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Biochimica et Biophysica Acta 1459 (2000) 77–87

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Binding of detergents and inhibitors to bovine complex I - a novel purification procedure for bovine complex I retaining full inhibitor sensitivity

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Received 23 December 1999; received in revised form 7 March 2000; accepted 15 March 2000

Abstract

Mitochondrial complex I exhibits some peculiar and poorly understood features regarding the effects of detergents on activity and sensitivity to hydrophobic inhibitors that are not seen with other membrane complexes using ubiquinone as a substrate. Therefore, we investigated the interaction of complex I from bovine heart mitochondria with different types of detergents by monitoring activity, degree of inhibition and inhibitor binding in the presence of increasing concentrations of detergent. It is shown that apart from their nature as solubilizing and delipidating agents the polyoxyethylene-ether detergents Triton X-100, Brij-35 and Thesit act as specific inhibitors of complex I and compete with classical complex I inhibitors for a common binding domain. These findings were used to develop a novel large-scale chromatographic procedure for isolation of inhibitor-sensitive NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria. The enzyme was purified by selective solubilization in Triton X-100 and subsequent hydroxylapatite, ion-exchange and gel-exclusion chromatography. By switching detergents from Triton X-100 to dodecylmaltoside after hydroxylapatite chromatography the procedure yields highly pure, monodisperse and fully inhibitor-sensitive enzyme.

1. Introduction

The proton-pumping NADH:ubiquinone oxidoreductase (EC 1.6.99.3, complex I) is the first membrane-bound electron transport complex of the mitochondrial respiratory chain and accounts for 40% of the proton translocating capacity across the inner mitochondrial membrane. In mammals, it consists of 43 subunits with a molecular mass of about 944 kDa [1].

A prerequisite for functional analysis of complex I is a preparation which retains the enzyme in its native state. For crystallization of this large membrane

Abbreviations: CC44, *N*-methyl-*N*-(3,4-dimethoxybenzyl)-4-(*p*-tert-butylphenoxy)benzamide; Chaps, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate; CMC, critical micelle concentration; DQA, 2-decyl-4-quinazolinyl-amine; EGTA, ethylene glycol-bis(β-aminoethylether) *N*,*N*,*N*′,*N*′-tetraacetic acid; FeS, iron–sulfur cluster; HAR, hexammineruthenium(III)-chloride; NBQ, *n*-nonylubiquinone; NFQA, (6-methoxy-1,2,3,4-tetrahydronaphth-2-yl)-(8-fluoroquinazolin-4-yl)amine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SMP, submitochondrial particles; Tris, tris-(hydroxymethyl) aminomethane

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0005-2728/00/$ – see front matter
PII: S0005-2728(00)00115-8

protein complex, the isolated enzyme should not only be native and highly pure, but also in a monodisperse and homogeneous state and have a low lipid content [2]. However, one of the commonly observed features of complex I is its susceptibility to solubilization during various purification procedures as monitored by inhibitor insensitivity and loss of catalytic activity. The standard procedure to purify complex I from bovine heart mitochondria by Hatefi and Rieske [3] retains inhibitor sensitivity, but uses cholate as a detergent that is not monodisperse and therefore not suited for crystallization experiments [4]. This preparation also has a rather high lipid content and contains significant amounts of transhydrogenase and bc_1 complex as contaminants [3,4]. On the other hand the excellent monodisperse preparation of Finel et al. [4] has irreversibly lost much of its activity and is inhibitor insensitive. A similar preparation from the same laboratory by Buchanan and Walker was reported to be inhibitor sensitive, but no information on the amount of rotenone needed and the incubation times was given [2]. The situation is in stark contrast to the cytochrome bc_1 complex which requires exhaustive delipidation for inactivation leaving only a few molecules of cardiolipin bound to the complex; this inactivation is still fully reversible [5] and has little effect on inhibitor binding [6]. This remarkable difference between the two enzyme complexes highlights the need to understand the effect of detergents especially on inhibitor binding to complex I.

Using fluorescence quench titration and radioligand binding assays [7] we could recently demonstrate that complex I has only one extended inhibitor binding domain with overlapping binding sites for all its classical hydrophobic inhibitors.

Here we analyze the influence of various detergents on activity and inhibitor binding. We demonstrate by a simple kinetic model that polyoxyethylene-ether detergents act as inhibitors and compete for the same binding domain in complex I as other hydrophobic inhibitors.

Based on these results we have developed a novel isolation procedure for bovine complex I which yields the enzyme in a pure, homogeneous, monodisperse and fully inhibitor-sensitive state.

2. Materials and methods

2.1. Chemicals

The fluorescent complex I inhibitor NFQA (6-methoxy-1,2,3,4-tetrahydronaphth-2-yl)(8-fluoroquinazolin-4-yl)amine; AE C631392) was a kind gift from Hoechst Schering AgrEvo Chemical GmbH (Frankfurt am Main). *n*-Nonylubiquinone (NBQ) was prepared essentially following the protocol of Wan et al. [8]. Hydroxylapatite was prepared according to [9] as modified by Schägger [10]. CC44, *N*-methyl-*N*-(3,4-dimethoxybenzyl)-4-(*p*-tert-butylphenoxy)benzamide [11] was a kind gift from H. Miyoshi, Kyoto University.

Peroxide-free Triton X-100 was purchased from Serva (Heidelberg) and dodecylmaltoside was obtained from Biomol Feinchemikalien GmbH (Hamburg). All other chemicals were purchased from Sigma Aldrich Chemie GmbH (Deisenhofen) or Carl Roth GmbH and Co. (Karlsruhe) in analytical quality.

2.2. Analytical methods

Protein was determined according to a modified Lowry protocol [12]. Redox spectra of cytochromes were recorded on a Shimadzu UV-300 spectrophotometer using the extinction coefficients reported in [13].

Low temperature EPR spectra were obtained on a Bruker ESP 300E spectrometer equipped with a liquid helium continuous flow cryostat, ESR 900 from Oxford Instruments. The samples were mixed with NADH in the EPR tube and frozen in liquid nitrogen after 30 s reaction time.

The phospholipid content was determined as organic phosphate content following the protocol of Chen et al. [14].

Acid extraction of flavin was performed essentially as described by Koziol [15] and measured fluorometrically by adding an internal standard of FMN (excitation wavelength 455 nm, emission wavelength 530 nm). In the tested range of 0.1–1 μM the relation between concentration and fluorescence intensity (counts per second) was linear. Data were analyzed by using the Psiplot software package version 4.61 (Poly Software International).

Determination of ubiquinone content was performed according to Kröger and Klingenberg [16] using the 'rapid extraction method'. About 10 mg of isolated complex I were used for extraction. Tricine SDS–PAGE was performed according to Schägger and von Jagow [17].

2.3. Determination of catalytic activity

NADH:NBQ activity was measured using a Shimadzu UV-300 spectrophotometer in the dual wavelength mode by following NADH oxidation at 340–400 nm ($\varepsilon = 6.10$ mM^{-1} cm^{-1}) at 30°C [7]. 100 μM NADH and 50 μg of bovine submitochondrial particles (SMP) or fractions from different preparation steps or purified complex I were added to buffer containing 50 mM Tris/HCl, pH 7.4, 5 μM Kresoxim-Methyl Brio (to inhibit cytochrome bc_1 complex) and 2 mM KCN (to inhibit cytochrome *c* oxidase). The catalytic reaction was started by addition of 60 μM NBQ. SMP were prepared essentially as described in [7]. In order to test the effect of different detergents on NADH:NBQ oxidoreductase activity of complex I in SMP, these compounds were added as aqueous solutions before dilution of SMP. The inhibition of the residual rate was assayed with 2 μM of NFQA or CC44 dissolved in ethanol which was added after starting the catalytic reaction with 60 μM NBQ.

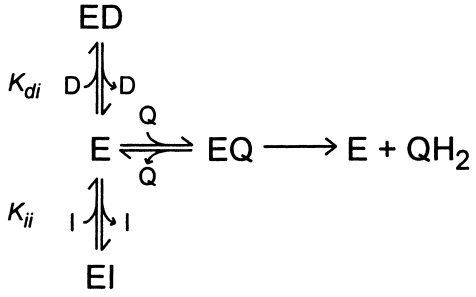
Detergent- and inhibitor-insensitive NADH:HAR activity was measured using a Shimadzu UV-300 spectrophotometer in the dual wavelength mode by following NADH oxidation at 340–400 nm ($\varepsilon = 6.10$ mM^{-1} cm^{-1}) at 30°C [18].

2.4. Effect of detergents on binding of NFQA to complex I in bovine submitochondrial particles

Fluorescence quench titrations were performed using the novel fluorescent complex I inhibitor NFQA (excitation wavelength 316 nm and emission wavelength 360 nm) at 25°C as described previously [7]. Data were analyzed by fitting the following parameter directly to the titration curve: specific fluorescence of the bound and free inhibitor, K_d of NFQA and the number of binding sites. Detergent was added to the SMP solution before starting the titration.

2.5. Kinetic model for concurrent competitive inhibition by detergent and inhibitor

Assuming that some detergents act as competitive complex I inhibitors the concurrent interaction of the enzyme with the detergent and another competitive inhibitor can be described in the framework of the Michaelis–Menten theory by the following reaction scheme:



(1)

with E, enzyme; D, detergent; I, inhibitor; Q, ubiquinone; QH_2; ubiquinol, K_{ii}, dissociation constant for the inhibitor; K_{di}, dissociation constant for the detergent.

Then the catalytic rate is given by the following equation:

$$v = \frac{V_{max} \times [NBQ]}{K_m \times \left(1 + \frac{[I]}{K_{ii}} + \frac{[D]}{K_{di}}\right) + [NBQ]} \quad (2)$$

with K_m, Michaelis–Menten constant for NBQ; v, catalytic rate; V_{max}, maximal catalytic rate.

The residual rate v_i at a given concentration of inhibitor [I] in the absence of detergent is given by:

$$v_i = \frac{V_{max} \times [NBQ]}{K_m \times \left(1 + \frac{[I]}{K_{ii}}\right) + [NBQ]} \quad (3)$$

It follows that the percentage I_R by which this residual rate v_i at a given concentration [I] is inhibited by increasing concentrations of detergent [D] is:

$$I_R = 100 - \frac{v}{v_i} \times 100 \quad (4)$$

Provided that all concentrations, K_m for NBQ and the I_{50} values for the inhibitor and the detergent are known and the detergent and the inhibitor both act as competitive inhibitors, these equations should allow simulation of the experimental data.

For a given set of experimental conditions I_{50} values that are determined as the concentration of inhibitor or detergent required to reduce the NADH oxidation rate to 50% of the uninhibited rate can be converted to the corresponding dissociation constants:

$$K_{ii} = \frac{K_m \times I_{50}^{inhibitor}}{[NBQ] - K_m} \quad (5)$$

and

$$K_{di} = \frac{K_m \times I_{50}^{detergent}}{[NBQ] - K_m} \quad (6)$$

2.6. Purification of bovine complex I

All preparation steps were performed at 4°C. Bovine heart mitochondria which were prepared according to [19], were diluted in 50 mM Tris/HCl, 2 mM KCN, pH 7.4 to a protein concentration of 35 mg/ml (or 17.5 μM cytochrome aa_3, cytochrome aa_3 content in mitochondrial membranes is 0.5 nmol/mg). After adding Triton X-100 to a final concentration of 1.75% (20% stock solution; w/v) and NaCl to a final concentration of 600 mM (4 M stock solution) the suspension was stirred for 2 min and then centrifuged for 60 min at 180 000 × *g*. The supernatant contained matrix proteins, cytochrome *c*, most of succinate dehydrogenase (complex II) and ATP synthase (complex V). The pellet was resuspended in 300 mM sucrose, 2 mM EGTA, 10 mM K-phosphate, 10 mM Tris/HCl, pH 7.4, homogenized and adjusted to a protein concentration of 40 mg/ml total protein (or 30 μM cytochrome aa_3, cytochrome aa_3 content in pre-extracted membranes is 0.7 nmol/mg). Then an equal volume of 4% Triton X-100, 1.2 M NaCl, 300 mM sucrose, 20 mM Tris/HCl, pH 7.4 was added. After stirring for 2 min the extract was centrifuged for 60 min at 180 000 × *g*. The red supernatant contained most of cytochrome bc_1 complex (complex III) and NADH:ubiquinone oxidoreductase (complex I). The green pellet contained the cytochrome *c* oxidase (complex IV). The red supernatant was adjusted to a final concentration of 50 mM Na-phosphate (500 mM stock solution) and additional 1% Triton X-100. The extract was bound to an equal volume of hydroxylapatite which was equilibrated with 0.05% Triton X-100, 250 mM NaCl, 50 mM Na-phosphate, pH 7.4. For reducing the salt concentration and further delipidation the hydroxylapatite column was washed with one volume of 0.1% Triton X-100, 250 mM NaCl, 50 mM sodium phosphate, pH 7.4. Elution of complex I and complex III occurred simultaneously at 200 mM K-phosphate, 0.5% Triton X-100, pH 7.4. The fractions containing complex I were pooled and diluted with 0.1% Triton X-100, 20 mM Tris/HCl, pH 7.4 to 50 mM K-phosphate. This pool was applied to a DEAE Biogel A (100–200 wet mesh; Bio-Rad Laboratories) column equilibrated with 0.1% Triton X-100, 50 mM NaCl, 20 mM Tris/HCl, pH 7.4. For detergent exchange the bound protein was washed with one column volume of 0.1% dodecylmaltoside, 50 mM NaCl, 20 mM Tris/HCl, pH 7. Complex I eluted at 200 mM NaCl, 0.1% dodecylmaltoside, 20 mM Tris/HCl, pH 7.4. Under these conditions the cytochrome bc_1 complex remained bound to the column as it required 280 mM NaCl for elution. The resulting yellowish fractions were pooled according to NADH:ubiquinone oxidoreductase activity. For further delipidation and elimination of residual cytochrome *c* oxidase the Biogel A pool was applied to a TSK G 4000 SW column (Toso-Haas) and was eluted in 200 mM KCl, 0.1% dodecylmaltoside, 20 mM Tris/HCl, pH 7.4. The pool containing pure complex I was concentrated by ultrafiltration through a YM-100 membrane (Amicon) and was stored in liquid nitrogen.

2.7. Protein sequencing

For amino-terminal sequencing the subunits of isolated complex were separated by tricine SDS–PAGE [17]. After transfer onto a polyvinylidene-difluoride membrane individual subunits were sequenced by automated Edman degradation, in an Applied Biosystems 473 A pulsed-liquid protein sequencer.

3. Results

3.1. Effects of detergents on inhibitor binding and inhibition

The interaction of various detergents with complex

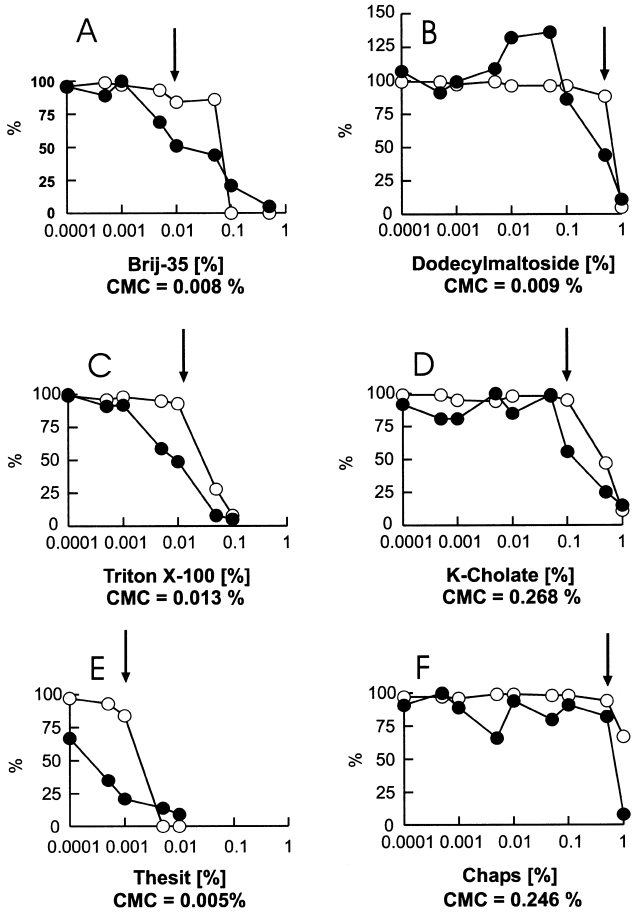


Fig. 1. Effect of detergents on inhibitor binding. NADH:NBQ activity (●) was measured as described in Section 2 at pH 7.4 and 30°C. Inhibition of the residual rate (○) was assayed by the addition of 2 μM NFQA to the assay. Black arrows indicate the loss of NFQA binding as determined by fluorescence quench titration.

I in bovine submitochondrial particles was analyzed. Three different polyoxyethylene-ether detergents (Triton X-100, Thesit, Brij-35), the sugar detergent dodecylmaltoside and two different bile acid derivatives (K-cholate, Chaps) were included in this study. Within the group of polyoxyethylene-ethers Triton X-100 and Thesit are known to be strongly delipidating while Brij-35 is not capable of extracting proteins from a membrane.

In general, loss of NADH:NBQ oxidoreductase activity and loss of sensitivity of the residual rate towards NFQA inhibition caused by increasing detergent concentrations were found to develop in parallel (Fig. 1). As a rule, higher concentrations of detergents were needed to decrease inhibition of the residual activity (Fig. 1, open circles) than for inhibition of the catalytic rate (Fig. 1, filled circles). Although eventually all detergents abolished the catalytic activity of complex I, a concentration of 0.01% or less of the polyoxyethylene-ether detergents was sufficient for 50% inhibition, but between 0.1 and 1% of dodecylmaltoside and the bile acid derivatives was necessary to achieve the same effect. Strikingly, the polyoxyethylene-ethers, especially Triton X-100 and Thesit exert their effect on complex I at concentrations below their CMC while on the other hand dodecylmaltoside and Chaps decrease the catalytic activity and inhibitor sensitivity only at concentrations well above their CMC. For Brij-35 we observed a significant loss of activity at low concentrations and a complete insensitivity to NFQA already at 0.1% compared to 1% for dodecylmaltoside, while the CMC for both detergents are quite similar. The molar I_{50} value was 5.8 μM for Thesit and 150 μM for Triton X-100. It should be noted that this I_{50}

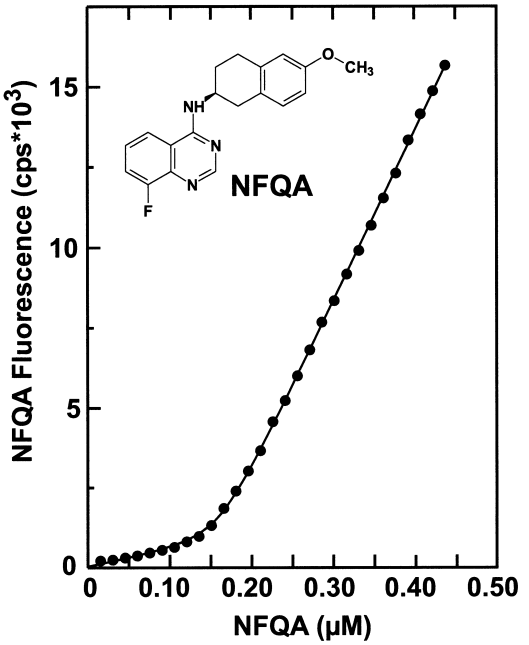


Fig. 2. Binding characteristics of NFQA. Submitochondrial particles from bovine heart were diluted in 1 mM EDTA, 1 mM $MgCl_2$, 75 mM Na-phosphate, pH 7.4 to a final complex I concentration of 0.15 mM [7]. NFQA (structure see insert) was added from a 15 mM stock solution in ethanol. The excitation maximum at 316 nm and the emission maximum at 360 nm were used to follow NFQA binding. The fluorescence was quenched by 92 ± 5% upon binding to complex I, the apparent K_d was 2.4 ± 0.5 nM (n = 12).

value for Triton X-100 is considerably higher than the value reported by Ushakova et al. [20]. However, when K_{di} was calculated using Eq. 6 and thereby taking into account the different experimental conditions of the two studies the resulting values are rather similar.

The effect of the same detergents on equilibrium binding of NFQA was studied by fluorescence quench titration. NFQA (Fig. 2) is a fluorescent inhibitor that shows a marked quench of fluorescence by $92 \pm 5\%$ upon binding to complex I (Fig. 2). In the absence of detergents an apparent K_d of 2.4 ± 0.5 nM ($n = 12$) was determined and one binding site per complex I for NFQA was found by this method. Compared to the related compound DQA [7] binding of NFQA is tighter and its fluorescence is quenched to a much greater extent. Therefore, it was better suited for this study. In the presence of increasing concentrations of detergents the fluorescence quench of NFQA gradually disappeared indicating loss of binding of the inhibitor to its binding site in complex I. The black arrows in Fig. 1 indicate the approximate concentration of detergent up to which NFQA binding could still be followed by this method. These concentrations have to be considered only as estimates and the experiment is performed at much higher concentrations of complex I than the kinetics. Nevertheless, for all detergents tested the concentrations were in the same range, above which significant effects on the inhibition of the residual catalytic activity in the presence of a given concentration of the detergent were observed (Fig. 1, open circles), and above which equilibrium binding was lost. This indicates that the detergent-induced loss of inhibition was caused by impairment of inhibitor binding and not by some structural change generating a bypass route for electron transfer onto ubiquinone.

To further investigate the inhibitory mechanism of the polyoxyethylene-ether detergents, we systematically varied the Triton X-100 to protein ratio and measured the specific NADH:NBQ oxidoreductase activity of complex I (Fig. 3). The inhibitory effect was found to be strictly dependent on the absolute detergent concentration and not affected by the detergent to protein ratio. This further excludes lipid extraction as the cause for the inhibitory action of Triton X-100.

Overall we concluded from these results that Triton X-100 and Thesit should be considered as specific inhibitors of complex I. They seemed to interact with complex I as single molecules and obviously delipidation played no significant role at concentrations where the detergents display their inhibitory effect. On the other hand, inhibition by dodecylmaltoside, K-cholate and Chaps that was observed at much higher concentrations was probably at least partly due to more general detergent effects like phase partitioning and delipidation.

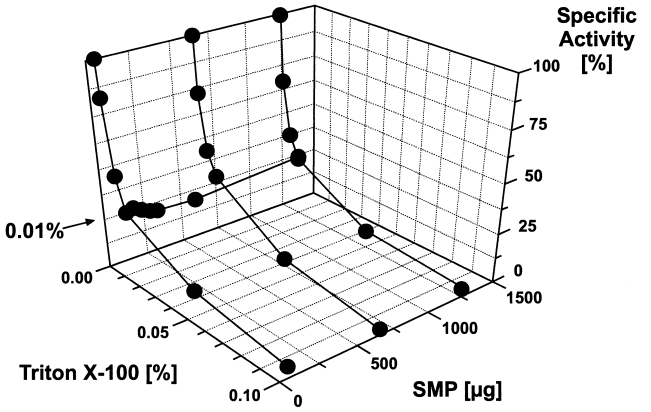


Fig. 3. Effect of Triton X-100 on NADH:ubiquinone oxidoreductase activity. NADH:NBQ activity was measured as a function of the concentrations of Triton X-100 and amount of SMP in the cuvette.

To test our conclusion that the polyoxyethylene detergents behaved like other complex I inhibitors we treated the experiments shown in Fig. 1C and E as a situation where two inhibitors compete for the same substrate binding site. The corresponding Michaelis–Menten type kinetic model is depicted in Eq. 1. This scheme is based on the assumption that the inhibitor and the detergent exhibit competitive inhibition. As only CC44 and other derivatives of Capsaicin show competitive inhibition [11], we measured catalytic activity and degree of inhibition by CC44 in the presence of increasing concentrations of Thesit. The competing effects are illustrated by the curves shown in Fig. 4 that depict the extent of inhibition of the *residual* rate in the presence of a fixed concentration of CC44 by increasing amounts of Thesit. Assuming concurrent binding of detergent and inhibitor according to Eq. 1 the data were fitted with Eq. 4 using independently determined, fixed values for the K_m of NBQ (2.2 μM), the dissociation constant for Thesit K_{di} (0.22 μM, calculated from I_{50} accord-

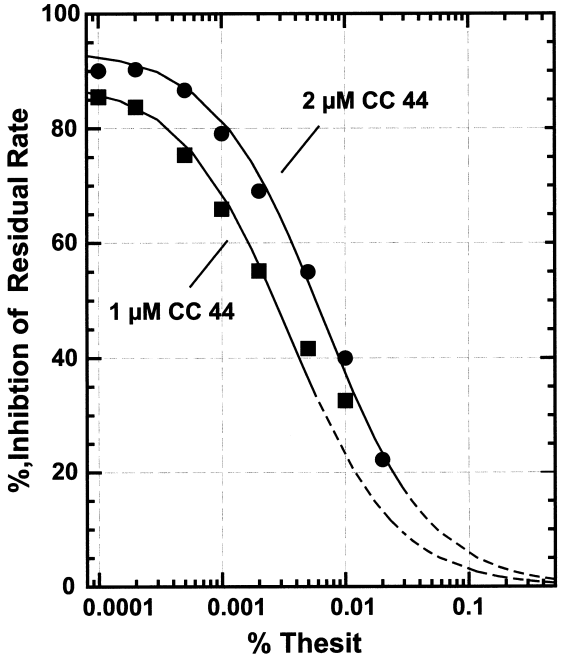


Fig. 4. Concurrent competition of Thesit and CC44 for the NBQ binding site. The degree of inhibition of the residual NADH:NBQ oxidoreductase activity of SMP by 1 μM (■) and 2 μM (●) CC44 in the presence of increasing concentrations of the polyoxyethylene-ether detergent Thesit was measured. The lines represent the least-square fit of K_{ii} for CC44 based on Eq. 4. The following parameters were used to calculate both curves: K_m(NBQ) = 2.2 μM, [NBQ] = 60μM, K_{ii}(CC44) = 4.7 nM and K_{di}(Thesit) = 0.22 μM (see Section 2 for details).

ing to Eq. 6) and 60 μM NBQ. For 1 and 2 μM CC44 the best numerical fit shown in Fig. 4 gave an I_{50} value for CC44 of 120 nM which is in good agreement with directly obtained values [7]. Fig. 4 shows that the experimental data for 1 and 2 μM CC44 were described very well by the kinetic model of Eqs. 2–4. At higher CC44 concentrations (>2 μM) the overall line shape remained unchanged and the curve was shifted in the expected direction, but obviously complex partitioning phenomena started to cause some deviation from the kinetic model (data not shown). If compounds exhibiting non-competitive inhibition like rotenone and piericidin A were used in the same experiment, Eq. 4 could not be applied. However, qualitatively comparable results were observed reflecting a more complex but essentially similar situation for these inhibitors (data not shown).

3.2. Purification of inhibitor-sensitive bovine complex I

Our findings about the effect of detergents on catalytic activity, inhibition and inhibitor binding were instrumental for the development of a novel large-scale chromatographic preparation method for bovine complex I. A summary of a typical preparation is presented in Table 1.

The detergent Triton X-100 was found to be indispensable for selective pre-extraction (0.5 g Triton X-100/g total protein) of bovine heart mitochondria which removes complexes II and V. For extraction of complex I a Triton X-100 to protein ratio of 1 g/g was used which leaves more than 98% of complex IV in the membrane fraction. Apart from resulting in a 2–3-fold increase of specific HAR activity (see Table 1), the hydroxylapatite column allowed efficient delipidation and reduction of the salt concentration, thereby avoiding ammonium sulfate precipitation steps common in other complex I preparations [2–4].

The key step to restore NADH:NBQ oxidoreductase activity and inhibitor sensitivity of complex I was the exchange of detergent from Triton X-100 to dodecylmaltoside on the DEAE Biogel A column:

Table 1
Yield and purity of complex I during preparation

Preparation step/detergent	Total protein (mg)	Specific activity (μmol min^{-1} mg^{-1})	Total units (%)
Membranes	7490	3.4	100
Pre-extraction sediment/Triton X-100	5100	5.1	102
Extraction supernatant/Triton X-100	2830	9.6	106
Hydroxylapatite pool/Triton X-100	670	24	63
Biogel A pool/dodecylmaltoside	230	45	40
TSK pool/dodecylmaltoside	48	49	9

The specific activity was measured as electron-transfer rate from NADH (200 μM) to the artificial electron acceptor HAR (2 mM) at 340 nm minus 400 nm (30°C).

Table 2
Characterization of isolated complex I

Preparation step	FMN (nmol/mg)	Phospholipid (nmol/mg)	Ubiquinone (nmol/mg)	Specific activity (μmol min^{-1} mg^{-1})	Inhibition (%)
Biogel A pool	0.8 ± 0.1 ($n = 3$)	≈500	0.10	1.1 ± 0.1 ($n = 3$)	>90
TSK pool	1.1 ± 0.1 ($n = 3$)	≈100	not detectable	0.45 ± 0.05 ($n = 3$)	>90

FMN, phospholipid and ubiquinone contents were determined as described in Section 2. The specific activity was measured at pH 7.4 and 30°C as electron-transfer rate from NADH (100 μM) to NBQ (60 μM) at 340 nm minus 400 nm. Inhibition was tested by adding 2 μM of DQA or piericidin A to the assay mixture (see Fig. 7).

While the pooled fractions from the hydroxylapatite column in Triton X-100 exhibited only a very low rate of ubiquinone reduction that was not affected by the addition of hydrophobic inhibitors (data not shown), the eluate from the Biogel A column in dodecylmaltoside exhibited a substantial recovery of NADH:NBQ oxidoreductase activity that was fully sensitive to DQA (Table 2).

The yield after this step was 40% as judged by HAR activity compared to mitochondrial membranes and complex I was 80–90% pure (Table 1). This is sufficient for most kinetic and spectroscopic studies. The remaining contaminant was found to be mostly cytochrome *c* oxidase. Assuming an average molecular mass of 750 g/mol for the phospholipids in the inner mitochondrial membrane [21], the phospholipid content of complex I at this stage (Biogel A pool) was ≈500 nmol/mg and ubiquinone content was about 0.1 nmol/mg (Table 2).

The final size exclusion chromatography with a TSKgel G 4000 SW column removes most of the residual cytochrome oxidase and polymers of complex I. After this step the preparation contained about 100 phospholipid molecules and 1.1 ± 0.1 FMN per mg of protein (Table 2). This is very close to the value expected for pure enzyme based on a total molecular mass of around 940 kDa. The ubiquinone content was below the detection level. Upon re-chromatography on the same column complex I elutes as a single and symmetric peak, indicating a monodisperse state of the enzyme (data not shown). The SDS–PAGE of subsequent preparation steps shown in Fig. 5 illustrates that no significant contamination remains after the TSKgel column. However, optical spectra of the isolated enzyme still identified cytochrome oxidase as a very minor contaminant of 1–2% of the total protein (data not shown) which is about the same as in the preparation by Finel et al.

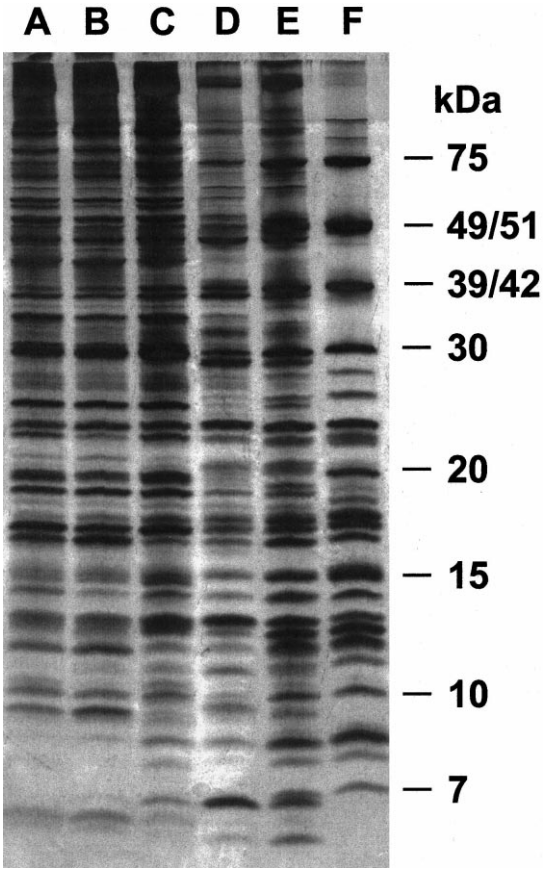


Fig. 5. Stages of complex I purification. Silver-stained tricine SDS–PAGE of isolated complex I and different stages of preparation was performed according to [17]. The protein samples (5 μg total protein, see Table 1) were incubated for 30 min at 35°C in 4% SDS (w/v), 2% mercaptoethanol, 10% glycerol, 0.01% Serva blue G, 50 mM Tris/HCl, pH 7.0. The numbers indicate molecular masses. The six largest hydrophilic proteins of complex I were identified by N-terminal sequencing. Lane A, membranes; lane B, sediment of pre-extraction; lane C, supernatant of extraction; lane D, hydroxylapatite pool; lane E, Biogel A pool; lane F, TSK pool. See also Table 1.

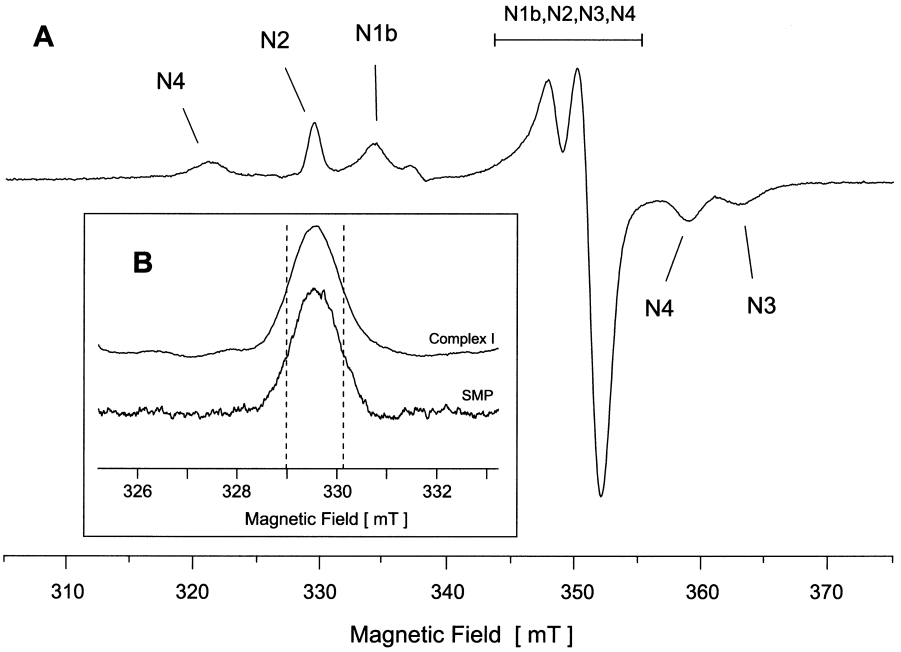


Fig. 6. EPR spectra of purified complex I. A: EPR spectrum of purified complex I (12.7 mg/ml, TSK pool) reduced with 6.5 mM NADH. The spectrum shows signals typical of the four FeS centers N1b, N2, N3, N4, denoted according to the nomenclature of Ohnishi [22]. The corresponding g values (g_x, g_y, g_z) were N1b: 1.92, 1.94, 2.02, N2: 1.93, 1.93, 2.05, N3: 1.86, 1.93, 2.04, N4: 1.89, 1.94, 2.11. B: Comparison of the linewidth of the g_z signal of cluster N2 in isolated complex I (same sample as A) and SMP (protein concentration, 21.4 mg/ml, reduced with 10 mM NADH). SMP from bovine heart mitochondria were prepared according to published procedures [34,35] Both spectra were corrected for a baseline drift, mainly due to the g_z signal of cluster N3. EPR conditions for A and B: sample temperature, 12 K; microwave frequency, 9.48 GHz; microwave power 0.1 mW; modulation amplitude 0.64 mT.

(see Fig. 4 in [4]). These same authors have observed a loss of the 42 kDa subunit in their preparation upon chromatography on a Mono Q column. To exclude this inhomogeneity for our preparation we have performed N-terminal sequencing of the band in the SDS gel consisting of the 39 and 42 kDa subunits (see Fig. 5). The intensity of the PTH amino acid peaks in the HPLC following Edman degradation suggested that nearly equal amounts of the two subunits are present in the purified enzyme.

EPR spectra of the isolated enzyme at 12 K (Fig. 6A) show signals from four iron–sulfur centers (N1b, N2, N3, N4) as reported for other less pure preparations of complex I [22]. At lower temperature (5 K) and high microwave power the fifth FeS center N5 [22] was identified with $g_{x,y,z}$ 1.90, 1.93, 2.07 (data not shown). N2 is known to be sensitive to detergent-induced structural changes of complex I [4]. Therefore, we compared the lineshape of the g_z signal of cluster N2 in isolated complex I and SMP (Fig. 6B). There was neither a shift nor a line broadening detectable in our purified enzyme.

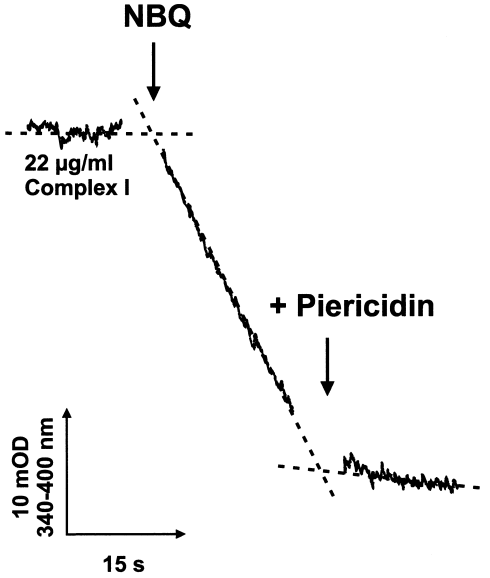


Fig. 7. Instant inhibition of purified complex I. The complex I (TSK pool) concentration in the assay mixture was 22 µg/ml corresponding to about 25 nM complex I. About 95% of the catalytic activity was inhibited within a few seconds after addition of 2 µM piericidin A from an ethanolic stock solution during the assay. No preincubation or addition of phospholipid was needed.

The NBQ reductase activity of isolated complex I (Biogel A pool, cf. Table 2) was 1.1 μmol min^{-1} mg^{-1} and could be increased to 2.2 μmol min^{-1} mg^{-1} in the presence of 0.10 mg/ml egg yolk phospholipid. This specific activity is significantly higher than comparable preparations of similar purity [2]. After further purification and delipidation the activity decreased to 0.45 μmol min^{-1} mg^{-1} (TSK pool) while inhibitor sensitivity of 95% was preserved (Fig. 7). It was not possible to increase this activity by simply adding phospholipid to the assay mixture.

4. Discussion

Complex I is the largest and least understood multiprotein complex of the mitochondrial respiratory chain. Its investigation has been severely hampered in the past by the sensitivity to detergents during enzyme purification evident in particular as a loss of inhibitor sensitivity. This study analyzes the interaction of different detergents with complex I to develop an isolation procedure for pure, monodisperse and inhibitor-sensitive enzyme.

Our results demonstrate that in contrast to dodecylmaltoside and derivatives of bile acids, some polyoxyethylene-ether detergents are specific inhibitors of complex I. These compounds are of similar or even higher potency as well known inhibitors like amytal [23] or capsaicin [24]. The inhibitory action of these detergents was found to be independent of their solubilizing and delipidating properties. Inhibition of complex I by Triton X-100 was reported earlier [20,25,26], but the polyoxyethylene-ether detergent Thesit studied here was found to be 25-fold more efficient than Triton X-100. In fact inhibitor potency is obviously linked to the chain length of both the alkyl- and the polyoxyethylene-ether moiety [27].

Binding analysis by fluorescence quench titration [7] using the novel quinazoline type inhibitor NFQA (cf. Fig. 2) revealed that the detergent-induced loss of sensitivity of the residual activity to other potent inhibitors of complex I was in fact due to loss of binding and not caused by the creation of an electron bypass to ubiquinone. We have shown that the loss of inhibitor sensitivity of residual activity in the presence of detergent can be quantitatively described (Fig. 4) by a simple Michaelis–Menten type kinetic model assuming that the inhibitor CC44 and the detergent Thesit act as competitive inhibitors for ubiquinone (see Eq. 1).

In summary, our study demonstrates that polyoxyethylene-ether detergents act as specific, competitive complex I inhibitors. This seems to be a major cause for the loss of activity and inhibitor sensitivity in many complex I preparations. However, at higher detergent concentrations additional effects may become important, as unspecific delipidation or disintegration of the complex.

These results prompted us to develop a novel purification procedure which yields bovine complex I in active and inhibitor-sensitive state. Triton X-100 is one of the best non-ionic, non-denaturing detergents for differential extraction of mitochondrial membrane proteins [10]. On the other hand we had learned that this detergent is unsuitable to isolate complex I in an active and inhibitor-sensitive form as it acts as a specific inhibitor. However, this effect was reversible and complex I regained its full inhibitor sensitivity by exchanging the detergent from Triton X-100 to dodecylmaltoside after the first chromatographic step in our purification protocol. This further supports the notion that Triton X-100 is a reversible inhibitor of complex I.

The phospholipid content in purified complex I was found to have a significant effect on catalytic activity, as the specific activity of the preparation on the level of the Biogel A pool could be doubled by addition of external phospholipid. Furthermore we observed a decrease of activity after extensive delipidation (500 → 100 mol phospholipid/mg complex I) on the TSKgel G 4000 SW column. In contrast to the preparation described in [4] even after this extensive delipidation our purified enzyme was fully sensitive to hydrophobic complex I inhibitors and inhibition required no added phospholipid or preincubation steps.

The purified enzyme was found to contain 1 mol FMN/mol complex I. Thus, we found no evidence for a doubling of the flavin-containing 51 kDa subunit as has been proposed by Albracht and de Jong [28]. Five different iron–sulfur clusters were identified in the purified complex I by EPR spectroscopy. We could not detect a shift or broadening of the g_z line of the cluster N2 signal caused by our purification protocol. Therefore, we conclude that detergent and

delipidation had no effect on the environment of this iron–sulfur center.

The preparation of pure and fully inhibitor-sensitive complex I will prove useful for further studies of this enzyme. However, while in the same range as other comparable preparations, the turnover number for ubiquinone reductase activity of the isolated complex I in dodecylmaltoside was still less than 10% when compared to submitochondrial particles. As we excluded detergents and a general depletion of phospholipids as reason for this loss of activity, one has to consider more specific factors to explain this phenomenon. For example, it would be possible that complex I needs a specific type of phospholipid, e.g. cardiolipin, for full activity. Another attractive idea would be a loss of a tightly bound quinone in the course of the purification procedure. Prosthetic quinones have been proposed as a component of several hypothetical mechanistic schemes for complex I [29–33]. It is therefore tempting to speculate that the low ubiquinone content of our preparation might explain the low specific activity. Ubiquinone reconstitution experiments with the isolated enzyme will be needed to test this hypothesis.

Acknowledgements

We are indebted to Hideto Myoshi for his generous gift of CC44. We thank Franz J. Streb and Kathy Pfeiffer for excellent assistance in performing the experiments. This work was supported by grant BR 1633/1, SFB 472, Graduiertenkolleg 145/12 of the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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