Orientation of cytochrome c oxidase molecules in the two populations of reconstituted vesicles resolved by column chromatography on DEAE-Sephacryl

Yu-Zhong Zhang a, Roderick A. Capaldi a,*, Pieter R. Cullis b and Thomas D. Madden b

a Institute of Molecular Biology, University of Oregon, Eugene, OR 97403 (U.S.A.) and b Department of Biochemistry, University of British Columbia, Vancouver, BC (Canada)

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Vesicles reconstituted with bovine heart cytochrome c oxidase and dioleoylphosphatidylcholine can be resolved into two populations by column chromatography in DEAE-Sephacryl (Madden, T.D. and Cullis, P.R. (1984) J. Biol. Chem. 259, 7655–7658). These two fractions (I and II) were treated with two proteases. These are trypsin, which has been found to cleave subunit IV in the M domain of the cytochrome c oxidase molecule, and chymotrypsin, which has been found to cleave subunit III in the C domain. These studies show that fraction I vesicles contain cytochrome c oxidase orientation with the M domain outside, i.e., in the same topology as in submitochondrial particles, while fraction II vesicles contain enzyme molecules with their C domain outside, and thus in the same orientation as in mitochondria.

Cytochrome c oxidase is the terminal component of the respiratory chain, catalyzing the vectorial transfer of electrons from ferrocytochrome c on the cytoplasmic side of the mitochondrial inner membrane to molecular oxygen [1]. The enzyme can be isolated by cycles of detergent solubilization and ammonium sulfate precipitation steps [2], and then reconstituted into membrane vesicles by adding purified phospholipid and dialyzing away the detergent [3]. Vesicles prepared by dialysis can be relatively large and have up to 90% of the cytochrome c oxidase molecules inserted with their cytoplasmic domain (C domain) facing outwards [4].

Recently, Madden et al. [5,6] have described a reconstitution procedure which results in the preparation of small vesicles containing an average of only one cytochrome c oxidase molecule per vesicle. Briefly, this method involves preparing unilamellar vesicles by extruding a mixture of lipids and cholate as detergent through polyacarbonate filters under nitrogen pressure before adding cytochrome c oxidase and dialyzing away the detergent. These cytochrome c oxidase-containing vesicles can be resolved into two fractions based on their binding affinity for DEAE-Sephacryl [6]. One fraction (fraction II) shows electron-transfer activity with externally added cytochrome c, indicating that the oxidase molecules in this fraction are oriented with their C domain outermost. The other fraction (fraction I) shows latent cytochrome c oxidase activity; electron transfer occurring only between added cytochrome c and the oxidase when detergent is present to disrupt the vesicles. Based on functional assays alone, cytochrome c oxidase in this latter population of vesicles appears to be oriented with the C domain inside, i.e., in the same orientation as submitochondrial particles. The pre-
sent study was undertaken to examine the orientation of protein in the two populations of cytochrome c oxidase vesicles more definitively. Protease digestion experiments are described which give an unambiguous orientation of cytochrome c oxidase molecules in these membranes.

Fig. 1 shows the effect of trypsin on these two populations of vesicles. Trypsin has been shown to cleave subunit IV of bovine heart cytochrome c oxidase and remove the N-terminal seven residues of the polypeptide [7]. This cleavage occurs in submitochondrial particles, which have the M face of the inner membrane available for protease digestion, but not in mitochondria or mitoplasts (mitochondria from which the outer membrane has been removed), and which have the C domain of cytochrome c oxidase outermost [7]. Thus trypsin cleavage is a valuable indicator of the exposure of the M domain of cytochrome c oxidase at the surface of vesicles.

Cleavage of subunit IV ($M_r$, 17000) to the smaller fragment ($M_r$, 16000) was essentially complete in fraction I vesicles under conditions where there was very little cleavage of the subunit in fraction II vesicles. This is evident from the Coomassie blue-stained gels (Fig. 1a) and from antibody blot experiments which follow the fate of subunit IV specifically (Fig. 1b). Interestingly, it required more trypsin and longer periods of incubation to obtain protease digestion in both sets of vesicles (I and II) than in vesicles prepared by the conventional dialysis method. This was true of chymotrypsin as well, and may have to do with the small size of the vesicles prepared by the high pressure method or with the fact that the lipid/protein ratio is much greater in these vesicles (molar ratio of 50000:1; c.f. 5000:1) than in previously described reconstitutions.

The amount of enzyme oriented with M domains out in fraction II vesicles was 5-10% as judged from a quantitation of the trypsin cleavage by densitometric measurements of Coomassie blue-stained gels.

Fig. 2 shows the effect of chymotrypsin treatment on the two populations of cytochrome c oxidase-containing vesicles. Previous studies have established that chymotrypsin cleaves subunit III in the C domain [4]. Chymotrypsin was found to cleave subunit III in fraction II vesicles, but not fraction I vesicles, as monitored both by the disappearance of the polypeptide from Coomassie blue-stained gels and more reliably by the altered migration of $[^{14}C]$dicyclohexylcarbodiimide-labeled subunit. As shown in Fig. 2, chymotrypsin cleavage in the C domain generates a fragment containing $[^{14}C]$DCCD which comigrates with
subunit IV (the reagent binds at Glu 90 [8]). This fragment is not generated in the fraction I vesicles. Fraction II vesicles thus behave in protease digestion experiments like mitoplasts, and identically to reconstituted vesicles prepared by conventional dialysis procedures of reconstitution [4]. They have the C domain outermost and M domains extending into the interior of the closed membranes from activity measurements.

Activity measurements have been taken to indicate that fraction I vesicles are oriented with cytochrome c oxidase in the opposite orientation, i.e., with M domains outermost and C domain in the lumen of the vesicle [6]. This is established unambiguously by the present structural study. The protease cleavage experiments show that the N-terminus of subunit IV, and therefore the M domains, extend from the outside of the vesicles. This rules out the possibility that the latent activity is due to oxidase molecules being trapped inside the liposome, lying in the inner surface without being integrated in the membrane. It also rules out the possibility that a large majority of the protein is in multilamellar structures and thereby unable to react with cytochrome c.

The inaccessibility of subunit III, and by inference, the C domain to chymotrypsin rules out the possibility that the protein is bound on the outer surface of the vesicles, not integrated into the bilayer, but inactive without detergent present. In summary, the combined data indicate that cytochrome c oxidase is transmembranous in fraction I vesicles, oriented as in submitochondrial particles with M domains outermost and C domain in the lumen of the vesicle. Such ‘inside-out’ vesicles offer exciting possibilities for both structural and functional studies of cytochrome c oxidase.

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References

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