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THE EFFECT OF CHLOROPLAST COUPLING FACTOR ATP SYNTHETASE ($CF_1 \cdot CF_0$) RECONSTITUTION ON FLUIDITY PROPERTIES OF ISOLATED THYLAKOID LIPID VESICLES

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The reconstitution of chloroplast coupling factor ATP synthetase ($CF_1 \cdot CF_0$) with thylakoid lipids by cholate dialysis produced vesicles that displayed higher steady-state anisotropy (r_s) values for both 1,6-diphenyl-1,3,5-hexatriene (DPH) and trimethylammonium-diphenyl hexatriene fluorescence than the pure lipid alone. This is interpreted as meaning that the insertion of protein into the lipid bilayer brings about an increase in the ordering of acyl chains. This ordering effect became more obvious as the protein-to-lipid ratio was increased. Time-resolved decay analyses of DPH fluorescence anisotropy confirmed the conclusion drawn from the steady-state measurements, but further indicated that the dynamic motion of the probe was also slightly restricted after $CF_1 \cdot CF_0$ incorporation. The restriction of DPH motion and the change in the half-angle for its cone of rotation was observed at relatively low protein-to-lipid ratios as compared with other reconstituted or biological membranes, suggesting that perhaps lipid-protein interactions occur with the inserted $CF_1 \cdot CF_0$ complex.

Introduction

It seems that the thylakoid membrane system of higher plant chloroplasts possesses considerable lateral heterogeneity in the arrangement of its components [1–3]. Photosystem II and light-harvesting chlorophyll *a/b* complexes are located principally in the appressed membrane regions, whilst Photosystem I complexes, and possibly the cytochrome b_6f complexes [3,4], are restricted to the unappressed membranes. In view of this arrangement, lateral diffusional processes must be of considerable importance in mediating both linear electron transport and energy transfer [1,2,5] and it is, therefore, necessary to consider the biochemical composition and biophysical nature of the thylakoid lipid matrix.

The thylakoid membrane is unusual in that approx. 75% of its lipids are galactolipids [6], which possess a high degree of unsaturation, the principal fatty acyl chain being $C_{\Delta 9,12,15}$ (18 : 3) α-linolenic acid [6]. In view of the high unsaturation level it is not surprising that thylakoid membranes [7,14] and bilayers constructed from isolated chloroplast lipids [12] are extremely fluid as monitored by steady-state and time-resolved anisotropy studies using the fluorescence probe DPH. Recently, evidence has been obtained that a mechanism exists for regulating thylakoid membrane fluidity in response to growth temperature which involves alteration of the intrinsic membrane protein-to-lipid ratio [8–11,23]. Therefore, in the pre-

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *p*-toluene sulphonate; DOPC, L-α-dioleylphosphatidylcholine; DMPC, L-α-dimyristoylphosphatidylcholine; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DMPC, 1,2-dimyristoyl-*sn*-glycerophosphocholine.

sent study we have used a model system to investigate the effect of protein insertion on the fluidity properties of isolated thylakoid lipids. The approach has been to use the fluorescence anisotropy properties of DPH and also its analogue TMA-DPH to monitor the fluidity of a reconstituted thylakoid lipid system in which the amount of thylakoid protein complex, ATP synthetase ($CF_1 \cdot CF_0$), was varied.

Materials and Methods

Thylakoid lipids were extracted from broken pea thylakoid membranes, purified by reverse-phase high-performance liquid chromatography and pigment-depleted by thin layer chromatography as previously described [12]. Coupling factor ATPase ($CF_1 \cdot CF_0$) was prepared from market spinach as outlined by Pick [13] and incorporated into thylakoid lipid vesicles using the following cholate dialysis method. Isolated monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), phosphatidylglycerol (PG) and sulphoquinovosyldiacylglycerol (SQDG) in $CHCl_3$ were combined in the molar ratio 4 : 2 : 1 : 1, respectively (this mixture will be referred to as mixture I) to give 600 nmol lipid for steady-state fluorescence measurements or 1 μmol lipid for time-resolved fluorescence measurements. After removal of all traces of $CHCl_3$ by a stream of N_2, the resultant lipid film was dissolved in 30 or 50 μl of 2% (w/v) sodium cholate for steady-state or time-resolved measurements, respectively, and an appropriate amount of sucrose density-gradient purified $CF_1 \cdot CF_0$ was added. The $CF_1 \cdot CF_0$ mixture was made

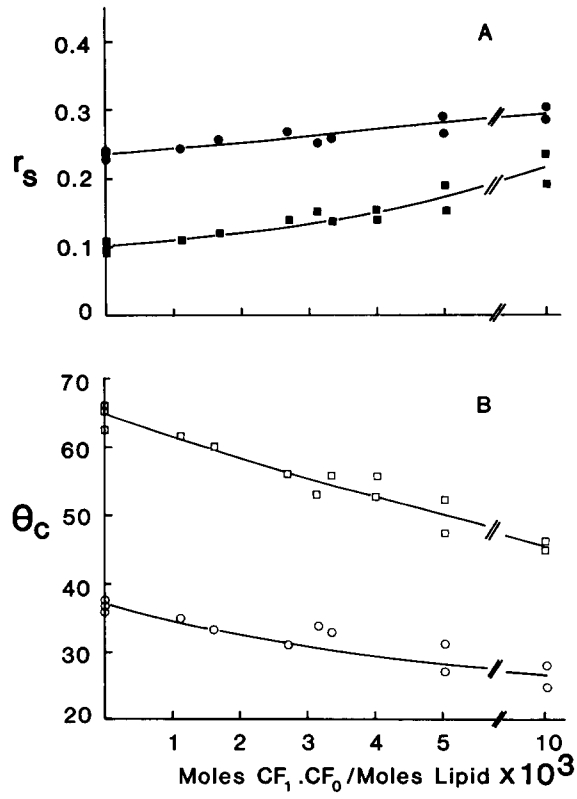

Fig. 1. (A) Steady-state anisotropy and (B) cone angle, θ_c, for DPH (squares) and TMA-DPH (circles) in reconstituted $CF_1 \cdot CF_0$/thylakoid lipid vesicles. Vertically or horizontally polarized excitation light was provided at 360 nm, 10 nm slit width, through a Polaroid HN'B polarizer. Fluorescence was monitored at 460 nm, slit width 8 nm, through a 390 nm cut-off filter and a second polarizer orientated parallel or perpendicular to the plane of polarization of the excitation light. Anisotropy (r_s) was calculated as described in Materials and Methods, while θ_c was calculated from r_s according to Pottel et al. [18]. Probe-to-lipid molar ratio was 1 : 400.

TABLE I

FLUORESCENCE LIFETIMES OF DIPHENYLHEXATRIENE IN TOTAL THYLAKOID LIPID AND RECONSTITUTED $CF_1 \cdot CF_0$/LIPID VESICLES

α_i, fractional preexponential term where $\alpha_i = A_i / \Sigma_i A_i$ and τ_i, lifetime for double exponential fit of fluorescence decay. Average fluorescence lifetime $\langle \tau \rangle = \Sigma_i (A_i \tau_i^2) / \Sigma_i (A_i \tau_i)$. Results are an average of three experiments for mixture I and two experiments for mixture I + $CF_1 \cdot CF_0$. Standard deviations are given in parenthesis. The protein-to-lipid molar ratio for the $CF_1 \cdot CF_0$ reconstituted vesicles was 1 : 250.

	α_1	τ_1	α_2	τ_2	$\langle \tau \rangle$	χ^2
Mixture I	0.37 (0.03)	2.17 (0.54)	0.63 (0.03)	6.65 (0.60)	5.92 (0.28)	1.5–1.8
Mixture I + $CF_1 \cdot CF_0$	0.57 (0.01)	1.71 (0.21)	0.43 (0.01)	6.89 (0.49)	5.60 (0.39)	2.7–3.8

to 1.5 ml in a medium comprising 0.2 M sorbitol/5 mM KCl/5 mM Tricine-KOH (pH 8.0) (LSB) and left to incubate for 30 min at 4°C before making the volume up to 3 ml with the same medium. The $CF_1 \cdot CF_0$/lipid/cholate mixture was then dialysed for 20 h against 4 l of LSB. Dialysis was carried out in a stirred 250 ml flask with constant input of fresh medium at approx. 200 ml/h. All manipulations involving $CF_1 \cdot CF_0$ were carried out at 4°C or less. Following dialysis, the lipid vesicles were labelled at 4°C for 1 h with DPH or TMA-DPH at a probe to lipid molar ratio of 1 : 400. During dialysis, the dialysis medium was bubbled with O_2-free N_2 and for labelling with fluorescence probes and fluorescence measurements the sample cuvettes were gassed with N_2 and protected from light.

Steady-state fluorescence anisotropy measurements were made using a Perkin-Elmer MPF-44A equipped with Polaroid HN'B polarizers, linked to an Apple microcomputer. Vertically polarized excitation light at 360 nm was passed through a UG-1 filter while fluorescence emission was monitored through a 390 nm cut-off filter and a polarizer orientated parallel or perpendicular to the orientation of the exciting light (see legend to Fig. 1). The steady-state anisotropy, r_s, was calculated according to Ref. 12, whilst the DPH cone half-angle, θ_c, was calculated from r_s according to Pottel et al. [18].

Time-resolved fluorescence anisotropy measurements were made using an Applied Photophysics SP-70 single photon-counting, nanosecond spectrofluorimeter as previously described [12,14]. Collection of horizontally ($F_\perp(t)$) and vertically ($F_\parallel(t)$) polarized DPH fluorescence following excitation with vertically polarized light was carried out by collecting fluorescence for 5 s intervals at each emission polarizer orientation.

As the analysis of time-resolved fluorescence data has previously been described [12,14–16] a brief summary only will be given. Total fluorescence ($F_T(t)$) and difference fluorescence ($F_D(t)$) data were created where:

$$F_T(t) = F_\parallel(t) + 2F_\perp(t)G \quad (1)$$

and:

$$F_D(t) = F_\parallel(t) - F_\perp(t)G$$

and G is a correction factor for partially polarized excitation light (see Refs. 12 and 14). Decay data were then analysed assuming exponential decay as follows. Firstly $F_T(t)$ was deconvoluted from the excitation pulse assuming:

$$F_T^\delta(t) = A_1 \exp\frac{-t}{\tau_1} + A_2 \exp\frac{-t}{\tau_2} \quad (2)$$

where δ indicates a response to an infinitely short pulse of light and A_i, τ_i are preexponential factors and lifetimes, respectively. The latter parameters were then utilized in a second deconvolution procedure to fit $F_D(t)$ where anisotropy was assumed to decay according to:

$$r^\delta(t) = (r_0 - r_\infty)\exp\frac{-t}{\phi} + r_\infty \quad (3)$$

Here, r_∞ is the residual anisotropy and ϕ, the rotational correlation time of the DPH molecule. The initial anisotropy, r_0, was assumed to be 0.39 [12,14–16]. Static order and dynamic motion

TABLE II

DYNAMIC AND STATIC FLUORESCENCE ANISOTROPY DECAY PARAMETERS FOR DIPHENYLHEXATRIENE IN TOTAL THYLAKOID LIPIDS AND RECONSTITUTED $CF_1 \cdot CF_0$/LIPID VESICLES

r_s(meas) and r_s(cal) are measured and calculated steady-state anisotropies; r_∞, residual anisotropy; ϕ, rotational correlation time; S, order parameter; θ_c, cone half-angle; D_w, wobbling diffusion coefficient and η_c, viscosity within cone. Standard deviations are given in parenthesis. The protein-to-lipid molar ratio for the $CF_1 \cdot CF_0$ reconstituted vesicles was 1 : 250.

	r_s (meas)	r_s (cal)	r_∞	ϕ (ns)	S	θ_c^0	D_w (ns^{-1})	η_c (P)
Mixture I	0.096 (0.002)	0.080 (0.004)	0.011 (0.004)	1.391 (0.090)	0.118 (0.061)	78.6 (5.1)	0.184 (0.010)	0.209 (0.010)
Mixture I + $CF_1 \cdot CF_0$	0.142 (0.012)	0.130 (0.011)	0.054 (0.016)	1.483 (0.095)	0.369 (0.051)	59.9 (3.0)	0.145 (0.001)	0.278 (0.001)

parameters (see Table II) were calculated from r_∞ and ϕ as described [12,14–16].

Results and Discussion

$CF_1 \cdot CF_0$ prepared according to Pick [2] was found, on performing SDS-polyacrylamide gel electrophoresis, to possess polypeptide bands corresponding to the $CF_1 \cdot CF_0$ subunits as reported [13]. Our preparation often possessed a further polypeptide at approx. 36 kDa, of unknown identity. Incorporation of the $CF_1 \cdot CF_0$ complex into thylakoid lipid vesicles appeared to result in a single population of reconstituted liposomes, as judged by the presence of a single narrow band following centrifugation of the dialysed $CF_1 \cdot$ CF/lipid vesicles on a 7–40% (w/v) sucrose-density gradient. While the residual cholate present was not estimated, it seems probable that for the rigorous dialysis procedures employed they were rather less than 30 μg cholate/mg protein as indicated in other studies employing the cholate-dialysis procedures [20,21].

Fig. 1A shows the effect of $CF_1 \cdot CF_0$ incorporation into thylakoid lipid vesicles, on the steady-state anisotropy (r_s) of DPH and TMA-DPH fluorescence. Both probes almost certainly align with the lipid acyl chains [15,20,22]. However, DPH would be expected to probe somewhat deeper within the acyl chain region of the lipid bilayer than TMA-DPH, since the latter carries a positive charge on its trimethylammonium moiety,and for this reason will partition so that its polar head is in the surface region of the lipid bilayer. As can be seen in Fig. 1A, higher r_s values were found with TMA-DPH as compared with DPH at all the protein-to-lipid ratios used. This is in agreement with electron-spin resonance studies employing spin-labels placed at different depths within lipid bilayers [23–25] which have shown that the lipid-acyl chains in the head group region are more ordered and motionally restricted than those in the midplane region of the bilayer. As pointed out by Van der Meer and co-workers [18,26], r_s is often dominated by the order parameter r_∞ when it has a value greater than 0.12. For this reason we have used the relationship in Ref. 18 to derive r_∞ and hence the cone half-angle, θ_c, for DPH and TMA-DPH motion (see Refs. 12 and 14–16) assuming an initial anisotropy, r_0, of 0.39. It can be seen in Fig. 1B that incorporation of $CF_1 \cdot CF_0$ into the thylakoid lipid bilayer caused a substantial decrease in θ_c with increasing protein incorporated, indicating an enhanced restriction in the range of probe motion. Such a restriction of DPH motion, indicative of acyl-chain ordering, has been observed both in reconstituted [20] and natural [16] membranes. In the case of DPH fluorescence anisotropy in vesicles devoid of $CF_1 \cdot CF_0$, the value for θ_c calculated from r_s was somewhat lower than found by time-resolved measurements (see Table II). However, this discrepancy almost certainly arises due to the poor correlation between r_s and r_∞ at low r_s values [18].

Table I shows the time-dependent fluorescence decay parameters for DPH incorporated into mixture-I vesicles with $CF_1 \cdot CF_0$ absent or present at a protein-to-lipid molar ratio of 1 : 250. The fluorescence decay was always best fitted by assuming a double exponential, as has been found for DPH fluorescence in a number of membrane systems [14,16,20] including those using pure lipids [12,17,27,28]. In the present study, the incorporation of $CF_1 \cdot CF_0$ into thylakoid lipid vesicles caused a slight decrease in the average fluorescence lifetimes, $\langle \tau \rangle$, which reflects changes in the short- and long-lifetime components. Similar changes in $\langle \tau \rangle$ and relative proportions of α_1 and α_2 were noted by Kinosita and colleagues [20] in reconstituted cytochrome oxidase/DMPC vesicles at temperatures above the phase transition and were explained in terms of energy transfer from DPH to the heme *a* of the oxidase. However, pure $CF_1 \cdot CF_0$ is devoid of chromophore groups and the alterations in $\langle \tau \rangle$, α_1 and α_2, respectively, may reflect a change in the hydrophobicity of the DPH molecule's local environment [29], or alternatively slight contamination of our $CF_1 \cdot CF_0$ preparation with thylakoid pigments (see Refs. 12 and 14).

Table II shows the effect of $CF_1 \cdot CF_0$ reconstitution into thylakoid lipid vesicles on static and dynamic anisotropy decay parameters of DPH fluorescence. Clearly, the incorporation of $CF_1 \cdot CF_0$ to a protein-to-lipid molar ratio of 1 : 250 has a substantial effect in limiting both the range and the rate of probe motion. In the case of the static parameters, r_∞ was increased 5-fold which resulted in a decrease of θ_c from 78.6° to 59.9°. However,

the 25% decrease in θ_c is significant, since this represents a change from an almost isotropic situation, as is found for unsaturated lipids [30] (including isolated thylakoid lipids [12]), to one in which a substantial degree of lipid order exists. For comparison, in other biological membranes, θ_c was found to be 53.2° and 53.1° for sarcoplasmic reticulum and mitochondrial inner membrane, respectively [15], at 35°C, while for intact thylakoid membranes $\theta_c = 48°$ at 25°C [14]. In a reconstituted cytochrome oxidase/DOPC system [20] θ_c decreased from 80° in the absence of protein to 60° at a protein-to-lipid ratio of 2 : 100 (at 35°C).

In the case of the dynamic anisotropy parameters, the incorporation of $CF_1 \cdot CF_0$ into thylakoid lipid bilayers had relatively small effect (see Table II), with the rotational correlation time, ϕ, being increased by 6.5%, which resulted in a decrease of D_w by 21%. This is in contrast to the report of Kinosita et al. [20] who found no restriction of the rate of DPH motion in DOPC vesicles by cytochrome oxidase. Whilst D_w did decrease when $CF_1 \cdot CF_0$ was present, the actual value found at 25°C (0.145 ns^{-1}) was still of the same order as found with unsaturated pure lipid systems [30] and indicates a highly fluid environment for the DPH molecule. In the case of intact biological membranes which have been investigated by time-resolved DPH fluorescence anisotropy, D_w values have indicated less fluid environments [14,20] than found in this present reconstitution, allowing for the difference in the experimental temperatures employed. For example, the D_w values for sarcoplasmic reticulum and mitochondrial inner membrane were 0.139 and 0.146 ns^{-1}, respectively (monitored at 35°C) and D_w for thylakoid membrane, at 25°C, was 0.12 ns^{-1}.

The results presented above clearly indicate that incorporation of protein into isolated thylakoid lipids brings about substantial changes in both the ordering and dynamic properties of the acyl chains, as judged from the anisotropy of DPH and TMA-DPH fluorescence. The main features of the results are that the ordering of the lipid acyl chains is more greatly affected than its dynamic motion and these changes are observed at lower protein-to-lipid ratios than found with other reconstituted [20] and biological [14,16] membranes. The precise reasons for these effects are unclear but could reflect the use of $CF_1 \cdot CF_0$ complex chosen for the reconstitution study. The reason for using the $CF_1 \cdot CF_0$ protein is that it is a natural component of the thylakoid membrane and, more importantly, is devoid of any chromophore which could alter the fluorescence characteristics of the probe by energy transfer. However, it has been recently suggested by Mörschel and Staehelin [19] that when $CF_1 \cdot CF_0$ is reconstituted in chloroplast lipids there is a strong interaction between the complex and the lipid matrix. This was deduced from freeze-fracture pictures which indicated that particle sizes, after reconstitution, were substantially larger than found for the native complex. The DPH and TMA-DPH technique would not detect the suggested local immobilisation of the lipid, but rather express this effect as an average for the whole lipid matrix. To detect the proposed heterogeneity of lipid ordering requires the use of phase-selective probes, such as *trans*-parinaric acid and its analogues [31]. Nevertheless, the results presented in this paper support the general contention that modification of lipid: protein ratios could regulate the overall fluidity of the in vivo membrane [10]. However, our results also indicate that it is probably necessary to define more precisely which specific proteins are changing their concentration in response to changes in environmental factors. At present, our understanding of lipid association with intrinsic thylakoid membrane proteins is rather poor, but already evidence is accumulating that such specific interactions do occur. For example, in the context of the present work, it has recently been shown by Pick et al. [32] that the $CF_1 \cdot CF_0$ complex has maximum synthetase and ATPase activity when associated with certain thylakoid lipids, particularly unsaturated MGDG.

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