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Influence of surface charge on the incorporation and orientation of cytochrome *c* oxidase in liposomes

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Cytochrome *c*-oxidase is usually oriented 80–90% right-side-out when reconstituted with asolectin by the cholate dialysis method. Transformation of positively charged lysine groups at the matrix domain into negatively charged groups with succinic anhydride results in random orientation. A random orientation is also found after reconstitution in phosphatidylcholine, which can be changed into predominant right-side-out orientation by addition of cardiolipin. It is concluded that electrostatic interaction between positively charged groups of cytochrome *c*-oxidase with negative groups of phospholipids determines the asymmetric orientation of the enzyme in liposomes.

Liposome; Cytochrome *c*-oxidase; Orientation; Protein modification; Succinic anhydride; Cardiolipin

1. INTRODUCTION

The amphipathic y-shaped COX complex [1] is located in the inner mitochondrial membrane in an asymmetrical manner. The lower part of the y, containing the binding domain for cytochrome *c*, is oriented to the cytosolic side; the two upper arms to the matrix side of the membrane [1,2]. Reconstitution of the isolated enzyme in liposomes by the cholate dialysis method [3] results in 70–90% right side-out orientation [4]. The reason for the predominant right-side-out orientation is not known. In contrast, reconstitution by the freeze-thaw sonication procedure results in equal distribution of right-side-out and inside-out orientations [5].

Mammalian COX is composed of 13 subunits [6]. From hydropathic plots and the deduced membrane orientation of the 3 mitochondria-encoded subunits [7], and from 6 of the 10 nucleus-encoded subunits [2], the distribution of charged amino acids of COX on both sides of the inner mitochondrial membrane was calculated [8]. The calculation revealed a strong dipole in the membrane penetrating enzyme complex, containing an excess of 15 negative charges at the cytosolic side and 20 positive charges at the matrix side [8].

In the present paper, the influence of charged groups on the surface of COX on its orientation in liposomes after reconstitution by the cholate dialysis method was investigated. The surface charge was modified by lysine-specific reagents. It will be shown that charged groups are essential for incorporation into the membrane and that positive lysine groups on the matrix side determine the dominant right-side-out orientation of the enzyme after reconstitution by the cholate dialysis method.

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Abbreviations: COX, cytochrome *c*-oxidase; CCCP, carbonyl cyanide *m*-chorophenyl-hydrazone; RCR, respiratory control ratio

2. MATERIALS AND METHODS

2.1. *Chemicals*

L-α-Phosphatidylcholine (type II, from soybean, asolectin) and cytochrome *c* (type VI, from horse heart) were obtained from Sigma; valinomycin and CCCP from Boehringer, dodecyl-β-D-maltoside (laurylmaltoside) form Calbiochem. Na-cholate and succinic acid anhydride were purchased from Serva; acetic anhydride from Fluka. All other reagents were of the highest purity grade commercially available.

2.2. *Isolation of COX and preparation of COX vesicles*

COX was prepared from beef heart mitochondria as described by Errede et al. [9]. The enzyme was reconstituted into liposomes by the cholate dialysis method [3].

2.3. *Modification of COX with succinic acid anhydride and acetic anhydride*

30 μM COX was incubated with 0- 25 mM succinic acid anhydride or acetic anhydride in 100 mM K-Hepes, pH 8.5, and 0.0225% laurylmaltoside by addition of different amounts of a 0.5 M stock solution of anhydride in DMSO. Before the treatment, the corresponding acid and DMSO were added to the samples in order to obtain the same final concentration of DMSO and the corresponding acid after hydrolysis of the anhydride in each sample. After 1 h incubation, the samples were diluted with a sonicated asolectin solution to obtain the final concentrations of 3 μM COX and 40 mg asolec-

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tin/ml in 100 mM-K-Hepes, and 1.5% Na-cholate, pH 7.5, which was used for preparation of COX vesicles.

2.4. *Activity measurements*

COX activity was measured polarographically with a Clark-type electrode according to Ferguson-Miller et al. [10] in 10 mM K-Hepes, pH 7.4, 40 mM KCL, 0.1 mM EDTA, 25 mM ascorbate, 0.02 μM COX, and 50 μM cytochrome *c* in the absence and presence of 1 μg/ml valinomycin and 3 μM CCCP at 25°C.

2.5. *Measurement of orientation of COX in the vesicles*

The orientation of COX in the vesicles was calculated from the reduced spectrum obtained by impermeant (ascorbate + cytochrome) and permeant (TMPD) reducing agents in the presence of cyanide, as described by Casey et al. [11],

Table 1

The influence of modification of COX vesicles with succinic acid anhydride on the re-reconstitution after solubilization of the modified COX vesicles with cholate

	Right-side-out	Inside-out
Control	73%	27%
10 mM succinic acid anhydride	71%	29%
20 mM succinic acid anhydride	78%	22%

3 μM of reconstituted COX in 100 mM K-Hepes, pH 7.6, were treated with 10 and 20 mM succinic acid anhydride, as described in section 2. For solubilization, the modified COX vesicles were incubated for 1 h with cholate (final concentration 3%) and then re-reconstituted by the cholate dialysis method

3. RESULTS

The surface charge of isolated bovine heart was modified by incubation with succinic anhydride, a lysine-specific reagent [12]. Succinic anhydride converts positively charged lysine amino groups into negatively charged groups. After reconstitution in liposomes, the amount of incorporated enzyme and its orientation was measured. Modification of COX by succinic anhydride decreases the respiratory control ratio of the reconstituted enzyme, and its orientation from about 75% right-side-out to 45%, as shown in fig.1. This change of orientation is apparently due to modification of matrix-oriented lysine amino groups, because no change of orientation was found if the enzyme was first reconstituted in liposomes and then reacted with succinic anhydride. After solubilization in cholate and reconstitution, the modified COX did not reveal a change of its right-side-out orientation percentage, as shown in table 1.

In contrast to succinic anhydride, which converts positively into negatively charged groups, acetic anhydride neutralizes positively charged lysine amino groups. Treatment of isolated COX with 25 mM acetic anhydride results only in a small decrease of right-side-out orientation of the reconstituted enzyme from about 80% to 60% (fig.2). This result further supports the assumption that the charge distribution at the two hydrophilic domains of the complex is responsible for the asymmetric orientation of COX in liposomes. Acetic acid anhydride decreased the RCR of the reconstituted enzyme to a similar extent as succinic anhydride.

The inner mitochondrial membrane contains the negatively charged phospholipid cardiolipin, which was demonstrated to be essential for the activity of COX [13], and the ADP/ATP carrier [14]. The presence of cardiolipin in liposomes also influences the orientation of reconstituted COX as demonstrated in fig.3. Proteoliposomes prepared with purified phosphatidylcholine in the absence of cardiolipin do no show a preferred orientation. Phosphatidylcholine is a neutral zwitterionic phospholipid. With increasing concentrations of cardiolipin, the right-side-out orientation of COX increases from 49% in the absence to 79% in the presence of 20% cardiolipin in liposomes. The acidic phospholipid has only a negligible influence on the RCR of the reconstituted enzyme.

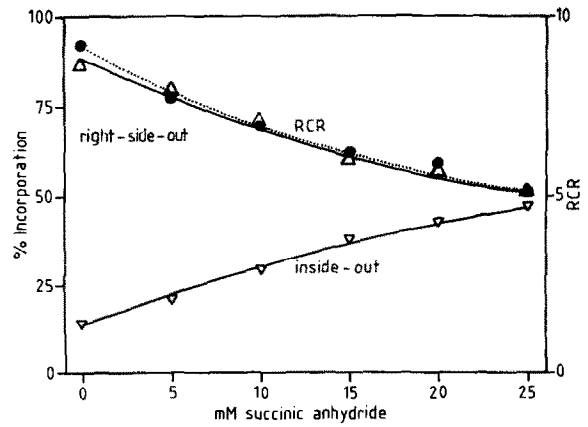

Fig.1 Incorporation of succinic anhydride-modified COX into liposomes. (△) right-side-out incorporated; (▽) inside-out incorporated; (•) respiratory control ratio.

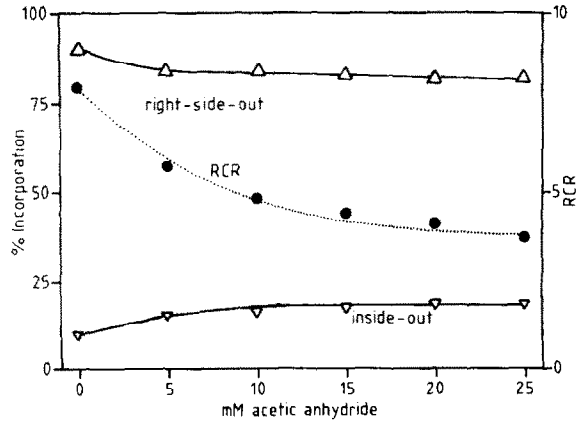

Fig.2 Incorportation of acetic anhydride-modified COX into liposomes. (△) right-side-out incorporated; (▽) inside-out incorporated; (•) respiratory control ratio.

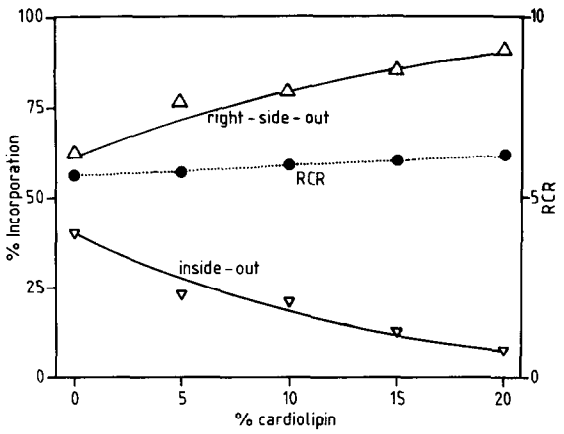

Fig.3 Incorporation of COX into liposomes prepared with purified phosphatidylcholine in the presence of different amounts of cardiolipin. (△) right-side-out incorporated; (▽) inside-out incorporated; (●) respiratory control ratio.

4. DISCUSSION

The results of this investigation indicate a strong influence of positive charges on the surface of COX on its asymmetric incorporation into phospholipid vesicles by the cholate dialysis method. They can be best explained by assuming different charge densities on the inner and outer phospholipid layer in liposomes [15]. Because of the curvature of liposomal membranes, the density of the charged head groups is assumed to be higher on the inner than on the outer side. Asolectin contains excess negatively charged lipids [13]. The excess of positively charged groups at the two matrix domains of COX [8] are assumed to orient these domains to the inner liposomal phase of higher negative charge density. This assumption is supported by fig.3, where in liposomes with pure phosphatidylcholine, an electroneutral zwitterion, COX showed no preferential orientation. With increasing content of the negatively charged cardiolipin in the membrane, COX became asymmetrically oriented with the matrix domain to the inside. Similarly, the exchange of positively into negatively charged groups at the matrix domains of COX by increasing concentrations of succinic anhydride (fig.1) decreases the asymmetric orientation of the enzyme and finally results in a random distribution of inside-out and right-side-out oriented complexes.

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