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Rapid purification of cytochrome *c* oxidase from *Paracoccus denitrificans*

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ABSTRACT

Two methods are described for the purification of cytochrome *c* oxidase from Triton X-100 extracts of the periplasma membrane of *Paracoccus denitrificans*. The first is a large-scale procedure for the preparation of 100–250 nmol of cytochrome *c* oxidase (10–20 mg) in 1 week. The second is a rapid procedure for isolating up to 25 nmol in 2–3 days. Owing to the high yields given by fast protein liquid chromatography (FPLC) on Mono Q columns, the overall yield is about 20%, whereas the yield in many other previously published procedures does not exceed 10%. The use of FPLC on Mono Q also offers a considerable saving of time.

INTRODUCTION

Oxidative phosphorylation in procaryotes is achieved through a variety of terminal oxidases [1]. Among them, cytochrome *o*(*bo*) appears to be the most widespread enzyme [2]; the best studied respiratory components, however, are the aa_3-type oxidases [1]. The first procedures for the isolation of this type of oxidase were described by Yamanaka and Fukumori [3], Ludwig and Schatz [4] and Fee *et al.* [5]. Since then, procedures for the isolation of aa_3-type oxidases from a large number of prokaryotes have been described [6–8]. All these procedures have in common that they are time consuming and that the yields are low. Therefore, in an attempt to increase the overall yield and to decrease the time requirement, we developed a procedure based on fast protein liquid chromatography (FPLC) on Mono Q columns. Two methods will be discussed: a large-scale procedure for the isolation of 100–250 nmol and a more rapid, small-scale procedure for amounts up to 25 nmol.

EXPERIMENTAL

Cells of *Paracoccus denitrificans* (ATCC 13543) are grown in large-scale fermenters (100–3000 l) at the Gesellschaft für Biotechnologische Forschung (Braunschweig, F.R.G.) with succininic acid as carbon source. After collection of the cells, the biomass is frozen at −70°C.

Membranes of *Paracoccus denitrificans* are prepared essentially as described in

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ref. 4. After thawing of 1 kg of wet biomass, the suspension obtained is diluted to about 2 l with 10 m*M* potassium phosphate (pH 7.4) and homogenized in a Dyno-Mill, using 0.3-mm glas beads and a rotation speed of 4500 rpm. The temperature is kept as low as possible, in any case below 10°C. The cell suspension is passed twice through the homogenizer and whole cells are sedimented by a 30-min centrifugation at 5000 rpm (Beckman J2-21 with JA-20 rotor). The supernatant is then centrifuged overnight to sediment the crude membranes at 8000 rpm and 4°C[a]. The supernatant of this centrifugation step is checked for haeme a and protein contents. About 10% of the total haeme a is lost in this step. As the haeme a-to-protein (h/p) ratio of this solution is below 0.1 nmol/mg, this fraction is discarded. The membrane is then pre-extracted with deoxycholate and KCl (final concentrations: 0.1% deoxycholate and 0.2 *M* KCl). After a 1-h stirring period, the suspension is centrifuged overnight at 8000 rpm[a]. The supernatant is checked again for haeme a and usually discarded. Only 2–3% of the haeme a is lost in this step. The membrane suspension is frozen in 100-ml portions.

Preparation of the Triton X-100 extract

Depending on the desired procedure, one or more portions of membrane suspension are thawed. The intrinsic membrane proteins are solubilized by addition of Triton X-100–potassium phosphate (pH 7.8)–EDTA to the final concentrations of 2%, 0.1 *M* and 0.1 m*M*, respectively. The suspension is homogenized in a Potter–Elvehjem homogenizer and centrifuged for 1 h using a Beckman JA-10 rotor. The supernatant is saved and the pellet is resuspended in 2% Triton X-100–0.1 *M* potassium phosphate (pH 7.8)–0.1 m*M* EDTA and treated as above. The combined supernatants are characterized and concentrated by ultrafiltration.

Large-scale protocol

About 100 ml of Triton X-100 extract, corresponding to *ca.* 2000 nmol of haeme a and *ca.* 6000 mg of protein, are applied to an Ultrogel AcA-34 column (90 × 10 cm I.D.) and chromatographed in 50 m*M* potassium phosphate (pH 7.6)–0.2% Triton X-100–20 m*M* KCl–0.1 m*M* EDTA. The eluate is monitored at 280 and 420 nm; the haeme a-to-protein (h/p) ratio of selected fractions are determined. Fractions in which the h/p ratio is clearly higher than the initial value are pooled for further purification. This solution is concentrated and freed from salts by ultrafiltration and applied to a Q-Sepharose column (20 × 5 cm I.D.), equilibrated with 10 m*M* potassium phosphate (pH 7.8)–1% Triton X-100–0.1 m*M* EDTA. A smaller part of the proteins, mostly cytochrome *b*, is not bound and appears in the effluent. All cytochromes with reduced minus oxidized difference absorption bands at 550, 590 and 605 nm are firmly bound to the anion exchanger under these conditions. Proteins are eluted by a linear gradient from 0 to 1 *M* NaCl. Again, the eluate is monitored at 280 and 420 nm, and fractions are checked for their specific h/p ratios. Fractions with twice the initial h/p ratio are pooled and used for further purification on Mono Q.

[a] The centrifugation time can be reduced considerably when the preparation is performed on a smaller scale and ultracentrifuges are used.

Small-scale protocol

A 20-ml volume of Triton X-100 membrane extract containing *ca.* 200 nmol of haeme a and having an h/p ratio of *ca.* 0.3 nmol/mg are applied to a small Ultrogel AcA-34 column (30 × 4 cm I.D.) and chromatographed overnight in the same buffer as in the large-scale procedure. Fractions with a substantially improved h/p ratio are used for further purification steps on Mono Q. The crude cytochrome *c* oxidase solution is applied in two portions of about 60 nmol of haeme a to a Mono Q HR 10/10 column. In two consecutive runs cytochrome *c* oxidase is further purified and the cytochrome *c* oxidase fractions are again pooled and used for two or three subsequent runs on Mono Q.

Fast Protein Liquid Chromatography

FPLC is performed on a Mono Q HR 10/10 column (Pharmacia). This column is coupled to an LKB 2150 GTI HPLC pump, an LKB 2152 controller, a LKB 2158 Uvicord SD monitor with a titanium flow cell, an LKB 2212 Helirac fraction collector and an electromagnetic valve to form the gradient. The buffers used in these FPLC experiments were (A) 25 m*M* potassium phosphate (pH 7.8)–1% Triton X-100–0.1 m*M* EDTA and (B) A containing 1 mol/l NaCl. In some experiments potassium phosphate was replaced with 20 m*M* Tris–HCl (pH 7.8) in order to allow determinations of phophorus by inductively coupled plasma atomic emission spectrometry (ICP-AES). The Mono Q column was washed after every 4–8 runs according to the specifications of the manufacturer (Pharmacia). Prior to a series of runs on Mono Q, the crude cytochrome *c* oxidase solutions were filtered through a 0.2-μm filter (Sartorius 11107 25 N).

Ultrafiltration

This was carried out using a Minitan tangential flow ultrafiltration system (Millipore) with membranes having a cut-off of 30 kilodalton (Minitan PTTK, Millipore).

Haeme a

Haeme a was determined spectrophotometrically according to ref. 9. Haeme a concentrations were calculated using a molar absorption coefficient of 12000 l/mol · cm for the difference absorbance at 605 nm between dithionite-reduced and hexacyanoferrate(III)-oxidized cytochrome *c* oxidase. This value has been adopted from bovine heart cytochrome *c* oxidase [9] and has so far given satisfactory results. Cytochromes *b*, *c* and a_1 were identified by their reduced minus oxidized difference absorbance bands at 560, 550 and 590 nm, respectively [1].

Protein contents

Protein contents were determined with the bicinchoninic acid assay (BCA) of Pierce [10].

RESULTS

Two methods are described for the purification of cytochrome *c* oxidase from *Paracoccus denitrificans*. The use of these procedures is not restricted to the isolation of cytochrome *c* oxidase but may also be used for the purification of other intrinsic

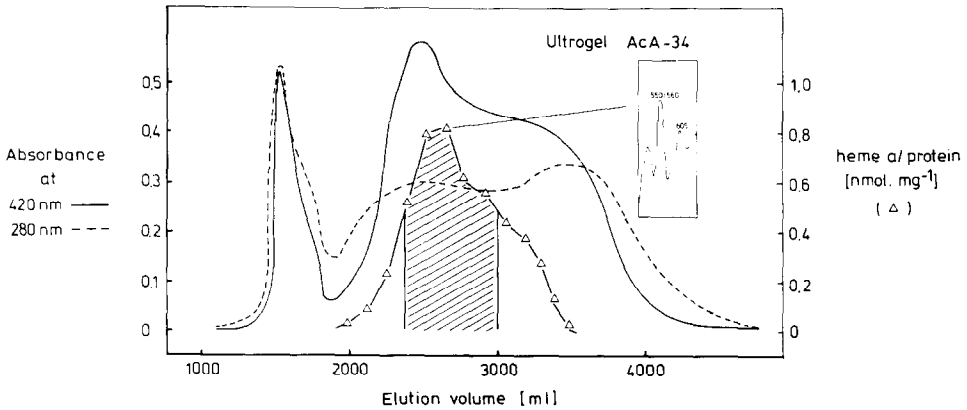

Fig. 1. Gel chromatography of Triton X-100 membrane extract of *Paracoccus denitrificans* on Ultrogel AcA-34. The fractions corresponding to the shaded area were used for subsequent purification on Q-Sepharose.

membrane proteins, such as cytochromes *b*, *c*, *o*(*bo*) and a_1. We have already successfully purified cytochrome *c* oxidase (caa_3) from *Thermus thermophilus* by the same procedure [11].

Large-scale purification

The large-scale procedure starts with the solubilization by Triton X-100 of an appropriate sample of membrane suspension, usually 200–300 ml, as described under Experimental. The h/p ratios of these membrane extracts vary from 0.2 to 0.6 nmol haeme a/mg. Fig. 1 shows the result of the first chromatography of concentrated membrane extract on Ultrogel AcA-34. As shown in Table I, this procedure leads to a loss of about 45% in terms of haeme a.

TABLE I

LARGE-SCALE PURIFICATION OF CYTOCHROME *c* OXIDASE FROM *PARACOCCUS DENITRIFICANS*

Step	Haeme a (nmol)[a]	Protein (mg)	Haeme a/protein (nmol/mg)	Yield (%)[b]	Overall yield (%)
Triton X-100 membrane extract	2.100	6.550	0.3	100	100
Ultrogel AcA-34	1.200	1.714	0.7	57	57
Q-Sepharose	715	477	1.5	60	34
	150	100[c]	1.5		
Mono Q (run a)	123	27	4.6	82	28
Mono Q (run b)	111	8.6	12.9	90	25
Mono Q (run c)	98	4.7	20.8	88	22

[a] 2 nmol of haeme a correspond to 1 nmol of cytochrome *c* oxidase.

[b] Yield as a percentage of haeme a in Triton X-100 extract.

[c] As the binding capacity of Mono Q HR 10/10 is limited, only 100-mg portions are used for further purification.

The fractions corresponding to the shaded area are pooled and concentrated by ultrafiltration. The salt concentration is reduced by alternating ultrafiltration and dilution steps prior to the next purification step on Q-Sepharose. When the specific conductivity of the sample has attained the value of the starting buffer [25 mM potassium phosphate (pH 7.8)–1% Triton X-100–0.1 m*M* EDTA], the material is bound to the anion exchanger and, after washing with 300 ml of starting buffer, eluted with a linear gradient from 0 to 1 *M* NaCl in the same buffer. The result of this experiment is shown in Fig. 2. The resolution in this step is already much improved in comparison with the gel chromatographic step on Ultrogel AcA-34. As judged from the reduced minus oxidized difference spectra (see insets in Fig. 2), several discrete fractions are observed, including cytochrome *b* or *o*(*bo*) (560 nm), cytochrome a_1 (590 nm), cytochrome *c* (550 nm) and cytochrome *a* (605 nm). After characterization, the fractions are pooled according to their specific h/p ratios. Again, a substantial loss is observed and the overall yield drops to 36% (Table I).

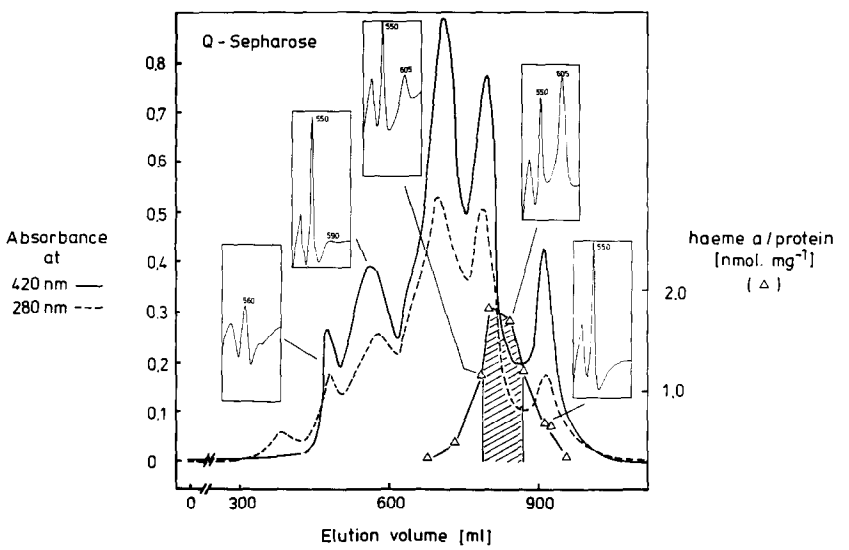

Fig. 2. Ion-exchange chromatography on Q-Sepharose. The reduced minus oxidized difference absorbance spectra in the insets indentify the cytochrome components (see Results). The material corresponding to the shaded area was used for further purification by FPLC on Mono Q.

The pooled fractions from the Q-Sepharose chromatography with an h/p ratio of 1.5 nmol/mg (Fig. 2 and Table I) are concentrated and dialysed by ultrafiltration and, in portions of 100 mg, corresponding to 150 nmol of haeme a, bound to the Mono Q column. Under the conditions used, all the protein material binds to the matrix. In the first Mono Q run (Fig. 3a), the gradient used is 0–5 min 0% B, 5–15 min 0–20% B, 15–65 min 20–60% B, 65–75 min 60–100% B, 75 min 100% B, 75–80 min 100–0% B and 80–90 min 0% B, at a flow-rate of 2 ml/min; in the subsequent runs (Fig. 3b and c) the same gradient is used.

In three consecutive Mono Q runs, which can be performed in the course of one day, pure cytochrome *c* oxidase (h/p ratio *ca.* 22 nmol/mg) is obtained. The molecular

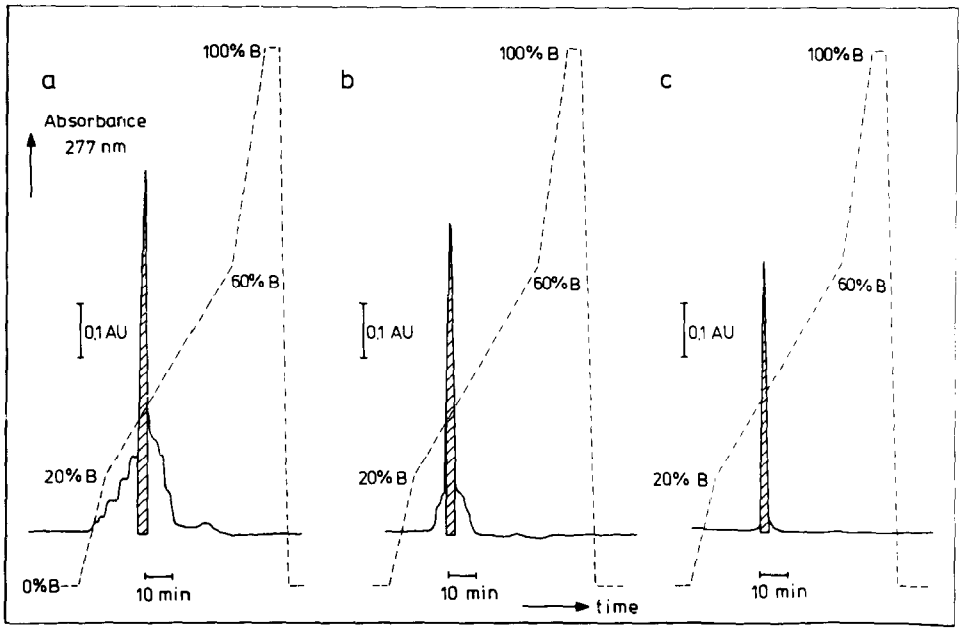

Fig. 3. Series of consecutive chromatographic steps on Mono Q. In experiment (a) the crude product from the Q-Sepharose with an h/p ratio of 1.5 nmol/mg is further purified; (b) and (c) show the chromatograms (absorbance monitored at 277 nm) of the subsequent experiments on Mono Q. The material corresponding to the shaded area was used for further purification and/or final characterization.

mass of the two-subunits complex is, on the basis of the sequence, calculated to be 90 kilodalton [12,13]; the theoretical h/p ratio is then 22.2 nmol/mg. The step-by-step yields of the Mono Q runs are considerably higher than those in the two preceding purification steps, ranging from 82 to 90%. Thus, according to this method, cytochrome *c* oxidase from *Paracoccus denitrificans* can be obtained in relatively high yields and with a substantial saving of time.

A possible explanation for the low purification rate and the low yield in the initial step on Ultrogel AcA-34 may be that cytochrome *c* oxidase, at least in part, shares the same micelles with other contaminating membrane proteins. Thus, in the early phase of the preparation, cytochrome *c* oxidase is eluted together with contaminating membrane proteins, giving rise to the observed low purification rate and low yield. Apparently, the strong binding of the proteins to the anion-exchange groups of both Q-Sepharose and Mono Q causes a more differentiated partition of the various membrane proteins over the micelles, leading to increased resolution between cytochrome *c* oxidase and contaminating proteins.

Small-scale purification

For the small-scale purification, *ca.* 20 ml of Triton X-100 membrane extract with *ca.* 200 nmol of haeme a and an h/p ratio of 0.3 nmol/mg is applied to a smaller Ultrogel AcA-34 column and chromatographed overnight. Fractions are monitored and checked for their individual h/p ratios. They are then pooled as described for the

TABLE II

RAPID, SMALL-SCALE PURIFICATION OF CYTOCHROME *c* OXIDASE FROM *PARACOCCUS DENITRIFICANS*

Step	Haeme a (nmol)	Protein (mg)	Haeme a/protein (nmol/mg)	Yield[a] (%)	Overall yield (%)
Triton X-100 membrane extract	200	670	0.3	100	100
AcA-34	118	197	0.6	59	59
Mono Q (run a)[b]	90	69	1.3	76	45
Mono Q (run b)	72	16	4.5	80	36
Mono Q (run c)[c]	51	3.9	13.1	71	25
Mono Q (run d)	35	1.6	21.9	69	17

[a] Yield as a percentage of haeme a in Triton X-100 extract.
[b] In two runs.
[c] Not shown in Fig. 4.

large-scale protocol. In order to speed up the purification procudure, the Q-sepharose chromatograhic steps are omitted in the small-scale protocol. Only fractions with substantially improved h/p ratio are used for subsequent purification steps on Mono Q. Thus, *ca.* 120 nmol of haeme a with an h/p ratio of *ca.* 0.7 nmol/mg is, after dialysis, applied to the Mono Q column (Table II). This crude cytochrome *c* oxidase solution still contains cytochrome *b*, most of which does not bind to the Mono Q column under these conditions and thus appears in the effluent together with the excess of Triton

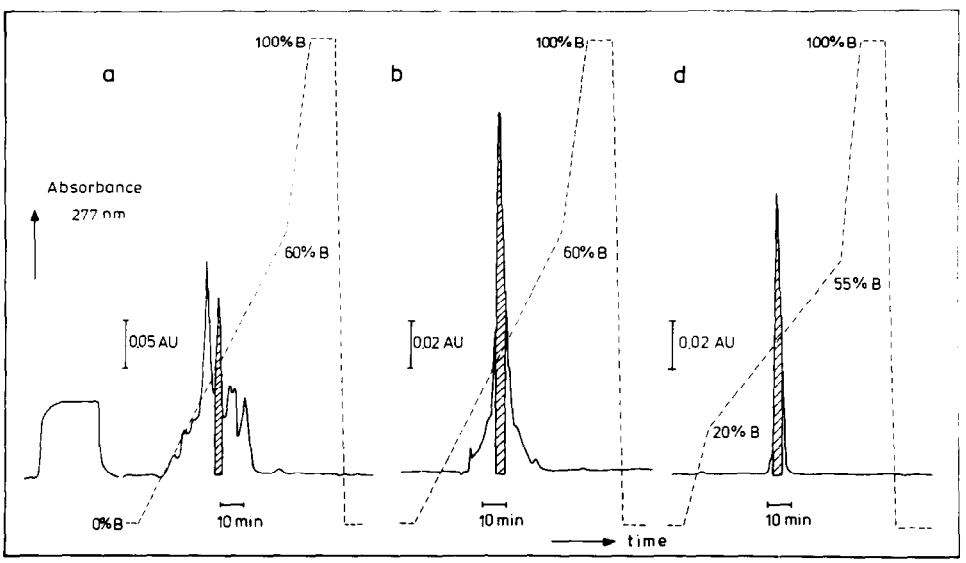

Fig. 4. Series of consecutive chromatographic steps on Mono Q. In (a) the crude product of the small-scale AcA-34 chromatographic step (not shown) with an h/p ratio of 0.6 nmol/mg is further purified; (b) and (d) show the chromatograms (absorbance monitored at 277 nm) of the subsequent experiments on Mono Q. The chromatogram of experiment (c) (see Table II) is omitted. The material corresponding to the shaded area was used for further purification and/or final characterization.

X-100 accumulated during the ultrafiltration process. The cytochrome *b* and the excess of Triton X-100 cause a shift of the baseline on loading of the Mono Q column (Fig. 4a). In the first experiment the membrane proteins are eluted with the following gradient: 0–5 min 0% B, 5–65 min 0–60% B, 65–70 min 60–100% B, 70–80 min 100% B, 80–85 min 100–0% B and 85–95 min 0% B, at a flow-rate of 2 ml/min. The second peak was identified by spectral investigation as cytochrome *c* oxidase. At least three other membrane-bound cytochrome *c* components are observed in this Mono Q run. They were, however, not investigated further. The yield amounts to 76% and a substantial purification result is obtained (Tabel II). The cytochrome *c* oxidase fraction (shaded area in Fig. 4a) is applied to the Mono Q column and rechromatographed under the same conditions (Fig. 4b). Again, a good yield is observed and the h/p ratio has increased to 4.5 nmol/mg. The next two chromatographic steps in which less steep gradients (0–5 min 0%B, 5–15 min 0–20% B, 16–65 min 20–55% B, 65–70 min 55–100% B, 70–80 min 100% B, 80–85 min 100–0% B and 85–150 min 0% B) are used, lead to pure cytochrome *c* oxidase with an h/p ratio of 22 nmol haeme a/mg.

Some characteristics of the purified cytochrome c oxidase

The purity of the membranous enzyme was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) [10]. In agreement with the results of Ludwig and Schatz [4], two bands are observed, corresponding to proteins with apparent molecular weights of $45 \cdot 10^3$ and $28 \cdot 10^3$ dalton. However, recently, Haltia *et al.* [14] have shown that cytochrome *c* oxidase from *Paracoccus denitrificans* can be isolated as a three-subunit complex by using detergents other than Triton X-100. This complex is far less stable than the two-subunit complex and therefore not suitable for the investigation of structure–function relationships. The genes for these three proteins have been cloned and sequenced [12,13].

Using ICP-AES, a technique which allows the simultaneous determination of a large number of elements, we could show that cytochrome *c* oxidase from *Paracoccus denitrificans*, as its mitochrondrial counterpart from bovine heart, is characterized by the stoichiometric presence of three copper ions per two haeme irons [15]. The presence of three copper ions per two haemes thus appears to be a general property of all aa_3-type cytochrome *c* oxidases, Whereas the Zn and Mg stoichiometries in bovine heart cytochrome *c* oxidase preparations are close to unity and vary only slightly from preparation to preparation, the same elements very considerably in the *Paracoccus* enzyme. In the absence of phosphate (use of Tris–HCl buffers), the stoichiometry of phosphorus in these preparations can be determined by ICP-AES and directly correlated with the number of bound phospholipids: a varying number from one to five phospholipid molecules remain bound in these preparations [15]. Thus, our preparations, although homogeneous in SDS-PAGE and pure in terms of its h/p ratio, clearly show inhomogeneities with respect to the bound metals and bound phospholipids. Future research will focus on the elimination of these inhomogeneities.

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