CHAPTER TWENTY-SIX

Measurement of Superoxide Formation by Mitochondrial Complex I of *Yarrowia Lipolytica*

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Abstract

Complex I (NADH: ubiquinone oxidoreductase) is generally regarded as one of the major sources of mitochondrial reactive oxygen species (ROS). Mitochondrial membranes from the obligate aerobic yeast *Yarrowia lipolytica,* as well as the purified and reconstituted enzyme, can be used to measure complex I–dependent

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generation of superoxide ($O_2^{\bullet -}$). The use of isolated complex I excludes interference with other respiratory chain complexes and matrix enzymes during superoxide dismutase–sensitive reduction of acetylated cytochrome *c*. Alternately. hydrogen peroxide formation can be measured by the Amplex Red/horseradish peroxidase assay. Both methods allow the determination of complex I–generated ROS, depending on substrates (NADH, artificial ubiquinones), membrane potential, and active/deactive transition. ROS production by *Yarrowia* complex I in the "forward mode" is essentially independent of catalytic turnover, membrane potential, and the presence of inhibitors or the active/deactive transition.

1. Introduction

Within the respiratory chain, complex I (NADH: ubiquinone oxidoreductase) and the cytochrome bc_1 complex (complex III, ubiquinol: cytochrome *c* oxidoreductase) have been identified as the main sources of "reactive oxygen species" (ROS; Fridovich, 1978; Turrens, 2003). While complex III produces superoxide *in vitro* preferably under conditions of "oxidant-induced reduction" (i.e., in the presence of the specific center N inhibitor antimycin A, sufficient amounts of reducing equivalents, and an oxidized downstream respiratory chain), complex I has been shown to generate superoxide under various conditions at high rates and also in the absence of inhibitors. For this reason, most groups working in the ROS field regard complex I as the more important mitochondrial source of ROS as either a signaling or a deleterious agent. Over the years, all major cofactors involved in the electron transfer chain of complex I have been proposed as sites for superoxide production: flavine-mononucleotide (Galkin and Brandt, 2005; Kussmaul and Hirst, 2006; Liu *et al.*, 2002; Vinogradov and Grivennikova, 2005), iron-sulfur clusters N2 (Genova *et al.*, 2001) and N1a (Kushnareva *et al.*, 2002), and a semiquinone radical formed on ubiquinone reduction (Lambert and Brand, 2004; Ohnishi *et al.*, 2005b). Although contributions from other redox centers still cannot be excluded entirely, it now seems clear that reduced FMN is the major source of electrons for superoxide formation in mitochondrial complex I (Galkin and Brandt, 2005; Kussmaul and Hirst, 2006). Bacterial complex I seems to produce mainly H_2O_2 by direct reduction of O_2 with two electrons from $FMNH_2$ (Esterhazy *et al.*, 2008). This variation may be due to the marked difference in the redox potential of iron-sulfur cluster N1a, which is much more negative in the mitochondrial enzyme.

In mitochondrial membranes superoxide production by complex I can be measured under two different conditions (Kushnareva *et al.*, 2002; Lambert and Brand, 2004; Liu *et al.*, 2002; Votyakova and Reynolds, 2001): when mitochondria respire on NADH-generating substrates (e.g., malate/glutamate or malate/pyruvate), superoxide production is low

and is stimulated by the addition of inhibitors blocking electron transfer. This is due to increased reduction of upstream components of the respiratory chain, including the redox cofactors of complex I. The highest rates of superoxide production are measured under conditions of succinate-supported reverse electron transfer from complex II via of ubiquinone and complex I onto NAD^+ (Hinkle *et al.*, 1967; Vinogradov and Grivennikova, 2005; Votyakova and Reynolds, 2001). Under these conditions FMN and the other redox centers of complex I become even more reduced. This state and, therefore, ROS formation is highly sensitive to even a small drop in the protonmotive force.

This chapter describes methods to measure the superoxide and hydrogen peroxide (H_2O_2) production by purified complex I from the obligate aerobic yeast *Yarrowia lipolytica*, an organism that was established in our laboratory as a convenient model for the structural and functional analysis of mitochondrial complex I (Kerscher *et al.*, 2002).

2. Materials, Sample Preparation, and Purification of Complex I from *Yarrowia Lipolytica*

This section summarizes the materials and the methods that are required to obtain a highly pure and active preparation of complex I from *Y. lipolytica*. The use of purified complex I for the investigations of ROS production excludes interference with other respiratory chain complexes and mitochondrial matrix enzymes.

2.1. Materials

Asolectin (total soy bean extract with 20% lecithin) phosphatidylethanolamine (purified from egg yolk), and phosphatidylcholine (purified from egg yolk) were purchased from Avanti Polar Lipids (Alabaster, AL). *n*-dodecyl-*ß*-D-maltoside was obtained from Glycon (Luckenwalde, Germany), and octyl-*ß*-D-glucopyranoside was from Biomol (Hamburg, Germany). Oxonol VI (bis-(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol) and Amplex Red (*N*-acetyl-3,7-dihydrophenoxazine) were purchased from Invitrogen/Molecular Probes (Eugene, OR), and decylubiquinone (DBQ) was from Alexis Biochemicals (Lausen, Switzerland). Superoxide dismutase (SOD, from bovine liver), horseradish peroxidase (HRP), cytochrome *c* (from horse heart), deamino-NADH (dNADH), cardiolipin (sodium salt, from bovine heart), and all other chemicals were from Sigma. Acetylated cytochrome *c* was prepared by treatment with acetic anhydride as described (Galkin and Brandt, 2005; Kakinuma

and Minakami, 1978). DQA (2-n-decyl-quinazolin-4-yl-amine, SAN 549) was obtained from AgrEvo (Frankfurt, Germany). DBQ, rotenone, DQA, stigmatellin, and Amplex Red were dissolved in DMSO. Bio-Beads SM-2 (20 to 50 mesh) were from Bio-Rad Laboratories (München, Germany) and activated by methanol as described (Dröse *et al.*, 2005). Superoxide and H_2O_2 production were determined with either a diode array spectrophotometer (MultiSpec 1501, Shimadzu) or a Spectra Max PLUS^{384} plate reader spectrophotometer (Molecular Devices, München). The diode array spectrophotometer was generally used when different activities (e.g., NADH-oxidation, ROS-production, generation of $\Delta\psi$) were to be measured simultaneously. After optimization of the assay with a conventional spectrophotometer, routine measurements can be performed with a microplate reader setup, which consumes less material and saves time because multiple samples can be analyzed in parallel.

2.2. Cell growth and preparation of mitochondrial membranes

The *Y. lipolytica* strain PIPO was grown overnight at 27° in a 10-L Biostat E fermenter (Braun, Melsungen) in modified YPD medium (2.5% glucose, 2% Bacto peptone, 1% yeast extract). This strain contains a chromosomal copy of the modified *NUGM* gene, encoding a C-terminally his-tagged version of the 30-kDa subunit of complex I (Kerscher *et al.*, 2002). Mitochondrial membranes were prepared following the protocol of Kerscher *et al.* (1999) with the modification detailed in Kashani-Poor *et al.* (2001).

2.3. Purification of complex I, lipid-activation, and reconstitution into proteoliposomes

Purification of *n*-dodecyl-*β*-D-maltoside solubilized complex I was achieved by Ni-affinity chromatography, followed by gel-filtration as detailed before (Kashani-Poor *et al.*, 2001). Chromatography and gel filtration removes most phospholipids and endogenous Q_9 from the protein (Dröse *et al.*, 2002): only between 26 and 66 mol "organic" (i.e., chloroform/methanol extractable) phosphate per mol complex I and substoichiometric amounts of Q_9 (0.2 to 0.4 mol/mol) remain bound to the enzyme. Delipidation results in a concomitant loss of the NADH: ubiquinone oxidoreductase activity, which can be fully reversed by adding back the phospholipids either as detergent-solubilized micelles (Dröse *et al.*, 2002) or by reconstitution into proteoliposomes (Dröse *et al.*, 2005). A comparable dependence between lipid content and ubiquinone reductase activity was reported later for purified complex I from bovine heart (Sharpley *et al.*, 2006) and *Escherichia coli* (Sinegina *et al.*, 2005; Stolpe and Friedrich, 2004).

For the preparation of lipid-activated complex I that was used for superoxide detection by acetylated cytochrome *c*, 0.5 mg/ml complex I

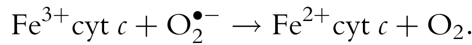
were mixed with 10 mg/ml lipids (76% phosphatidyl-choline/19% phosphatidyl-ethanolamine/5% cardiolipin in 2.3% octylglucoside; Dröse *et al.*, 2002) and dialyzed for 24 h against measuring buffer (50 m*M* Na^+/MOPS, pH 7.0, 1 m*M* EDTA, 20 m*M* NaCl). Alternately, for reconstitution of complex I into proteoliposomes, 0.3 to 0.5 mg/ml complex I was mixed with 10 mg/ml asolectin and 1.6% octylglucoside and dialyzed under the same conditions (Galkin and Brandt, 2005). To compensate for ubiquinone depletion of the complex I preparations, natural ubiquinone (Q_9 in the case of *Y. lipolytica*) can be added to the lipid/protein/detergent mixture during reconstitution of the enzyme. Q_9 retention can be checked spectrophotometrically at 275 nm after solubilization of the final suspension of the proteoliposomes and reduction by sodium borohydride.

For the parallel measurement of NADH oxidation, H_2O_2 generation and the buildup of a membrane potential by reconstituted complex I, tightly coupled proteoliposomes were prepared with Bio-Beads for removal of detergents (Dröse *et al.*, 2005); 10 mg/ml asolectin solubilized in 16 mg/ml octylglycoside (resulting in "total solubilization" of lipids) was mixed at a protein/lipid ratio of 1:50 (w/w) with purified complex I (usually 200 to 400 μg) in 20 m*M* K^+/MOPS, pH 7.2, 50 m*M* KCl. The mixture was incubated at 4° on an Eppendorf Mixer 5432, and the detergents were removed by the stepwise addition of a 20-fold excess of Bio-Beads (by weight) in four equal aliquots at 30-min intervals. We recently observed a decrease in complex I activity with some batches of Bio-Beads when the proteoliposomes were in prolonged contact with the beads. Therefore, the original protocol (Dröse *et al.*, 2005) was optimized by minimizing the incubation time with Bio-Beads while retaining the tightness of the proteoliposomes formed. Proteoliposomes were collected by centrifugation (90,000*g* for 1 h) and resuspended in a small volume of 20 m*M* K^+/MOPS, pH 7.2, 50 m*M* KCl (usually 1 μl/μg of protein applied). After ultracentrifugation, the resuspended proteoliposomes were incubated for at least 2 h on ice to allow for sedimentation of aggregated protein and bead fragments. Then the supernatant was carefully removed and used for subsequent measurements. This procedure usually resulted in proteoliposomes with a respiratory control ratio of ~4 that was stable for more than 24 h at 4°.

3. Detection of Superoxide ($O_2^{\bullet -}$) by Acetylated Cytochrome *c*

Superoxide detection was based on the acetylated cytochrome *c* assay (Azzi *et al.*, 1975). Reduction of ferricytochrome *c* can be followed spectrophotometrically at 550 nm ($\varepsilon_{550\text{-}539\ nm}$ = 21.5 mM^{-1} cm^{-1}) to

measure rates of superoxide formation (overview in Tarpey and Fridovich, 2001):



$$Fe^{3+}\text{cyt } c + O_2^{\bullet -} \rightarrow Fe^{2+}\text{cyt } c + O_2.$$

Cytochrome *c* is not a specific agent for superoxide detection, because it can also become reduced by cellular components like ascorbate, glutathione, or cellular reductases that enzymatically catalyze cytochrome *c* reduction (Tarpey and Fridovich, 2001). However, if the contribution by such side reactions is not too high, the superoxide-dependent rate can be deduced as the difference between the cytochrome *c* reduction rates in the absence and presence of superoxide dismutase. A high rate constant (10^6 $M^{-1}s^{-1}$) of the reaction with superoxide anion and the marked decrease of enzymatic reduction and oxidation rates by respiratory chain complexes III and IV on acetylation of cytochrome *c* greatly improve the sensitivity of the assay. In general, AcCyt *c*, as is the case for HRP and Amplex Red (see section 4), have to be in excess so that the rates of reduction and oxidation can be treated as zero order and depend only on the rate of superoxide production.

3.1. Superoxide production by mitochondrial membranes of *Y. lipolytica*

For the measurement of complex I activity in mitochondrial membranes from *Y. lipolytica* (0.1 to 0.2 mg protein/ml in the assay), deamino-NADH (dNADH) was supplied as electron donor, because this yeast also contains an alternative NDH2-type NADH: ubiquinone oxidoreductase that can use NADH but not its deamino-form (Kerscher *et al.*, 2008). It should be noted that strains lacking the NDH2-type enzyme are also available (Kerscher *et al.*, 1999). dNADH oxidation was followed spectrophotometrically at 340 to 400 nm in 40 m*M* Na^+/MOPS, pH 7.0, 0.2 m*M* EDTA, and 20 m*M* NaCl at 28°. The oxidation of 100 μM dNADH was determined in the absence or presence of 60 μM *n*-decyl-ubiquinone (DBQ), 1 μM stigmatellin, and 10 m*M* sodium azide. In the first case, dNADH oxidation relies on the presence of endogenous Q_9 and the ubiquinol-oxidating activities of the downstream respiratory chain complexes III and IV. In the second case, the complex I–dependent activity depends on the presence of the substrate analog DBQ, whereas the activities of complexes III and IV are blocked by the specific inhibitors stigmatellin and cyanide, respectively. Note that DBQ forms micelles in aqueous solutions above a concentration of ~100 μM (log partition coefficient cyclohexane/water ~7; Fato *et al.*, 1996). As typically observed for fragmented membrane preparations of mitochondria, the dNADH oxidation rate was two to three times lower than the dNADH: DBQ oxidoreductase activity

(Table 26.1). This was largely due to loss of endogenous cytochrome *c* during membrane preparation. The measurement of the dNADH-dependent superoxide formation was monitored as the SOD-sensitive (15 U/ml) reduction of 27 μM acetylated cytochrome *c* dissolved in the aforementioned buffer, with a SpectraMax PLUS^{384} plate reader at 28°. Prior to our experiments, our detection system was tested by monitoring cytochrome *c* reduction by xanthine oxidase (15 mU/ml of enzyme and 50 μM of hypoxanthine). In such conditions addition of 5 U/ml of SOD completely inhibited the reduction of the cytochrome. In *Yarrowia* membranes, the SOD-insensitive production of superoxide was ~50% of the total rate, whereas the superoxide formation rate was ~0.15% of the dNADH oxidation rate in the absence of inhibitors and increased sevenfold on addition of 0.9 μM of the complex III Q_i site (center N) inhibitor antimycin A. Under these conditions, superoxide is formed at the Q_o site (center P) of complex III and at complex I. A block of center P by 1 μM stigmatellin, the ubiquinone binding site of complex I by 2.2 μM DQA or cytochrome oxidase by 10 mM sodium azide resulted in a twofold to threefold increase in superoxide generation. It can be concluded that a higher reduction level of the redox centers in complex I was responsible for the increase of the superoxide production, regardless of whether the electron-transfer chain was blocked at its own ubiquinone binding site or at downstream respiratory chain complexes. Note that stigmatellin inhibition excluded any contribution of complex III to superoxide generation.

3.2. Superoxide production by reconstituted complex I

Detection of the superoxide generated by reconstituted complex I can be carried out with a multiplate reader. Under the conditions described (27 μM acetylated cytochrome *c* and 10 μg/ml proteoliposomes) one measurement takes approximately 4 to 5 min and only 5% of the rate of cytochrome *c* reduction is SOD insensitive. The rates of the NADH-dependent activities catalyzed by complex I–containing proteoliposomes are shown in Table 26.2. Superoxide generation in the absence of any acceptor was only 0.15% of the NADH: DBQ reductase reaction (accounted as 2 e^--transfer). It should be noted that DBQ is a much more reliable substrate for complex I (i.e., for the determination of the catalytic activity and for ROS measurements) than the more hydrophilic Q_1. Addition of hydrophobic ubiquinone analogs does not significantly affect the superoxide generation rates; however, hydrophilic Q_1 increases the production eightfold. Presumably, Q_1 acts as a redox mediator between molecular oxygen and the enzyme, mostly at a nonphysiologic site in the hydrophilic domain of complex I (Galkin and Brandt, 2005). It seems most likely that in this case the source of superoxide is the autooxidation of reduced or semireduced Q_1 formed by complex I turnover (James *et al.*, 2005).

Table 26.1 dNADH-dependent activities of *Y. lipolytica* mitochondrial membranes[a]

dNADH oxidation			O_2^- generation				
HAR[b]	O_2	DBQ[b]	No additions	Antimycin	DQA	Stigma tellin	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] Data from Galkin and Brandt (2005).
[b] In the presence of sodium-azide and stigmatellin.

Table 26.2 NADH-dependent activities of complex I–containing proteoliposomes[a]

	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

[a] Data from Galkin and Brandt (2005).

This reaction occurs in the aqueous phase and, therefore, is less pronounced with hydrophobic ubiquinone derivatives. In fact, it was shown that the rate correlates with ubiquinol hydrophilicity and is greatly reduced in the presence of phospholipid membranes (James *et al.*, 2005). Consequently, the addition of phospholipids or the reconstitution into proteoliposomes not only recovers the maximal catalytic activity of purified complex I but also reduces unwanted side reactions. Enrichment of proteoliposomes by ubiquinone Q_9 (~60 mol/mol enzyme) does not affect any of the complex I activities.

All NADH-dependent activities of complex I, including superoxide generation, can be inhibited effectively by diphenyleneiodonium (DPI)—a covalent inhibitor of flavoproteins. Because DPI reacts only with reduced FMN, proteoliposomes have to be incubated with inhibitor (20 mol DPI per mol of the enzyme for ~90% inhibition) on ice for at least 60 min in the presence of a reductant such as NADH in micromolar concentrations. If incubated with oxidized enzyme, DPI does not inhibit complex I at concentrations up to 5 μM. It should be noted that during long incubations in the reduced state, the activity of complex I always decreases, probably because of dissociation of the reduced flavine from the enzyme (Gostimskaya *et al.*, 2007).

3.3. pH profile for ubiquinone reduction and superoxide formation

The absolute NADH: ubiquinone oxidoreductase activity of reconstituted complex I is pH dependent, showing a classical bell-shaped curve with an optimum at approximately pH 7.5 (Fig. 26.1). However, the rate of

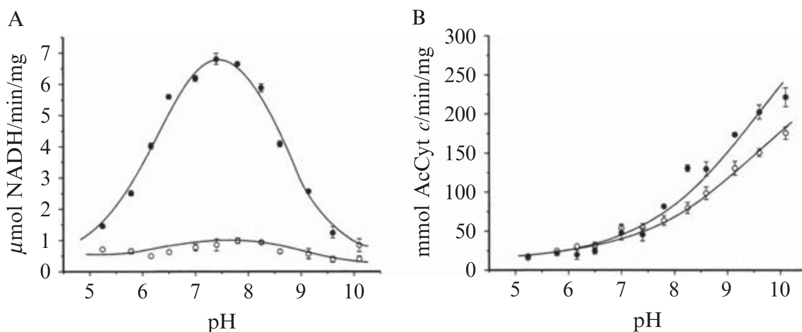


Figure 26.1 pH dependence of ubiquinone reduction and superoxide formation. Complex I was activated by a mixture of 76% phosphatidyl-choline/19% phosphatidyl-ethanolamine/5% cardiolipin. Shown are the pH dependence of the NADH: DBQ oxidoreductase activity (A) and the pH dependence of superoxide generation by complex I (B) in proteoliposomes with (○) and without (•) the addition of 1 μM DQA. Note that the pH profiles for ubiquinone reduction and superoxide formation are different.

superoxide production gradually increases with alkalinization of the medium and is 10 times faster at pH 10 than at pH 6.0. This increase is mostly due to a rise in the overall rate of cytochrome *c* reduction, whereas the SOD-insensitive component is increased only slightly. Above pH 9.5 the initial rate can be measured for a short time only, most likely because of the instability of the enzyme in highly alkaline medium. The pH dependence of the superoxide generation rates corresponds to the accumulation of the fully reduced flavine.

4. Detection of Hydrogen Peroxide by the Amplex Red/HRP Assay

With the Amplex Red/HRP assay, the generation of superoxide is detected indirectly by measuring H_2O_2 formation. Superoxide dismutates spontaneously to H_2O_2, but the reaction is greatly accelerated by superoxide dismutases. In the presence of horseradish peroxidase, H_2O_2 reacts with Amplex Red in a 1:1 stoichiometry to produce the red-fluorescent oxidation product resorufin (Zhou *et al.*, 1997).

4.1. Spectral properties of resorufin

It has to be noted that absorption and fluorescence of resorufin are both pH dependent. Below the pKa (~6.0), the absorption maximum shifts to ~480 nm, and the fluorescence quantum yield is markedly lower. In addition, the Amplex Red reagent is unstable at high pH (>8.5). For these reasons the reactions should be performed at pH 7 to 8. For the calculation of H_2O_2 generation the extinction coefficient $\varepsilon_{571\text{nm}} = 54\ \text{cm}^{-1}\ \text{m}M^{-1}$ can be used, or the assay can be calibrated with known concentrations of H_2O_2 that must be prepared freshly from a 3 to 30% stock solution. The exact H_2O_2 concentrations can be determined by a catalase assay with an oxygen electrode. Note that the excitation/absorption maximum of 563 nm given by Zhou *et al.* (1997) is not correct. We could confirm the maximum given by the manufacturer (571 nm). Nevertheless, if the resorufin formation is followed by fluorescence measurements, the samples should rather be excited at a wavelength of about 560 nm because of the small Stokes shift (absorption and fluorescence emission maxima are 571 nm and 585 nm, respectively). The emission wavelength should be set to ~590 nm.

4.2. Parallel measurement of NADH oxidation, H_2O_2 generation, and buildup of a membrane potential by reconstituted complex I

To study the influence of membrane potential and catalytic turnover on superoxide formation, it would be desirable to assay NADH-oxidation (or NADH: DBQ oxidoreductase activity), ROS-production by the Amplex

Red/HRP assay, and generation of a membrane potential by oxonol VI simultaneously in a diode array spectrophotometer. However, we observed an interference of oxonol VI with the Amplex Red/HRP assay (not shown). Therefore, H_2O_2 generation with the Amplex Red/HRP assay and generation of a membrane potential must be measured in parallel (Fig. 26.2). Because HRP has a measurable NADH-oxidase activity and also catalyzes a NADH-dependent oxidation of Amplex Red (see following), these background activities must be determined in the absence of complex I. Approximately 15 μg of reconstituted complex I was added to 20 m*M* K^+/MOPS, pH 7.2, 50 m*M* KCl at 30° for each measurement. To determine the membrane potential formed, 2 μM oxonol VI was present. Subsequently 100 μM NADH, 100 μM DBQ, and 1 μM FCCP were added as indicated in Fig. 26.2. NADH-oxidation was followed at 340 − 400 nm ($\varepsilon_{340\text{-}400} = 6.1$ mM^{-1} cm^{-1}), and oxonol VI accumulation in the proteoliposome lumen corresponding to the buildup of a membrane potential was monitored at 623 − 603 nm. The increase in oxonol VI absorption was abolished when the membrane potential was collapsed by the addition of FCCP.

Complex I turnover and membrane potential can be attenuated by the addition of inhibitors of the ubiquinone binding pocket like DQA or rotenone (not shown). Alternately, the active/deactive (A/D) transition of complex I (Vinogradov, 1998) can be used conveniently for this purpose: if complex I is preincubated for 20 min at 30°, it switches to the ‘‘deactive’’- or D-form that is stabilized by bivalent cations like Ca^{2+} and Mg^{2+} (Kotlyar *et al.*, 1992). Zn^{2+} has been reported to inhibit complex I at even lower concentrations (Sharpley and Hirst, 2006), and we found with *Yarrowia* complex I that this is also due to stabilization of the D-form (Dröse and Brandt, unpublished results). The low concentration needed to inhibit complex I turnover by Zn^{2+} avoided the formation of metal hydroxides that could form at higher concentrations of Ca^{2+}, Mg^{2+} at pH > 7, and disturb the assay by clouding. When the NADH: DBQ oxidoreductase activity was attenuated by incubation of the deactivated complex with increasing concentrations of $ZnCl_2$ (Fig. 26.2C), a parallel decrease in the membrane potential formed was observed (Fig. 26.2A). For the parallel determination of H_2O_2 production, 20 μM Amplex Red, 0.2 U/ml HRP, 400 U/ml SOD, and the preincubated proteoliposomes were added to the assay buffer. Note that some preparations of SOD have a significant H_2O_2 disproportionating activity because of tiny impurities of highly active catalase. This can easily be checked by oxygen release after adding H_2O_2 to an SOD solution in an oxygraph chamber or by investigating the effect of the SOD on H_2O_2 standards in the Amplex Red/HRP assay. The rather high concentration of SOD was necessary to accelerate the dismutation of superoxide to hydrogen peroxide and to suppress the NADH-dependent oxidation of Amplex Red catalyzed by HRP (Chen and Schopfer, 1999) that causes an unspecific background in the absence of SOD (Votyakova

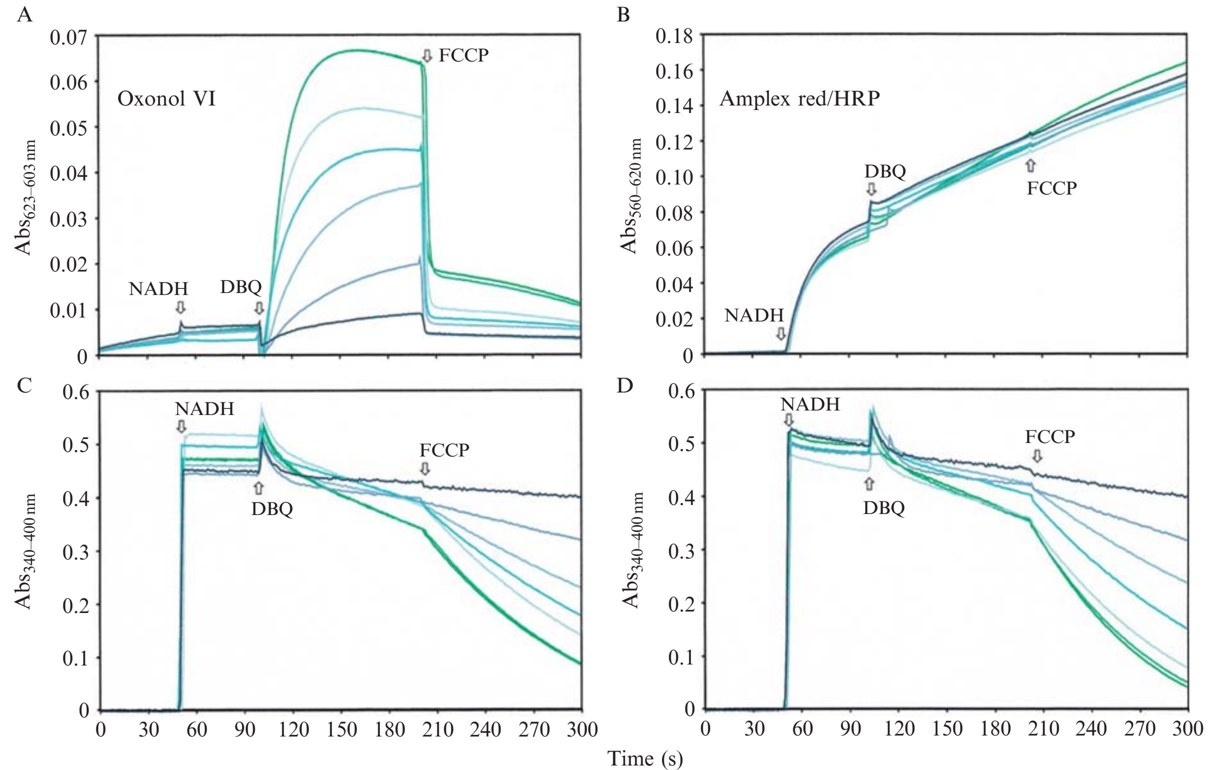


Figure 26.2 Parallel measurement of NADH oxidation, formation of membrane potential, and H_2O_2 generation. By use of proteoliposomes with reconstituted complex I from *Y. lipolytica*, Zn^{2+}-ions were applied to inhibit complex I turnover; increasing Zn^{2+} concentrations (0, 1, 5, 10, 20, 50, and 100 μM) are indicated by different shades of grey. (A) Generation of $\Delta\psi$ as absorbance of oxonol VI; B, rate of H_2O_2 generation measured by the Amplex Red/HRP assay. The rates of NADH oxidation measured simultaneously to the oxonol VI assay are shown in (C) and the rates of NADH oxidation measured simultaneously to the Amplex Red/ HRP assay are shown in (D).

and Reynolds, 2004). It must be stressed that H_2O_2 detection by the Amplex Red/HRP assay is highly dependent on the concentration of HRP and Amplex Red and that the "optimal conditions" have to be determined empirically for each experimental setup.

A comparison of the NADH-oxidation rates measured in the Amplex Red/HRP assay (Fig. 26.2C) with those measured with oxonol VI (Fig. 26.2D) revealed that both dyes reduced the coupling ratio of the proteoliposomes somewhat. In the example shown in Fig. 26.2, the addition of Amplex Red already reduced the coupling ratio from 4.9 to 3.8. When oxonol VI was added, it went down further to ~2.7.

Neither the size of the membrane potential nor the catalytic rate of complex I markedly affected the rate of H_2O_2 generation (Fig. 26.2 B). Our observation that ROS production by complex I was essentially independent of catalytic turnover is in good agreement with published measurements with purified complexes from *Yarrowia* (Galkin and Brandt, 2005), bovine heart mitochondria (Kussmaul and Hirst, 2006), and *E. coli* (Esterhazy *et al.*, 2008). However, it is in stark contrast with several reports showing that complex I inhibitors stimulate ROS generation in mitochondrial membranes and intact mitochondria (Lambert and Brand, 2004; Ohnishi *et al.*, 2005a; Votyakova and Reynolds, 2001). There are several possible explanations for this discrepancy: (1) in intact mitochondria, ROS production may be partly due to activity of $NAD(P)^+$-dependent matrix enzymes that are stimulated by the increase in nucleotide reduction; (2) the application of hydrophilic analogs of the substrate ubiquinone facilitates nonphysiologic side reactions that do not occur with the endogenous, much more hydrophobic, ubiquinones Q_9 and Q_{10} (see earlier); (3) fast consumption of NADH by complex I in submitochondrial particles or mitochondrial membranes is prevented by complex I inhibition, thereby affecting ROS production that is controlled by the $NADH/NAD^+$ ratio (Kussmaul and Hirst, 2006). Note that the latter effect renders endpoint measurements inadequate for determining the effect of inhibitors on complex I–dependent ROS generation.

5. Conclusions

Isolated complex I from *Y. lipolytica* generates superoxide during oxidation of NADH in the absence and presence of ubiquinone by direct transfer of an electron from the reduced flavine to oxygen. The generation of superoxide in the forward NADH: ubiquinone oxidoreductase reaction is not sensitive to protonmotive force and essentially is not dependent on catalytic turnover of the enzyme nor on the presence of specific inhibitors.

When measuring NADH-dependent superoxide production by isolated complex I, several factors should be taken into consideration:

- The enzyme has to be reactivated by phospholipids.
- Superoxide generation rates should be given in relation to other NADH-dependent activities of complex I (i.e., NADH: ubiquinone oxidoreductase and NADH: HAR oxidoreductase).
- Superoxide production can be measured directly by superoxide-sensitive probes or indirectly by measuring H_2O_2 formation in presence of excess SOD.
- Concentrations of the components of coupled systems (acetylated cytochrome *c*/SOD or Amplex Red/peroxidase) have to be newly established for any given experimental setup.
- Side reactions of HRP have to be considered when the hydrogen peroxide production is monitored by the Amplex Red/HRP assay.
- For measuring superoxide generation during NADH: ubiquinone oxidoreduction, the use of decylubiquinone is recommended to minimize nonspecific reactions with oxygen.
- Any NADH-dependent activities of complex I including superoxide generation can be blocked by inhibition of the enzyme with diphenyleneiodonium under reducing conditions.
- Special care should be taken to control the active/deactive transition of complex I in preparations of the eukaryotic enzyme.

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