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Effect of neutral and acidic phospholipids on mitochondrial ATP synthase secondary structure

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The secondary structure of delipidated and egg phosphatidylcholine or asolectin reconstituted mitochondrial ATP synthase complex from beef heart was investigated by Fourier transform infrared spectroscopy. Upon reconstitution, the infrared spectra of ATP synthase revealed an increase in turns and a concomitant decrease in β-sheet content which occurred to a larger extent in the presence of asolectin rather than in the presence of egg phosphatidylcholine. These data correlate with kinetic data showing a higher ATPase activity of the asolectin reconstituted enzyme protein than the egg phosphatidylcholine reconstituted or delipidated enzyme complexes.

ATP synthase; Infrared spectroscopy; Protein secondary structure; Egg phosphatidylcholine; Asolectin; Liposome

1. INTRODUCTION

The mitochondrial ATP synthase from bovine heart is a phospholipid-dependent multi-subunit complex which consists of two domains: a hydrophilic moiety endowed with ATPase activity, termed F_1, and a membranous domain, termed F_0 which contains a proton channel [1,2]. The F_1 moiety is composed of five different, well-characterized subunits whereas, at present, nine subunits of unknown stoichiometry have been attributed to the F_0 sector and to the stalk connecting the two domains [3]. Several reports have dealt with the isolation of the ATP synthase complex from the bovine heart [3–7]. Depending on the isolation method, the ATP synthase preparations differ for the residual phospholipid content and protein contaminants [4,8]. In this paper we report data on the effect of neutral (egg-PC) and acidic (asolectin) phospholipids on the activity as well as the secondary structure of reconstituted ATP synthase complex. Information on the aggregation state of the enzyme protein in reconstituted systems is also reported. Among the various preparations of the ATP synthase complex we have chosen that of Serrano et al. [4] since this preparation is well characterized [4,9–11], easy to prepare, electrophoretically and enzymatically highly reproducible and phospholipid-dependent.

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Abbreviations: egg-PC, egg phosphatidylcholine; ATPase, delipidated ATP synthase; aso/ATPase, asolectin-reconstituted ATP synthase; egg-PC/ATPase, egg-PC-reconstituted ATP synthase; ANC, adenine nucleotide carrier.

2. MATERIALS AND METHODS

All buffers, inhibitors, reagents and asolectin were obtained from Sigma; acrylamide and bisacrylamide from Biorad; Deuterium oxide (99.9% ^2H_2O) was purchased from Aldrich. Phosphatidylcholine was obtained from egg yolk (egg-PC) as previously described [12].

2.1. *Preparation of F_0F_1 ATP synthase*

ATPase complex was prepared from Mg-ATP submitochondrial particles [7] as originally described by Serrano et al. [4] and defined as fraction 38–45p. For simplicity, this preparation will be named throughout the paper as ATP synthase, isolated according to Serrano [4].

2.2. *Analytical procedures*

ATP hydrolytic activity was measured at 37°C by monitoring spectrophotometrically at 340 nm the oxidation of NADH in a coupled lactate dehydrogenase-pyruvate kinase regenerating system in the presence or absence of oligomycin according to Stiggal et al. [7].

Gradient (13–19%) SDS-PAGE of ATP synthase complex was performed according to Montecucco et al. [13]. After staining with Coomassie blue, the gel pattern was recorded by scanning with a Shimadzu dual chromato-scanner CS 930 at 570 nm.

Protein concentration was determined according to Lowry et al. [14].

Analysis of ATP synthase residual phospholipids was performed after extraction with chloroform/methanol as described in [9].

2.3. *Reconstitution of ATP synthase complex in phospholipid membranes for infrared and ATP hydrolysis measurements*

The delipidated ATP synthase complex (ATPase) was reconstituted in phospholipid membranes according to the cholate-dialysis method [15]. In particular, 10 μmol of egg-PC or asolectin were hydrated with 140 μl of 10 mM tricine/NaOH, 1 mM 1,4-dithio-L-threitol (DTT), pH 8, and then 60 μl of 10% Na-cholate (w/v), pH 8, were mixed with the hydrated phospholipids. After vortexing, a clear solution of mixed micelles containing phospholipid-cholate was obtained. The buffer and cholate solutions were prepared in H_2O or in ^2H_2O and buffered to pH or p^2H 8 (p^2H = pH + 0.4) [16]. Afterwards, 3 mg of ATP synthase, dispersed in 100 μl tricine/DTT buffer, were added to the

mixture of phospholipid-cholate and, after a short incubation (40 min at 0°C), the sample was subjected to dialysis for at least 24 h at 4°C with several changes of tricine/DTT buffer.

ATP synthase was also subjected to the same exhaustive dialysis procedure with tricine/DTT buffer pH or p^{2}H 8 without the addition of phospholipid micelles. After dialysis the phospholipid-reconstituted or delipidated ATP synthase complex was recovered by centrifugation at 10,000 rpm for 10 min in a Beckman microfuge 12.

For the measurement of the ATP hydrolytic activity of the delipidated or phospholipid reconstituted ATP synthase complex, samples were withdrawn after dialysis or after incubation (40 min at 0°C) and, in the latter case, subjected to reconstitution in liposomes by cholate-dilution directly in the spectrophotometer cuvettes [4].

2.4. *Infrared spectra*

The protein samples were analyzed using a Perkin-Elmer 1760-x Fourier transform infrared spectrometer as described in [17]. Second derivative spectra were calculated over a 9-data point range (9 cm^{-1}). Spectral deconvolution was performed using the Perkin-Elmer ENHANCE function which is analogous to the method developed by Kauppinenn et al. [18]. Deconvolution parameters were set with the half-bandwidth at 18 cm^{-1} and a resolution enhancement factor of 2.75.

The estimation of the ATP synthase secondary structure was made by curve-fitting of the deconvoluted spectrum (amide I band) as previously described [17].

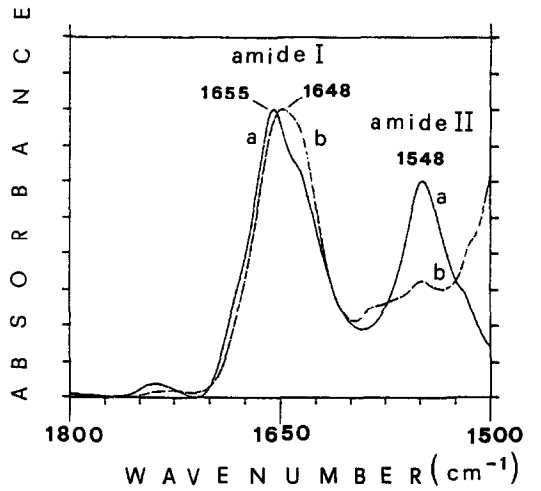

Fig. 1. Infrared absorbance spectra in the 1800–1500 cm^{-1} region of delipidated mitochondrial ATP synthase complex. Spectra (a) (amide I maximum at 1655 cm^{-1}) and (b) (amide I maximum at 1648 cm^{-1}) were obtained at 20°C in a H_2O and a ^2H_2O medium, respectively upon subtraction of buffer spectra from sample spectra.

3. RESULTS AND DISCUSSION

3.1. *Characterization of the ATP synthase complex*

In order to gain information on the correlation between functional and structural parameters of mitochondrial ATP synthase we determined: (a) the subunit composition, (b) the ATPase activity, (c) the residual phospholipid content of delipidated enzyme complex. SDS-PAGE densitometric traces of Serrano ATP synthase revealed that the F_0 and F_1 subunits represents 80% of the purified protein complex. A protein of approximately 30 kDa co-purifies with the ATP synthase; this polypeptide was previously identified as adenine nucleotide carrier [4,8] (data not shown).

Table I shows the ATPase activity and the sensitivity to oligomycin of the delipidated and reconstituted enzyme complex. The ATP synthase preparation is more efficiently reconstituted with acidic liposomes of asolectin (which is a crude mixture of soy bean phospholipids containing up to 20% of negatively charged phospholipids), than with liposomes of egg-PC. A lipid-to-protein ratio of higher than 3 μmol/mg protein did not improve the reconstitution of the ATP synthase complex with egg-PC liposomes in agreement with previous reports [4,5,9]. Qualitatively similar results were obtained when ATP hydrolysis of the ATP synthase complex was measured after exhaustive enzyme dialysis, though the activities were reduced to approximately one-third by such treatment (data not shown).

Table I

ATPase activity of delipidated and phospholipid reconstituted ATP synthase complex isolated according to Serrano et al. [4]

ATP synthase preparation	ATPase activity (μmol/mg protein)	Oligomycin sensitivity
Delipidated complex	2.3	90%
Egg-PC/ATPase	4.7	95%
Aso/ATPase	9.2	98%

ATP hydrolytic activity of the fraction 38–45p was determined spectrophotometrically after cholate-dilution as described in Materials and Methods. Phospholipid to protein ratio was 3 μmol/mg protein. Oligomycin concentration was 2 μg/ml.

The delipidated Serrano ATP synthase complex was found to contain residual phospholipids with distribution of lipid classes as in submitochondrial particles [9] (data not shown).

3.2. *Infrared spectra of delipidated and phospholipid reconstituted ATP synthase complex*

The infrared spectra of the ATPase in H_2O and ^2H_2O show maxima (amide I band) at 1655 cm^{-1} and 1648 cm^{-1}, respectively (see Fig. 1). The shift to lower wavenumbers of the amide I band maximum and the decrease in intensity of the amide II band at 1548 cm^{-1} (observed in a ^2H_2O medium) is due to the exchange of the amide hydrogens with deuterium [19,20]. Fig. 1 also shows a small peak at about 1740 cm^{-1} due to the C=O stretching vibration of phospholipids [21]. These lipids constitute those which are strictly associated to the membrane protein and therefore difficult to remove completely.

The reconstitution of the mitochondrial ATP synthase complex (ATPase) in egg-PC (egg-PC/ATPase) or asolectin (aso/ATPase) model membranes leads to changes in the amide I band shape as shown in Fig. 2 (for the protein in a H_2O medium) and in Fig. 3 (for the protein in a ^2H_2O medium). In particular, Fig. 2 shows

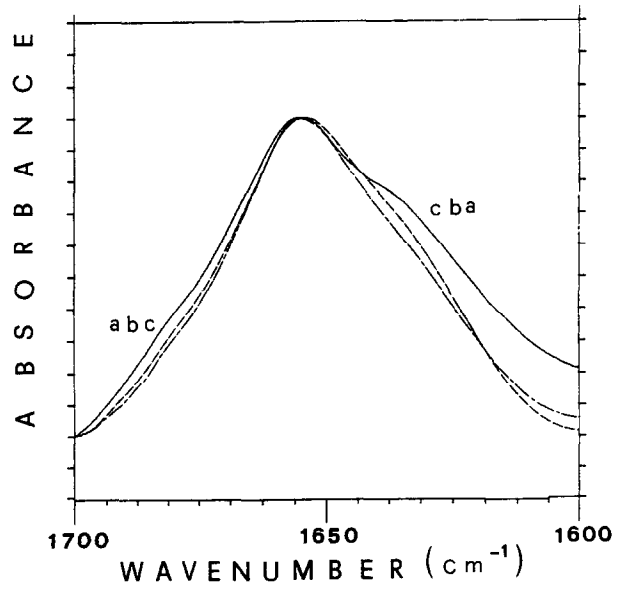

Fig. 2. Infrared absorbance spectra (amide I band) of delipidated and reconstituted mitochondrial ATP synthase. These spectra refer to the protein in a H_2O medium at 20°C. Spectra (a), (b) and (c) refer to the delipidated, egg-PC reconstituted and asolectin reconstituted ATP synthase complex, respectively. The spectra were obtained by subtraction of the buffer spectra from the sample spectra.

that the shoulder at about 1640 cm^{-1} present in the delipidated protein decreases in intensity upon reconstitution in the phospholipid bilayers. Such a decrease, which is higher in aso/ATPase than in egg-PC/ATPase can be attributed to a decrease in β-sheet content in the protein. Fig. 3 shows differences in the position (see also legend to Fig. 3) and in the relative intensities of the β-sheet and α-helix bands which are more pronounced in aso/ATPase than in egg-PC/ATPase as compared to ATPase. Such differences in the infrared spectra indicate different secondary structures and in particular a lower β-sheets content in the reconstituted ATP synthase complex.

Table II shows the quantitative analysis of infrared spectra and confirms that the reconstitution of ATP synthase induces a decrease in β-sheets content which occurs to a higher extent in the presence of asolectin. The decrease in β-sheets is accompanied by a parallel increase in turns, while the α-helix content is not significantly affected by the reconstitution in phospholipid bilayers. Since only the F_0 sector of the enzyme complex is inserted in liposomes [13,22–24], the last result suggests that the residual phospholipids, strongly associated to F_0, maintain the original α-helix content in this portion of the enzyme. However, we cannot exclude the influence of the co-purified adenine nucleotide carrier (ANC) in the preservation of the F_0 secondary structure since this transmembrane hydrophobic protein, which is in contact with subunits 1(b), 2(a) and 7 of F_0 sector [13], stabilizes the ATP synthase complex preserving the sensitivity to oligomycin [5,10,13]. Although ANC and the ATP synthase are obtained as separate entities and perform independently their functions in reconstituted systems, their functional association in situ is likely [23].

A large number of studies have been carried out on the importance of the polar head charge and on the degree of saturation of the phospholipid acyl chains for the catalytic activities of the mitochondrial ATP synthase complex [9–11,22,25,26]. Nevertheless, the mechanisms of interaction of acidic and isoelectric phospholipids with the ATP synthase complex are still not yet completely defined. The more dramatic influence on the enzyme activities as well as the secondary structure is played by the reconstitution of the delipidated complex with liposomes of acidic phospholipids. In aso/ATPase the β-sheet and turn contents are about half and double, respectively, as compared to the delipidated enzyme preparation. Since integral membrane proteins usually show predominantly an α-helical secondary structure and since the mass of the F_0 sector is about 1/3 of the total mass of the enzyme [3], it is reasonable to hypothesize that the large decrease in β-sheet content upon reconstitution of the enzyme in asolectin bilayers could involve also the F_1 sector. Because photolabelling experiments with azido-phospholipids strongly argue against a direct contact between the phospholipid bilayer and the F_1 portion [13], the conformational change of F_1 could probably be triggered by the F_0 subunits involved in the connecting stalk. Unfortunately, at present the topology of F_0 and F_1 subunit is far for being resolved. However, as recently demonstrated by Hakerman et al. [27] using antibody and proteolytic enzymes, several F_0 subunits protrude in the matrix space and could be in contact with F_1 subunits and phospholipid polar head. Moreover, a direct involvement in the F_0 and F_1 binding has been demonstrated for F6, OSCP (oligomycin sensitivity conferring factor) and β subunits [27,28].

Reconstitution of delipidated ATP synthase complex in liposomes induce other effects besides changes in the protein secondary structure. Fig. 3 (see especially second derivative spectra) shows that the band at about 1618 cm^{-1}, which is not imputable to a particular pro-

Table II

Estimation of the secondary structure of ATP synthase complex in the delipidated and reconstituted forms

Secondary structure	Serrano ATP synthase		
	delipidated	egg-PC	asolectin
α	42%	45%	42%
β	40%	35%	23%
t	9%	11%	21%
r	9%	9%	14%
α/β	1.05	1.28	1.82

The symbols α, β, t and r stand for α-helix, β-sheet, turns and random coil (unordered structures), respectively.

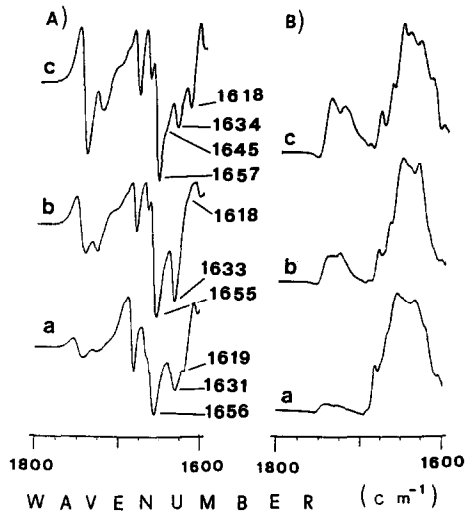

Fig. 3. Second derivative and deconvoluted infrared spectra of delipidated and reconstituted ATP synthase complex. The spectra were obtained at 20°C in a ^2H_2O medium. (Panel A) Second derivative spectra. (Panel B) Deconvoluted infrared spectra. (a), (b) and (c) refer to spectra of delipidated, egg-PC reconstituted and asolectin reconstituted ATP synthase complex, respectively. Bands due to α-helix are visible at 1656, 1655 and 1657 cm^{-1} in spectra (a), (b) and (c), respectively. Bands due to β-sheets are visible at 1631, 1633 and 1634 cm^{-1} in spectra (a), (b) and (c), respectively. Bands at 1681 and 1666 cm^{-1} (1667 and 1668 cm^{-1} in spectra (b) and (c), respectively) are due to turns, the bands at 1603 cm^{-1} and around 1618 cm^{-1} are not imputable to a particular protein secondary structure. Unordered structures are seen as a shoulder at about 1645 cm^{-1} in spectrum (c). The bands at about 1728 and 1743 cm^{-1} are due to the C=O stretching vibration of the phospholipid molecules. Band positions similar to those reported above can be observed in the deconvoluted spectra. The assignments of the amide I component bands to a particular secondary structure have been made according to Byler and Susi [33].

tein secondary structure, is almost absent in egg-PC/ATPase. Bands around 1620 cm^{-1} can often be found in the spectra of thermally [17,29,30] or solvent [30–32] denatured proteins and they were attributed to the formation of a β-sheet type structure as a consequence of intermolecular interactions, that is, aggregation. The spectra reported in Fig. 3 hence indicate protein aggregation in ATPase and aso/ATPase but not in egg-PC/ATPase. At present it is not possible to discriminate between different intermolecular interactions (e.g. subunit–subunit, domain–domain, F_0–F_1). However, the kinetic and infrared data suggest that the nature of phospholipids play an important role in such interactions. In particular, egg-PC (whose liposomes are more fluid than asolectin liposomes) seems to be able to prevent protein aggregation; however, the low ATPase activity of egg-PC/ATPase suggests an incorrect or incomplete incorporation into liposomes. On the contrary, asolectin is not able to avoid protein aggregation but, the high ATPase activity of aso/ATPase suggests a more correct insertion in liposomes. Probably, the correct insertion in liposomes is more important than prevention of protein aggregation for the maintenance of enzyme activity.

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