Activation of Isolated NADH:Ubiquinone Reductase I (Complex I) from *Escherichia coli* by Detergent and Phospholipids. Recovery of Ubiquinone Reductase Activity and Changes in EPR Signals of Iron−Sulfur Clusters†

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ABSTRACT: NADH:ubiquinone oxidoreductase (NDH-1 or complex I) from *Escherichia coli* was purified using a combination of anion exchange chromatography and centrifugation in sucrose density gradient. The dependence of enzyme activity on detergent and phospholipids was studied. Artificial hexaammineruthenium reductase activity was not affected by dodecyl maltoside (DDM) and asolectin. Ubiquinone reductase activity had a bell-shape dependence on DDM concentration; 7−10-fold activation could be achieved. Treatment with asolectin subsequently yields additional 2-fold activation with a corresponding increase in the apparent V_{max} and without significant changes in apparent K_m. Comparative EPR studies of complex I reduced with NADH, "as prepared" and "activated by asolectin" showed an increase in the signals derived mainly from two [4Fe-4S] clusters in the activated enzyme. One of these signals could be simulated with an axial spectrum with g values of $g_{xyz} = 1.895, 1.904, 2.05$, which corresponds to the parameters reported for the N2 cluster. This data indicates conformational rearrangements of catalytic importance in complex I upon binding of phospholipids.

The energy-transducing NADH:quinone oxidoreductase (NDH-1, or complex I) is the first member of the respiratory chain of mitochondria and many bacteria. The mitochondrial enzyme has been under investigation for more than 40 years already. However, progress has been strongly hampered by the extraordinary size of the complex, >900000, and its complexity (up to 46 subinits) (*1*, *2*). The main stumbling stone has been a low ubiquinone reductase activity of the purified enzyme, the natural activity of complex I. In vivo, complex I catalyzes NADH oxidation by ubiquinone, but a number of artificial electron acceptors, such as FeCy[1] and HAR, have been used, the reduction of which does not require the whole enzyme, but only three water-soluble subunits, and which is not coupled to transmembrane translocation of H^+ [see ref *3* for a review]. The ratio between the artificial and natural activities reflects the intactness of the enzyme. The turnover of complex I purified from bovine mitochondria was found to be 900 s^{-1} with FeCy and 30 s^{-1} with ubiquinone (*4*). Hence, the ratio artificial/natural activity was 30 for purified complex I whereas this number is 1.5−2.5 for complex I in the mitochondrial or bacterial membrane [*5*, *6*, present work]. A breakthrough was expected from studying bacterial analogues of the eukaryotic complex I, which are only half the size and composed of only 13−14 subunits. These enzymes perform the same function, have the same cofactors, and show significant similarity in amino acid sequence to the core subunits of eukaryotic complex I [see for reviews refs *7*−*9*]. Unfortunately, few protocols for purification of bacterial complex I are as yet published. Up to now, bacterial complex I has been purified only from two species, *Escherichia coli* (*10*−*12*) and the hyperthermophilic bacterium *Aquifex aeolicus* (*13*). Complex I isolated from *E. coli* keeps its integrity upon isolation; however its quinone reductase activity is low (*11*), and the ratio between artificial and natural activity is in the range of 30−40. It is not clear whether the loss of small membrane subunits, essential phospholipids, or partial denaturation is responsible for the low quinone reductase activity, or whether the drop of the activity is irreversible. Recently this problem was largely overcome by the treatment of the isolated enzyme with phospholipids. The ubiquinone reductase activity of complex I isolated from *Yarrowia lipolytica* mitochondria and *E. coli* was strongly increased by treatment with phospholipids; the obtained turnover numbers approached approximately 100 s^{-1} (*14*) and 200 s^{-1} (*12*), respectively. This treatment necessarily also requires addition of detergent without which there was no interaction between complex I and phospholipids (*12*). The role of detergent in this aspect was not studied although it was reported that detergent itself did not activate complex I under the employed conditions.

We purified complex I from *E. coli* and optimized the conditions for observation of its quinone reductase activity. Surprisingly, the detergent dodecyl maltoside (DDM) itself strongly stimulated this activity, but this occurred only in a narrow range of DDM concentrations. Phospholipids caused further activation. Analysis of the kinetic properties and

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[1] Abbreviations: HAR, hexaammineruthenium(III) chloride; DQ, decylubiquinone; DDM, *n*-dodecyl β-D-maltoside; FeCy, ferricyanide; PMSF, phenylmethanesulfonyl fluoride; SDH, succinate dehydrogenase.

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inhibitor sensitivity of the ubiquinone reductase activity and EPR studies of the [Fe-S] clusters of complex I were performed to find out the possible role of the detergent and phospholipids in activation.

MATERIALS AND METHODS

Bacterial Growth and Membrane Preparation. E. coli strain MWC215 (Sm^R *nhd*::Cm^R) (*15*) was grown aerobically in a 25 L fermentor in LB medium, 37 °C. The cells were harvested at the second half of the exponential phase of growth, washed with 0.5 M KCl, 10 mM tris/HCl, pH 7.8, frozen in pellet, and stored at −20 °C until use.

Membrane Preparation. Approximately 100 g of thawed cells was suspended in 200 mL of buffer containing 50 mM MOPS/KOH pH 7.0, 100 mM KCl, 0.5 mM EDTA, 0.5 mM PMSF using UltraTurrax. Traces of DNase I were added to the suspension, and the cells were broken by passing through an APV Gaulin homogenizer at a pressure of 450 bar for 7−10 min. Unbroken cells and cell debris were spun down by centrifugation at 11000*g* for 20 min. The supernatant was centrifuged at 200000*g* for 2 h. The obtained membrane pellet was suspended in 25 mM MOPS/KOH pH 7.0, 100 mM KCl, spun down again, and suspended in 25 mM MES/BTP, pH 6.0, 10 mM betaine at a protein concentration 60−80 mg/mL. The membrane suspension was frozen in 1.5 mL aliquots in liquid N_2 and stored at −80 °C until use.

Complex I Purification. Thawed *E. coli* membranes were diluted to a protein concentration of 6 mg/mL in buffer containing 50 mM MES/NaOH, pH 6.0, 0.5 mM PMSF and solubilized by addition DDM to 0.5% (w/v). After 10 min incubation at 4 °C, nonsolubilized material was removed by centrifugation for 1 h at 140000*g*. The supernatant was passed through a 0.45 μm filter and adjusted to 100 mM NaCl by dropwise adding 5 M NaCl. The chromatography steps were carried out using the ÄKTAprime chromatography system (Amersham Biosciences). Solubilized membranes (approximately 90 mL) were applied to a 30 mL bed volume DEAE-Trisacryl M (BioSepra) anion exchange column equilibrated with 50 mM MES/NaOH, pH 6.0, 100 mM NaCl, 0.05% DDM at a flow rate of 2.7 mL/min. The column was washed with 85 mL of buffer, and retained material was eluted with a 180 mL linear gradient of 100−150 mM NaCl, followed by a 60 mL step at 150 mM NaCl and then with a 300 mL linear gradient of 150−300 mM NaCl in the above buffer. Fractions with NADH/HAR reductase activity eluting at 120−150 mM NaCl were pooled, adjusted to 100 mM NaCl by dilution with 50 mM MES/NaOH, pH 6.0, 0.05% DDM, and concentrated to 4−5 mg/mL of protein using Amicon Ultra-15 centrifugal filter devices (M_r cutoff 100 kDa). The concentrated sample was applied onto 10.5 mL gradients of 15−40% sucrose in 50 mM MES/NaOH, pH 6.0, 100 mM NaCl, 5% glycerol, 0.05% DDM and centrifuged for 20 h at 270000*g*. The NADH/HAR active fractions harvested from sucrose gradients were combined, followed by cycles of dilution twice with 50 mM MES/NaOH, pH 6.0, 100 mM NaCl, 0.05% DDM and concentration in Amicon Ultra-15 centrifugal filter devices until a volume of about 10 mL. Finally, complex I containing sample was applied to a 5 mL bed volume of Source 15Q (Pharmacia) equilibrated with 50 mM MES/NaOH, pH 6.0, 100 mM NaCl, 0.05% DDM and eluted at a flow rate of 2 mL/min with a 350 mL multistep gradient from 100 to 370 mM NaCl in the above buffer. Fractions containing NDH-I (140−190 mM NaCl, volume about 140 mL) were pooled and adjusted to 100 mM NaCl with 50 mM MES/NaOH, pH 6.0, 0.05% DDM by concentration and dilution in Amicon Ultra-15. Finally, sample volume was about 500 μL with protein concentration of 4−5 mg/mL. Purified complex I was stored in small aliquots at −80 °C.

Activation of Complex I. Asolectin (soybean phospholipid, L-α-phosphatidylcholine, type II-S, Sigma) was used for activation of purified complex I. Routinely 13.6 mg of solid asolectin was sonicated on ice in 1 mL of 10 mM MES/NaOH, pH 6.0, 50 mM NaCl, containing 2.7% DDM until the solution was clear. Purified complex I was added to the DDM/phospholipid suspension to a concentration of 1.8 mg/mL, and the mixture was incubated on ice for 20 min. Maximal increase of NADH/DQ activity was observed when the ratio protein:detergent:phospholipid was approximately 1:8:4 (w/w/w).

Activity Measurements. Complex I activity was measured at 30 °C in 1.4 mL of buffer containing 25 mM HEPES/BTP, pH 7.5 with constant stirring using a USB2000 UV−vis spectrophotometer (Ocean Optics, Inc). HAR and DQ reduction were followed by NADH oxidation at 340 nm ($\epsilon = 6.2\ mM^{-1}\ cm^{-1}$). HAR or DQ reduction was measured in the assay buffer containing 200 μM NADH, 350 μM HAR or 50−60 μM DQ, 3.5 mM KCN and initiated by the addition of 1−5 μg of protein. When the effect of DDM on the DQ reductase activity was studied, the detergent was added to the reaction mixture before the protein addition. All experiments on the activity characterization were reproduced 3−4 times. Individual curves are presented.

Other Analytical Procedures. Protein concentrations were determined by the BCA Protein Assay Reagent kit (Pierce) with bovine serum albumin as a standard. Precast polyacrylamide minigels containing 10−20% acrylamide gradient (Pierce) were used according to the manufacturer's instructions. Flavin was extracted by acid (*16*), and its content was determined fluorometrically ($\lambda_{ex} = 378$ nm, $\lambda_{em} = 530$ nm) using a fluorescence spectrophotometer (F-4000, Hitachi).

EPR Spectroscopy. X-band EPR measurements (9.4 GHz) were performed with a Bruker EMS EPR spectrometer, equipped with an Oxford Instruments ESR900 helium flow cryostat with an ITC4 temperature controller. The field modulation frequency was 100 kHz; modulation amplitude was 1.27 mT. The spectra shown are normalized for temperature, gain, and microwave power (*17*) and corrected for baseline. The spectral simulation was performed using Bruker Analytic GmbH software WinEPR SimFonia version 1.26 (beta). Samples of purified complex I (150 μL, 3−4 mg protein mL^{-1}) were mixed with NADH (10 mM final concentration), transferred into EPR tubes, and frozen in liquid nitrogen.

RESULTS

Complex I Purification. For isolation of complex I the *E. coli* strain MWC215 was used. This strain was obtained from wild type by a knockout of the alternative NADH dehydrogenase, NDH-2, (*15*), which yields a 2-fold increase of complex I content in cytoplasmic membranes. Purification of complex I was based on procedures published by Leif et

Table 1: Purification of Complex I from *E. coli*[a]

fraction	protein (mg)	NADH:HAR activity		yield (%)
		μmol min^{-1}	μmol min^{-1} mg^{-1}	
membranes	1718	3762	2.2	100
membrane extract	1648	3609	2.2	96
DEAE-Trisacryl	57.9	1390	24	37
sucrose gradient	21.3	1010	48	26
source 15Q	8.4	737	85−90	19

[a] Values based on 100 g wet weight of cells.

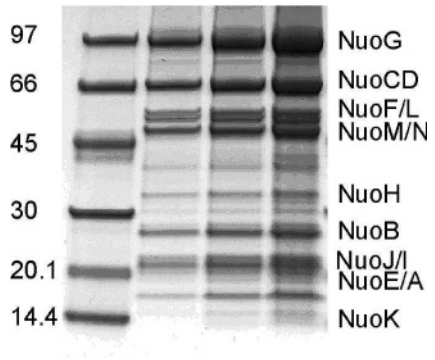

FIGURE 1: Coomassie Blue-stained SDS−PAGE gel of purified complex I. Lane 1: molecular mass markers (kDa). Lanes 2−4: increasing amounts of complex I (15 μg, 30 μg, 50 μg). The assignment of the observed bands to individual subunits of complex I is given on the right.

al. and David et al. (*10*, *11*). Complex I was extracted from *E. coli* membranes using DDM and purified by two ion chromatography steps: weak anion exchanger, DEAE-Trisacryl, and strong anion exchanger, 15Q source, with intermediate purification on sucrose density gradient. The used protocol yields in approximately 8 mg of complex I with HAR reductase activity of 80−90 μmol NADH mg^{-1} min^{-1} from 100 g wet weight of cells (Table 1).

The content of noncovalently bound FMN was determined at 1.0 nmol/mg protein. SDS PAGE showed the bands that correspond to all subunits of complex I (Figure 1); also some additional bands were detected at 30−40 kDa.

One contaminant band at 30 kDa probably derives from the iron−sulfur protein of SDH, as found by David et al. (*11*) and confirmed by EPR measurements (see below).

Dependence of Ubiquinone Reductase Activity on Phospholipids and Dodecylmaltoside. The ubiquinone reductase activity of purified complex I had strong dependence on the DDM concentration in the assay. However, it does not depend significantly on detergent concentration in stock solution of the enzyme as long as it is not too low to precipitate complex I and not too high to denature it (not shown). The dependence on DDM concentration in the assay was bell shaped with a maximum at 0.005% DDM when the protein concentration was lower than 2 μg/mL. Total increase of activity at optimal DDM concentration was over 10-fold from the value without added DDM, and the activity dropped four times when the DDM concentration was an order of magnitude higher than at optimum (Figure 2, circles).

The ubiquinone reductase activity of complex I treated with phospholipids was also dependent on DDM concentration in the same way, but the optimal detergent concentration

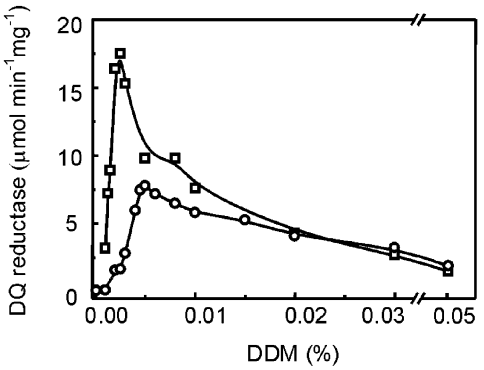

FIGURE 2: Dependence of NADH:ubiquinone oxidoreductase activity of "as prepared" and activated by phospholipids purified complex I on DDM concentration. Circles: "as prepared" complex I. Squares: activated complex I.

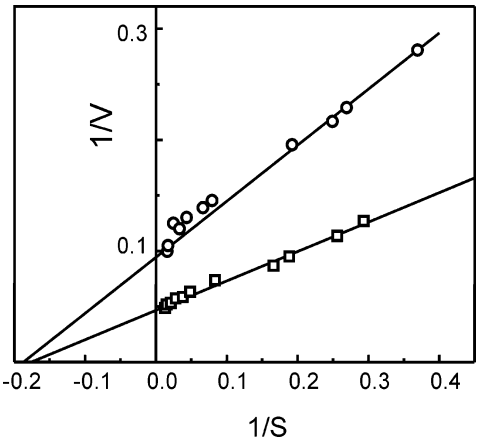

FIGURE 3: Kinetic analysis of the ubiquinone reductase activity of "as prepared" and activated by phospholipids complex I. Apparent K_m and V_{max} were determined as 5.4 and 5.7 μM DQ and 10.6 and 22 μmol mg^{-1} min^{-1} for "as prepared" (at optimal DDM concentration, 0.005%, circles) and activated (at optimal DDM concentration, 0.0025%, squares) complex I, respectively.

was shifted to a lower value of 0.0025% DDM (Figure 2, squares). At optimum DDM concentration the ubiquinone reductase activity reached 8 and 18 μmol NADH mg^{-1}min^{-1} (corresponding to turnovers 130 and 300 s^{-1} when calculated per flavin) in "as prepared" and activated enzyme, respectively. The HAR reductase activity was 80−90 μmol NADH mg^{-1} min^{-1} (turnover 1300−1500 s^{-1}) in both preparations, and it was not affected by DDM in the assay. Therefore the ratio artificial, HAR reductase, to natural, ubiquinone reductase, activities was approximately 10 in "as prepared" enzyme and reached 4 for the activated complex I. This ratio as measured in bacterial membranes was 1.5−2.

Kinetic Analysis of "As Prepared" and "Activated" Ubiquinone Reductase Activity. The ubiquinone reductase activity of "as prepared" and activated complex I was titrated with DQ in the high affinity area at an optimal concentration of DDM, which was 0.005% for "as prepared" and 0.0025% for the activated enzyme (Figure 3).

The apparent K_m was virtually the same for both preparations; its value was determined as 5.4−5.7 μM of DQ. The difference in V_{max} was 2-fold; V_{max} values of 10.6 and 22 μmol NADH mg^{-1} min^{-1} were found for "as prepared" and activated complex I, respectively.

Sensitivity of Purified Complex I to Rolliniastatin. The sensitivity of purified complex I to rolliniastatin was tested

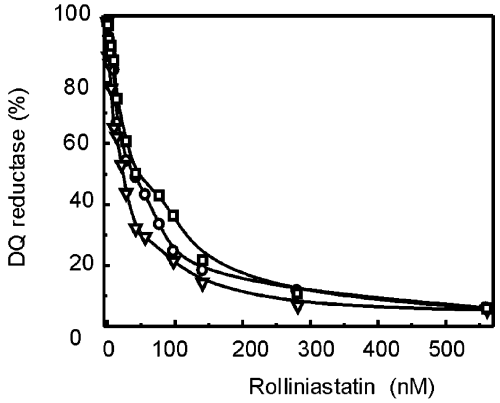

FIGURE 4: Inhibition of NADH:ubiquinone oxidoreductase activity of membranes and purified complex I by rolliniastatin. Triangles: membranes, no DDM in assay. Circles: "as prepared" complex I, 0.005% DDM in assay. Squares: activated by phospholipids complex I, 0.0025% DDM in assay.

at optimal DDM concentration in the assay (see above) and 60 μM DQ. Rolliniastatin strongly inhibited the ubiquinone reductase activity of "as prepared" and activated complex I almost in the same way (Figure 4, circles and squares). Titration of the ubiquinone reductase activity of complex I in membranes by rolliniastatin was also performed (Figure 4, triangles).

Comparison of the obtained titration curves shows that the rolliniastatin sensitivity of complex I is not significantly different whether the enzyme was in cytoplasmic membranes or solubilized.

EPR Spectra of "As Prepared" and Activated Complex I. Oxidized preparations of purified complex I showed the signal of typical shape and position of the [3Fe-4S] S3 cluster of SDH, which is due to some contamination with the SDH iron−sulfur protein (not shown). S3 was only partially reduced by NADH, and it still contributed to some extent to the signal at $g = 2.17$ at low temperature in the NADH-reduced preparation. The spectra of NADH-reduced "as prepared" (dotted line) and activated (solid line) complex I are shown in Figure 5. Binuclear Fe-S clusters of complex I can be detected at 45 K; the obtained signals are similar to those reported by Leif et al. (*10*) and most probably derive from three [2Fe-2S] clusters, including the one that is specific for the *E. coli* enzyme (*18*) with $g_z = 1.994$ (Figure 5) although the presence of only two binuclear Fe-S clusters in *E. coli* complex I was reported recently (*19*, *20*). The signals from the [2Fe-2S] clusters in activated complex I were slightly smaller than in "as prepared"; however, the difference was insignificant. At low temperature, 10 K, and low microwave power, i.e., under conditions when bi- and tetranuclear clusters are seen by EPR, the signals at $g = 1.93$ and 1.9 were stronger in activated complex I but the difference was also small. At 10 K and high microwave power, 63 mW, when mainly fast relaxing [4Fe-4S] clusters can be detected, differences at $g = 2.05$ and 1.9 became evident. The signal at $g = 1.9$ of activated complex I is 50% stronger than the signal of "as prepared" enzyme. Some fast relaxing [4Fe-4S] clusters can be well observed only at very low temperatures. Comparison of 5 K spectra of activated and "as prepared" complex I preparations showed that there also the signal at 1.93 is significantly increased in the activated complex (Figure 5).

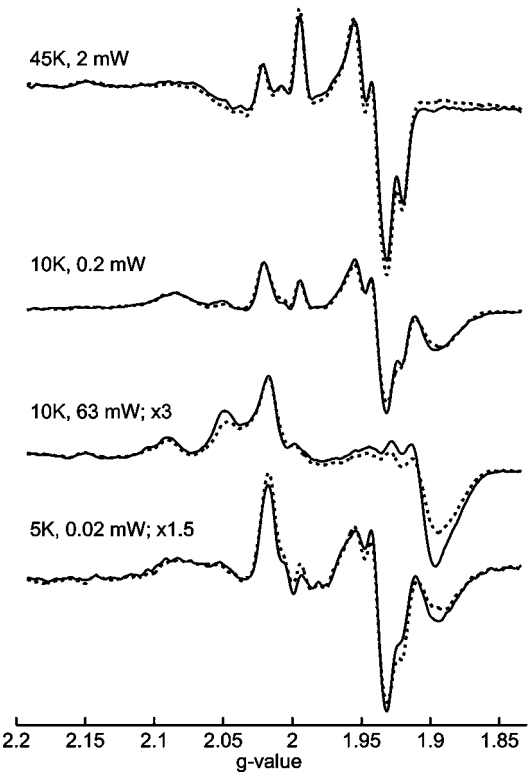

FIGURE 5: EPR signals of NADH reduced "as prepared" (dotted line) and activated (solid line) complex I. Temperature and microwave powers incident to the cavity are given on the left.

Figure 5 gives only a rough impression of the changes in EPR spectra upon activation of complex I. The difference spectra obtained by subtraction of "as prepared" spectra from those of the activated enzyme at different temperature and microwave power are shown in Figure 6. All difference spectra taken at 5 K and 10 K show that the signals at $g = 2.05$ and 1.9 were increased in the activated complex I. These signals are not saturated at high microwave power at 10 K, and they dominate in the spectrum taken at 10 K and 63 mW, which may be simulated using the following parameters: $g_{xyz} = 1.895$, 1.904, 2.05 and width (xyz) = 40, 21, 18.5 G (Figure 6, 10 K, 63 mW, dotted line). The other feature of the difference spectra is a narrow trough at $g = 1.93$. This signal can be observed at 5 K and 0.2 mW, and also at 10 K, but it is relatively readily saturated and broadened practically beyond detection at 63 mW (Figure 6). Saturation and temperature behavior of the observed difference signals lead us to suggest that both of them derive from [4Fe-4S] clusters, one less fast relaxing ($g_y = 1.93$) than the other ($g_z = 2.05$, $g_{xy} = 1.9$).

DISCUSSION

Complex I purified from *E. coli* using the present protocol has a specific activity with HAR of 80−90 μmol NADH $mg^{-1}min^{-1}$ which is slightly higher than reported elsewhere (*11*). However, it still is contaminated by the SDH iron−sulfur protein, which most probably is due to formation of supercomplexes of the enzymes of the respiratory chain in vivo, as it was found in mitochondria and *Paracoccus denitrificans* (*21*, *22*). The bell-shape dependence of the ubiquinone reductase activity of purified complex on DDM concentration in the assay (Figure 2) indicates a multiple

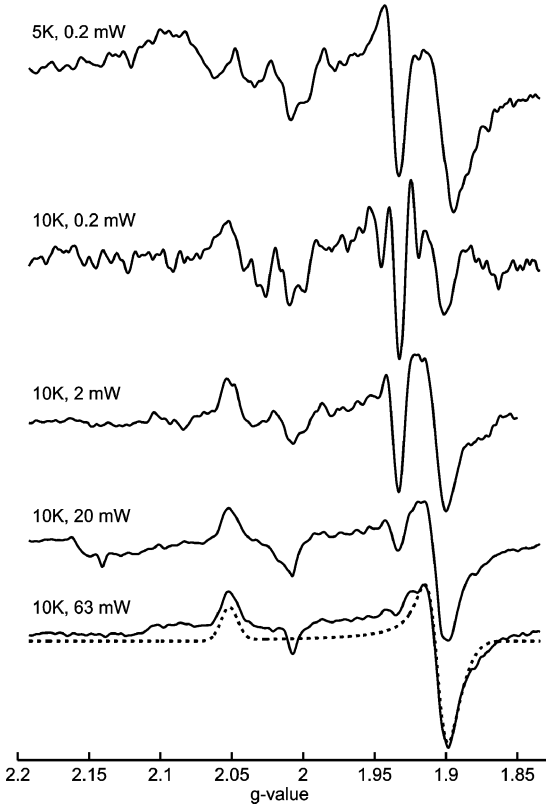

FIGURE 6: The changes in NADH induced EPR spectra caused by the treatment of complex I with phospholipids. The spectra were obtained by subtraction the spectra of "as prepared" from the spectra of activated complex I, taken at different temperature and microwave powers incident to the cavity (shown on the left). The bottom difference spectrum was simulated with parameters $g_{xyz} = 1.895$, 1.904, 2.05 and width (*xyz*) = 40, 21, 18.5 G (dotted line).

effect of the detergent. The descending part of the dependence curve should be a result of partial denaturation of the enzyme by the detergent. The ascending part of the curve may be explained in two different ways. First, DDM micelles in which complex I is embedded may work as a relatively hydrophobic phase to increase the local ubiquinone concentration. Second, binding the additional DDM molecules at the surface of complex I possibly let the enzyme, which was in a strained conformation after the extraction from the membrane, relax to a more native state that may result in increase of accessibility of the quinone binding site or/and improvement of electron transfer from [Fe-S] clusters to ubiquinone. If the first alternative is valid, the apparent K_m for ubiquinone should increase with decreasing DDM concentration, but that was not observed. The titration of the activity by decylubiquinone at half-optimal detergent concentration (not shown) did not differ significantly from that at optimal detergent concentration (Figure 3, circles). Therefore, the second alternative of a direct effect of DDM on complex I is more likely. The same question should be addressed regarding the additional stimulation of ubiquinone reductase activity by phospholipids, i.e., whether they operate as a lipid phase to accumulate decylubiquinone in the vicinity of complex I or not. Determination of the apparent K_m for DQ showed that it practically did not change upon activation of complex I by phospholipids. All the activation was due to increase of V_{max}, which was approximately 2-fold higher in activated complex I. This data shows that phospholipids also affect the enzyme directly, and not via a higher accessibility toward its substrate, ubiquinone.

At optimal conditions the ubiquinone reductase activity of purified complex I reached a turnover of 300 s^{-1}. It was not possible to compare this activity directly to that of the membrane-bound enzyme, because the content of complex I in the membranes could not be reliably quantified. However, some estimation can be done. The ratio HAR reductase/ubiquinone reductase reached about 4 in activated complex I, whereas for *E. coli* membranes this value was determined to be 1.5−2. On the assumption that the artificial HAR reductase activity, which requires only three water soluble subunits of complex I, stays virtually the same in membrane-bound and solubilized enzyme, one can suggest that the ubiquinone reductase activity of purified and activated enzyme is only twice less than that of membrane-bound. This difference could be due to different properties of the natural substrate, Q8, and DQ used for the present activity measurements. Thus we propose that the purified complex I may adopt a state close to the native one upon activation with phospholipids.

The possibility that the enzyme conformation has been changed upon the treatment of complex I with phospholipids and detergent in a way that some artificial binding sites for ubiquinone become accessible was tested using rolliniastatin, the inhibitor that belongs to the annonaceous acetogenin family (*23*), and found to occupy the quinone binding center of complex I (*23*−*25*). The titration of NADH:ubiquinonone reductase activity of *E. coli* sub-bacterial vesicles by rolliniastatin showed high sensitivity of *E. coli* complex I to this inhibitor (Figure 4). Purified complex I was inhibited by rolliniastatin in a similar way regardless of its activation state (Figure 4), which indicates that changes in accessibility or modification of the quinone binding site are not involved in the phospholipid-dependent stimulation of the activity. The reason for the observed activation phenomenon could be a fragile interaction between hydrophilic and membrane fragments of complex I, which may be improved by phospholipids. This suggestion could be tested by EPR measurements. If there are any obstacles in electron transfer from the Fe-S clusters to ubiquinone, or conformational changes around these clusters, it is possible that some changes in EPR spectra of "as prepared" and activated complex I could be detected.

All Fe-S clusters in complex I are located in nonmembrane subunits. Cluster N2 is believed to interact directly with ubiquinone, and is located in one of two amphiphilic subunits, NuoI or NuoB, which are part of the fragment of complex I that connects the hydrophilic domain to the memrane domain [see ref *26* for a review]. A close topographical relationship between N2 and ubisemiquinone was found on the basis of the splitting of the N2 g_z signal due to magnetic interaction with semiquinone, and the spin-relaxation enhancement effects of cluster N2 on a fast-relaxing ubisemiquinone species. The distance between cluster N2 and the fast-relaxing ubisemiquinone was estimated to be 8−11 Å. (*27*−*29*). The hydrophobic isoprenoid chain of ubiquinone is typically located near the middle of the membrane with the benzol ring close to the surface; therefore it is reasonable to suggest that the location of N2, although in water soluble subunits, may be only a short distance from the membrane plane. In contrast to this, N2 was recently reported to reside far away (40 Å) from the

membrane surface on the basis of antibody visualization experiments [see ref *30* for a review]. On the other hand, a significant conformational change during the catalytic cycle of complex I has been proposed that might bring this domain close to the membrane (*31*). The paramagnetic properties of Fe-S clusters are very sensitive to their surroundings. Hence, the effect of phospholipid binding on the EPR signals of complex I, particularly [4Fe-4S] shown here, would be expected in case of conformational changes in the enzyme upon activation. Indeed almost no difference in the signals of binuclear centers (spectrum at 45 K) in "as prepared" and activated complex I was detected, which seems reasonable since these clusters reside in the distal part of the enzyme, in the water soluble subunits NuoE and NuoG, which are hardly expected to interact with phospholipids. The activation of complex I resulted in increase of signals that belong to two [4Fe-4S] clusters.

The difference signal of one fast-relaxing cluster could be simulated by an axial spectrum with the parameters (g_{xyz} = 1.895, 1.904, 2.05) which are close to those reported for N2 in *E. coli* (g_{xyz} = 1.905, 1.91, 2.05) (*10*), and therefore tentatively assigned to this center, although more data, especially redox titrations, are necessary to validate this conclusion. The difference signal of the other cluster (g = 1.93) could be better detected at 5 K and 10 K at low microwave power, which indicates a longer relaxation time; assignment of this signal is an open question as yet. The increase in signals of [4Fe-4S] clusters upon activation of the enzyme could be due to an increase in their redox potential or/and magnetic decoupling of their spins from each other or another EPR-silent paramagnetic species. Both effects can occur by the rearrangements in the ligands or immediate surroundings of the Fe-S clusters.

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