Biochimica et Biophysica Acta 808 (1985) 219–224
Elsevier

BBA 41778

Preparation of reconstituted cytochrome oxidase vesicles with defined trans-membrane protein orientations employing a cytochrome *c* affinity column

Thomas D. Madden and Pieter R. Cullis

Department of Biochemistry, The University of British Columbia, Vancouver, British Columbia, V6T 1W5 (Canada)

(Received January 29th, 1985)

Key words: Cytochrome oxidase; Membrane reconstitution; Protein orientation; Affinity chromatography; Cytochrome *c*

Reconstituted cytochrome oxidase systems in which the majority of the vesicles contain a single oxidase dimer can be prepared. It is shown that, when these are passed through a cytochrome *c* affinity column, only those vesicles oriented outwards (such that the active site is available to external cytochrome *c*) are bound to the support matrix. Protein-free vesicles and vesicles containing an inwardly oriented enzyme are eluted in the void volume. Subsequently, vesicles containing an outwardly oriented enzyme can be eluted from the column at high salt concentrations. This protocol has been used successfully to resolve vesicles of either oxidase orientation when the enzyme is reconstituted with a variety of lipid mixtures. The recovery of oxidase activity from the column ranged between 75 and 94%.

Introduction

Cytochrome *c* oxidase, the terminal component of the mitochondrial electron-transport chain, is one of the best characterized integral membrane proteins. It is a *Y*-shaped multisubunit enzyme where the two arms of the *Y* span the inner mitochondrial membrane, and the stalk (which contains the cytochrome *c* binding site) extends from the cytoplasmic side [1]. Following detergent solubilization, it can be purified and reconstituted into vesicles of a defined lipid composition.

While initially, such reconstituted systems were claimed to be asymmetric with all of the oxidase molecules oriented with their cytochrome *c* binding sites facing outwards [2], later workers reported a random orientation based on heme reduction experiments [3]. More recently, several groups have reported values for the percentage of outwardly oriented enzyme ranging from 55% to 85% [4–6]. Such a random orientation complicates defined subunit labelling of the protein or studies on the position of the heme moieties or studies of vectorial transport. Recently, however, we demonstrated that when oxidase is reconstituted at very high lipid-to-protein ratios, the resultant vesicles contain predominantly a single oxidase dimer [7]. When these reconstituted membranes are applied to a DEAE column, vesicles containing inwardly oriented cytochrome oxidase are eluted in the void volume, together with protein-free vesicles, while those containing outwardly oriented oxidase are retarded on the column [7,8]. The protein orientation of these two vesicle preparations was recently confirmed by proteolytic digestion of specific subunits [9]. It is likely that the ability of DEAE chromatography to resolve between reconstituted oxidase vesicles with different protein

Abbreviations: DOPC, dioleoylphosphatidylcholine; PS, phosphatidylserine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TMPD, *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine dihydrochloride; DPPC, dipalmitoylphosphatidylcholine.

transmembrane orientations relies on the interaction between the resin and negatively charged groups present on the large polar region on the C-side of the oxidase molecule. In turn, this would suggest that if the reconstituted vesicles were prepared containing negatively charged lipids, the ability of DEAE chromatography to resolve between the two protein orientations might be impaired. We show in this paper that this is indeed the case and have therefore adapted a method utilizing a cytochrome *c* affinity column originally developed by Bill et al. [10] to separate vesicles containing outwardly oriented oxidase (such that the enzyme can interact with external cytochrome *c*) from protein-free and inwardly oriented oxidase vesicles.

Materials and Methods

Materials

Cytochrome *c* (horse heart, type VI), cytochrome *c* (*Saccharomyces cerevisiae*, type VIII), cardiolipin (bovine heart, sodium salt), sodium cholate, Tween 80 and asorbic acid were obtained from Sigma. Activated thiol-Sepharose 4B-CL was purchased from Pharmacia, while dithiothreitol was obtained from BioRad. ^{3}H-dipalmitoylphosphatidylcholine (^{3}H-DPPC) was purchased from Amersham.

Dioleoylphosphatidylcholine (DOPC) and egg phosphatidylserine (PS) were synthesized as described previously [11,12], and were at least 99% pure as determined by thin layer chromatography.

Preparation of cytochrome oxidase vesicles

Cytochrome *c* oxidase was prepared by the method of Kuboyama et al. [13]. The oxidase was finally resuspended in 1% Tween 80, and stored in liquid nitrogen at a protein concentration of approx. 50 mg/ml.

For reconstitution experiments 15 mg of phospholipid and 7.5 mg of cholate were hydrated in 1 ml of 150 mM sucrose, 75 mM potassium phosphate (pH 7.4). Unilamellar vesicles were then prepared according to the LUVET procedure [14] as described previously [8]. Cytochrome oxidase (100 μg) was added and the mixture dialyzed (Spectrapor 2, 10 mm diameter dialysis tubing) for 24 h against three changes of 400 vol. 150 mM sucrose/75 mM potassium phosphate (pH 7.4). Prior to application of the vesicles to the cytochrome *c* affinity column, they were dialyzed for 2 h against 250 mM sucrose/25 mM potassium phosphate (pH 7.4).

Preparation of cytochrome c affinity column

Cytochrome *c* from *Saccharomyces cerevisiae* was coupled to activated thiol Sepharose 4B-CL by a modification of the method of Bill et al. [10]. Cytochrome *c* (25 mg) was first reduced overnight in 1 ml 100 mM NaCl/1 mM EDTA/20 mM dithiothreitol/50 mM Tris-HCl (pH 7.4). The protein was then passed down a column (1.2 × 8 cm) of Sephadex G-25 (fine) equilibrated with 100 mM NaCl/1 mM EDTA/50 mM Tris-HCl (pH 7.4) to remove dithiothreitol. Washed activated thiol Sepharose 4B-CL (2 g) was equilibrated with the same buffer, and then mixed with the cytochrome *c* in a dialysis bag (25 mm diameter; molecular weight cut-off 3400). The mixture was then dialyzed against 1 l 100 mM NaCl/1 mM EDTA/50 mM Tris-HCl (pH 7.4) for 24 h at 4°C with one change of buffer. The solution was stirred vigorously to ensure that the Sepharose remained dispersed within the dialysis tubing. After 24 h, a further 25 mg of reduced cytochrome *c* was added, and the dialysis continued with fresh buffer for 24 h. The coupling efficiency was greater than 95%.

Separation of reconstituted vesicles on cytochrome c affinity column

Prior to the application of the vesicles to the affinity column (0.9 × 10 cm), the bound cytochrome *c* was reduced with 4 ml 40 mM ascorbate/50 mM potassium phosphate (pH 7.4). The column was then washed with 5 ml 100 mM potassium phosphate (pH 7.4) and equilibrated with 250 mM sucrose/25 mM potassium phosphate (pH 7.4). The reconstituted oxidase vesicles were eluted with the same buffer at a flow rate of 2–4 ml · h^{-1} and bound vesicles were then eluted with 200 mM potassium phosphate (pH 7.4).

Assay procedures

Cytochrome oxidase activity was measured spectrophotometrically by following the oxidation of ferrocytochrome *c* (bovine heart, type VI) as described previously [6]. The orientation of the re-

constituted oxidase was determined by the method of Nicholls et al. [3]. Vesicles were allowed to go anaerobic in the presence of 2.5 μM cytochrome *c*/10 mM ascorbate/1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP)/0.4 μM valinomycin. The extent of reduction of the heme groups (605–630 nm) was then compared to the fully reduced state upon addition of 250 μM *N*, *N*, *N'*, *N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD). In some experiments sodium cyanide (1 mM) was added to prevent any re-oxidation of the heme groups [5].

Results

When cytochrome oxidase is reconstituted at very high lipid-to-protein ratios ($\geq$ 50000 : 1, molar ratio) the resultant vesicles are either protein-free or contain predominantly only one oxidase [7]. We have shown previously that if this mixture is chromatographed on DEAE-Sephacel, protein-free vesicles and those containing inwardly oriented oxidase (such that the cytochrome *c* binding site is on the interior of the vesicle) are eluted in the void volume, while vesicles containing an outwardly oriented enzyme are retarded [7]. The ability of DEAE chromatography to resolve these populations presumably relies on the negatively charged groups on the larger polar region of the protein interacting with the resin. It might be predicted therefore that when negatively charged lipids are present in the vesicles, the resolution obtained between these populations might be impaired. That this is the case is illustrated in Fig. 1 which shows the elution profile on DEAE Sephacel of reconstituted oxidase vesicles composed of DOPC/EPS (10 : 1). The main vesicle peak where the majority of the vesicles is protein-free) is broad and overlaps the elution peak containing oxidase activity.

To overcome the limitations of the DEAE procedure, we developed a cytochrome *c* affinity column technique which would allow vesicles with external cytochrome *c* binding sites to interact with the immobilized ligand, thus permitting their separation from protein-free vesicles and those in which the oxidase is oriented facing inwards. Cytochrome *c* from *Saccharomyces cerevisiae* contains a free sulfhydryl group on a cysteine residue close to the N-terminus. Coupling of the protein to

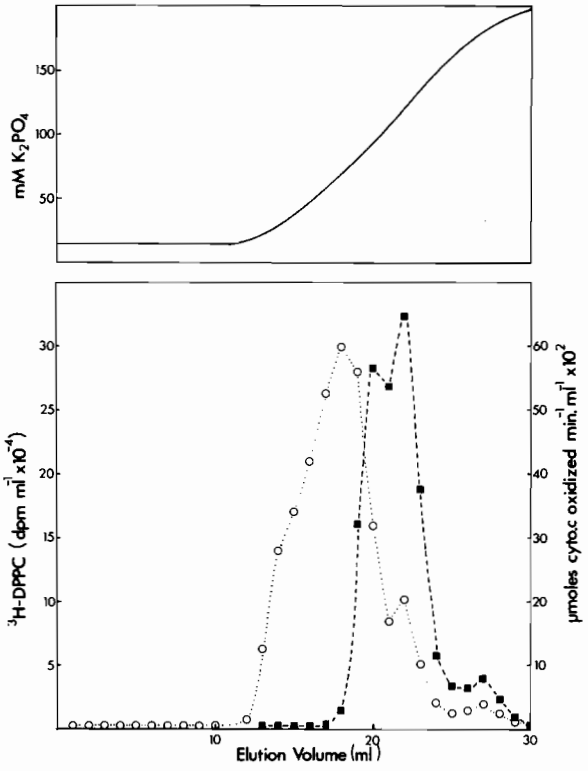

Fig. 1. Elution profile on DEAE-Sephacel of reconstituted cytochrome oxidase vesicles composed of DOPC/egg PS (10 : 1). The elution of ^{3}H-DPPC (○ · · · ○) was used as a marker for all vesicles, and oxidase activity (□--□) as an indication of vesicles containing outwardly oriented enzyme. The ionic strength of the elution medium was increased from 20 mM to 200 mM potassium phosphate (pH 8.3).

Sepharose 4B via this group is preferable to using CNBr-activated Sepharose which probably cross-links to lysine residues necessary for oxidase binding [15]. The elution profile of oxidase reconstituted with DOPC on the cytochrome *c* affinity column is shown in Fig. 2. The major vesicle peak is eluted in the void volume, but shows virtually no oxidase activity. Vesicles containing outwardly oriented cytochrome oxidase are bound to the column, and are eluted with 200 mM potassium phosphate (pH 7.4).

That some of the vesicles eluted in the void volume contain inwardly oriented oxidase was demonstrated by addition of the detergent octyl β-D-glucopyranoside (Fig. 3). Upon solubilization of the vesicles latent oxidase activity is observed in agreement with previous results [8].

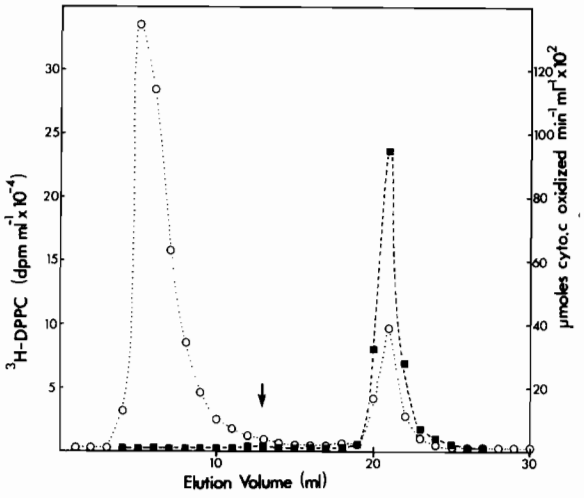

Fig. 2. Elution profile of cytochrome oxidase reconstituted with DOPC on a cytochrome *c* affinity column. The elution of ^{3}H-DPPC (○ · · · ○) and oxidase activity (□---□) is shown. The arrow indicates the point at which 200 mM potassium phosphate (pH 7.4) was applied to the column. The recovery of vesicles (^{3}H-DPPC) was 99% and recovery of oxidase activity, 75%.

Resolution between the different vesicle populations can also be achieved when cytochrome oxidase is reconstituted with a mixture containing negatively charged lipids. In Fig. 4 the elution profiles are shown for oxidase reconstituted with DOPC/egg PS (10 : 1) and DOPC/cardiolipin

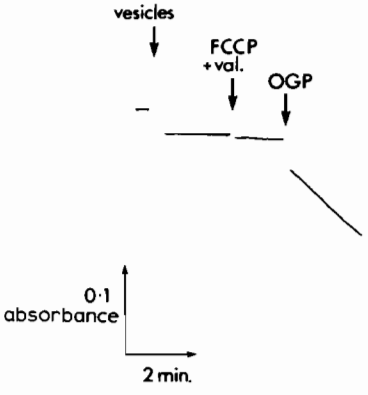

Fig. 3. Spectrophotometric assay of cytochrome oxidase activity. Vesicles eluted in the void volume on the cytochrome *c* affinity column as shown in Fig. 2 were assayed for enzyme activity. The assay cuvette contained 30 μM ferrocytochrome *c* in 50 mM potassium phosphate (pH 7.4) at 25°C. Valinomycin and FCCP were added to a final concentration of 0.4 μM and 1 μM, respectively and octyl B-D-glucopyranoside (OGP) to a concentration of 25 mM.

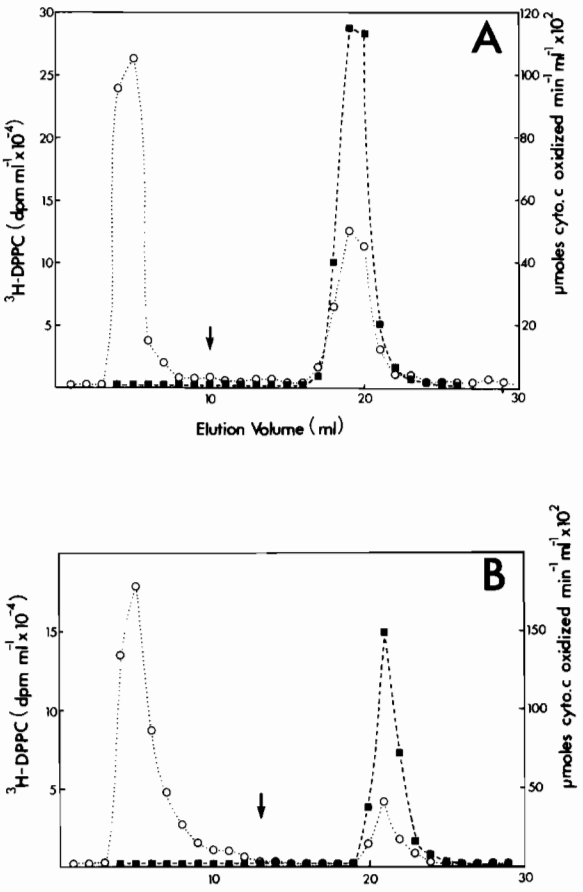

Fig. 4. Elution profiles of reconstituted cytochrome oxidase vesicles containing acidic phospholipids on cytochrome *c* affinity column. (A) The elution of ^{3}H-DPPC (○ · · · ○) and oxidase activity (□---□) is shown for vesicles composed for DOPC/egg PS (10 : 1). The recoveries of vesicles (^{3}H-DPPC) and enzyme activity were 98% and 94%, respectively. (B) The elution of ^{3}H-DPPC (○ · · · ○) and oxidase activity (□---□) is shown for vesicles composed of DOPC/cardiolipin (20 : 1). The recoveries of vesicles (^{3}H-DPPC) and enzyme activity were 99% and 92%, respectively. In both profiles the arrow indicates the point at which 200 mM potassium phosphate was applied to the column.

(20 : 1). When negatively charged lipids are present some interaction between the protein-free vesicles and the affinity column was observed. This is presumably due to the positive lysine residues on the cytochrome *c*. The flow rate of the column was adjusted up to 4 ml · h^{-1} to bring off vesicles which were not attached via outwardly oriented oxidase molecules and 200 mM potassium phosphate (pH 7.4), then applied to elute these oxidase containing vesicles. The recovery of oxidase activ-

TABLE I
ORIENTATION OF RECONSTITUTED CYTOCHROME OXIDASE

Sample	Lipid composition	Orientation of oxidase	
		% facing out	% facing in
Mixture prior to separation on affinity column	DOPC	58	42
Vesicles eluted with 200 mM K_2PO_4	DOPC	98	2
Vesicles eluted with 200 mM K_2PO_4	DOPC/egg PS (10:1)	94	6
Vesicles eluted with 200 mM K_2PO_4	DOPC/cardiolipin (20:1)	92	8

ity from the column varied between 75 and 94%.

Freeze-fracture electron microscopy was used to determine the size of reconstituted vesicles prepared by the LUVET procedure [16]. A fairly homogeneous distribution was observed with mean diameters of 35–45 nm dependent upon the lipid composition. Preliminary experiments indicated that when larger reconstituted vesicles of DOPC were prepared by the sonication procedure [6], average diameter 65 nm, a substantial proportion of the outwardly-oriented oxidase vesicles did not adhere to the affinity column and were eluted in the void volume.

The spectroscopic procedure of Nicholls et al. [3] was used to quantitate the orientation of the oxidase. Prior to passage of the reconstituted vesicles through the affinity column approx. 60% of the oxidase molecules were oriented facing outwards, and 40% facing inwards as shown in Table 1. However, the vesicles eluted from the column with high salt concentrations show an asymmetric orientation with up to 98% of the oxidase molecules oriented outwards. The small percentage of inwardly oriented oxidase molecules presumably arise from vesicles containing more than one oxidase where one faces out and can bind to the immobilized cytochrome *c*.

Discussion

The complexity of most biological membranes makes it impractical to study individual proteins in situ. Routinely, the protein is solubilized in detergent, purified and then reconstituted with pure phospholipids. While such reconstituted systems have proved extremely useful, they often suffer from one disadvantage. In native membranes the proteins exhibit a defined orientation across the bilayer, whereas in reconstituted vesicles the proteins do not usually exhibit a defined orientation. This random insertion necessarily complicates studies on the structural and functional properties of the enzyme.

Some examples of reconstituted membrane proteins exhibiting an asymmetrical orientation have been reported. Helenius et al. [17] demonstrated that when the spike glycoprotein from Semliki Forest virus was reconstituted into phosphatidylcholine vesicles employing the nonionic detergent octyl β-D-glucopyranoside, vesicles with virtually all (95%) of the protein pointing outwards could be obtained. These authors suggested that this non-random incorporation was the result of protein insertion into preformed membranes. On the basis of electron microscopy Herbette et al. [18] have proposed that reconstituted vesicles containing Ca^{2+}-ATPase from sarcoplasmic reticulum prepared with a molar ratio of lipid/protein of $\geq 90:1$ are predominantly asymmetric with the majority of the Ca^{2+}-ATPase oriented outwards and thus available to substrate added to the external medium. These reconstituted vesicles display Ca^{2+} uptake rates comparable to the native sarcoplasmic reticulum. In addition, when bacteriorhodopsin is reconstituted from deoxycholate with either soybean lipids or its own endogenous lipids, all of the protein carboxy-termini are exposed on the outside of the vesicle, i.e., the proteins have the opposite orientation to that observed in the intact cell membrane [19]. Such asymmetric systems are, however, the exception rather than the rule and most reconstituted proteins exhibit both orientations.

Our approach to the problem of protein asymmetry has been to separate vesicles of random orientation on the basis of charged group dif-

ferences [6] or as detailed here by binding to the active site via an immobilized ligand. The use of an affinity column has two major advantages over ion-exchange columns. First, negatively charged lipids can be included in the reconstituted vesicles without impairing resolution of the vesicle populations. This is obviously an advantage as it allows lipid mixtures which more closely resemble those found in native membranes to be employed in the reconstitution. Second, while cytochrome oxidase vesicles can be resolved by DEAE chromatography, this is likely due to the fact that the polar region of the protein exposed on the C-side of the mitochondrial membrane (containing the cytochrome *c* binding site) is considerably larger than the polar region on the M-side. However, using an affinity column, any membrane enzyme, irrespective of its trans-membrane geometry, for which an immobilized ligand can be prepared can potentially be resolved into populations in which the active site is inwardly directed or where it faces the exterior of the vesicle.

In summary, the results presented here demonstrate that reconstituted vesicles containing an outwardly oriented oxidase molecule can be bound to a cytochrome *c* affinity column. That such an interaction can retard a moiety of the size of the reconstituted vesicle system is remarkable. This interaction has been used to resolve populations of oxidase vesicles where the protein is oriented facing inwards from those where the cytochrome *c* binding site is oriented facing outward. It is likely that this procedure will be of general utility for the preparation of asymmetric reconstituted vesicles using a variety of membrane enzymes.

Acknowledgements

This work was supported by a grant from the British Columbia Health Care Research Foundation. T.D.M. was the recipient of a Postdoctoral Fellowship from the Medical Research Council of Canada, while P.R.C. is a Medical Research Council of Canada Scientist.

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