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SHORT REVIEW

New Molecular Aspects of Energy-Transducing Protein Complexes[1]

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Introduction

The main function of biological membranes is to separate spaces in the living cell. Since the solute composition in the various spaces has to be different, all the biological membranes function in energy conduction. The nature of this energy conduction is determined by the structure, the polarity, and the specialized activities of the membrane. Most of these properties are designated by multisubunit protein complexes. Lipid bilayers are largely impermeable to most solutes, including small ions and polar molecules (Danielli and Davson, 1935; Singer, 1974). The permeability of membranes is introduced by specific proteins that by so doing determine the nature of energy conduction across the system. For example, the nucleus of eukaryotic cells is surrounded by a very permeable membrane. The large pores in the membrane are constructed of a protein complex that possesses ATPase activity (Aaronson and Blobel, 1975; Kondor-Koch *et al.*, 1982). The permeability of outer membranes of mitochondria and gram-negative bacteria is maintained by a specific protein, the induced conductance of which is affected by membrane potential (Rosenbusch, 1974; Schindler and Rosenbusch, 1978, Zalman *et al.*, 1980; Freitag *et al.*, 1982). Therefore, even the most permeable membranes are involved in energy-transducing processes.

[1]Abbreviations: DCCD; dicyclohexylcarbodiimide; F_1, the catalytic sector of the mitochondrial proton ATPase; F_0, the membrane sector of the proton ATPase complex; CF_1, the catalytic sector of the chloroplast proton ATPase; CF_0, the membrane sector of the chloroplast proton ATPase; BF_1, the catalytic sector of the bacterial proton ATPase.

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In the last decade, it became apparent that most of the biological membranes maintain a high degree of asymmetry (Mitchell, 1968; Racker, 1976). This property is essential for the proper activity of the various functional systems in the membrane. The degree of asymmetry varies from uneven distribution of lipids in the bilayer up to absolute polarity of large protein complexes in the membrane. Therefore, one of the main functions of protein complexes is to keep the strict functional asymmetry of energy-transducing membranes (Racker, 1976).

Of all the biological sciences, membrane bioenergetics is studied by the most versatile approaches. Although considerable information about energy-transducing protein complexes was obtained from work with whole cells and membrane fragments, it was the development of new approaches and techniques that advanced the understanding of the structure and function of protein complexes. As enormous amounts of techniques are employed, it is impossible to include most of them in this paper. We elected to discuss in this review only the techniques of resolution and reconstitution of membrane proteins, planar lipid bilayers, immunological studies, and biogenesis of membranes. As examples of specific protein complexes we shall mention the acetylcholine receptor, proton-ATPases, cytochrome oxidase, and the photosystem I reaction center.

A protein complex can be defined as the minimal structure that catalyzes a characterized biochemical reaction. Along this line photosystem I reaction center of higher plants was defined as the minimal structure that catalyzes the photoreduction of ferredoxin by reduced plastocyanin (Bengis and Nelson, 1975, 1977). It was isolated as a protein complex containing seven different polypeptides that were designated as subunits I to VII in the order of decreasing molecular weights from 70,000 to 8,000 respectively (Nelson and Notsani, 1977; Hauska *et al.*, 1980). A photosystem I reaction center was isolated from green algae and cyanobacteria with only four different polypeptides (Nechushtai and Nelson, 1981a,b; Nechushtai *et al.*, 1983).

Cytochrome oxidase can be defined as the minimal structure that catalyzes the vectorial oxidation of reduced cytochrome *c* by oxygen coupled to the formation of a protonmotive force across the membrane (Racker 1976). Cytochrome oxidase isolated from mitochondria contains at least seven subunits, designated as subunits I to VII in the order of decreasing molecular weights of 42,000 to 6,000 respectively (Mason *et al.*, 1973; Cabral and Schatz, 1978; Casey *et al.*, 1980; Winter *et al.*, 1980). However, a cytochrome oxidase that fits the same definition was isolated from *Paracoccus denitrificans* containing only two different subunits (Ludwig and Schatz, 1980; Ludwig, 1980). The proton-ATPase complex is the minimal structure that, upon reconstitution into lipid vesicles, can form a protonmotive force by hydrolyzing ATP and can form ATP at the expense of a protonmotive force

(Kagawa and Racker, 1971; Pick and Racker, 1979). In this complex enzyme a proton flux should be coupled with a phosphorylation reaction. These two catalytic events take place in separate sectors of the enzyme. A membrane sector functions in proton conduction, while the catalytic sector which protrudes from the membrane is functioning in the phosphorylation reaction (Senior, 1973). The simplest enzyme was isolated from bacteria and chloroplasts (Nelson *et al.*, 1974; Nelson *et al.*, 1980a; Foster and Fillingame, 1979; Fillingame, 1981). It is composed of eight different polypeptides, five of which are constituents of the catalytic sector, F_1 (subunits α, β, γ, δ, ϵ), and three belong to the membrane sector F_0 (subunits I, II, and III). The mitochondrial enzyme contains extra polypeptides.

Acetylcholine receptor is the minimal structure that, upon binding of acetylcholine, brings about the opening of ion channel across the membrane. It should also possess pharmacological characteristics like desensitization and sensitivity to specific inhibitors (Eldefrawi and Eldefrawi, 1982; Moreau and Changeux, 1976). Acetylcholine receptor from Torpedo electric organ has been isolated with four kinds of glycopeptide subunits (Reynolds and Karlin, 1978; Lindstrom *et al.*, 1979; Raftery *et al.*, 1980). The monomeric receptor consists of two subunits α (M.W. 38,000) and one each of β (50,000), γ (57,000), and δ (64,000) subunits that for a change were designated in the order of increasing molecular weights.

Protein complexes also have physical identity caused by the tight binding of their various subunits. However, the integrity of a polypeptide in a protein complex can be ensured only when its specific function is resolved.

Resolution and Reconstitution of Protein Complexes

Two main approaches are used for resolution-reconstitution studies in biological membranes. One is to aim at the depletion or inactivation of a single component in the membrane—consequently a given activity is inhibited—then to look for restoration of the activity of the membrane by the addition of the purified factor. The second approach is to isolate a functional protein complex from a given membrane, and to study its properties in solution and after reconstitution into lipid vesicles of planar bilayers.

Upon isolation of the chloroplast inner membrane, ferredoxin is readily liberated, and thereby NADP photoreduction is inactivated. In looking for the lost factor, the function of ferredoxin was discovered (San Pietro and Lang, 1958; Tagawa and Arnon, 1962). Ferredoxin was purified and crystallized and its function in electron transport between photosystem I and the enzyme ferredoxin-NADP reductase was established (Shin and Arnon, 1965).

Cytochrome *c* is the most loosely bound component of the mitochondrial

electron transport chain (Jacobs and Sanadi, 1960). It can be removed by salt washing from the inner membrane, and thereby the electron transport between the cytochrome b–c_1 complex and cytochrome oxidase is inhibited (Lenaz and MacLennan, 1966). Upon addition of purified cytochrome *c*, the electron transport activity of the chain is restored. This line of investigation not only helped to establish the function of cytochrome *c*, but also was used as a tool for the study of the mechanism of electron transport in cytochrome b–c_1 complex and cytochrome oxidase (Bowyer and Trumpower, 1981).

Unfortunately for the scientists, but fortunately for the efficiency of energy transduction, most of the proteins are bound to the membrane with varying degrees of tightness. Plastocyanin is an example of a protein which is hydrophilic in nature, and is bound quite firmly to the chloroplast membrane. However, upon brief sonication it can be liberated, and electron transport and photophosphorylation are inhibited in the depleted particles (Hauska *et al.*, 1971). These two activities can be restored by sonication of plastocyanin into the chloroplast vesicles. This observation led to the conclusion that plastocyanin is situated on the internal side of the thylakoid membrane and that electrons must cross the membrane for the photoreduction of ferredoxin by the photosystem I reaction center (Nelson *et al.*, 1972a; Trebst, 1974).

The same line of investigation was the first to be employed in the study of the function of the proton-ATPase complex in oxidative phosphorylation (Racker, 1976). The initial studies of this enzyme were based on the liberation of the catalytic sector-F_1 from the membrane by a mechanical force and the investigation of the properties of the depleted membranes and the isolated F_1. Subsequently, the system was reconstituted by the addition of the isolated F_1 to the depleted membranes and by so doing, the oxidative phosphorylation activity of the system was restored (Pullman *et al.*, 1960; Penefsky *et al.*, 1960). The activity of the partially depleted system could be reconstituted not only by the addition of native F_1 but also by inactivated enzyme (Fessenden and Racker, 1966; Racker, 1967). Therefore, it was concluded that the enzyme fulfills a structural role as well as a catalytic role. Similar results were obtained from experiments with isolated chloroplasts. Jagendorf and Smith (1962) observed that EDTA treatment caused uncoupling in isolated chloroplasts. Soon thereafter Avron (1963) demonstrated that photophosphorylation activity of the EDTA-treated chloroplasts can be reconstituted upon addition of their supernatant. Subsequently, the catalytic sector of the chloroplast proton-ATPase complex was purified, and it was shown that this enzyme catalyzes photophosphorylation (Vambutas and Racker, 1965; McCarty and Racker, 1966). These studies promoted the thorough investigation of the structure and function of the catalytic sectors of proton-ATPases from various sources (Senior and Brooks, 1970; Lambeth and Lardy, 1971; Senior, 1973; Nelson, 1976; Kagawa, 1978).

The stoichiometry of the subunits in the enzyme is still a matter of

debate, but most of the recent observations indicate a ratio of 3:3:1 for the distribution of α, β, and γ subunits of the enzymes from mitochondria, *E. coli,* and chloroplasts (Senior, 1979; Bragg and Hau, 1975; Fillingame, 1981; Foster and Fillingame, 1982; Yoshida *et al.,* 1979; Kagawa, 1978; Todd *et al.,* 1979; Merchant *et al.,* 1983). The function of each individual subunit was studied by a combination of methods (Nelson, 1976, 1981a). Studies of partial resolution of the enzyme have contributed to the following notions: The α subunit may possess the regulatory nucleotide binding sites (Ohta *et al.,* 1980). The active site of the enzyme might be situated in the β subunit of the enzyme (Deters *et al.,* 1975; Philosoph *et al.,* 1977; Douglas *et al.,* 1977; Dunn and Futai, 1980; Yoshida *et al.,* 1977a, b). The γ subunit may serve as a proton-gate within the catalytic sector (Yoshida *et al.,* 1977a, b, but see Khanashvili and Gromet-Elchanan, 1982), and it may function in the assembly of α and β subunits to form active ATPase (Larson and Smith, 1977; Dunn and Futai, 1980). The δ subunit functions in the binding of CF_1 and BF_1 to the membrane sector (Nelson and Karny, 1976; Younis *et al.,* 1977; Sternweis and Smith, 1977; Sternweis, 1978). The ϵ subunit functions in the binding of BF_1 to the membrane (Sternweis and Smith, 1977; Sternweis, 1978; Kagawa *et al.,* 1979). The ϵ subunit of CF_1 inhibits the ATPase activity of CF_1 (Nelson *et al.,* 1972b). A specific ATPase-inhibiting protein regulates the ATPase activity of F_1 (Pullman and Monroy, 1963; Pederson *et al.,* 1981). In mitochondria the oligomycin-sensitivity conferral protein (OSCP) and F_6 function in the binding of F_1 to the membrane (MacLennan and Tzagoloff, 1968; Senior, 1973). Many more "coupling factors" were identified in the mitochondrial coupling device, but even the most unambiguous one—OSCP—proved to be analogous to the δ subunit of BF_1 (Walker *et al.,* 1982a). Neither cytochrome oxidase nor acetylcholine receptor could be dissociated into reconstitutable active subunits. In photosystem I reaction center subunit I was isolated in a pure form and it was shown to contain the P700, the primary electron acceptor and primary light-harvesting antenna. Subunit III can be depleted from the complex and it was indicated that this polypeptide might function in facilitating the electron transport from reduced plastocyanin to the oxidized P700 (Bengis and Nelson, 1977). Even though a detailed picture of the structure and function of the protein complexes was deduced from those studies, it could not be completed without turning to the second approach of isolation and reconstitution of the purified protein complexes into lipid vesicles.

In a landmark paper, Kagawa and Racker (1971) reported on reconstitution of solubilized mitochondrial membrane, containing the proton-ATPase complex, into phospholipid vesicles. The membranes were solubilized in sodium cholate solution and upon addition of phospholipids and removal of the detergent by dialysis, active reconstituted phospholipid vesicles were formed. This method of reconstitution by cholate dialysis is widely used for reconstitu-

tion of membrane proteins, but it is not suitable for several protein complexes, and the resulting vesicles are of a small size (Racker, 1976). Therefore, several techniques were developed in order to meet the special requirements of the various protein complexes and to control the size of the vesicles. Detergent dilution (Racker *et al.*, 1975), protein incorporation (Eytan *et al.*, 1975), reconstitution by sonication (Racker and Stoeckenius, 1974), and reconstitution by freezing and thawing (Kasahara and Hinkle, 1977) served as partial answers to the above-mentioned problems (Eytan, 1982). Even though reconstitution of isolated membrane proteins into phospholipid vesicles has become one of the most successful methods of elucidating the mechanism of action of complex biological systems, it still needs a touch of art in its practice. The membrane protein has to be purified under conditions that will preserve it to be reconstitutively active (Racker, 1976), and the phospholipids must be suitable for the reconstitution of the specific protein (Sigrist-Nelson *et al.*, 1978; Niggli *et al.*, 1981). There is no way to predict it, and the only way to go about it is to keep trying and use one's intuition. Sometimes it is helpful to use crude phospholipids or lipids from the membranes from which the protein was extracted (Nelson *et al.*, 1977; Huganir *et al.*, 1979). Quite often these difficulties prompt the researchers to work with impure proteins or to replace the proper reconstitution procedure by studies with detergent micelles. They surely miss the excitement of having reconstituted a single and pure protein complex into active lipid vesicles.

The initial studies of partial resolution of chloroplast and mitochondrial membranes have led to new concepts in bioenergetics and contributed to the general acceptance of the chemiosmotic hypothesis (Mitchell, 1966, 1968; Jagendorf, 1967, 1975). However, only by reconstitution of the proton-ATPase complex into phospholipid vesicles could its complete independence from the electron transport be demonstrated (Kagawa and Racker, 1971; Pick and Racker, 1979; Racker and Stoeckenius, 1974). It was shown that the reconstituted proton-ATPase complex can pump protons coupled to ATP hydrolysis, it can produce ATP from ADP and phosphate by a preformed protonmotive force, and it can catalyze the reaction of ATP-P_i exchange. Direct measurements of proton and ion conductances became possible and bacteriorhodopsin was elected as a tool and model for proton pumping (Lanyi and Oesterhelt, 1982). The membrane sector of proton-ATPase complexes was isolated, and it was demonstrated that it acts as a DCCD-sensitive proton channel (Okamoto *et al.*, 1977; Schneider and Altendorf, 1980, 1982). Subunit III (proteolipid) was isolated from chloroplast membranes and, upon reconstitution into lipid vesicles containing galactolipids, DCCD-sensitive proton conductance was demonstrated (Nelson *et al.*, 1977; Sigrist-Nelson and Azzi, 1980). The subunits of the membrane sector of proton-ATPase from thermophilic bacteria were isolated, and it was shown that reconstitution of

two of them into phospholipid vesicles was required for the creation of proton flux (Okamoto *et al.*, 1977). It was proposed that the membrane sector of the proton-ATPase complex from bacteria and chloroplast is composed of three different subunits (Schneider and Altendorf, 1980, 1982; Nelson *et al.*, 1980a). In chloroplasts, subunit I might serve as a binding site for CF_1, subunit II might function in the assembly of six copies of subunit III, and subunit III functions as a specific proton channel (Nelson, 1980, 1981a). However, studies with mutants of *E. coli* have indicated that the complete assembly of the three F_0 subunits was required for its proper function (Friedl *et al.*, 1983; Hoppe *et al.*, 1983). Subunit II (M.W. 17,200) of F_0 was proposed as the binding site for the catalytic sector.

Cytochrome oxidase was reconstituted into phospholipid vesicles (Hinkle *et al.*, 1972; Jasaitis *et al.*, 1972). The oxidation rate of reduced cytochrome *c* was modulated by the membrane potential in a fashion reminiscent of the respiratory control in intact mitochondria. These studies emphasized the importance of the membrane architecture for energy transduction, a fact that was not generally accepted only a decade ago (Chance *et al.*, 1970). Recently it became apparent that protein complexes of energy-transducing membranes are involved in proton translocation that is not directly linked to the mechanism of the electron transport (Papa, 1976, 1982; Hauska *et al.*, 1983). Studies with reconstituted cytochrome oxidase indicate that the enzyme is a redox-linked proton pump (Wikstrom and Krab, 1979). Casey *et al.* (1979) showed that protons are ejected from reconstituted vesicles in a ratio of $0.9H^+/e^-$. DCCD abolishes the proton pumping activity of cytochrome oxidase and binds specifically to subunit III of the enzyme (Casey *et al.*, 1980). Recently similar results were obtained with cytochrome oxidase from the thermophilic bacterium PS 3 (Sone and Hinkle, 1982). This enzyme contains three subunits, pumps protons, and is inhibited by DCCD which appears to bind to subunit III. Cytochrome oxidase from *Paracoccus denitrificans,* which was shown to consist of only two subunits, is also active in proton pumping (Solioz *et al.*, 1982). Yet binding studies with DCCD and subsequent inhibition experiments have not been carried out.

Purified photosystem I reaction center was reconstituted into phospholipid vesicles (Orlich and Hauska, 1980). When phenazine methosulfate was added, a light-dependent proton uptake was recorded. By further reconstitution of purified proton-ATPase complex, cyclic photophosphorylation was obtained (Hauska *et al.*, 1980). Therefore, the isolated photosystem I reaction center was found to be active not only in NADP photoreduction but also in generation of protonmotive force which comprises all of the known biochemical activities of the system.

Neurotransmission at the vertebrate neuromuscular junctions is mediated by the release of acetylcholine from the nerve terminal and its

binding to the acetylcholine receptor on the postsynaptic membrane. Electrophysiological experiments have shown that binding of the neurotransmitter to its receptor triggers the opening of large cation-selective channels that subsequently close up, and desensitization of the receptor takes place. The opening of the channels is suppressed by several pharmacological inhibitors including α-bungarotoxin and curare. Epstein and Racker (1978) reported on reconstitution of crude acetylcholine receptor into phospholipid vesicles in which all the above-mentioned effects were preserved. Continuous presence of phospholipids during solubilization of the receptor in cholate was essential for preserving the integrity of the channel. Subsequently, intact acetylcholine receptor was purified and reconstituted into phospholipid vesicles (Huganir *et al.*, 1979; Changeux *et al.*, 1979; Wu and Raftery, 1979; Lindstrom *et al.*, 1980). The reconstitution studies provided evidence that the four subunit receptors contain not only the binding sites for acetylcholine but also the active cation channel, and the activated receptor by itself brings about the response of agonist-induced membrane permeability. This is different from the β-adrenergic receptor in which the receptor must interact with another membrane protein in order to transmit the signal (Citri and Schramm, 1980, 1982).

Incorporation of membrane proteins into liposomes provided the possibility of studying vectorial processes with purified protein complexes. The ability to reconstitute the functional asymmetry of energy-transducing membranes by isolated components promises further excitement in the future.

Study of Proteins in Planar Lipid Bilayers

Reconstituted bilayer vesicles advanced our knowledge of the mechanism of energy transduction by biological membranes. Unfortunately, their inner space is small and is inaccessible for direct electrical measurements across the membrane. On the other hand, planar bilayers are an ideal system for that particular purpose and, moreover, the composition of solutes and the imposed electric potential are readily controlled in the two sides of the membrane. Planar lipid bilayers are formed by lifting two lipid monolayers in two sides of a thin Teflon septum with a small hole in the middle (Montal and Mueller, 1972). Reconstitution of proteins in the lipid bilayer is based on the incorporation of the protein into the monolayers, and, upon the formation of the bilayer from the two monolayers, the protein is reconstituted into the bilayer (Montal *et al.*, 1981). The weakness of the system lies in its instability, and one should be very cautious to conclude only from results taken from stable membranes, and not immediately before their breakage.

Formerly, the formation of the monolayers was performed by placing a

drop of phospholipids solubilized in hexane on the surface of the water solution. In order to incorporate proteins into the monolayers, they had to be first introduced into inverted lipid micelles in decane or hexane (Gitler and Montal, 1972). Some complex membrane proteins could not survive this treatment and were inactivated (Nelson *et al.*, 1980b).

For this reason, Schindler (1979) has developed a procedure that does not require the use of organic solvents for the formation of protein-containing monolayers. It was observed that phospholipid vesicles and even native membranes having the right lipid composition and a certain size spontaneously formed monolayers containing the membrane proteins of the vesicles (Schindler, 1980; Schindler and Quast, 1980). This study opened up the possibility of reconstituting purified functional proteins and comparing their properties with the same activity from native membranes. Another way of circumventing the use of organic solvents is the fusion of preformed vesicles with preformed planar bilayers (Miller and Racker, 1976; Miller, 1978). The vesicles interact with planar bilayers in the presence of a negatively charged lipid, calcium, and osmotic conditions that cause the swelling of the vesicles.

Planar bilayers are suitable for the study of fast transmembrane events, involving charge transfers across the membrane (see Schonfeld *et al.*, 1979). It is a superb technique for the study of ion channels, but quite sluggish in the investigation of carriers and enzymes with slow turnover in the reconstituted membranes. The technique requires expertise, precision, and a great deal of patience. Therefore, it is not widely used in laboratories that study the reconstitution of energy-transducing systems in biological membranes. Nevertheless, in the last decade impressive results have been achieved by the employment of planar bilayer techniques (Montal *et al.*, 1981). However, in the future the patch-clamping technique, developed several years ago, might replace most of the studies involving bilayers. For that goal, the formation of bilayers from monolayers containing proteins should be solved. Before the ink of the last sentence was dry, the formation of lipid bilayers formed from monolayers at the air–water interface in the tip of patch-pipettes was elegantly demonstrated in three different laboratories (Schuerholz and Schindler, 1983; Hanke *et al.*, 1983; Suarez-Isla *et al.*, 1983).

Photosystem I reaction center and cytochrome oxidase are protein complexes that function in electron transport across the membrane. Photosystem I reaction center has not been studied in planar bilayers. Schonfeld *et al.* (1979) have reconstituted the reaction center from photosynthetic bacteria into planar lipid bilayers. They observed light-induced membrane potentials and used the direct measurements to evaluate the interaction of this protein complex with electron donors and acceptors. Cytochrome oxidase was also reconstituted into planar bilayers (Montal, 1974). In the presence of reduced cytochrome *c* (by ascorbate) on one side of the bilayers, a membrane potential

was formed. Now is the right time to extend this observation, in the light of the discovery of proton pumping activity of the enzyme (Wikstrom and Saari, 1977; Krab and Wikstrom, 1978). The possible involvement of a DCCD-sensitive proton channel can be better assessed by studies of lipid bilayers.

The involvement of a proton channel in the membrane sector of the chloroplast coupling device was indicated by McCarty and Racker as early as 1966. A channel can be defined as a membrane structure that facilitates a fast transport of components (ions) across the membrane. Racker and Hinkle (1974) proposed that channels should not be drastically affected by the phase transition of the membrane. Indeed, antibiotics that were well characterized as channels behaved accordingly. Okamoto *et al.* (1977) tested the reconstituted membrane sector of the proton-ATPase complex from thermophylic bacteria, and it was shown to be affected by the phase transition of the membrane, as proton carriers should behave. Moreover, the reported turnover of proton movements was too small to account for a channel mechanism. On the other hand, the calculated proton conductance through the open chloroplast membrane sector justifies the assumption of a channel mechanism in CF_0 (Nelson, 1980, 1981a). A safeguard mechanism that partially closes the channel upon removal of the catalytic sector was proposed.

Direct evidence for the possible involvement of a proton channel in the proton conductance within the proton-ATPase complex came from lipid bilayer studies (Schindler and Nelson, 1982). The proteolipid (equivalent to subunit III of CF_0 and BF_0) was isolated from yeast mitochondria and reconstituted into planar bilayers via phospholipid vesicles. Electric conductance through membrane channels, which were highly specific for protons, was demonstrated. At pH 2.2 and an applied voltage of 100 mV, a single channel of 12 pS was measured. The formation of the proton channel required the association of at least two polypeptide chains. The number of proton channels was sharply increased by elevating the pH from 4 to 5, and it was proposed that a glutamic or aspartic residue might be involved in this transition phenomenon. The possibility that proton-ATPase contains three self-associated proton channels was raised. These observations were performed under nonphysiological conditions. Follow-up of this research with isolated membrane sectors of proton-ATPase complexes is desired for further assessment of the mechanism of proton conduction. Study of excised patches from reconstituted vesicles containing the purified components and the use of this technique to remove patches of chloroplast and mitochondrial membranes might advance our knowledge (see Tank *et al.*, 1982).

Acetylcholine receptor was recently studied in phospholipid bilayers that were formed from native membrane vesicles (Schindler and Quast, 1980), and reconstituted vesicles containing purified receptor from the Torpedo electric organ (Nelson *et al.*, 1980b). In the presence of carbamylcholine a dose-

dependent increase in membrane conductance, which exhibited desensitization, was recorded. This conductance was decreased in the fashion of competitive inhibition by curare. Asymmetric bilayers formed from phospholipid vesicles containing the acetylcholine receptor in only one of the chambers showed asymmetric responses to carbamylcholine. When the agonist was applied to the compartment containing the vesicles without the receptor, small or no changes in conductance resulted. Upon subsequent addition of the agonist to the receptor side, the characteristic response was obtained. The single-channel conductance and the lifetime of the opened channels were in fair agreement with the values estimated by fluctuation analysis. These studies opened up new possibilities of direct measurements of multiple effects and events in a single receptor molecule. Indeed recently it was demonstrated that the acetylcholine receptor channel displays two kinetically distinct open states, which are different in their mean open times but have similar channel conductances (Labarca *et al.*, 1983). The strategies described for the acetylcholine receptor may be extended to other excitable channels.

Planar bilayers are like handsome princes—when they are not turning into frogs one might even enjoy suffering from them.

Immunological Studies

Immunological studies are extensively used in modern membrane research. Antibodies are excellent tools for approaching specific targets on the membranes, or to probe newly synthesized polypeptides on their way to the membranes. Antibodies are very specific ligands, and therefore immunological cross-reactivity is an excellent indication for chemical identity in the cross-reacting antigens. Due to the sensitivity of the immune system, the use of as pure antigens as possible is advocated for obtaining antibodies. The decision whether to inject native or denatured protein depends on the kind of experiment in which the antibody has to be used. Injections of native proteins increase the probability of obtaining antibodies directed against sites on the surface of the antigen. These antibodies are more likely to inhibit partial reactions when assayed *in situ*. Some of the antigenic sites might be dependent on the tertiary structure of the protein and, after denaturation, they are no longer recognized by the antibody (Lien *et al.*, 1972). Injection of native enzymes increases the risk of getting contaminating antibodies, and therefore it is advisable to inject polypeptides that were cut off SDS-gels and electroeluted into dialysis bags (Nelson *et al.*, 1973, Nelson, 1983). If the antigen is prepared from cells that are evolutionarily far distant from the animal in which the antibody was raised, it is quite possible that antibodies would be obtained that are directed against sites which are buried inside the protein as

well as antibodies that are directed to sites on its surface. The latter might inhibit biochemical reactions *in situ* (Nelson *et al.*, 1973).

If the denatured antigen is prepared from cells closely related to the host (enzyme from rat injected into mice or rabbits), antibodies directed exclusively against hidden sites that are exposed only upon denaturation of the enzyme are very likely to be obtained. Those antibodies recognize only denatured proteins and do not interact with the native enzyme. Therefore, they might serve in cross-absorbing of denatured proteins out of enzyme solutions. Even antigens that were prepared from purified enzymes and subsequently eluted out of SDS-gels might be contaminated by foreign proteins. The presence of contaminating antibodies that are directed against polypeptides having the same molecular weight as the antigen can be revealed by combination assay using partial cleavage and immunodecoration (Nelson, 1983).

When the antigen is ready, there are several ways to get the proper antibody, and the procedure might be selected according to the special requirements of the system to be tested. For obtaining large quantities of antibodies, injection of goats or rabbits might be preferred. If only small amounts of antigen are available, injection of mice and the induction of ascitic fluid might be used (Lamb *et al.*, 1978). Another way to circumvent the need for large amounts of antigen is the procedure of direct injection of the rabbit's lymph nodes (Goudie *et al.*, 1966; Louvard *et al.*, 1982). The development of the technique for generating monoclonal antibodies opened up imaginative new possibilities for immunological studies (Kohler and Milstein, 1975). The monoclonal antibody is specific for a single determinant; it can be obtained at high titers, and unlimited supplies of this specific antibody can be produced (Kohler and Milstein, 1976). Therefore, superficially, it would seem as if everyone should turn to monoclonal antibodies and stop using regular antibodies. The main reason that prevents this is the fact that monoclonal antibodies are a poor choice for experiments requiring immunoprecipitation of the antigens. In these experiments people reached the ridiculous stage of mixing five different monoclonal antibodies in order to get decent immunoprecipitation. The second obstacle is the fact that in immunoassays the signals obtained are rather low. The third drawback is due to the tendency to use crude membranes as antigens for raising the antibodies. This is due to the fact that any monoclonal antibody is monospecific by definition and, for technical reasons, selection of the antibodies favored the isolation of pure antigens. It seems to us that the reverse procedure might give much better results, and it is recommended not only to purify the antigen to homogeneity but also to isolate fragments of it by limited proteolysis and to use them as antigens. This procedure should increase the probability of obtaining the desired monoclonal antibodies.

When the desired antibody is available, there are numerous ways to use it in the study of energy-transducing membranes. One of the most useful techniques is electrotransfer of polypeptides from slab gels to nitrocellulose paper, followed by immunodecoration by a specific antibody and specific ligand to identify it. The procedure of electrotransfer and immunodecoration by ^{125}I-protein A was developed by Towbin *et al.* (1979), and its use in energy-transducing membranes has grown constantly since then (Ludwig and Schatz, 1980; Rott and Nelson, 1981). The procedure is very convenient and requires very simple instrumentation. Specific proteins can be detected and their quantity in crude cell extracts can be estimated. It is based upon the transfer of proteins by electrophoresis from the gel to a nitrocellulose paper under conditions in which a replica of the original gel pattern is obtained. After blocking all the additional binding sites on the paper, specific proteins are detected by sequential incubation with specific antibodies. Following proper washing of the unbound globulins, the bound antibody is decorated with ^{125}I-protein A or a second antibody labeled with ^{125}I, fluorescent markers, or enzymes that give color reactions. Markers with specific affinity like ^{125}I-lectins or ^{32}P-labeled DNA can be used instead of the antibody.

Even though inhibiting antibodies were successfully used in the study of energy-transducing membranes (Berzborn, 1980), it seems the identification of polypeptides by immunodecoration and immunoprecipitation is more widely employed. The latter was markedly advanced by the introduction of fixed *Staphylococcus aureus* cells for immunoprecipitation (Kessler, 1975). A combination of peptide-specific antibodies, monoclonal antibodies, immunodecoration, and immunoprecipitation is an attractive recipe for ever-increasing use of immunological techniques in the study of energy-transducing processes.

An antibody that was raised against purified photosystem I reaction center was found to be specifically directed against subunit I (Nelson and Bengis, 1975). The antibody inhibited the ferredoxin-dependent reactions in isolated chloroplasts, and it was concluded that part of subunit I protrudes from the external side of the thylakoid membrane. Antibodies were also raised against each individual subunit that was electroeluted from SDS-gels. They were used for the localization of their genes on the chloroplast DNA (Westhoff *et al.*, 1981, 1983) and for the study of immunological cross-reactivity of the subunits of photosystem I reaction centers from various sources (Nechushtai *et al.*, 1983). The antibody that was raised against subunit I of Swiss chard photosystem I reaction center cross-reacted with subunits I of reaction centers from higher plants (spinach), green algae (chlamydomonas), and cyanobacterium (Mastigocladus). Several more plants and algae were tested recently, and all of them gave the same results (Nechushtai and Nelson, unpublished). Antibodies that were raised against

subunits II of Swiss chard or spinach photosystem I reaction centers were found to cross-react with the corresponding subunits II from the above-mentioned sources. Antibodies against subunits III to VII showed little or no cross-reactivity. It is noteworthy that while subunit I of eukaryotes is a chloroplast gene product, subunit II is synthesized by cytoplasmic ribosomes (Nechushtai *et al.*, 1981). Therefore, these immunological studies indicated the common evolutionary source of photosystem I reaction centers and that subunit II has preserved its structure upon transfer of its gene from the prokaryotes to the nucleus of the eukaryotes.

Antibodies against individual subunits of cytochrome oxidase were used mainly for biogenesis studies. Perhaps the most dramatic event in this field was the rise and the fall of the "polyprotein" as a precursor for the cytoplasmically made subunits of cytochrome oxidase (Poyton and McKennie, 1979a,b; Lewin *et al.*, 1980; Mihara and Blobel, 1980). The discovery of the "polyprotein" was probably due to the presence of contaminating antibody against polypeptide with a molecular weight of about 55,000. The antigens that were isolated on columns may contain highly antigenic polypeptide with a molecular weight of about 55,000. The antibodies against the subunits of cytochrome oxidase that were prepared following electroelution from SDS-gels did not contain contaminating antibodies (Nelson, 1983).

Antibodies prepared the same way were used for the assay of cross-reactivity with the two subunits of cytochrome oxidase from *Paracoccus* (Ludwig, 1980). It was shown by the technique of electrotransfer and immunodecoration that subunit I of the *Paracoccus* enzyme cross-reacted with the antibody against subunit I of cytochrome oxidase from yeast mitochondria. Similarly, subunit II of the *Paracoccus* cytochrome oxidase cross-reacted with anti-subunit II from yeast mitochondria. Therefore, it appears that the two subunits of *Paracoccus* cytochrome oxidase correspond to two of the mitochondrially made subunits, an observation which is in line with the view that mitochondria have evolved from symbiotic prokaryotes.

Antibodies were extensively used in the study of proton-ATPase complexes from various sources. Antibodies against seven subunits of the chloroplast enzyme were raised, and some of them inhibited photophosphorylation by isolated chloroplasts (Nelson *et al.*, 1973). In that study it was concluded that the α subunit of CF_1 contains the tight nucleotide binding sites functioning in the regulation of the enzyme and that the γ subunit possesses the binding site for the ϵ subunit. Similarly, antibodies against individual subunits of the proton ATPase of *E. coli* indicated that part of the γ subunit might be required for proper assembly of α and β subunits with ATPase activity (Larson and Smith, 1977; Dunn and Futai, 1980). Antibodies raised against β subunits of the enzymes from the thermophylic bacteria PS3 and the photosynthetic bacteria *Rhodospirillum rubrum* inhibited ATPase and phos-

phorylation activities of the enzyme (Yoshida *et al.*, 1979; Khananshvili and Gromet-Elhanan, 1982). Antibodies against individual subunits of proton-ATPase complexes played a major role in studies of its biogenesis (Maccecchini *et al.*, 1979). Immunoprecipitation with the help of fixed *Staphylococcus aureus* cells is used throughout most of the recent biogenesis studies (Chua and Schmidt, 1979).

The technique of electrotransfer and immunodecoration was used to look for structural similarities among subunits of proton-ATPase complexes from various sources (Rott and Nelson, 1981). From all the combinations of antigen–antibody examined, only the antibodies against β subunits cross-reacted with the corresponding subunit of all the proton-ATPase complexes tested. It was proposed that the results indicated similarities in the amino acid sequences in the β subunits of all of the proton-ATPase complexes. Recently, this was confirmed by DNA sequencing of β subunits from *E. coli* membranes, bovine mitochondria, and spinach chloroplasts (Walker *et al.*, 1982b, 1982c; Zarawski *et al.*, 1982). The method of immunodecoration was used to detect contaminating proton-ATPase complex in chromaffin granules, and a novel ATPase was discovered in their membranes (Cidon and Nelson, 1982, 1983). The observations that the β subunits of proton-ATPase complexes have preserved their structure in all of the prokaryotes and the eukaryotes showed that this kind of proton-translocating enzyme evolved only once and strict structure has to be maintained in order to preserve its function.

Injection of rabbits with purified *Electrophorus electricus* acetylcholine receptor resulted in neuromuscular blockade concomitant with the production of antibody directed against the receptor (Patrick and Lindstrom, 1973). This was a fine demonstration of self-recognition by an antibody directed against receptor from an evolutionarily remote vertebrate. Since then, antibodies against acetylcholine receptors have proven to be useful in the elucidation of structure, function, and metabolism of this protein complex (Eldefrawi *et al.*, 1977; Lindstrom *et al.*, 1978; Karlin *et al.*, 1978; Kao and Drachman, 1977). Tzartos and Lindstrom (1980) have checked the cross-reactivity of monoclonal antibodies directed against specific sites on acetylcholine receptor. They identified sites which were unique to α or β subunits, and antigenic similarities between α and β subunits and between γ and δ subunits were also recorded. A small region on the α subunit dominated the immune response to the native Torpedo receptor in rats, and a monoclonal antibody to this part caused passive experimental autoimmune myasthenia gravis. It was proposed that the antigenic similarities might reflect structural homologies among the various subunits of the receptor and with subunits of acetylcholine receptors from various sources. Raftery *et al.* (1980) showed amino acid homology among the various subunits of acetylcholine receptor from Torpedo by

sequencing analysis. Recently the primary structures of α, β, and δ subunits of the Torpedo acetylcholine receptor were obtained from cDNA sequences (Noda *et al.*, 1982, 1983). The amino acid sequence homology among these three subunits was corroborated, and information on specific domains in the subunits was further advanced. Bovine acetylcholine receptor was recently purified by affinity chromatography on toxin coupled to agarose (Einarson *et al.*, 1982). Like the receptor from fish, it is composed of four glycoprotein subunits. The subunits cross-reacted with antibodies against the corresponding subunits of Torpedo acetylcholine receptor. It was found that immunization of rats with receptors from bovine and human muscles was very active in induction of experimental autoimmune myasthenia gravis. The evidence presented suggests a common functional structure in acetylcholine receptors from fish electric organ and mammalian muscle.

The work on acetylcholine receptor is a fine demonstration of useful employment of immunological techniques for the study of structure and function of complex membrane proteins. Monoclonal antibodies directed against sites which are in close proximity to the acetylcholine binding site, but do not interfere with the function of the receptor, might help in fighting certain autoimmune diseases of the acetylcholine receptor.

Biogenesis Studies

How to use the biogenesis of cell organelles for bioenergetic studies? The ultimate goal should be to use an *in vitro* system in which isolated genes will serve as templates for the synthesis of individual polypeptides, which will eventually be imported and assembled into functional protein complexes in preformed lipid bilayers. In this kind of system, the powerful genetic engineering techniques could be used for generating controlled changes in the peptides, and by studying the changes in their properties, the mechanism of action of the complexes might be solved.

In the last few years, genetic engineering has become a biological technique that has opened up new horizons. The developments in this area are so rapid that almost every general remark becomes obsolete by the time it is printed. However, the study of membrane bioenergetics is going to use extensively the new developments in this field. So far, the most useful information has come from sequencing data that gave full information on the amino acid sequences of all the products of mitochondrial genes (Thalenfeld and Tzagoloff, 1980; Coruzzi *et al.*, 1981; Bonitz *et al.*, 1982). A few chloroplast proteins were sequenced, and the information on several more of them will soon be available (Westhoff *et al.*, 1981, 1983). Several nuclear gene products are under current investigation, and not before too long, the

amino acid sequences of most of the chloroplast and mitochondrial proteins will be in hand.

Another approach that is going to yield practical results as well as scientific adventures is transformation of eukaryotic cells and overproduction of certain polypeptides. So far, transformation of eukaryotic cells is limited to a few instances like yeast, but soon, this technique will be available for plant cells. In this case, the technology of going back from cell cultures to fertile plants is very advanced (Murashige, 1980). This will open up the possibility of studying the effect of overproduction of certain chloroplast polypeptides on various activities of the system.

Cytogenetic studies have developed to a stage in which cybrids can be produced by fusion of plant cell followed by plant regeneration. By this technique properties that are linked to chloroplasts or mitochondria can be transferred from one plant species to another (Belliard *et al.*, 1979; Aviv and Galun, 1980).

The development of yeast mitochondria is enhanced in the presence of oxygen (Schatz and Mason, 1974). In most instances the development of chloroplasts is light dependent (Ohad, 1975). These properties were extensively used in the study of the biogenesis of the organelles. Moreover, valuable information was obtained on the sequential events that lead to the formation of the functional membranes. For example, it was shown that while the appearance of the two photosystems in the chloroplast membranes is light dependent, substantial amounts of cytochrome b_6–f complex and proton-ATPase complex are present in etiolated chloroplasts (Bradbeer, 1981). Employing pulse labeling and especially electrotransfer and immunodecoration techniques, the sequential assembly of the various subunits within a single protein complex can be followed (Nechushtai and Nelson, 1983). By correlation with the appearance of a specific reaction, catalyzed by the partially assembled complex, valuable information on the function and mode of action of individual subunits can be obtained.

Figure 1 depicts a schematic representation of the four protein complexes that were discussed in this review. The actual complexity of each peptide is much more involved than imagination can envisage or computer can predict. It is the biogenesis of each protein complex that correctly assembles the functional units in the various membranes.

There are three main ways in which membrane proteins are synthesized and incorporated into membranes. One involves synthesis on free polysomes followed by spontaneous incorporation into the membrane without further chemical modification of the protein. The proteins of the mitochondrial outer membrane are synthesized by this route (Gasser and Schatz, 1983; Freitag *et al.*, 1982). The second way is by vectorial translation via bound polysomes, with the help of signal sequences and signal recognition system (Blobel and

Dobberstein, 1975a, b; Walter and Blobel, 1981a,b; Meyer *et al.*, 1982). The third way is by vectorial processing in which the polypeptide is synthesized by free polysomes, usually as a larger precursor, and is imported into or across the membrane concomitantly with chemical modification (Chua and Schmidt, 1979; Schatz, 1979).

The biogenesis of acetylcholine receptor is a typical vectorial-translation process (Anderson and Blobel, 1981, Anderson *et al.*, 1982). Signal sequences of 24, 17, and 21 amino acids were identified for the α, γ, and δ subunits,

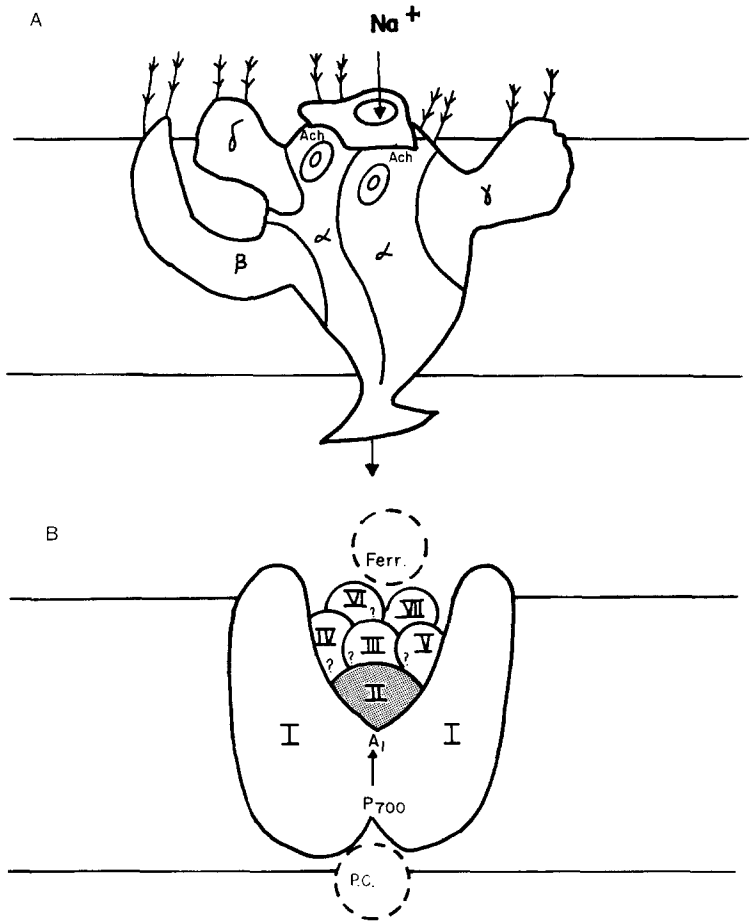

Fig. 1. Schematic representation of the protein complexes in the membranes. (A) A fish as representative of acetylcholine receptor. The various subunits are presented and the structures in the outer side of the membrane (Ψ) indicate the glycosylated moieties. (B) Higher plant Photosystem I reaction center. (C) Mitochondrial cytochrome *c* oxidase. (D) Chloroplast proton-ATPase. The shaded subunits are products of cytoplasmic ribosomes.

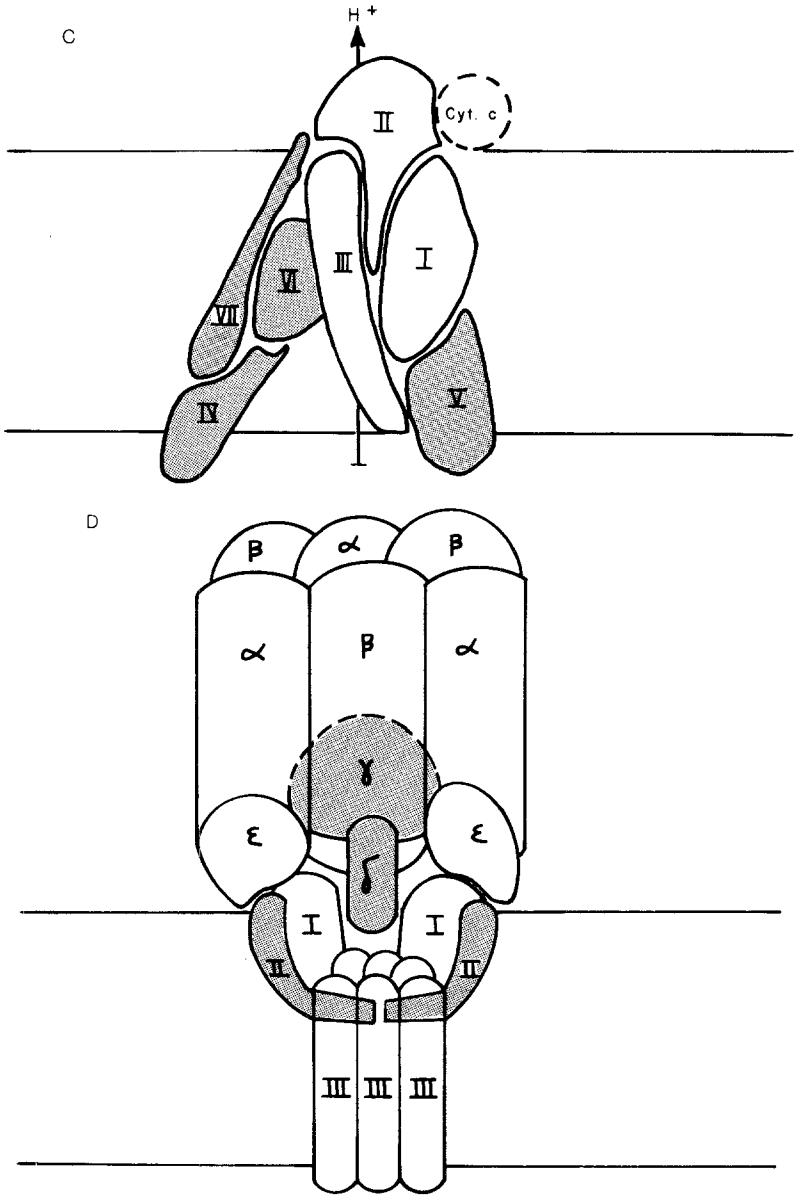

Fig. 1. Continued.

respectively (Noda *et al.*, 1983; Claudio *et al.*, 1983; Anderson *et al.*, 1982). The synthesis of the N-terminal signal peptide is inhibited by a "signal recognition protein (SRP)" (Walter and Blobel, 1981a). This elongation arrest is released after attachment of the polysomes to the "docking protein" on the endoplasmic reticulum (Walter and Blobel, 1981b; Meyer *et al.*, 1982). The nascent chain is then elongated across the membrane, and glycosylation takes place in the distal side of the membrane. Then the receptor is assembled and transported via the Golgi apparatus by fusion of the microsomal vesicles with the plasma membrane. The glycosylated parts are now facing the outer side of the plasma membrane. The final shape of the postsynaptic membrane is formed by association with cytoskeletal proteins inside the cell.

Mitochondria and chloroplasts retain control over synthesis of several of their proteins via their own unique DNA and RNA molecules and protein-synthesizing machinery. The synthesis of polypeptides inside the organelles should be synchronized with the production and import of polypeptides that are coded for by nuclear genes (Nelson *et al.*, 1980a). Overproduction of certain polypeptides is feasible providing that their accumulation will not impair the functionality of the system. In energy-conducting membranes the biosynthesis processes should proceed while the impermeability to ions should be strictly maintained (Nelson, 1981b). Most of the mitochondrial and chloroplast proteins that come from outside the organelle are synthesized as larger precursors (Schatz, 1979; Neupert and Schatz, 1981). The precursors are transported into the organelle via an energy-dependent process by a typical vectorial processing mechanism (Nelson and Schatz, 1979; Grossman *et al.*, 1980; Gasser *et al.*, 1982). In photosystem I reaction center subunit I was identified as a chloroplast product while subunit II is synthesized by cytoplasmic ribosomes as a larger precursor (Chua and Schmidt, 1979; Nechushtai *et al.*, 1981; Nechushtai and Nelson, 1981a). It was suggested that subunit II serves as a template for the assembly of photosystem I reaction center in the chloroplast membrane and the nuclear control over this process is mediated by subunit II. Strict homology in the amino acid sequences has been preserved in subunits I and II throughout evolution (Nechushtai *et al.*, 1983).

The three large subunits of cytochrome oxidase are coded for by the mitochondrial genome and synthesized on the organelle ribosomes (Schatz and Mason, 1974). The four small subunits of the enzyme are synthesized on cytoplasmic ribosomes as larger precursors and imported into the inner mitochondrial membrane via vectorial processing mechanism (Lewin *et al.*, 1980; Mihara and Blobel, 1980). Cytochrome oxidase from *Paracoccuse* contains only two subunits which correspond to subunits I and II of the mitochondrial enzyme (Ludwig, 1980). There is no clear answer as to why the mitochondria has furnished its cytochrome oxidase and proton-ATPase with

several more subunits than are required by the minimal functional complex. One of the reasons has to do with the transfer of genes from the organelle to the nucleus, and we predict that some of the extra subunits will be found to function in the proper assembly of the protein complexes.

The proton-ATPase complex is perhaps the most versatile enzyme in nature. It is present in every living cell tested so far. In eukaryotic cells it is present exclusively in organelles with a semiautonomous genetic system and its synthesis is shared by organelle and cytoplasmic ribosomes. In mitochondria only two out of about 12 subunits are produced by its ribosomes, while the rest of the subunits are synthesized on cytoplasmic ribosomes as larger precursors and imported into the organelle by vectorial processing (Schatz, 1979). The chloroplast proton-ATPase complex contains eight different subunits, five of which are the organelle products and three are synthesized on cytoplasmic ribosomes (Nechushtai *et al.*, 1981; Nelson, 1981a). It was proposed that this situation leaves the control of assembly in the hand of the nuclear genes and leads to the synchronization of the formation of proton channels with the production of CF_1 that should gate the proton leak through them (Nelson *et al.*, 1980a; Nelson, 1981b).

Biogenesis studies are now widespread in several laboratories. There are many more open questions than answers. We still do not know exactly how a polypeptide can be transported across a membrane, and we have not the slightest idea what governs the correct assembly of protein complexes in the membrane.

It is a combination of privilege and challenge for the bioenergeticist to cope with methods ranging from genetics to biophysics in a single system. However, within this wide variety of techniques, one can find various elements that provide the scientist with continual excitement.

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