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Three Proton Pumps, Morphology and Movements[1]

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Abstract

The diameter of F_1 coupling factor and the distance it protrudes from the membrane of bovine heart submitochondrial particles were measured quantitatively using horse spleen ferritin as a standard. Employing the freeze-etch technique, particles of similar size were found on membranes of submitochondrial particles and on membranes of particles first depleted by F_1, then reconstituted by addition of F_1. The extramembranous size of F_1 is 9.7 nm and F_1 protrudes from the membrane surface by about 13.6 nm. Bacteriorhodopsin and cytochrome oxidase were incorporated into lipids derived from membranes of extremely thermoacidophilic microorganisms by the octylglucoside dilution method. The bacteriorhodopsin pump was fully functional provided high concentrations of valinomycin were added. With decanoyl-*N*-methylglucamide as detergent the pump was very active in the absence of valinomycin. Concentrations of gramicidin that collapsed the ΔpH in bacteriorhodopsin liposomes prepared with soybean phospholipid had little or no effect on these rigid proteoliposomes. Very high concentrations (30 μg per ml) were partially effective, suggesting a mechanism other than formation of a gramicidin dimer channel. Cytochrome oxidase lost virtually all activity when incorporated into these rigid liposomes but was fully reactivated on addition of suitable detergents.

Key Words: F_1 ATPase; freeze-etch electron microscopy; proton pumps; reconstitution; bacteriorhodopsin; cytochrome oxidase; lipids of thermoacidophilic bacteria.

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[1]Abbreviations: SMP, submitochondrial vesicles prepared from bovine heart mitochondria exposed to sonic oscillation in the presence of pyrophosphate; F_1, the water-soluble coupling factor of the mitochondrial ATPase complex; CF_1, the water-soluble coupling factor of the chloroplast ATPase complex; ASU vesicles, submitochondrial vesicles prepared from bovine heart mitochondria disrupted by sonic oscillation in ammonia, then passed through Sephadex and treated with urea; OSCP, oligomycin sensitivity-conferring protein; Mega 8, 9, and 10 for octoyl, nanoyl, and decanoyl-*N*-methylglucamide; 1799, bis-(hexafluoroacetonyl)acetone; PMS, *N*-methylphenazonium methosulfate.

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Introduction

David Green has taught us many things. His monograph *The Mechanism of Biological Oxidation* had an important impact on enzymology in the United States. It served as the midwife in the birth of the first "Enzyme Club" in New York City in 1942, a creation of David's vision (Siekevitz, 1983). David was thinking big. Those who didn't, he called "small potatoes." The method developed in his laboratory for the mass production of mitochondria opened the way to the resolution of the respiratory chain into four complexes (Hatefi *et al.*, 1962), the decisive work on the isolation and function of coenzyme Q (Green, 1961), the isolation of F_1, the mitochondrial ATPase (Pullman *et al.*, 1960), and the reconstituion of oxidative phosphorylation (Racker, 1976). David Green was among the first to relate mitochondrial morphology to function with the aid of the electron microscope. Intuitively, he saw a potential role of the "elementary particles" of the inner mitochondrial membrane (later identified as F_1) in the process of oxidative phosphorylation. Although the details of his vision turned out to be inaccurate, he suggested that movements of these particles may be involved, a concept which has now been revitalized (Gresser *et al.*, 1982). Later he related morphological alterations in the mitochondria seen in the electron microscope to conformational changes associated with differences in the energized state.

We have therefore chosen to write about the morphology of F_1 and the role of movements of membrane proteins in this paper dedicated to his memory. It is a field still in its infancy of research, a subject always in the forefront of David's mind, a problem to which he has contributed pioneering experiments and visionary thinking.

Although there is general agreement that F_1 contains the active site of the mitochondrial, chloroplast, and microbial pumps, there are still questions raised about its extramembranous location, about the existence of the stalk, about the possibility of staining artifacts. Indeed in many old as well as recent reviews F_1 is schematically drawn without the stalk as a ball on the surface of the membrane. This representation may be partly motivated by chemical and genetic experiments (Hackney, 1980; Aris and Simoni, 1983) that suggest a rather intimate relationship between subunits of F_1 and components of the hydrophobic sector. Thus new and even double-breasted models of F_1 have been proposed at Gordon conferences on bioenergetics. These models include physical mobility of F_1 (Gresser *et al.*, 1982) and a mode of operation that involves three active cooperative sites (Gresser *et al.*, 1982; Cross and Nalin, 1982; Nalin and Cross, 1982; Penefsky, 1983).

With these new and important developments before us it seems imperative to emphasize that the morphology of F_1 on a protruding stalk is not just an artifact of negative staining. We shall point out that such a morphology is not

contradicted by the experiments with cross-linkers and with genetic mutants of *E. coli*. Moreover, it requires the formulation of a proton channel extended from the proteolipid via the stalk to the active site of F_1 and thus suggests more experimentation.

In addition to electron microscopy, experiments performed with mitochondrial and chloroplast membranes using immunological methods and radioactive labeling have provided additional evidence for the extramembranous location of F_1 (Racker, 1976). In this paper we describe new experiments that have yielded quantitative data on the dimensions of F_1 and its distance from the membrane. Accepting these data as evidence for the extramembranous location of F_1, we draw the following conclusion: The stalk must (a) serve as a proton channel and (b) it may, as suggested by cross-linking and genetic experiments, include some components of the so-called hydrophobic sector. The most likely candidate for such a component in the stalk is subunit B of the hydrophobic sector.

The two other proton pumps we want to discuss are bacteriorhodopsin and cytochrome oxidase. What restrictions can we impose on their movements? We have recently conducted reconstitution studies with a remarkable group of lipids that are constituents of the plasma membranes of some extremely thermoacidophilic microorganisms. They form rigid membranes that lack the liberties of movements granted to membranes by the free-floating fatty acid chains of ordinary phospholipid bilayers (Tornabene and Langworthy, 1979).

Materials and Methods

Submitochondrial vesicles (Racker, 1962), ASU vesicles (Racker and Horstman, 1967), F_1 (Horstman and Racker, 1970), OSCP (Senior, 1971), bacteriorhodopsin (Becker and Cassim, 1975), and cytochrome oxidase (Carroll and Racker, 1977) were prepared as described. Lipids from *Thermoplasma* and *Sulfolobus* were prepared as described (Langworthy, 1982). Octylglucoside and Mega 10 were from CalBiochem. Mega 8 and 9 were synthesized and donated by Dr. R. M. McCarty.

Negative staining was achieved with 2% phosphotungstate at pH 6.8. For freeze-etching the samples were suspended in 10 mM buffer, pH 7.4 (Tris-Cl or KP_i), placed on a clean gold specimen stub and rapidly frozen in liquid Freon 22 held at near liquid-nitrogen temperature. The frozen suspension of vesicles was cut with a cold knife mounted in a Balzers 360 M freeze-etch unit maintained at a vacuum of 2×10^{-4} Pa and a temperature of 171°K. The exposed cut surface of the frozen suspension was etched for 1–2 min before shadowing with platinum–carbon. Prior to shadowing, the platinum–carbon

filament was heated for 5–10 sec to remove moisture and adsorbed gases. The etched specimen surface was shadowed at a 45° angle with platinum–carbon for a period of 7 sec followed by a 10-sec evaporation with carbon. The thickness of the shadow material and duration of the platinum–carbon evaporation was controlled by a quartz crystal film thickness monitor. Following exposure to Chlorox overnight to remove adhering lipid, the replicas were rinsed in several changes of distilled water and picked up on 300- or 400-mesh copper grids. Photo recording was done in an AEI EM6B electron microscope operated at an accelerated voltage of 80 kV. Magnification was corrected using a carbon waffle type grating having 54,684 lines per inch.

Reconstitutions of bacteriorhodopsin and cytochrome oxidase were achieved by the detergent dilution method (Racker *et al.*, 1979) as described in the legends with either octylglucoside or Mega 10.

Results and Discussion

The ATP–Generating Proton Pump of Mitochondria; Morphology of F_1

In order to examine the morphology of F_1 and its relationship to the inner mitochondrial membrane it was thought necessary to use a method that is less drastic than negative staining and that would allow for a quantitative evaluation of the distance between F_1 and the membrane. Garber and Steponkus (1974) have used freeze-etching of subchloroplast particles reconstituted with CF_1 to demonstrate their presence on the membrane, and similar studies have been performed by Sikerwar and Malhotra (1979) with submitochondrial particles. To develop a more quantitative method that will allow determination of the size of F_1 as well as its distance from the membrane, we have used as a standard ferritin whose size, shape, and molecular weight are well established.

Ferritin and ASU Particles

To verify that the techniques used in this study produced reliable measurements for a particle of known size and molecular weight, horse spleen ferritin was used as a standard. The ferritin molecule has an inner core of ferric hydroxide micelles, about 5.5 nm in diameter (Farrant, 1954) surrounded by a shell of protein resulting in an overall molecular weight of 462,000–480,000 and a diameter of 11–12 nm as determined from x-ray data (Harrison, 1959, 1963). Figure 1 shows a representative field of horse spleen ferritin, negatively stained with 2% Na-phosphotungstate. The electron-dense core of ferric hydroxide micelles is surrounded by an electron-transparent

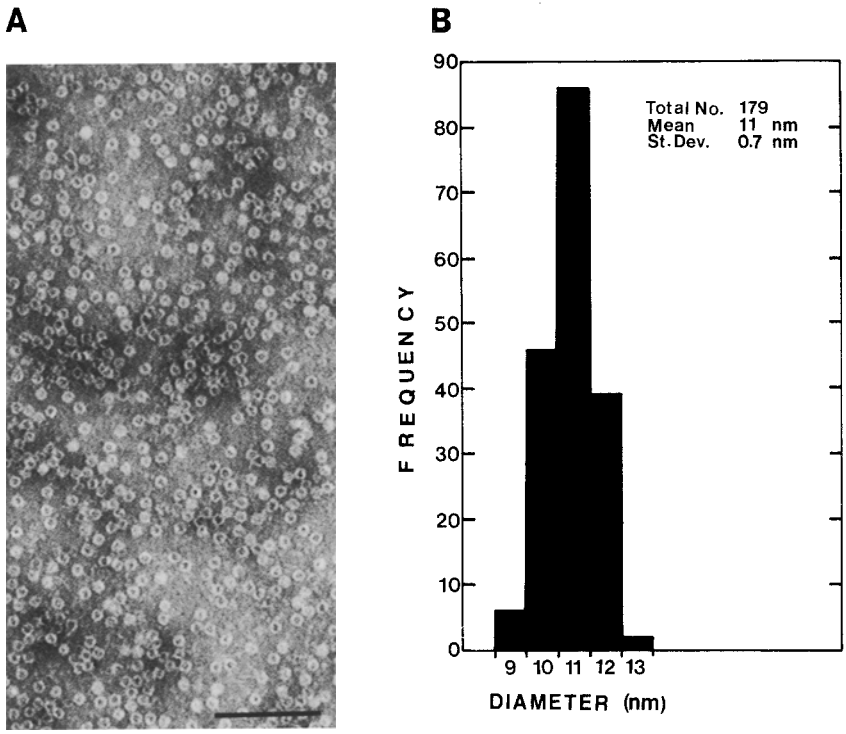


Fig. 1. Horse spleen ferritin. (A) Horse spleen was negatively stained with 2% sodium phosphotungstate, pH 6.8, as described under Materials and Methods. An electron-dense core of ferric hydroxide micelles (about 5.5 nm in diameter) is surrounded by an electron transparent protein shell. Calibration bar: 100 nm. (B) Histogram showing the frequency versus diameter size distribution of the negatively stained horse spleen ferritin.

protein shell. A mean diameter of 11 nm (S.D. = 0.7 nm) was obtained from the negative stain measurements for ferritin.

To ensure that the mitochondrial membranes were free of F_1, ASU vesicles were incubated with silicotungstate, which is known to remove F_1 (Racker, *et al.*, 1969). The stripped ASU vesicles were incubated with ferritin, and samples with and without ferritin were examined by negative staining, thin-sectioning, and freeze-etching. Figure 2a, b, c shows examples of ASU vesicles after negative staining, thin-sectioning, and freeze-etching, respectively. No F_1 was observed projecting from the membrane. The freeze-etched sample (Fig. 2c) shows the membrane to be free of F_1. Figure 2d, e, f shows ferritin adhering to the ASU vesicles in negatively stained, thin-sectioned, and freeze-etched samples. Although some free ferritin was observed in the negatively stained and thin-sectioned preparations, the majority of ferritin was closely associated with the ASU vesicle.

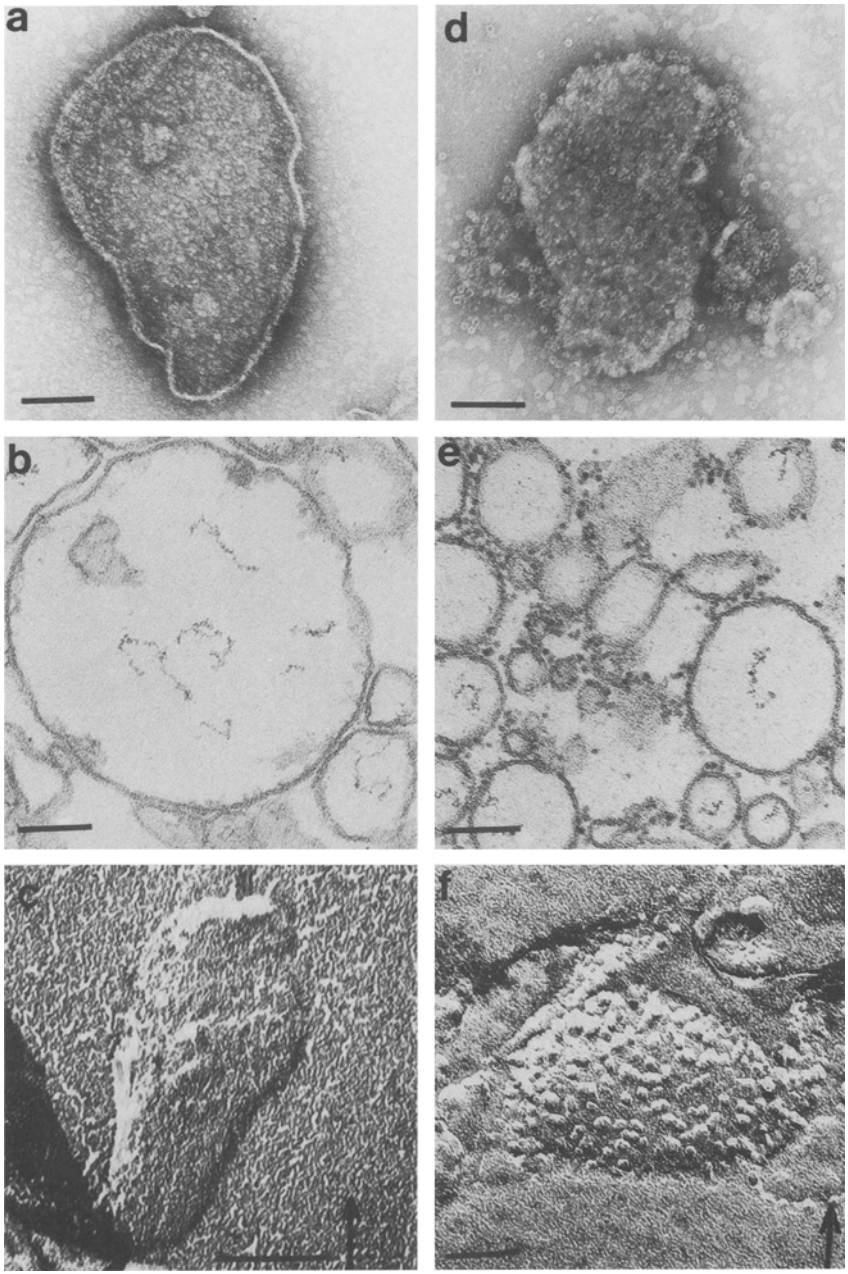


Fig. 2. ASU vesicles. The vesicles treated as described under Materials and Methods are shown before (a, b, c) and after (d, e, f) addition of ferritin as visualized by negative staining (a, d) with sodium phosphotungstate, by thin-sectioning (b, e), and by freeze-etching (c, f). Arrows in (c) and (f) indicate direction of shadowing. Calibration bar: 100 nm.

A purified sample of isolated F_1 was negatively stained and from the histogram of the frequency distribution the mean averaged diameter was found to be 9.7 nm (S.D. = 1 nm). This size agrees with the diameter of 9.5 nm calculated from the reported molecular weight of 365,000 for bovine heart mitochondria F_1 and is similar to the size of F_1 (10 nm) reported for insect muscle mitochondria F_1 (Younis *et al.*, 1978; Patil *et al.*, 1979).

Size Determination by Freeze-Etching

Hall (1960) has stated that shadow length can be a good measure of particle lengths. The distortion of shadow width can be estimated under standardized conditions (Ruben and Telford, 1980). From the freeze-etch experiment, the mean shadow length and mean shadow width of the ferritin on ASU vesicles were measured. Since the platinum–carbon electrode was positioned at an angle of 45° to the plane of the specimen holder, the relation of shadow width to specimen height above the etched surface should be the same. This also assumes that the etched face surface of the frozen sample is parallel with the plane of the specimen stage.

The mean shadow length of freeze-etched ferritin on ASU particles was calculated to be 11 nm (S.D. = 3 nm) (Fig. 3a) and the mean shadow width was 14 nm (S.D. = 3 nm) (Fig. 3b). The shadow length is in very close agreement with estimates from negative staining (Fig. 1) and with published data (Harrison, 1959) for ferritin. Since the measurements are in close agreement, it was concluded that the techniques and conditions used for this study were reliable and reproducible.

The increase in shadow width is due to the buildup of platinum–carbon material around the ferritin particle during the shadow width formation (Misra and Das Gupta, 1965). In this experiment, the mean shadow width is calculated to be 1.3 times greater than the mean shadow length, i.e., the real particle size.

Identification and Size Evaluation of F_1 on Freeze-Etched SMP and ASU Vesicles

Figure 4A shows a representative field of isolated F_1 freeze-etched and shadowed with platinum–carbon at a 45° angle. The mean shadow length of the isolated F_1 was found to be 10 nm (S.D. = 1 nm) (Fig. 4B, a). This agrees with the mean diameter of 9.7 nm calculated from negative stain measurements. The mean shadow width was calculated to be 15 nm (S.D. = 1 nm) (Fig. 4B, b). This agrees with the shadow width of 15 nm measured by Garber and Steponkus (1974) for freeze-etched spinach CF_1. The mean shadow width was greater in mean size by a factor of 1.5 over that of the mean shadow

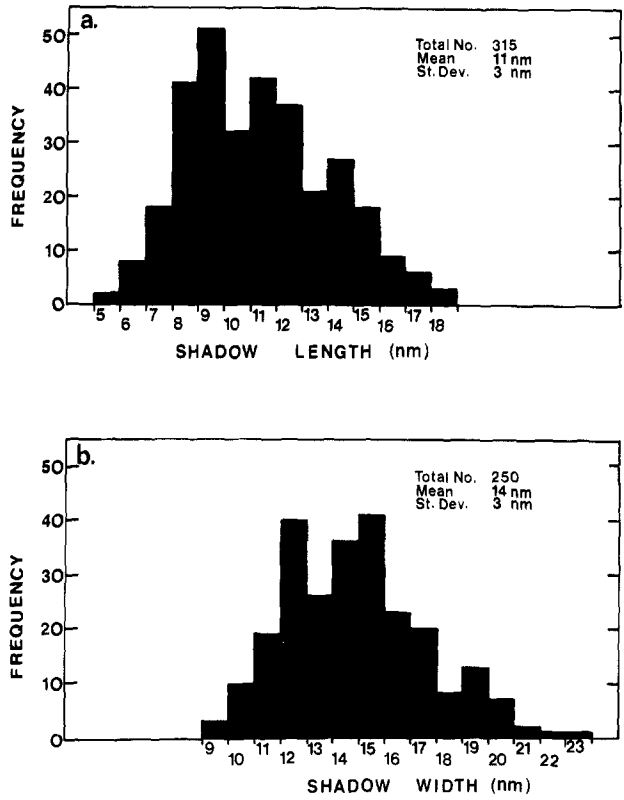


Fig. 3. Ferritin and ASU vesicles. Histograms showing the frequency versus shadow length (a) and shadow width (b) distribution of ferritin on freeze-etched ASU vesicles. Shadowing was performed as described under Materials and Methods.

length. This factor was used to measure and identify F_1 on the SMP and ASU membranes. Spheres casting distinct shadows were observed on the membrane surfaces of SMP (Fig. 5a) and of ASU vesicles (Fig. 2a, b, c) that had been incubated with F_1 (Fig. 5b).

Figure 6A shows histograms representing the distribution of shadow width measurements for F_1 on freeze-etched SMP and ASU + F_1, respectively. The mean shadow width of the spheres on SMP (Fig. 6A, a) was 14.5 nm (S.D. = 3 nm) and for particles on ASU + F_1 (Fig. 5A, b), the mean shadow width was 14.2 nm (S.D. = 3 nm). The mean shadow widths are in close agreement, indicating that spheres of similar size and orientation are present in both preparations. The real mean size dimensions of the spheres on SMP and ASU vesicles reconstituted with F_1 were calculated to be 9.7 and 9.5

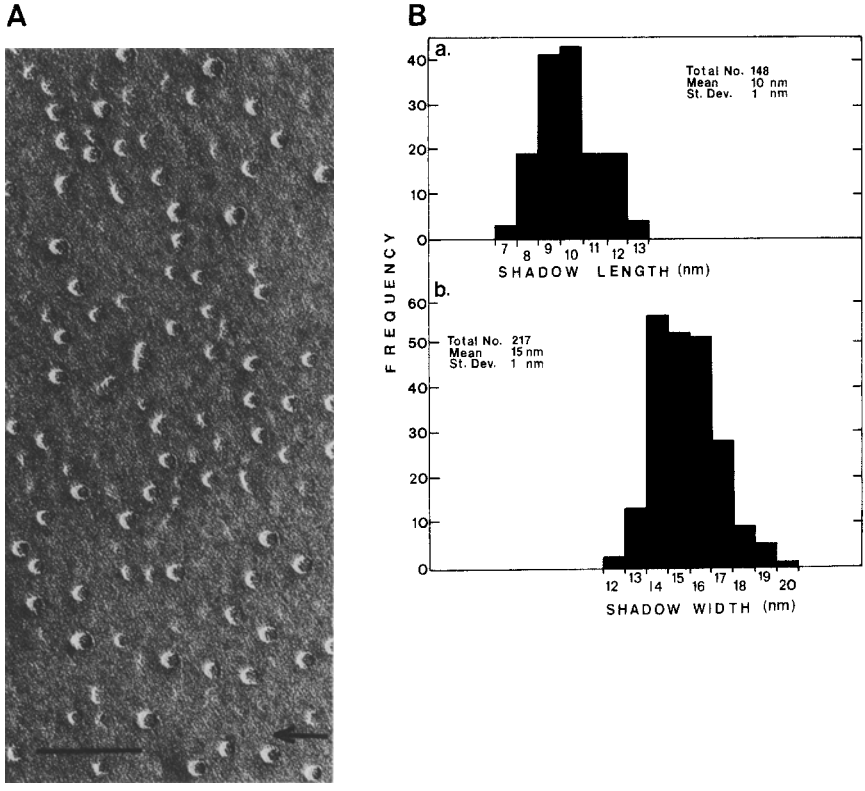


Fig. 4. Freeze-etching and shadowing of F_1. (A) Isolated F_1 ATPase from bovine heart mitochondria, freeze-etched and shadowed with platinum–carbon at an angle of 45° as described under Materials and Methods. Calibration bar: 100 nm. (B) Histogram of the frequency versus (a) shadow length and (b) shadow width of isolated F_1 ATPase freeze-etched with platinum–carbon at an angle of 45°.

nm, respectively, taking into account the increase in shadow width (×1.5). These dimensions agree with the negative staining and shadow length measurements for F_1.

The shadow lengths cast by the F_1 on the freeze-etched membranes were also measured. Figure 6B shows the histograms of shadow length distribution for F_1 on SMP and ASU vesicles, respectively. The mean shadow length of the F_1 attached to the SMP membranes (Fig. 6B, a) was 13.4 nm (S.D. = 3 nm). In freeze-etched preparations of reconstituted ASU vesicles (Fig. 6Bb), the mean shadow length was 13.8 nm (S.D. = 2 nm). We conclude from these findings that the size of F_1 is 9.7 nm and that it protrudes from the membrane by 13.6 nm, which suggests a stalk of about 4 nm in length.

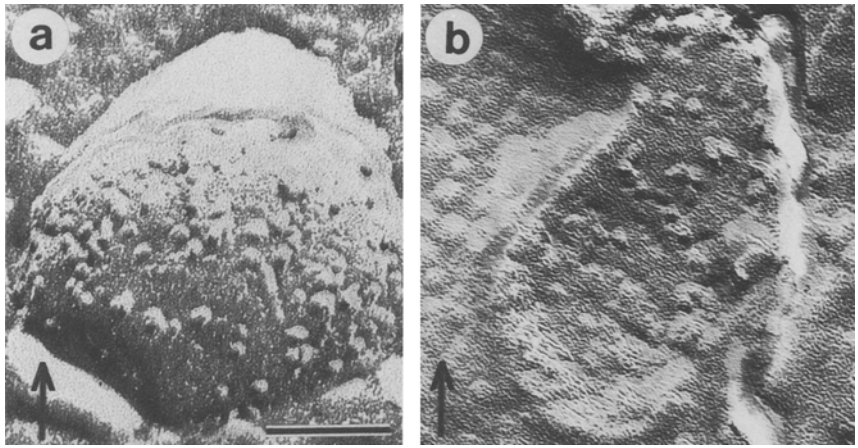


Fig. 5. Freeze-etching of SMP and reconstituted ASU vesicles. (a) SMP, freeze-etched and shadowed with platinum–carbon at a 45° angle, showing the presence of shadowed particles on the membrane surface, identified as F_1 ATPase. (b) ASU membranes reconstituted with F_1 ATPase were freeze-etched. Scattered shadowed particles on the membrane surface are identified as F_1 ATPase. Etching and shadowing were performed as described under Materials and Methods. Calibration bar: 100 nm.

Significance of Extramembranous Location of F_1

Although it is now almost universally agreed that the driving force for ATP generation is the proton flux generated during electron transport, as proposed by the chemiosmotic hypothesis (Mitchell, 1966), the intimate mechanism of ATP generation is still controversial (Boyer *et al.,* 1977). Recent observations of an intimate relationship between F_1 and components of the hydrophobic sector mentioned earlier have revived the view that the appearance of extramembranous F_1 is an artifact of negative staining (Sjostrand *et al.,* 1964).

The evidence presented in this paper, together with earlier morphological (Telford and Racker, 1973), radioactive labeling, and immunological experiments (Racker, 1976) strongly support the extramembranous location of F_1. This conclusion demands that the connecting stalk of about 4 nm play a critical role in the transmission of protons. It now appears that the composition of the stalk is more complex than has been proposed (Senior, 1973). OSCP and F_6 may be components of the stalk in view of their need for the attachment of F_1 to the membrane, and these coupling factors appear to interact with a protruding δ subunit of the protein (Racker, 1976). Indeed, electron micrographs (Younis *et al.,* 1978) of F_1 from insects reveal the presence of rudimentary stalks. Therefore, it seems likely that the stalk is a composite including the δ subunit of F_1, soluble factors (OSCP and F_6), as well

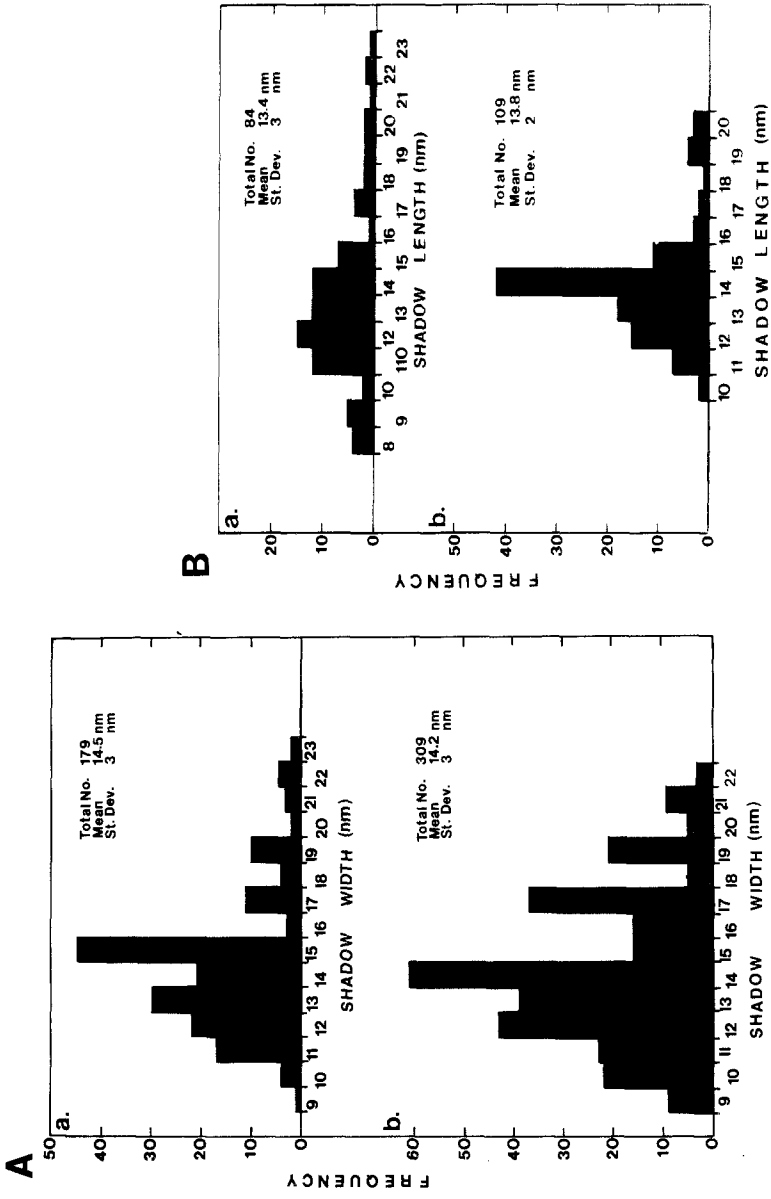


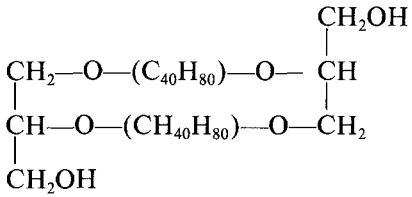
Fig. 6. Histograms of shadow width and length distribution of F_1. (A) Histogram of the frequency versus shadow width distribution of (a) F_1 ATPase on SMP membranes and (b) F_1 ATPase reconstituted with ASU vesicles. (B) Histogram of the frequency versus shadow length distribution of (a) F_1 ATPase on SMP membranes and (b) F_1 ATPase reconstituted with ASU vesicles.

as components of the hydrophobic sector that have hydrophilic sections that interact with F_1.

The extramembranous location of F_1 and the presence of a stalk thus calls for further exploration of the pathway of protons through the inner membrane, through the stalk to the active site of F_1 where the energy transformation takes place.

Reconstitution of Bacteriorhodopsin and Cytochrome Oxidase into Lipid Membranes of Thermoacidophilic Bacteria

Thermoacidophilic bacteria grow optimally at pH 2–3 at temperatures of 60°C or higher. Their lipids have been extensively studied. They contain ether linkages rather than fatty acid esters and form assemblies of diglycerol tetraethers that are unique (Tornabene and Langworthy, 1979). They are composed of two glycerol molecules connected by two identical, saturated, C-40 terminal alkyldiols as shown below.



This structure suggests that the membrane is assembled as an extremely rigid lipid monolayer rather than a typical bilayer.

We thought it would be of interest to examine whether or not proteins from a bilayer habitat could be incorporated into such membranes and what effect this would have on their function. Purified tetraether lipids from *Thermoplasma* and *Sulfolobus* (Tornabene and Langworthy, 1979; Langworthy, 1982) were employed.

Reconstitution of bacteriorhodopsin with the lipids of *Thermoplasma acidophilum* by the octylglucoside dilution method gave rise to some H^+ pumping on illumination. The rate and extent was slightly increased on addition of 0.1 μg of valinomycin, an amount sufficient to collapse the membrane potential generated by proton pumps in phospholipid bilayer liposomes. It can be seen from Fig. 7A that a maximal extent of the proton gradient was not achieved until 4 μg of valinomycin was added. Relatively high concentrations of nigericin (1 μg) or of uncouplers (data not shown) were also required to collapse the proton gradient. Similar data were obtained when the initial rate of proton pumping was measured as described previously (Racker and Hinkle, 1974), as shown in Fig. 7B. The rate of H^+ pumping with crude soybean phospholipids reconstituted by the same method and in the presence of optimal valinomycin was 2660 natoms $H^+ \times min^{-1} \times$ mg

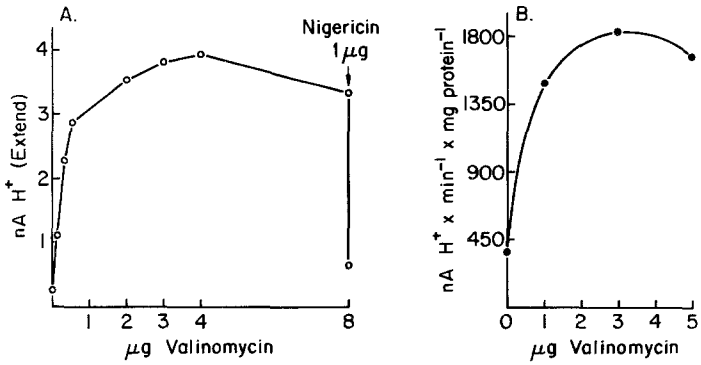


Fig. 7. Bacteriorhodopsin reconstituted with thermoacidophilic lipids. Lipids of *Thermoplasma acidophilum* at a concentration of 17.5 mg/ml in 0.15 M KCl were sonicated in a bath-type sonicator for 10 min. To 20 μl of this suspension were added, to a final volume of 30 μl bacteriorhodopsin (15 μg) and octylglucoside at a final concentration of 1.7%. After 10 min at room temperature the entire sample was tested for extent and rate of proton pumping in 1 ml of 0.15 M KCl as described previously (Racker and Hinkle, 1974).

protein^{-1}. Reconstitution of bacteriorhodopsin with lipids of *H. Halobium* has been described from Khorana's laboratory (Lind *et al.*, 1981), and marked stimulation by conventional amounts of valinomycin was observed. However, reconstitution worked well only when delipidated bacteriorhodopsin was used. For reconstitution with the lipids from thermoacidophilic microorganisms, octylglucoside concentrations between 1.5 to 2.2% were suitable. With higher concentrations of octylglucoside, the dependence on valinomycin was less marked. The most striking differences were seen with gramicidin. In bacteriorhodopsin liposomes constructed with dimyristoyl phosphatidylcholine the addition of 2 μg of gramicidin abolished the proton gradient even at 1°C when the lipids were in a frozen state (Racker and Hinkle, 1974). In proteoliposomes made with the C-40 lipids as much as 20 μg gramicidin lowered the ΔpH by only 11% when tested shortly after its addition, while 10 min later the inhibition increased to 44%. At 30 μg/ml or higher concentrations, inhibitions were observed shortly after addition of gramicidin. Considering the rigidity and the length of the C-40 chain lipids, it is not surprising that gramicidin dimer could not form a channel across the membrane. Indeed it is surprising that at the higher concentrations gramicidin was effective at all, suggesting that under these conditions an alternative mechanism was operative—perhaps that of a carrier.

Similar observations were made when C-40 lipids from *Sulfolobus acidocaldarius* were used for the reconstitution of bacteriorhodopsin by the octylglucoside dilution procedure. In the course of these experiments nonylglucoside was tried for reconstitution. The extent of proton pumping in the

Table I. Comparison of Octylglucoside and Mega 8, 9, and 10 Dilution Methods for Bacteriorhodopsin Reconstitution[a]

Methods of reconstitution	H^+ transport (extent) (natoms H^+)	
	Control	+ Valinomycin (0.2 μg)
Octylglucoside dilution	5.6	3.9
Mega 8 dilution	2.1	1.5
Mega 9 dilution	12.3	10.3
Mega 10 dilution	13.2	9.8

[a]The procedure of reconstitution of bacteriorhodopsin by detergent dilution was as described previously (Racker *et al.*, 1979) except that 1 mg soybean phospholipids, 10 μg bacteriorhodopsin, and 1.4% of the detergent in 0.15 M KCl were used during the incubation for 8 min at room temperature. The assays were all performed in 0.15 M KCl at a pH of about 6.3.

absence of valinomycin was somewhat greater than with octylglucoside, but addition of 2 μg valinomycin gave 50% inhibition rather than several fold stimulation.

A series of other neutral detergents of the *N*-D-gluco-N-methylalkanamide type (Hildreth, 1982) with 8, 9, and 10 carbons in the alkyl chain were then explored. As shown in Table I, with soybean lipids Mega 8 was less effective than octylglucoside but both Mega 9 and 10 gave about twice the extent of pumping. Valinomycin at 0.1 μg/ml slightly inhibited the extent of pumping. The initial rates of H^+ pumping were also about twice as fast with Mega 9 and 10 compared to octylglucoside.

A surprising observation was made when these detergents were used with *Sulfolobus* lipids. In contrast to octylglucoside proteoliposomes which were stimulated by valinomycin (Fig. 7), the vesicles reconstituted with Mega 10 were slightly inhibited even at 0.2 μg valinomycin/ml and about 80% inhibited at 2 μg valinomycin/ml (Table II). It appears that Mega 9 vesicles responded to valinomycin similar to octylglucoside vesicles, whereas Mega 10 vesicles responded similar to nonylglucoside vesicles.

The successful reconstitution of bacteriorhodopsin into the C-40 lipids of

Table II. Reconstitution of Bacteriorhodopsin with Lipids of *Sulfolobus*[a]

Method of reconstitution	Proton translocation (natoms H^+)	
	Control	+ Valinomycin (4 μg)
Octylglucoside dilution	1.2	3.7
Mega 9 dilution	1.8	2.3
Mega 10 dilution	4.9	1.2

[a]The experiments were performed as described in Table I except that 400 μg of *Sulfolobus* lipids were used instead of soybean phospholipids.

the thermoacidophilic bacteria suggests that all conformational changes in bacteriorhodopsin that occur during proton translocation can take place within the rigid cage of these membranes.

Reconstitution of cytochrome oxidase from bovine heart mitochondria with lipids of thermoacidophilic bacteria was of particular interest to us, since it may help in the evaluation of a hypothesis of proton pumping that invokes vertical movement of the transmembranous protein (Racker, 1985). This hypothesis is based on experimental observations (Carroll and Racker, 1977) that indicated profound conformational differences in cytochrome oxidase depending on whether it is in the reduced or oxidized form. When either no cytochrome *c* or reduced cytochrome *c* was present during reconstitution by the cholate dialysis procedure, vesicles were formed that were all right-side out. This was shown by comparing the increase of the rate of oxygen uptake in the presence of uncouplers with that elicited by permeabilization with suitable detergents that did not inhibit cytochrome oxidase and did not induce the chemical oxidation of the ascorbic acid that was used to reduce cytochrome *c* in the assay. In contrast, vesicles made in the presence of oxidized cytochrome *c* were "scrambled"; about half were right-side out and half were inside-out. Accordingly, uncouplers activated only about half the oxidation rate that was elicited by the detergent. It therefore seemed likely that a conformational change was induced in cytochrome oxidase by the presence of cytochrome *c* that increased its chances to be incorporated in the inside-out configuration. It was thought likely that oxidation and reduction of cytochrome oxidase embedded in the membrane may similarly induce conformational changes that could play a role in the expulsion of protons during electron transfer. To explore the possibility of such a contractile mechanism involving both the enzyme and the phospholipid bilayer, attempts were made to reconstitute cytochrome oxidase into the C-40 lipids. Incorporation of the enzyme into these lipids gave rather curious results. Virtually all cytochrome oxidase activity was lost with either *Thermoplasma* or *Sulfolobus* lipids when reconstitution was performed by either octylglucoside or Mega 10 dilution. Oxygen uptake could not be stimulated by uncouplers even in the presence of PMS yet was restored on addition of 0.1% Emasol (Table III). What does this mean? One explanation would be that all vesicles are inside-out. We shall therefore attempt to reconstitute oxidative phosphorylation with these lipids provided we succeed with the incorporation of the F_1F_0 complex. An alternative explanation is that the lipid cage is too large and the site for cytochrome *c* interaction not available at the surface. A more interesting explanation would be that the enzyme does not have enough mobility in the cage to function. We are currently testing these possibilities.

We are sure David Green would have thought of yet other possibilities. We are willing to try them all.

Table III. Reconstitution of Bovine Cytochrome Oxidase with Lipids of Thermoacidophilic Microorganisms[a]

Additions (sequential)	Oxygen uptake (natoms × min^{-1})
Experiment 1	
None	51
+ 1799 (4×10^{-4} M)	227
+ Emasol (0.2%)	227
Experiment 2	
None	18
+ Valinomycin[b]	18
+ Nigericin (1 μg)	18
+ PMS (10 μM)	18
+ Emasol (0.2%)	198

[a] In experiment 1, 1.5 mg of soybean phospholipids in 50 mM KP_i (that had been sonicated for 10 min) were incubated with 10 μg of cytochrome oxidase and octylglucoside (1% final concentration) for 12 min at 0°C and then assayed for activity as described previously (Carroll and Racker, 1977). In experiment 2, 10 μg of cytochrome oxidase were reconstituted as in experiment 1 except that 400 μg of *Thermoplasma* lipids were used and the octylglucoside concentration was raised to 1.5%.

[b] Valinomycin was added in increments to a final concentration of 4 μg/ml.

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