fact, no inhibitors are known to dissect the electron pathway in the region between FMN and cluster N2. Also, our observation that the more hydrophilic ubiquinone derivatives promote superoxide formation by acting as redox mediators (see above) strongly suggests that the electron input and output sites of complex I are the most likely points of electron escape to the oxygen. These points are FMN as the electron acceptor for NADH at one end and iron-sulfur cluster N2, considered as the immediate electron donor for ubiquinone, at the other end of the electron wire in complex I. To test directly whether cluster N2 could be the site of the electron leak to oxygen, we measured ROS production of mutant R141M of the 49-kDa subunits containing no detectable iron-sulfur cluster N2 (30). It turned out that despite the decreased specific ubiquinone reductase activity of the mutant enzyme, the rate of O_2^- production normalized to complex I content was unchanged (Table II). This result indicated that cluster N2 is not the redox center within complex I responsible for O_2^- generation.

The observed marked increase in O_2^- production by complex I above pH 7–8 (Fig. 3*B*) is likely to be caused by a concomitant decrease of the midpoint redox potential of the electron-donating redox group. The low pK value of the perhydroxyl radical of 4.8 (50) rules out significant changes in superoxide stability through effects on the recombination rate between HO_2 and O_2^- above pH 7. The pH dependence of the rate of superoxide production provides further evidence for excluding iron-sulfur cluster N2 as the site of ROS production, because its redox midpoint potential is pH-dependent in the range between pH 5 and 8 (51)[5] and, therefore, too low to explain the pH profile shown in Fig. 3*B*. Cluster N1a would be the only other candidate that exhibits a pH-dependent redox midpoint potential in bovine mitochondria (51), but no pH-dependent cluster N1 has been detected in *Y. lipolytica* complex I to date.[6] Having excluded all other candidates, this leaves us with FMN as a possible site of oxygen reduction within complex I.

In fact, our data provide good evidence for this option; the observed pH dependence of superoxide production (Fig. 3*B*) fits very well with the redox-Bohr properties of FMN. In general, it is known that generation of O_2^- by free flavine in solution (52, 53) or by flavodehydrogenases (54) is very pH-sensitive and rises steeply above neutral pH. A corresponding decrease in the slope of the pH dependence of the FMN midpoint potential is found between pH 7 and 10.5 (55), which matches the increased occurrence of semiquinone radical for this pH interval. Together with the overall drop in the midpoint potential of the FMN/$FMNH_2$ couple, this finding provides a good explanation for the observed increase in superoxide radical production (Fig. 3*B*). The observed strong stimulation of superoxide production by Q_1 also argues in favor of FMN as the reductant for oxygen, as it has been shown for other flavoenzymes that NADH and quinones may share a common binding pocket (56, 57). Thus, we conclude that FMN is the site of superoxide formation within complex I where electrons are transferred onto molecular oxygen either directly or via more hydrophilic ubiquinone derivates.

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Note Added in Proof—After the submission of this manuscript, an important paper on this subject appeared. In this article (Vinogradov, A. D., and Grivennikova, V. G. (2005) *Biochemistry* (*Mosc.*) **70,** 120–127), the authors characterize the superoxide production by complex I in bovine submitochondrial particles and independently suggest that the donor for one electron reduction of oxygen is the reduced form of flavine mononucleotide.

[5] K. Zwicker, A. Galkin, S. Dröse, S. Kerscher, and U. Brandt, manuscript in preparation.

[6] K. Zwicker, unpublished observation.

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