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UBIQUINOL-CYTOCHROME *c* REDUCTASE (EC 1.10.2.2)

ISOLATION IN TRITON X-100 BY HYDROXYAPATITE AND GEL CHROMATOGRAPHY

STRUCTURAL AND FUNCTIONAL PROPERTIES

WOLF DIETER ENGEL, HERMANN SCHÄGGER and GEBHARD VON JAGOW

Institut für Physikalische Biochemie der Universität München, Goethestrasse 33, 8000 München 2 (F.R.G.)

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Summary

1. A method for the isolation of a monodisperse ubiquinol-cytochrome *c* reductase (complex III) from beef heart mitochondria has been developed. The procedure consists of an enzyme solubilization in Triton X-100 followed by hydroxyapatite and gel chromatography.

2. The minimum unit of the isolated complex is composed of 9 polypeptide subunits with M_r of 49 000, 47 000, 30 000, 25 000, 12 000, 11 000 and 6000. It contains 8 μmol of cytochrome *b*, 4 μmol of cytochrome c_1, 7—8 μmol of nonheme iron, corresponding to 3.5—4 μmol of the Rieske iron-sulfur protein, less than 1.0 μmol of ubiquinone and about 60 μmol of phospholipids, per g of protein. The specific detergent binding amounts to 0.2 g of Triton X-100 per g protein.

3. Cytochrome *b* exhibits an α-absorbance maximum at 562 nm. In redox titrations it reveals two half-reduction potentials, i.e. —10 and +100 mV, at pH 7.0. The absorbance maximum of cytochrome c_1 lies at 553 nm and its half-reduction potential amounts to +250 mV.

4. The reductase reveals electron-transferring activity with ubiquinol-1, -2, -3, and -9 as donor and cytochrome *c* as acceptor. The activity with ubiquinol-9 was analyzed according to the surface dilution scheme developed for the action

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Abbreviations: Q_9, ubiquinone-9; Q_9H_2, ubiquonol-9; Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

of phospholipases. The molecular activity amounts to 75 mol of cytochrome *c* reduced per s at 20°C.

5. A dissociation constant K'_s of 5.5 mM has been determined for the Triton-solubilized enzyme: ubiquinol-containing micelle association. In this case the total concentration of ubiquinol plus Triton X-100 has been substituted for the concentration of binding areas on the ubiquinol-containing micelles. This substitution makes the reasonable assumption that the sum of ubiquinol concentration plus Triton X-100 is proportional to the number of available binding areas.

6. A K'_m value of 0.025 was found for ubiquinol-9. This is an analog to the Michaelis constant and is expressed as mol fraction of ubiquinol in the ubiquinol-Triton micelle.

Introduction

Ubiquinol-cytochrome *c* reductase is a unique multiprotein complex of the mitochondrial respiratory chain, in which electron transfer is linked to proton translocation [1].

The reductase of beef heart mitochondria has been isolated so far by salt fractionation in the presence of bile salts [2,3], that of *Neurospora crassa* mitochondria by affinity chromatography in Triton X-100 [4]. The latter method is not applicable to complex III of beef heart, since high salt concentrations, required for solubilization, disturb the affinity chromatography. An enzymatically inactive preparation of beef heart, termed bc_1-complex, has been isolated as a 400 000-dalton unit by hydroxyapatite chromatography in Triton X-100 [5,6]. This complex was stabilized during preparation by antimycin [7], a specific inhibitor of ubiquinol-cytochrome *c* reductase [8]. Under these conditions one polypeptide, the Rieske iron-sulfur protein [9,10], was lost. The published method has now been adapted for preparation of a complete and enzymatically active complex. Preservation of the functional structure required lowering of the Triton concentration during solubilization and chromatography. Under these conditions, however, the resolution capacity of hydroxyapatite is reduced. In order to obtain a highly purified complex, gel chromatography has to follow the hydroxyapatite chromatography.

Kinetics of enzymes with lipophilic substrates are complicated by the detergent required for solubilization of the substrate [11]. In the case of ubiquinol-cytochrome *c* reductase both the substrate and the hydrophobic enzyme have to be solubilized by detergents. The complex kinetics governed by micelle-micelle interactions have only been described qualitatively for complex III so far [12]. However, a reaction scheme for phospholipase, called surface dilution scheme, was recently developed [13,14]. This scheme is clearly suitable for a quantitative analysis of the kinetics of complex III in the presence of Triton X-100 when long side chain ubiquinols serve as substrate, as will be described further on.

Materials and Methods

Materials

Triton X-100 p.A. was purchased from Serva (Heidelberg, F.R.G.), an

average molecular weight of 650 was assumed [15]. Diaflo XM 100 membranes from Amicon (Witten, F.R.G.), Sepharose CL-6B from Pharmacia (Freiburg, F.R.G.) and cytochrome *c* from Boehringer (Mannheim, F.R.G.). All other chemicals were analytical grade from Merck (Darmstadt, F.R.G.). Hydroxyapatite we prepared ourselves [16]. Ubiquinone-9 was a gift from Hoffmann-La Roche (Basel, Switzerland).

Ubiquinones were reduced by vigorously shaking their solution in cyclohexane with an aqueous solution of sodium dithionite. The organic phase was washed with 0.5 M NaCl. After evaporation, the ubiquinol was dissolved in ethanol/acetic acid (50 : 1, v/v) and stored at −20°C.

Methods

Determinations

Protein, cytochrome contents, half-reduction potentials, nonheme iron and Triton X-100 were determined as described elsewhere [6,7].

Ubiquinone was determined by various procedures to ensure the validity of the data. Both the enzyme in solution as well as in a solid lyophilized state were extracted according to Ref. 17 by the normal and by the acid extraction method, difference absorbance spectra (oxidized minus reduced) were performed.

Phospholipids were determined according to Refs. 18 and 19. In order to determine tightly bound phospholipid the sediment of the chloroform-methanol extract was suspended in glacial acetic acid, brought to alkaline pH by 25% ammonia solution and further extracted by chloroform/methanol [20]. Since no tightly bound phospholipid could be detected by this method, the sediment of the chloroform-methanol extract was ashed, and the inorganic phosphate determined.

Dodecyl sulfate polyacrylamide gel electrophoresis was performed according to Neville and Glossmann [21] using 15% gels.

Ubiquinol-cytochrome *c* reductase activity was measured in a medium containing 250 mM sucrose, 2 mM KCN, 50 mM potassium phosphate, 0.2 mM EDTA, 0.1% bovine serum albumin, 100 μM ubiquinol-1, -2, -3 or -9, and 50 μM cytochrome *c* at pH 7.0 and 20°C. In order to solubilize ubiquinol-9, 1.5 mM Triton X-100 had to be added. The assay was started by adding the enzyme.

The reduction of cytochrome *c* was measured at a 550—540 nm wavelength pair in a Shimadzu UV 300 spectrophotometer [22]. For kinetic analysis the concentrations of Triton X-100 and ubiquinol-9 were varied as described in the text.

Isolation of ubiquinol-cytochrome c reductase

All operations were performed at 4°C. Mitochondria were prepared as described [23] and stored in liquid nitrogen.

For preextraction mitochondria were suspended in 20 mM Mops, pH 7.2, to a protein concentration of 35 mg/ml. Subsequently to 100 ml of the suspension 13.5 ml 0.31 M (≙20%) Triton X-100 and 20 ml of 4 M NaCl were added, giving final concentrations of 26 mg protein/ml, 31 mM Triton X-100 and 600 mM NaCl.

After 15 min stirring the suspension was centrifuged for 1 h at 80 000 × *g*. The pellet which contained complex III was resuspended in 300 mM sucrose, 20 mM Mops, pH 7.2, to a protein concentration of 35 mg/ml. It was diluted to twice the original volume by a buffer containing 60 mM Triton X-100, 1.2 M NaCl, 300 mM sucrose, 20 mM Mops, pH 7.2. After 15 min stirring the unsolved material was separated by centrifugation at 80 000 × *g* for 30 min. The reductase was bound in a batch procedure to hydroxyapatite that had been equilibrated with 80 mM sodium phosphate, 250 mM NaCl, 8 mM Triton X-100, at pH 7.2, and then packed into a column. Crude complex III was eluted by means of 200 mM sodium phosphate, 4 mM Triton X-100, pH 7.2. The fractions that contained more than 3.5 μmol of cytochrome *b* per g protein were pooled, concentrated by ultrafiltration through a Diaflo XM 100 membrane to a protein concentration of about 30 mg/ml, and applied to a Sepharose CL-6B column that had been equilibrated with a buffer containing 0.8 mM Triton X-100, 100 mM NaCl, 20 mM Mops, at pH 7.2.

The fractions obtained were analyzed for cytochrome and protein content.

For storage the enzyme was precipitated by dialysis against 10 mM Mops, pH 7.2 or by 25% acetone. The pellets were stored at −20°C. Alternatively the enzyme was stored in 50% glycerol (v/v) at −20°C.

Kinetic analysis

The activity of the reductase was analyzed according to the surface dilution scheme [13,14], applied in its simplest version (Fig. 1). The enzyme associates first with an ubiquinone-containing micelle. This step depends on the surface available for the enzyme on the mixed Triton-ubiquinol micelles. As no data regarding the mechanism of interaction between the enzyme and the ubiquinol-containing micelle are available at present, the surface areas have not been taken into consideration. In a first approximation the concentration of binding areas on the mixed micelles for the enzyme has been replaced by the total concentration of ubiquinol plus Triton X-100.

In a second step the enzyme-substrate complex is formed. This step depends on the concentration of ubiquinol in the micelles, expressed in the following as the mol fraction. In a third step the enzyme-substrate complex dissociates into free enzyme and the micelle that contains the oxidized ubiquinone. Steps 2 and 3 obey conventional enzyme kinetics.

The kinetics of cytochrome *c* are not taken into account in the reaction scheme. This simplification seems to be justified since the concentration of cytochrome *c* is held at substrate saturation.

The reaction scheme of Fig. 1 is described by the following set of equations:

$$v = \frac{V \cdot [A][B]}{K'_s \cdot K'_m + K'_m[A] + [A] \cdot [B]} \quad (1)$$

where

$[A] = [Tx] + [Q_9H_2]$

$[B] = [Q_9H_2]/([Q_9H_2] + [Tx])$

[Tx] = concentration of Triton in micelles (total Triton concentration − critical micelle concentration [15])

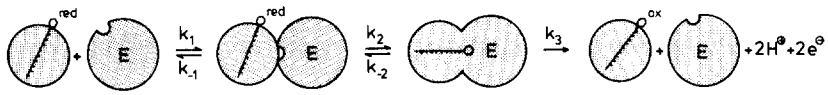

Fig. 1. Scheme of the reaction sequence of ubiquinol oxidation. The smaller circles symbolize the micelles containing the ubiquinones. The ubiquinone-9 molecule is represented by a simplified formula. The larger circles symbolize the Triton solubilized reductase (mol. wt. of 500 000), which possesses two ubiquinone-binding sites but for the sake of simplicity only one ubiquinone-binding site is pointed out. The involvement of cytochrome *c* is not regarded as described in the text.

$[Q_9H_2]$ = ubiquinol concentration

$$K'_s = k_{-1}/k_1$$

$$K'_m = \frac{k_{-2} + k_3}{k_2}$$

For practical purposes experimental series were performed in which the mol fraction [B] was held constant, but the total concentration [A] of Triton X-100 plus ubiquinol varied. Under these conditions Eqn. 2 is derived, leading to a linear plot of $[A]/v$ versus [A] [24].

$$\frac{[A]}{v} = \left(\frac{1}{V} + \frac{K'_m}{V \cdot [B]}\right) [A] + \frac{K'_s \cdot K'_m}{V \cdot [B]} \qquad (2)$$

Replotting the slopes for different mol fractions [B] vs. $1/[B]$ yields a linear plot which intersects the slope-axis at $1/V$ and the $1/[B]$-axis at $-1/K'_m$.

Replotting of the reciprocal intercepts with the [A]-axis vs. [B] also yields a linear plot that intersects the $1/[A]_{intercept}$-axis at $-1/K'_s$ and the [B]-axis at $-K'_m$.

Results and Discussion

Structure of the complex

Ubiquinol-cytochrome *c* reductase isolated in the nonionic detergent Triton X-100 has an advantage over that isolated by bile salts [2,3] in that it allows column chromatography with several materials. As described in the literature [25] and observed personally, chromatography of the complex in bile salts leads to a striking decrease of its enzymatic activity. The reasons for this are unknown so far. On the other hand, application of bile salts allows salt fractionation, which is not possible in the presence of polyoxyethylene detergents.

Hydroxyapatite, an excellent material for the separation of membrane enzymes in the presence of high concentrations of Triton X-100 [5,26], has a diminished separation capacity at Triton concentrations lower than 3 mM. However, high concentrations of Triton X-100 could not be used, since the Rieske iron-sulfur protein is then split off the complex [6]. The isolation procedure described for the antimycin-stabilized complex therefore had to be modified. Due to the lower enrichment after hydroxyapatite chromatography (see Table I), a gel chromatography had to be performed subsequently. The rationale for this step was the observation that the greater part of the impurities not separable by the hydroxyapatite chromatography are precipitated by

TABLE I

ENRICHMENT AND YIELD OF COMPLEX III DURING PREPARATION, USING CYTOCHROME *b* AS A MARKER

	Protein (mg)	Cyt. *b* (nmol)	Cyt. *b*/ protein (μmol/g)	Yield (%)	Enrich-ment (*n*-fold)
Mitochondria	12 570	6040	0.5	100	1.0
Preextracted mitochondria	7 030	5160	0.7	85	1.4
Extract	5 150	4820	0.9	80	1.9
Bound to hydroxy-apatite (HTS)	1 470	3915	2.7	65	5.6
Pooled HTS-fractions	680	3235	4.8	54	10.0
Pooled gel-column fractions	285	2160	7.6	36	15.8

lowering the ionic strength and Triton concentration [22]. The aggregated impurities and excess of Triton X-100 are separated on a Sepharose CL 6-B column (see Fig. 2). The preparation elutes from the column with a K_{av} value of 0.35 indicative of a M_r of about 500 000. The complex is present in a dimeric state, like the antimycin-stabilized complex [6].

Usually 30—40% of the original cytochrome *b* was obtained with a cytochrome *b*/protein content of more than 7.5 μmol/g protein. Considering that

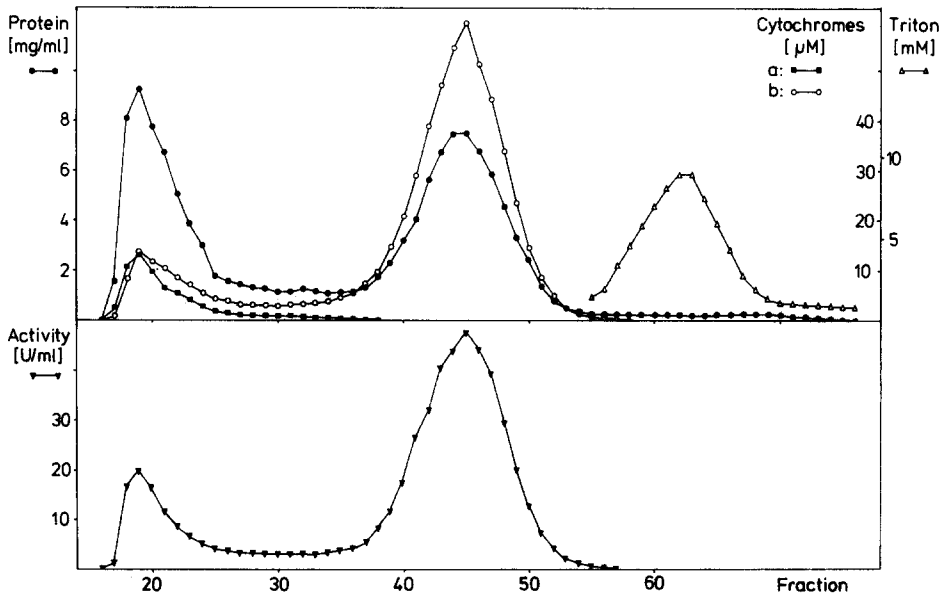

Fig. 2. Eluate profile of a Sepharose CL-6B column (710 ml). 17 ml of concentrate from the hydroxyapatite column containing 650 mg protein, 3150 nmol cytochrome *b* and 260 nmol cytochrome *a*, were applied on the column, and fractions of 5.5 ml were collected after 190 ml has been eluted. The fractions were analysed for ubiquinol-cytochrome *c* reductase activity, protein, cytochrome and Triton X-100 content.

only 80% of the mitochondrial cytochrome *b* belongs to complex III [27], the yield amounts to 40—50% with a 20-fold enrichment.

The enzyme is stable for several months without loss of cytochrome content or enzymatic activity when kept at −20°C in the presence of 50% (v/v) glycerol. The pellet obtained after precipitation by dialysis against 10 mM Mops has the same stability. The yield is somewhat lower, but the enzyme can be dissolved at high concentration in a buffer containing detergents, such as Triton X-100, or bile salts. Precipitation with acetone is less suitable, since 20% of the cytochrome *b* is lost.

The minimum unit of the pure complex shows 9 polypeptide subunits in the polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (see Fig. 3), assuming that the 30 000 band comprises the two cytochromes *b* and cytochrome c_1 [28]. The contents of Triton X-100, phospholipids, ubiquinone, nonheme iron and cytochromes are listed in Table II.

The ubiquinone contents are below the lower limit of detection (1.0 μmol/g protein). The reliability of the method in the presence of Triton X-100 was proved by detecting ubiquinone that was added to the preparation before determination (2.0 μmol/g protein). Thus the preparation gives no indication for the presence of tightly bound ubiquinone.

The phospholipid content of the described preparation (40—80 μmol/g protein) is lower than that of preparations obtained by ammonium sulfate precipitation in cholate (200—400 μmol/g protein) [1,29]. The main part of phospholipid and ubiquinone is lost from the complex before hydroxyapatite chromatography. Phospholipids and ubiquinone are present in the supernatant obtained after binding of the complex to the hydroxyapatite. The complex carries no tightly bound phospholipid.

A difference absorbance spectrum of the isolated complex is shown in Fig. 4.

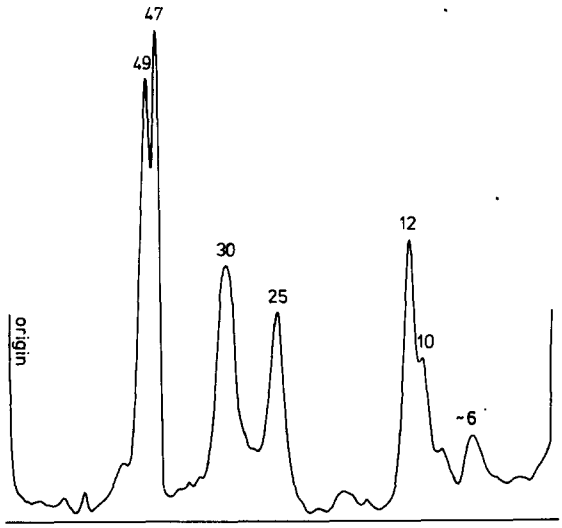

Fig. 3. Polypeptide pattern of the complex. Dodecyl sulfate polyacrylamide gel electrophoresis was performed as described in Methods. The gel was stained with Coomassie Blue R-250 and scanned at 578 nm. The numbers indicate the molecular weights in kilodaltons.

TABLE II

CONTENT OF COMPONENTS OF THE UBIQUINOL-CYTOCHROME *c* REDUCTASE ISOLATED IN TRITON X-100

Component	Content (μmol/g protein)	Molecular ratio
Cytochrome *b*	8	2.0
Cytochrome c_1	4	1.0
Nonheme iron	7— 8	1.9
Ubiquinone	<1.0	<0.2
Phospholipids	40—80	10—20
Triton X-100	350	95

The half-reduction potential of cytochrome c_1 is ±250 mV, the cytochromes b show two potentials of −10 mV and +100 mV at pH 7.0, respectively. The potentials of the *b*-cytochromes are pH-dependent due to a protonation-deprotonation reaction as shown by the following reaction sequence:

$$\text{Cytochrome } b^{3+} \overset{H^+}{\leftrightarrow} \text{cytochrome } b^{3+}H^+ \overset{e^-}{\leftrightarrow} \text{cytochrome } b^{2+}H^+$$

From redox titrations of the isolated complex (unpublished data) as well as of cytochrome *b* integrated in the membrane [30,31] a p*K* value of 6.9 for cytochrome b^{3+} and of >8.5 for cytochrome b^{2+} has been calculated [32].

Function of the complex

The electron-transferring activity of the reductase was analyzed in detail using ubiquinol-9 as electron donor. High activities are also shown with ubiquinol-1, -2 and -3, which do not have to be solubilized by Triton X-100, as has been described elsewhere [22]. No activity could be measured with succinate, succinate plus ubiquinone, or succinate plus artificial electron acceptors [33]. However, a succinate-cytochrome *c* reductase activity could be demonstrated in a system consisting of the described ubiquinol-cytochrome *c* reductase and a succinate-ubiquinone reductase prepared in Triton X-100 (unpublished data)

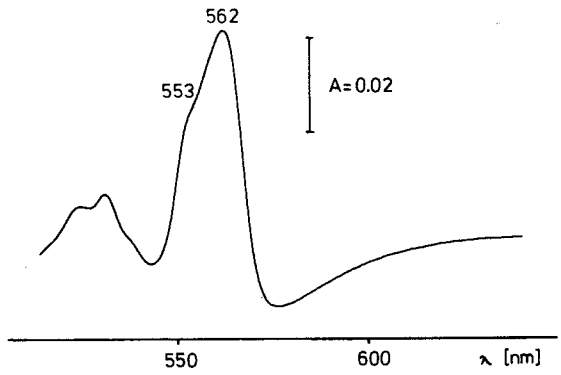

Fig. 4. Difference absorbance spectrum of the isolated complex III (0.26 mg protein/ml). The cytochromes were reduced by dithionite in the measuring side and oxidized by ferricyanide in the reference side.

in the presence of ubiquinone-3. The electron flow was sensitive to thenoyltrifluoroacetone and antimycin.

Ubiquinol-9, an analog of the mammalian ubiquinol-10, but shortened in the isoprenoid side chain by one unit, possesses quite similar physico-chemical characteristics. The electron-transferring activities were analyzed according to the surface dilution scheme, in contrast to recent studies [12,22] where conventional enzyme kinetics valid for water-soluble enzymes and substrates were used [34].

Fig. 5 shows a Hanes-plot for different mol fractions [B]. Replots of the slopes vs. 1/[B], and the reciprocal [A]-axis intercepts versus [B] are shown in Figs. 6 and 8. From these replots the following kinetic constants are derived:

V = 4.5 U/nmol cytochrome c_1 (molecular activity = 75 mol cytochrome c reduced per s)

K'_s = 5.5 mM([Q_9H_2] + [Tx])

K'_m = 0.025{[Q_9H_2]/([Q_9H_2] + [Tx])}

All measurements were made at 20°C and pH 7.0.

The values for K'_m and K'_s are comparable to the values obtained for enzymes that act on phospholipids [35].

The surface dilution scheme is applicable to the action of ubiquinol : cytochrome c reductase towards mixed ubiquinol-Triton X-100 micelles. The in vitro conditions may represent a model which is analogous to the conditions in the mitochondrial membrane where ubiquinol is 'solubilized' in the phospho-

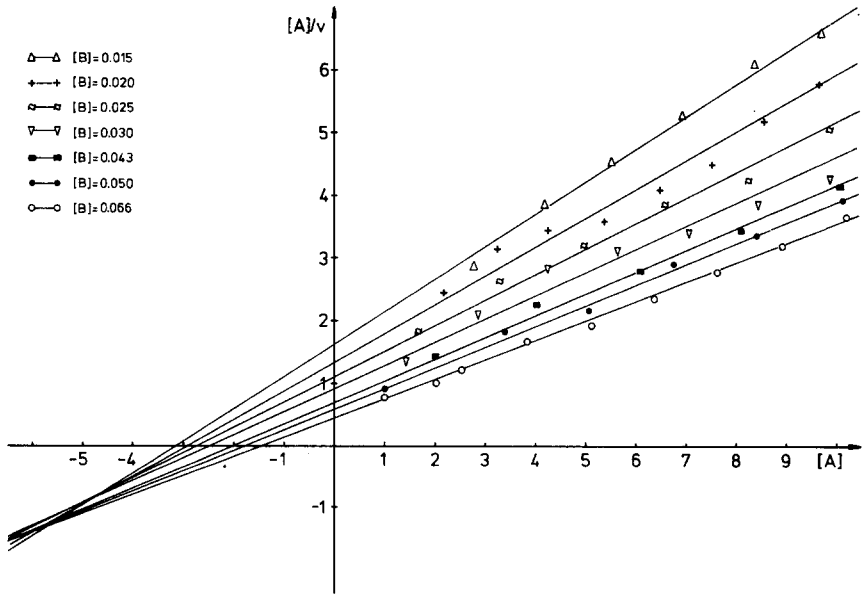

Fig. 5. Hanes plot of the ubiquinol-9-cytochrome c reductase activity of the complex for different mol fractions [B] as described in the text. The dimension of the total concentration of Triton X-100 plus ubiquinol-9 [A] is mM, that of the velocity is U/nmol cytochrome c_1.

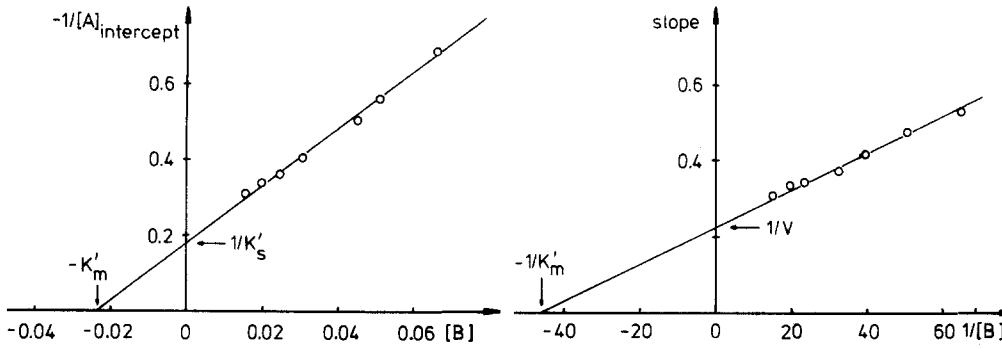

Fig. 6. Replot of the slope of the lines obtained by Fig. 5 as described in the text. Dimensions are the same as in Fig. 5.

Fig. 7. Replot of the reciprocal intercepts with the [A]-axis for different mol fractions [B] obtained from Fig. 5 as described in the text. Dimensions are the same as in Fig. 5.

lipid phase at a mol fraction of about 0.015.

The electron-transferring activity of the preparation can be inhibited by stoichiometric amounts of antimycin (1 antimycin/1 cytochrome c_1) to 10% of the original activity. At a 10-fold excess of antimycin the residual activity amounts to 2—3% (not shown).

In surface dilution kinetics the addition of Triton X-100 results in a lowering of the ubiquinol concentration expressed as mol fraction, diminishing the velocity of the reaction. Therefore it is clear that Triton X-100 is not an inhibitor of the enzyme, but a diluent of the substrate [22]. This effect mimics a competitive inhibition when the reaction is described in terms of conventional enzyme kinetics.

In a recent paper [12] an apparent K_m^* has been dealt with as a function of the Triton concentration. This K_m^* cannot be compared to the K'_m as defined in the surface dilution scheme. The results obtained for the ubiquinol-cytochrome *c* reductase cannot be fully interpreted by conventional enzyme kinetics, whereas the surface dilution scheme seems to describe the system satisfactorily. This is substantiated in the following: Since $[Tx] >> [Q_9H_2]$, it can be assumed that the mole fraction $[B] \approx [Q_9H_2]/[Tx]$ and $[A] \approx [Tx]$. By insertion into Eqn. 1, we derive Eqn. 1a:

$$v \approx \frac{V \cdot [Q_9H_2]}{K'_s \cdot K'_m + K'_m[Tx] + [Q_9H_2]} \tag{1a}$$

Comparison with the conventional equation for enzyme kinetics yields

$$K_m^* = K'_m \cdot [Tx] + K'_m \cdot K'_s \tag{3}$$

or

$$\frac{K_m^*}{[Tx]} = K'_m + \frac{K'_m \cdot K'_s}{[Tx]} \tag{4}$$

Eqn. 4 interprets well the published curves [12], specifically the independence of $K_m^*/[Tx]$ on the Triton *X*-100 concentration at high [Tx] since under these

conditions the K'_s-containing term becomes negligible. Thus a re-analysis of the published data yields about the same K'_s value for the *N. crassa* as for the beef heart complex, but the K'_m value for *N. crassa* complex III amounts to only 0.005.

The kinetic analysis of the electron flow from ubiquinol to cytochrome *c* so far does neither include the involvement of cytochrome *c*, nor the scalar proton release reaction taking place when the enzyme is robbed of its anisotropic orientation in the membrane.

A reembedding of the complex into its natural environment, namely into a phospholipid bilayer of a phospholipid vesicle is under study. It should be mentioned however, that under these conditions the surface dilution scheme is not applicable. Therefore a quantitative analysis of the enzyme kinetics becomes much more complicated.

Nevertheless, the described system opens new perspectives in the study of molecular mechanisms involved in electron transfer reactions of respiratory chain complexes. As the enzyme is pure and phospholipid-depleted, kinetic analysis is relatively simple. The present study provides a challenge for further projects revealing the action of substrates, inhibitors and (possibly) effectors acting on the machinery of respiration.

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