Bilayer thickness and enzymatic activity in the mitochondrial cytochrome *c* oxidase and ATPase complex

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1. INTRODUCTION

Lipid–protein interactions are of great importance for the correct function and location of membrane-bound enzymes [1,2]. Nevertheless very little is known about the structure and folding of the polypeptide chains directly involved in the interaction with lipids. So far a low-resolution picture of polypeptide arrangement in the membrane has been obtained only for bacteriorhodopsin [3]. Few of the many integral membrane proteins, which have been isolated in pure form, have been sequenced. Most of these sequences show uninterrupted stretches of uncharged amino acids ≥ 18 residues long [4]. Hydrophobic photolabelling with photoreactive lipids has been used to identify which of these segments are in contact with lipids and hence to define the protein surface of the hydrophobic sector [5–7].

A defined bilayer thickness is required for optimal activity of the sarcoplasmic reticulum Ca^{2+}-ATPase and the (Na^+, K^+)-ATPase [8,9]. This investigation was based on the use of a homologous series of unsaturated phosphatidylcholines of different fatty acid chain length, which form bilayers of varying thickness [8]. Thus some information on the vertical dimension of the hydrophobic sector and on the forces involved in the interaction between lipid and proteins were obtained.

As part of our effort toward the elucidation of the structure of cytochrome *c* oxidase and ATP-synthase we present here the extension of this approach to these most studied mitochondrial multi-subunit enzymes.

Abbreviations: PC, phosphatidylcholine; SDS, sodium dodecyl sulphate

2. MATERIALS AND METHODS

Bovine heart cytochrome *c* oxidase, prepared as in [10], had a heme content of 9.2–10.4 nmol heme *a*/mg protein. Lipid was lowered by repeated precipitation with ammonium sulphate in the presence of cholate to 5–9% (w/w) in different preparations. Enzymatic activity, measured polarographically in 50 mM phosphate buffer (pH 7.4) was <20 mol cytochrome $c \cdot s^{-1} \cdot$ mol heme aa_3^{-1} and raised to 145–160 in the presence of Tween 80. ATP-synthase was purified from bovine heart mitochondria both as in [11,12]. Their lipid contents were 11–16% (w/w) and 5–7% (w/w), respectively. The ATPase activity of the delipidated complexes, measured spectrophotometrically in Tris/acetate 20 mM, EDTA 0.5 mM (pH 8.0) was <0.4 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$ and could be increased several-fold by incubation with egg lecithin or soya-bean lipid vesicles as in [7,12]. Oligomycin sensitivity was $>85\%$ for all preparations. Protein concentration was estimated as in [13] and lipid phosphorus as in [14]. Lipid–protein complexes were formed in 50 mM phosphate buffer (Ph 7.4) for

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cytochrome *c* oxidase and in 20 mM Tris/acetate, 0.5 mM EDTA (pH 8.0) for ATP-synthases and diluted before assay as in [8]. di(14:1)PC and di(18:1)PC–protein complexes were analysed for lipid content after centrifugation on 10% sucrose buffer as in [8].

3. RESULTS AND DISCUSSION

Due to their low lipid content the activity of our preparations of cytochrome *c* oxidase and ATP-synthase was low. However, they could be activated many-fold by addition of di(18:1)PC in the presence of cholate and dilution. The values found (see legends) are similar to those determined when other methods to form lipid–protein complexes were used [6,7,12]. After 5 min incubation with the cholate–di(*n*:1)PC mixture both enzymes showed maximal effect on activity with all the di(*n*:1)PC used here. Therefore equilibration, mediated by cholate, between the residual endogenous lipids and the exogenous di(*n*:1)PC pool is attained very rapidly. This was also verified on PC–protein complexes isolated by centrifugation as in [8].

Fig.1 reports the effect of varying the PC fatty acid chain length from 9–23 carbon atoms on cytochrome *c* oxidase activity. Maximal activation was found with the di(18:1)PC–enzyme complex. Lowering or increasing the bilayer thickness with respect to this optimal value results in a progressive loss of enzymatic activity. The relatively higher activity exhibited by PC with hydrocarbon chains shorter than 13 carbon atoms is most probably related to a detergent-like effect. In fact it is known that these phosphatidylcholines are unable to form stable bilayer structures and that detergents such as dodecyl maltoside can support a very high cytochrome oxidase activity [15,16].

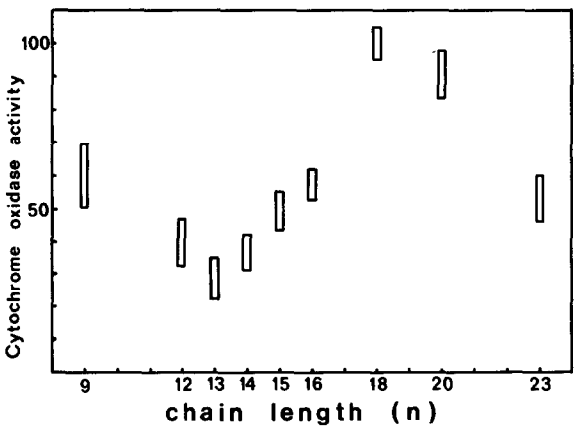

Fig.1. Effect of phosphatidylcholine hydrocarbon chain length on the activity of bovine heart mitochondrial cytochrome *c* oxidase. Activities are expressed as percentage of that found with di(18:1)PC in the same experiment. Bars represent the range of values found in different experiments with different enzyme preparations. Average activity with di(18:1)PC was 162 mol cyt $c \cdot s^{-1} \cdot$ mol heme aa_3^{-1}.

Another much studied mitochondrial transmembrane enzyme is the ATPase complex, which can be isolated in 2 forms differing for their specific activities and for the presence of a polypeptide (M_r 30 000), which is thought to be the adenine nucleotide carrier [11]. As reported in fig.2A the 2 di(*n*:1)PC–ATPase complexes show a similar dependence on bilayer thickness, but differently from cytochrome oxidase, their activities appear to be only slightly affected by the PC fatty acid chain length. This behaviour may be related to the unique structure of the mitochondrial ATPase complex, formed by two parts: a membranous F_0 domain, and an hydrophilic F_1-ATPase part which can be isolated in a watersoluble form [17].

When bound to F_0, the F_1-ATPase is sensitive to oligomycin, a specific antibiotic inhibitor, which binds to an unknown site on F_0 [17]. The oligomycin sensitivity of the F_1-ATPase activity is considered a very sensitive parameter of the correct coupling between F_1 and F_0 [18,19]. Fig.2B shows that oligomycin sensitivity of the ATPase complex is greatly affected by the thickness of the bilayer. It is very low for the short-chain PC–enzyme complexes and increases to reach its maximum for phosphatidylcholines with hydrocarbon chains >18 carbon atoms. We have tested the possibility that short chain PC alter the affinity of the oligomycin-binding site by using a whole range of oligomycin concentrations up to 250-times that able to fully inhibit the di(18:1)PC–ATPase complex and found no significant difference. This finding is in agreement and extends the observations [15,20] that di(9:0)PC and di(12:0)PC–ATPase complexes had a very low oligomycin sensitivity. This loss of sensitivity cannot be attributed to detachment of F_1 from F_0 since SDS–polyacrylamide gel electrophoresis after sucrose gradient centrifugation of a

di(12:1)PC–ATPase complex shows a staining pattern similar to that of a non-centrifuged control (not shown). A second possibility, irreversible inactivation of the ATPase complex by the short chain phosphatidylcholines, seems unlikely since an addition of di(18:1)PC–cholate to the di(12:1)-PC–ATPase complex, performed according to [8] leads to a recovery of oligomycin sensitivity of 55–65%. The same range of values is found when the di(12:1)PC is added to a di(18:1)PC–ATPase complex. The incomplete recovery of oligomycin sensitivity in these experiments is most probably related to the dilution of oligomycin the lipid phase. In fact it has been demonstrated that the addition of increasing amounts of lipids to the ATPase complex removes the oligomycin inhibition [21].

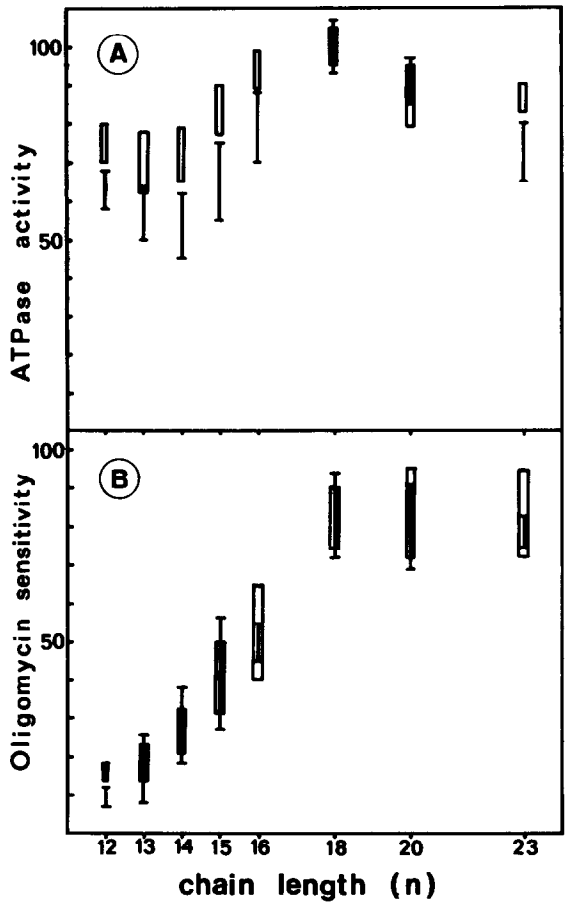

Fig.2.(A) Effect of phosphatidylcholine hydrocarbon chain length on the ATPase activity of bovine heart mitochondrial ATPase complex prepared according to [11] (▯) or to [12] (I). Activities are expressed as percentage of those found with di(18:1)PC in the same experiment. Average activities with di(18:1)PC were 4.4 mol ATP hydrolyzed . min^{-1} . mg $protein^{-1}$ for the ATPase complex prepared as in [11] and 16.5 mol ATP hydrolyzed . min^{-1} . mg $protein^{-1}$ for the ATPase complex prepared as in [12]. Bars represent the range of values found in different experiments with different enzyme preparations. (B) Oligomycin sensitivity of the ATPase activity of the 2 ATPase complexes in function of the varying fatty acid chain length of phosphatidylcholine; 8 μg oligomycin/9 μg enzyme was used in each assay; symbols as in (A).

A stable and fluid bilayer is required to support maximal activity of the mitochondrial ATPase complex [20]. The availability of a homologous series of unsaturated PC, whose transition temperatures are all well below the temperature of assay [8], has now allowed us to determine the activity profile in function of the fatty acid chain length of PC.

Taken together, the results in fig.2 indicate that the bilayer thickness does affect the ATPase complex resulting, rather than in a direct effect on ATPase-activity, in an uncorrect coupling between the F_1-ATPase and F_0.

Cytochrome *c* oxidase, sarcoplasmic reticulum Ca^{2+}-ATPase and (Na^+,K^+)-ATPase are all enzymes, whose catalytic unit(s) is present on a subunit(s), which has been shown to transverse the lipid bilayer [21,23,24]. This may be the reason for the large effect of bilayer thickness on their activities. On the contrary the mitochondrial ATPase complex is formed by two distinct, separable entities. Any change in the bilayer structure or dynamics will result in a parallel change of the ATPase activity only if the correct coupling between F_1 and F_0 is preserved. At variance from the Ca^{2+}-ATPase and the (Na^+,K^+)-ATPase, maximal activity for both mitochondrial enzymes is found with di(18:1)PC. It may be relevant to this finding that >55% of rat heart mitochondrial lipids contain an 18 carbon atom fatty acid chain [25].

Using a photoreactive phosphatidylcholine, which labels specifically those polypeptide regions interacting with the lipid polar head-group, we have identified in the subunit II of cytochrome *c* oxidase a group of charged amino acids followed in the sequence by an uninterrupted stretch of 18 uncharged residues [26]. This result and these data support the idea that the hydrophobic domain of a trans-

membrane protein is vertically delimited at both its upper and lower ends by 2 collars of charged amino acid residues, which interact with the polar head-group of lipids. The central part is composed of uncharged residues, which interact with the fatty acid portion of lipids. The length of the uncharged amino acid stretches, which interact directly with lipids, define for a transmembrane enzyme, the lipid bilayer thickness optimal for its activity. When the hydrophilic and hydrophobic surface portions of the hydrophobic domain do not match their lipid counterpart, as it occurs with short or long-chain phosphatidylcholines, this will result in low enzymic activity. The maximal activity exhibited by both mitochondrial enzymes with the di(18:1)PC would suggest that the polypeptides exposed to lipids are formed by stretches of 18–20 amino acid residues arranged vertically in an α-helical configuration as proposed for the subunit II of cytochrome *c* oxidase [26].

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