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Functional Organization of Intramembrane Particles of Mitochondrial Inner Membranes

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It is generally assumed that the high efficiency of electron transport and energy coupling in primary energy transducing membranes is the result of a special spatial arrangement of the interacting components within the membrane. Indeed, in recent years much evidence has been accumulated for an assymmetrical organization of the electron transport components of the inner mitochondrial membrane. Thus, cytochrome *c* appears to be localized at the outside surface of the inner membrane; ATPase at the inside surface; and cytochrome oxidase and cytochrome *b* within the hydrophobic membrane center. These concepts accord with the known sidedness of cytochrome *c* and ATPase reactivity and extractability; and with the disruption of the membrane structure upon extraction of cytochrome oxidase or cytochrome *b* (cf. ref. 1).

Based on current knowledge of biochemical and morphological studies a conceptual view of the possible orientation of mitochondrial membrane components was constructed as illustrated in Fig. 1. This shows the membrane to have distinct hydrophobic and hydrophilic regions. In certain areas the lipids are oriented in a bilayer structure, but in other areas the bilayer structure is perturbed by proteins that protrude into the center of the membrane. This is especially true for the large ATPase molecule (molecular weight 355,000 with its subunits) and for the lipoprotein electron transport components such as cytochrome $a + a_3$ (molecular weight 70,000–80,000), and cytochrome *b* (molecular weight 41,000). Permeability studies have shown that the dehydrogenases are located on the matrix side of the inner membrane.[2] Hence, it is necessary for substrates to permeate the membrane before they can donate electrons to the respiratory chain. The arrows in Fig. 1 show diagrammatically the possible direction of substrate translocation and electron flow, H^+ transport and ATP synthesis. The diagram does not imply any specific "boats and/or

bridges" hypothesis for the carrier mediated translocation of metabolites, inorganic ions, and adenine nucleotides, although these considerations must also be taken into account in developing a fuller comprehension of the functional organization of the inner mitochondrial membrane.

An important question is, how is it possible to determine the

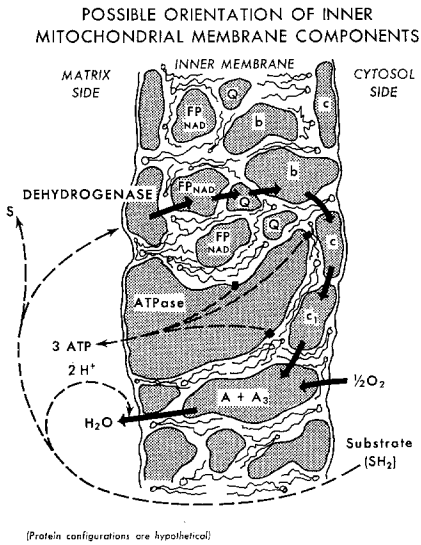

Figure 1. Illustration of possible orientation of inner mitochondrial membrane components. The membrane is visualized as combining the features of the lipid bilayer and subunit hypotheses of membrane structure (cf. Stoeckenius and Engelman[14]). Components which interact with the membrane predominantly by electrostatic forces (viz. cytochrome *c*) are surface components. Components organized in the center of the membrane are predominantly bound by strong hydrophobic forces. The large ATPase molecule, with its numerous subunits, is visualized to be bound by both hydrophobic and electrostatic forces each of which predominate in a different region of this large molecule. Excepting for the Q component the dark areas are for proteins and the light areas are lipid domains. The dimensions of the lipid and protein domains are hypothetical, drawn to emphasize functional organization (cf., Fig. 4, *bottom*).

components of the inner mitochondrial membrane by a direct experimental approach?

An approach to this problem was suggested in 1970 by Wrigglesworth, Packer and Branton,[3] who discovered the existence of particles in mitochondrial membranes. This has prompted our laboratory to carry out a detailed study of the factors which affect the distribution and dispersal, and identification of these particles in the membrane. Electron microscopy after freeze-cleavage is the only technique presently available which reveals the presence of structural components in the membrane interior.

I. *Interpretation of Electron Micrographs After Freeze Cleavage and Etching*

Freeze-cleavage and etching electron microscopy reveal components of the hydrophobic interior and membrane surface respectively. The possible orientation of lipids and proteins and their relationship to the fracture pattern and etch face are shown diagrammatically in Fig. 2. Lipids are generally thought to be present as bilayer structures. X-ray

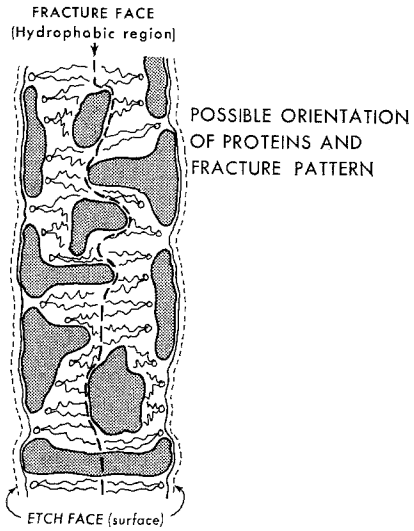

Figure 2. Possible orientation of membrane proteins and fracture patterns after freeze-cleavage. The dashed line shows the possible fracture face that might arise as a result of splitting the membrane down its hydrophobic center. This would cause exposure of components, which are revealed after the platinum shadowing to form a replica. In the case where proteins might entirely traverse the membrane the route that the fracture plane would take is uncertain; the fracture plane may either be around the component or it may itself break. The dashed line near the membrane surface is the etch face that would be revealed after sublimation of ice. Components on the etch face are revealed after platinum shadowing.

data of membranes suggest that the bilayer regions extend over at least a 400 Å field. In mitochondrial membranes fatty acids vary from about C-16 to C-22. This would vary the chain length from 19–27 Å respectively. It may be that shorter fatty acid chains preferentially are organized in those regions where proteins protrude more deeply into the membrane center. Accordingly, the fracture faces reveal protein components protruding into the hydrophobic, central region of the membrane; these are replicated by the platinum shadowing process. This interpretation, originally proposed by Branton,[4] has recently been established unequivocally by examination of complementary replicas of freeze-fracture specimens.[5] After fracturing, if the specimen

is warmed from liquid nitrogen temperature to −100°C, ice sublimes from the membrane surface at a rate of about 1000 Å/min. This process, known as etching, exposes the membrane surface.

II. *Structural Organization of Inner and Outer Mitochondrial Membranes Observed by Electron Microscopy After Freeze-Cleavage*

In collaboration with Dr. R. Melnick[6] outer and inner membranes of rat liver mitochondria have been separated and their structure examined by electron microscopy after freeze-cleavage. The photographs in Fig. 3 show concave and convex fracture faces observed on these membrane preparations. Note that the replica of the rat liver inner mitochondrial membrane preparation shows a large area of a concave fracture (left side) and a convex fracture face (right side). On the other hand the replica of the outer membrane preparation shows a large area of a concave fracture face (lower portion) and a smaller area of a convex fracture face (upper left).

The histograms prepared from these photographs (Fig. 4) show the distribution of particle sizes. On the average, inner membranes contain about twice the number of particles in convex as compared with concave fracture faces, while in outer membranes this ratio is about 1:4. The diagram (lower part) illustrates how the particles in the half membranes may be distributed after fracturing. The data suggest that most of the particles face either the cytoplasmic or the matrix compartments in the cell and that fewer of the particles are distributed in the areas facing the intermembrane space. Note that others have found that in plasma membrane the majority of particles are distributed on the side facing the inside of the cell. Secondly, the results accord with the idea that the proteins of the outer mitochondrial membrane are derived from a membrane of the eucaryotic cell and that the proteins of the inner mitochondrial membrane are vestiges of an early infection of a primitive nucleated cell by a procaryotic organism according to the endosymbiont hypothesis. The fatty acid components of the membranes are likely characteristic of the host because of the known ease with which environmental conditions lead to fatty acid exchange.

III. *Intramembrane Particles of Mitochondrial Inner Membranes—Dynamic State of Organization*

The experiments described above show that the outer and inner mitochondrial membranes contain a densely and a lightly particulated fracture face, designated "A" and "B": faces respectively. Such particle distribution, frequently seen in other membranes, represents

Figure 3. Membrane fracture faces observed after freeze-cleavage of inner and outer rat liver mitochondrial membranes; after Melnick and Packer.[6]

the distribution of particles in the half membranes. Particle-particle interaction may contribute to membrane cohesion, and may reflect the forces which bind the membrane together. The presence of two densely staining areas, i.e. "Railroad Tracks" in the "unit membrane" visualized by conventional electron microscopy after positive staining

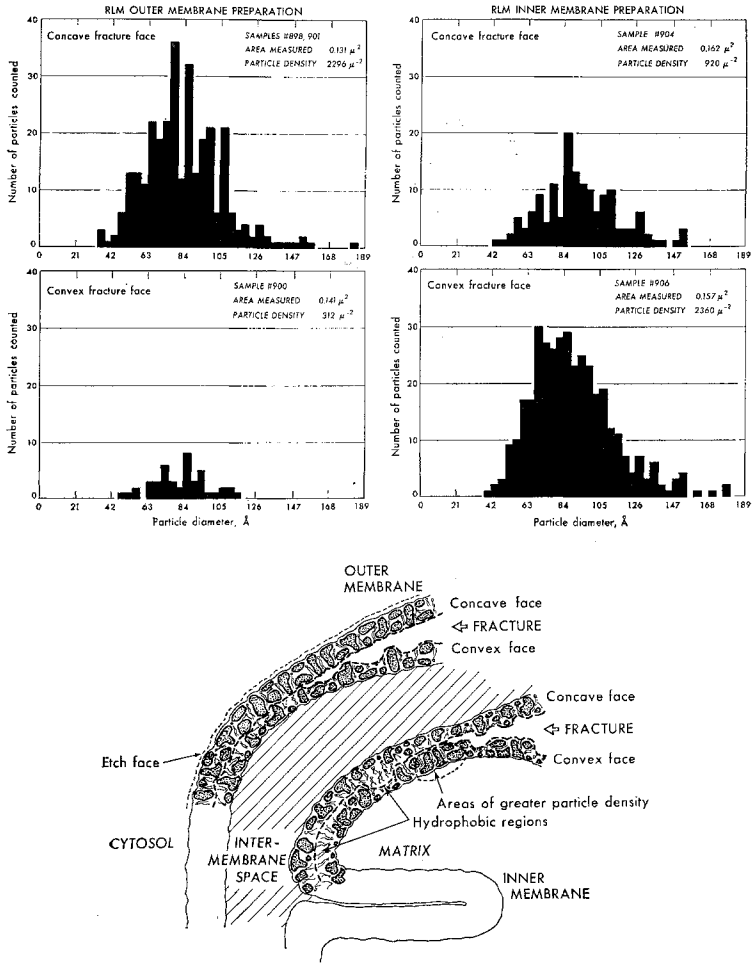

Figure 4. Analysis of particle size distribution observed in the fracture faces of inner and outer mitochondrial membranes and suggested distribution in these membranes. The measured particle sizes observed in the fracture faces after freeze-cleavage electron microscopy have been tabulated as a function of their frequency. In constructing these histograms no correction has been made for the thickness of the particles caused by the deposition of platinum during the shadowing process. An estimated correction is 20–30 Å for particles in this size range. Therefore, the actual particle diameters are 20–30 Å smaller in diameter. In the lower portion of the figure the diagram illustrates the possible organization of components in the half membranes that arise from inner and outer mitochondrial membranes.

with heavy metals suggests that the protein components predominate at the periphery. Since energy coupling requires a precise spatial relationship of membrane components, a vectorial dispersal of membrane components in the hydrophobic membrane phase may play a crucial role in the efficiency of energy coupling. It is important

to identify what factors govern the properties of the particles in these "half membrane" structures, e.g., are the particles fixed within the membranes or do they change their orientation?

A. *Membrane Dehydration Experiments*

We have recently observed that particle distribution in the half membranes varies with water content of the membranes (biological membranes generally have about 15% water content). These changes were noted after dehydrating the membranes using 2 M sucrose (and also by changing the relative humidity). Some results are shown in Table I. At 0·33 M sucrose the inner membrane particle density

TABLE I. Influence of sucrose concentration on the particle distribution observed in fracture faces of inner mitochondrial membranes

Inner membrane preparation	Area analyzed ($\times 10^{-1}\ \mu^2$)	Particle analysis: Total No.	Density $\times 10^{-1}\ \mu^2$	Particle clusters (% of total): 1	2	3	4	5
0·33 M sucrose, pH 7·2								
Convex	19·35	2675	1452	57·0	9·3	10·6	9·5	12·7
Concave	7·49	418	558	65·3	8·7	6·2	6·8	10·3
2·0 M sucrose, pH 7·2								
Convex	6·60	1525	2310	65·4	9·7	8·5	9·5	7·4
Concave	2·03	534	2630	78·9	9·0	7·4	3·2	0·0

Inner membranes were prepared as previously described and incubated under the conditions indicated in the Table before freezing in liquid nitrogen.

shows typical A and B faces, i.e. heavy and light distribution of particles in the convex and concave fracture faces. In contrast, at 2·0 M sucrose the density of particles in the hydrophobic center of the membrane is 60% greater. Also, the "typical" A face or B face are no longer apparent and both half membranes have an approximately equal distribution of particles.

It is noteworthy that under similar conditions of high sucrose concentration, Packer, Pollack, Munn and Greville[7] have found that the energy coupling is inhibited and that upon lowering the sucrose concentration to the isotonic range, normal electron energy transfer activity is restored. It remains to be shown, however, that the structural modifications seen by freeze-cleavage are also reversible.

Moreover, we have noted, as shown in Table II and in other experiments, that in the concave fracture face more single particles occur and are less aggregated with other particles than in the convex face. This approach may eventually lead to a better understanding of the factors which cause the components in the membrane to aggregate; a problem which is of particular relevance to the organization of the respiratory chain.

B. *Lipid Depletion Studies*

The above experiments show that by modifying the water content of the membranes we can change the particle distribution in the half

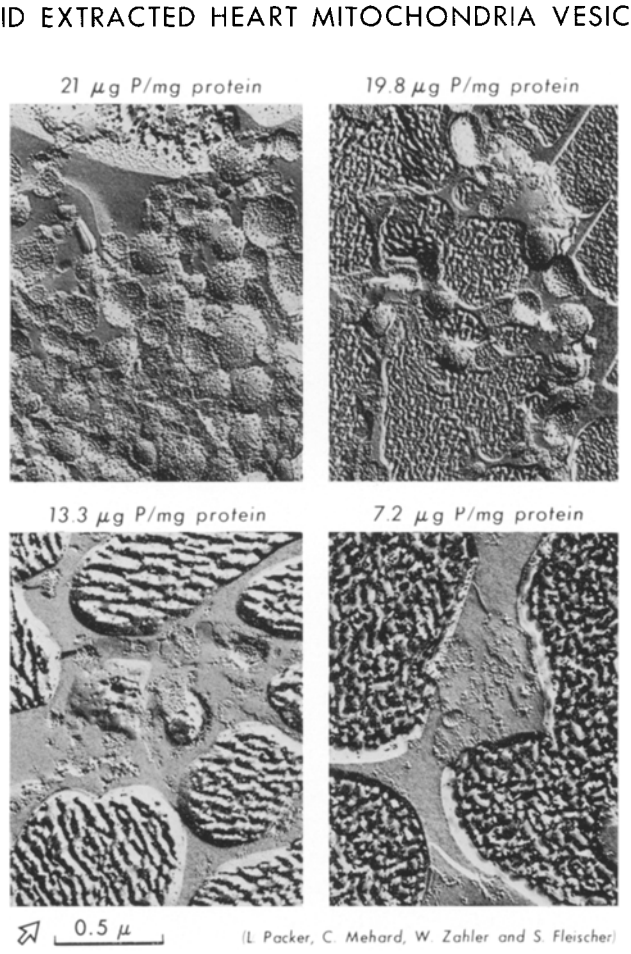

Figure 5. Effect of lipid depletion of beef heart submitochondrial particles on the particle distribution observed by electron microscopy after freeze-cleavage. Experiment carried out in collaboration with Dr. C. Mehard, Dr. W. Zahler, and Dr. S. Fleischer.

membranes. It was also of interest to determine the effect of lipid depletion.

In collaboration with Dr. S. Fleischer, Dr. C. Mehard and Dr. W. Zahler heart submitochondrial vesicles are being extracted in a stepwise manner with organic solvents. The extracted particles, of known lipid composition, are then examined by electron microscopy. Typical results are shown in Fig. 5. It has been established that after extensive extraction of lipid the membrane particles are disorganized and only an amorphous distribution pattern may be discerned. If lipids are removed gradually, then the smooth fracture faces disappear and only clusters of particles are deposited, and these are observed on the replica by such experiments. It should be possible to obtain evidence on the number of aggregated components in membrane vesicles. It is clear from these results that lipid plays a crucial role in establishing the proper conditions for dispersal of components in the membrane.

C. *Unsaturated Fatty Acid Depletion Studies*

To study effects of alteration *in vivo* of fatty acid composition, experiments were performed in which the unsaturated fatty acid composition of rat liver mitochondria was varied by dietary treatment.

The fatty acid composition of mitochondrial membrane is altered by growing rats on diets completely deficient in the essential unsaturated fatty acids.[8] In collaboration with Dr. M. A. Williams we have found (Table II) that the inner membrane preparations from

TABLE II. Particle densities in membrane fracture faces of mitochondria—Influence of unsaturated fatty acid composition

Unsaturated fatty acid composition	Sucrose in suspending medium mM	Fracture face	Area analyzed $\times 10^{-1}$	Particles		
				No.	Density/μ^2	A/B
EFA deficient	70	A	1·696	220	1333	1·62
		B	3·084	230	821	
	300	A	3·277	507	1317	1·14
		B	2·22	264	1170	
+ Linolenic acid	70	A	4·547	658	1491	2·18
		B	1·61	110	684	
	300	A	3·88	678	1742	1·86
		B	5·597	521	939	

Rat liver mitochondria were isolated from animals grown on diets deficient in essential unsaturated fatty acids or supplemented with linolenic acid for periods up to four weeks.[15] The mitochondria were suspended in the sucrose concentrations indicated in the Table prior to freezing in liquid nitrogen for preparation of samples for freeze fracturing.

fatty acid deficient, and linolenic acid fed rats show differences in the distribution of particles in the half membranes (A and B faces). The value for the ratio, particles in A face/particles in B face, is higher in the membranes from linolenic fed animals whose mitochondria also show a high degree of energy-coupling as compared with the fatty acid deficient preparations. These two types of mitochondria have remarkably different capacities for energy coupling as observed by

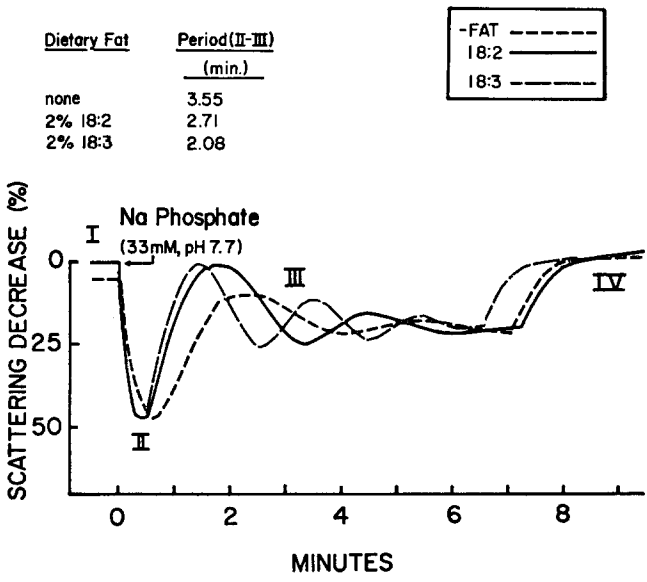

Figure 6. Effect of unsaturated fatty acids on the phase relationships of mitochondrial oscillations. Rat liver mitochondria were obtained from animals fed for a period of four weeks on a diet completely deficient in unsaturated fatty acids or supplemented with fatty acids of the type indicated in the Figure. Mitochondria were isolated as previously described,[15] suspended at pH 7·6 in an aerobic sucrose-EDTA test medium fortified with sodium succinate, the oxidizable substrate, at 25°C. Oscillations were started by adding a high concentration of permeant ions (33 mM sodium phosphate); experiments carried out in collaboration with M. B. Williams and R. C. Stancliff.

the period of the oscillatory state of ion transport (Fig. 6). It can be seen that the period of the oscillation is considerably lengthened in the mitochondria from the unsaturated fatty acid deficient animals. Under these conditions, the proportion of total unsaturated fatty acids in mitochondrial membranes does not change, but the unsaturated fatty acids contain fewer double bonds (i.e., their unsaturation index changes).[9] These experiments may provide direct evidence that the fluidity of the membrane is changing, and that this may be reflected by a changed pattern of distribution of particles in the half membranes that arise after freeze fracture. In this regard,

X-ray diffraction patterns suggest that membrane lipids are in bilayers with spatial distances of 4·8–5·0 Å. Therefore, the phospholipid molecules in the membranes must be packed closely together. Such tight packing suggests that the lipids would move in a coordinated manner.

Hence, the change in the hydrophobic center of the membrane after alteration of the unsaturated fatty acid content affects the pattern of membrane splitting during freeze-fracturing and can be detected by functional criteria, e.g., by measuring the period of the oscillatory state. From these results, together with the lipid depletion studies, it would appear that the inner mitochondrial membrane is in a liquid crystalline state where the proteins in the membrane center are in a dynamic state. These proteins can change their orientation in the membrane as the cohesive factors that bind them together, mainly hydrophobic forces, are modified by even small changes in the unsaturation index.

These interpretations have received support[10] from studies of mutant yeast mitochondria where the proportion of unsaturated fatty acids were lowered. When the unsaturated fatty acid content fell below 30% of the total fatty acids in the membrane, ATP synthesis was inhibited. Under these conditions, cytochrome content and ATPase activity were unchanged. Thus, these results suggest that the unsaturated fatty acid content of the membrane is important for energy coupling. The results can be rationalized by suggesting that the dispersal and orientation of the protein components are modified by changing the environment, and the flexibility inherent in the liquid crystalline state, which property is conferred upon the membrane by the presence of long chain unsaturated fatty acids which provide a milieu *par excellence* for protein dispersal.

D. *pH Dependence of Intraparticle Interactions*

Previous studies from this laboratory by Wrigglesworth and Packer[11] and House and Packer[12] with rat liver submitochondrial particles (SMP) have established that changes in pH markedly affect energy coupling and molecular conformations.

These studies revealed that, as the pH falls to 5·8, the following changes occur:

(a) Passive H^+ permeability of SMP decreases.
(b) The active H^+ uptake is increased.
(c) H^+/O ratio reaches a maximum of about 2 for choline or succinate oxidation.
(d) Respiration decreases.

These observations could be explained by changes in the structure of SMP. The main pH-dependent structural changes observed as the pH fell to 5·8 were:

(a) Volume changes seen by microscopy and packed volume.
(b) Increases in light scattering and ANS fluorescence.
(c) Changes in circular dichroism, suggesting alterations in quaternary protein structure, i.e., reversible particle aggregation.

In view of this it was of interest to determine if direct evidence could be obtained by electron microscopy after freeze-cleavage for reversible pH dependent intraparticle aggregation in the membrane. Indeed, we have observed large reversible aggregation effects of membrane preparation as the pH is lowered. Similar studies by Da Silva[13] on human erythrocyte ghosts have also demonstrated a reversible pH-dependent aggregation of membrane components.

IV. *Conclusions—Energy Coupling and Membrane Structure*

The above results indicate that respiration-dependent proton translocation markedly affects the molecular conformational state of functional proteins in the membrane by altering their mutual interaction. Since such changes are closely correlated with the degree to which the components are coupled to energetic processes, it seems that the dynamic organization of components in the membrane plays a crucial role in energy coupling in the inner mitochondrial membrane. These results are of interest with respect to the chemiosmotic and conformational hypotheses for energy coupling which state that the primary energetic event is the establishment of a H^+ gradient and/or conformational change. This is so because a change in H^+ gradient induced by electron transport would be anticipated to modify the aggregation state of the components involved in the tight coupling of energy from electron transport to oxidative phosphorylation or other energy-linked processes. At the molecular level of organization both are early events associated with energy coupling. Thus, a proton gradient can be viewed as causing a conformational change which brings the interacting proteins closer together, permitting an efficient chemical transfer of primary energy transduction into a form which can be conserved.

Other environmentally induced changes can also be considered as exerting a regulatory role on the energy coupling process by causing conformational changes. For example, changes in the concentration of bivalent cations such as calcium or magnesium would be expected

to affect the aggregation state and particle interactions. These might be due to direct effects upon protein, or, in the case of calcium, to stabilization of the phospholipid bilayer structure, thus reducing the mobility of protein components in the hydrophobic phase. Likewise, changes in the unsaturated fatty acid composition of the membrane brought about by dietary means or by changed fatty acid metabolism could modify energy coupling by affecting structure at the molecular level by affecting intramembrane particle interactions. Similarly, modifications in the protein components of the membrane, brought about by a changed pattern of biosynthesis and/or genetic regulation may also modify energy coupling through deletion and/or through a change in the structure of components of the integrated electron transfer and energy coupling system.

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