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Review

Reconstitution of ion-motive transport ATPases in artificial lipid membranes

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Key words: ATPase, Ca^{2+}-; ATPase, Na^+/K^+-; ATPase, H^+/K^+-; Lipid membrane

Contents

Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; AMP-PCP, adenosine 5′-(α,β-methylene)triphosphate; AMP-PNP, adenosine 5′-(α,β-imido)triphosphate; ANS, anilinonaphthalene sulfonate; Arsenazo III, 2,2′-[1,8-dihydroxy-3,6-disulfo-2,7-naphthalenebis(azo)] dibenzenearsonic acid; AS701, *N,N′*-diheptyl-*N,N′*-didiethyl ether; $C_{12}E_8$, octaethylene glycol dodecyl ether; $C_{12}E_9$, poly(oxyethylene) 9-lauryl ether; caged-ATP, P^3-1-(2-nitro)phenylethyladenosine 5′-triphosphate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone, CDTA, 1,2-(cyclohexylenedinitrilo)tetraacetic acid; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Cl_4F_3MeBzole, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole; Cyclex-2E, cyclo[Glu(OBz)-Sar-Gly-(*N*-cyclohexyl)Gly]$_2$; DCCD, *N,N′*-dicyclohexylcarbodiimide; DES, diethylstilbestrol; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DOCC, 3,3′-diethyloxadicarbocyanine; EDAC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; IAF, iodoacetamidofluorescein; morin, 2′,3,4′5,7-pentahydroxyflavone; NAP-taurine, *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonic acid; NBD-Cl, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole; NCCD, *N*-(2,2,6,6-tetramethylpiperidyl-1-oxyl)-*N′*-(cyclohexyl)carbodiimide; NEM, *N*-ethylmaleimide; octyl glucoside, *n*-octyl β-D-glucopyranoside; oxonol V, bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol; oxonol VI, bis(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol; PCB^-, phenyldicarbaundecaborane anion; percoll, poly(vinyl pyrollidone)-coated silica particles; PMS, *N*-methylphenazinium methyl sulfate; pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonic acid; quercetin, 3,3′,4′,5,7-pentahydroxyflavone; rutin, 3,3′,4′,5,7-pentahydroxyflavone 3β-D-rutinoside; S-13, *N*-(3-*tert*-butyl 5-chlorosalicilyl)-2-chloro-4-nitroanilide; SF6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidene malononitrile; SW 26, 2,2,2-trichloroethyl-3,4-dichlorocarbanilate; TCS, 3,3′,4′,5-tetrachlorosalicylanilide; TPB^-, tetraphenylboron anion; Triton X-100, poly(oxyethylene) ethers; Triton X-114, poly(oxyethylene) ethers; Tween 20, poly(oxyethylenesorbitan) monolaurate; Tween 80, poly(oxyethylenesorbitan) monooleate; 1,5-IAEDANS, *N*-iodoacetyl *N′*-(5-sulfo-1-naphthyl)ethylenediamine; 9-AA, 9-aminoacridine; 1799, bis(hexafluoroacetonyl)acetone.

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I. Summary and perspectives

Biological membranes are structurally and functionally complex asymmetrical barriers separating different cellular compartments or the cells from their environments and/or from other cells. The semifluid lipidic bilayers are provided with energy-transducing systems; transport systems for ions, metabolites and other molecules; intercellular communicating systems; cytoskeletal components and mechanical anchoring systems; receptors and recognition systems; signal transducers and perhaps other structures involved in processes of a different nature not yet discovered.

The structural organization [1–3] and lipid–protein interactions [4–7] in the membranes are aspects of great importance for understanding the different means of experimentally dissecting functional systems [8] that could later be reversibly reassembled in artificial model membranes [9–18] in order to study their functions in isolation.

The reconstitution of a purified enzyme into a planal membrane permits easy access of both sides of the membrane to experimental manipulation. Moreover, since the enzyme expands the membrane, it is possible to measure electrical phenomena directly. The formation of a planal membrane classically has been performed applying a mixture of oxidized cholesterol or phospholipids in an organic solvent to a small hole across two otherwise separated compartments. However, in order to increase the surface of the membrane, a similar procedure has been utilized applying the lipidic mixture to a large porous matrix, with multiple holes, between the two compartments.

The artificial vesicles are produced dispersing the appropriated lipids into an aqueous medium. This could be obtained in the absence of detergents, by sonication and/or by freeze-thaw cycles, by different mechanical pressure systems, or in the presence of detergents, that later are removed by dialysis, gel filtration, selective binding to a matrix or simple dilution to a lower concentration. The mechanism of vesicle formation has been recently reviewed [19].

The planal membrane system could be used also to fuse vesicles containing the reconstituted enzyme. In this hybrid system it is also possible to measure electrical events directly.

Fig. 1 presents schematics of the three different basic reconstitution systems used.

The incorporation of a heart mitochondrial F_1F_0-ATPase preparation, depleted of both phospholipids and cytochrome oxidase, by treatment with cholate and ammonium sulfate, into a soybean phospholipid membrane using a cholate-dialysis method [20] was the first achievement in a series of seminal works designed to put on ever more solid foundations the chemiosmotic principles of Peter Mitchell [21–24], and to open the path for a more effective reconstitution of other ion-motive ATPases.

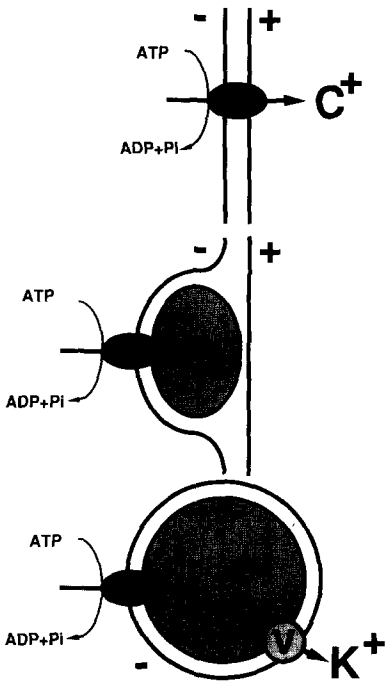

Fig. 1. Reconstitution methods. (Top) Reconstitution of an ATPase into a planal black lipid membrane. The translocated cation generates a membrane potential that can be measured with electrodes located in both compartments. (Middle) Reconstitution of an ATPase in a liposome later fused to a planal lipid membrane. The translocated cation to the lumen of the fused vesicle generates a membrane potential (half the value than before) that can be measured with electrodes located in both compartments. (Bottom) Reconstitution of an ATPase into a liposomal membrane. The translocated cation generates a membrane potential (positive inside) that can be measured with appropriate probes. The addition of the ionophore valinomycin (V) induces a compensatory movement of potassium ion.

The different ion-motive ATPases have a common evolutionary origin [18a,25,26], and their structural and functional similarities and differences became more apparent after their purification and reconstitution in artificial lipid membranes. However, I would like to emphasize that a large body of knowledge on the ATPases has been obtained by experiments using membrane fractions or solubilized and purified enzymes. Particularly useful has been inside-out vesicle preparation to study transport functions. However, the reconstitution technology allows us to study structural as well as functional aspects of these enzymes in the absence of interfering reactions. Nevertheless, obvious shortcomings of the reconstitution methods are the possible alterations of the enzymes during purification and reconstitution, the loss of essential components from the enzymes involved in the expression of important functional or regulatory properties, the alteration of the natural lipidic environment of the enzymes, and the absence of interaction of the enzymes with other relevant membrane or soluble systems. However, reconstitution of the different ion-motive ATPases has contributed dramatically to our understanding of the operation of these enzymes, and has complemented our knowledge on their physiological role in intact organisms.

The reconstitution methods have allowed us to study in detail and understand better several aspects of ion-motive ATPases, including: (i) determination of the minimal peptide composition required for transport functions: (ii) determination of specific roles of different subunits in the transport or regulatory processes; (iii) determination of the nature of the translocated ion(s); (iv) determination of the electrical properties of the transport mechanism; (v) determination of the mechanistic and coupling efficiency of the cation/ATPase ratio(s); (vi) determination of passive ionic fluxes of the whole enzyme, their subunits or their fragments in the absence or presence of some physiological ligands; (vii) detailed study of the interaction among the different subunits (if multiple); (viii) study of the supramolecular organization of the enzymes; (ix) study of the lipid–protein interactions; (x) analysis of

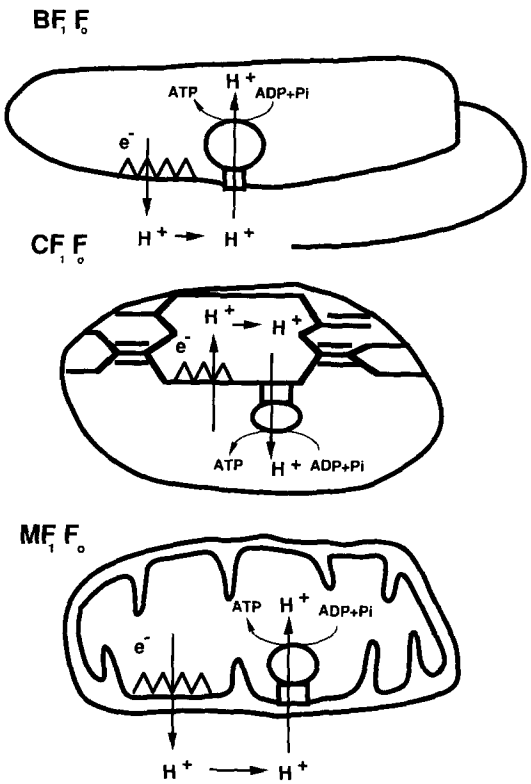

Fig. 2. F_1F_0-ATPases/synthases. (Top) The bacterial F_1F_0-ATPase/synthase (BF_1F_0) uses the electrochemical proton gradient (positive and acid outside) generated by the bacterial (aerobic or anaerobic) respiratory chain to synthesize ATP. (Middle) The chloroplast F_1F_0-ATPase/synthase (CF_1F_0) uses the electrochemical proton gradient (positive and acid inside the thylakoides) generated by the electron transport chain associated to the photosystems, to synthesize ATP. (Bottom). The mitochondrial F_1F_0-ATPase/synthase (MF_1F_0) uses the electrochemical proton gradient (positive and acid outside) generated by the respiratory electron transport chain, to synthesize ATP. The F_1F_0-ATPase/synthases are readily reversible, translocating protons in opposite directions during ATP hydrolysis.

regulatory mechanisms; (xi) studies of kinetic parameters and mechanisms of catalysis and transport; (xii) determination of topological features of the enzyme in the membrane; and (xiii) better understanding of complex cellular functions by co-reconstitution of ATPases with other transport systems.

The present review covers in a comprehensible manner the body of accumulated knowledge on the functions and regulations of ion-motive ATPases in prokaryotic and eukaryotic organisms using artificial membrane systems. The ATPases under consideration are involved in transport of Na^+, K^+, H^+ and/or Ca^{2+} and do not include ion-motive ATP-synthetases.

The F_1F_0-ATPase/synthetases from bacteria, chloroplasts and mitochondria (Fig. 2) have been excellently reviewed, and the reader is addressed to those papers for a detailed account of their properties and functions in reconstituted systems [27–31].

Since the earlier reconstitutions of the F_1F_0-ATPases, the Na^+/K^+-ATPase and the sarcoplasmic reticulum Ca^{2+}-ATPase, almost two decades ago, a large number of other ATPases have been studied in reconstituted systems. Particularly recent are the reconstitution of several H^+-ATPases of the vacuolar system of eukaryotic cells, distinct from the F_1F_0-ATPases and from the plasma membrane H^+-ATPase of lower eukaryotic cells, and the reconstitution of several cation transport ATPases from prokaryotic organisms.

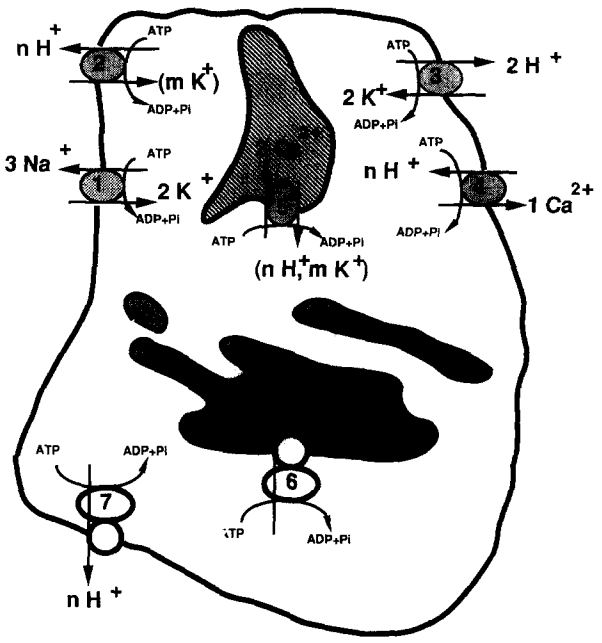

Fig. 3. Cellular location of eukaryotic phospho-ATPases and vacuolar type ATPases in an archetype cell. (1) Na^+/K^+-ATPase of animal cells. (2) H^+-ATPase of plasma membrane of lower eukaryotic cells. (3) H^+/K^+-ATPase of plasma membrane of gastric cells. (4) Ca^{2+}-ATPase from plasma membrane. (5) Ca^{2+}-ATPase from sarcoplasmic reticulum of muscle cells. (6) Vacuolar type H^+-ATPase located in different vesicular organelles. (7) Vacuolar type H^+-ATPase located in the plasma membrane.

II. Reconstitution of cation phospho-ATPases from eukaryotic cells

Eukaryotic organisms have several cation-translocating ATPases that form an acylphosphorylated intermediate during the catalytic cycle. These include the ubiquitous Na^+/K^+-ATPase [32–49] responsible for the extrusion of sodium ion and uptake of potassium ion by the cell, and the calmodulin-stimulated Ca^{2+}-ATPase [50–63] responsible for the extrusion of calcium ion from the cell in exchange for protons, both located in the plasma membrane. In addition, muscle cells have a different Ca^{2+}-ATPase located in the membrane from the sarcoplasmic reticulum [50,64–70], responsible for the transport of calcium ion from the myoplasm to the lumen of the sarcoplasmic reticulum network. Other Ca^{2+}-ATPases not yet fully characterized are located on the endoplasmic reticulum of non-muscle cells, the membranous structures from platelets and other specialized cellular structures such as synapses. Also the plasma membrane from the parietal cells from the stomach contains an H^+/K^+-ATPase [71–73] responsible for the extrusion of protons and the uptake of potassium ion. Finally, lower eukaryotic cells such as yeast/fungi and plants are equipped with H^+-ATPases [74–92] in the plasma membrane responsible for the extrusion of protons.

The localization of the different cation(s) phospho-ATPases from eukaryotic organisms are indicated in the upper part of the archetype cell represented in Fig. 3.

All the above-mentioned ATPases share a similar mechanism for the translocation of the different cation(s). In particular, it has been described that during the catalytic cycle the enzymes adopt two distinct conformations, denoted E_1 and E_2 states [93]. In addition, there is a real possibility that all these ATPases are capable of translocating two different cationic species in opposite directions, therefore working as exchangers of electroneutral and/or electrogenic nature. There are two ATPases from this group where the evidence for bicationic exchange is more controversial. One is the Ca^{2+}-ATPase from the sarcoplasmic reticulum, where protons and/or potassium ions have been proposed to be exchanged for calcium ions, and the other is the H^+-ATPase from the plasma membrane of yeast/fungi and higher plants, where potassium ions have been proposed to be exchanged for protons. Part of this controversy could be due to the fact that the proposed exchanges between the two cationic species appear to be only an optional and not an obligatory mode of operation of the enzymes in question.

The reconstitution of phospho-ATPases into artificial lipid membranes undoubtly has increased our knowledge on many issue of capital importance for the under-

standing of the physiological role of these enzymes. Some aspects of the reconstitution of the purified and intact enzymes, or part of the enzymes has been reviewed for the Na^+/K^+-ATPase [6,7,94–109], the Ca^{2+}-ATPase from the sarcoplasmic reticulum [6,7,94–102,104,109], and the Ca^{2+}-ATPase from the plasma membrane [55,59,60,101,104,110–112].

II-A. The Na^+/K^+-ATPase

The plasma membrane from eukaryotic cells is equipped with an electrogenic Na^+/K^+-ATPase responsible for the extrusion of Na^+ to the extracellular media and the uptake of K^+ by the cell (see Fig. 3, enzyme 1). This enzyme is composed of two distinct subunits: a 106 kDa α-subunit and a 38 kDa (after deglycosylation) β-subunit. The α-subunit is responsible for the hydrolysis of ATP, and forms an acylphosphate catalytic intermediate during its operation.

The Na^+/K^+-ATPase maintains both the resting chemical gradients of sodium and potassium ions across the plasma membrane ($1.4 \cdot 10^{-1}$ M Na^+ outside and 10^{-2} M Na^+ inside, and $4 \cdot 10^{-3}$ M K^+ outside and $1.6 \cdot 10^{-1}$ M K^+ inside). In addition, the enzyme also contributes to maintaining the electrical resting membrane potential of -75 mV to -95 mV (negative inside) across the plasma membrane.

The physiological role, molecular properties and mode of operation of the Na^+/K^+-ATPase from different origins have been extensively reviewed [32–49]. The factual accumulation of information about this enzyme and its functions, and the studies of the enzyme in reconstituted systems certainly helped to establish its pivotal importance in the physiology of the cell, dispelling claims made by some authors [113] suggesting that this enzyme was not responsible for the active transport of sodium ion.

II-A.1. Reconstitution in black lipid membranes

The first attempts to study the functions of the Na^+/K^+-ATPase in a reconstituted system were carried out in a black lipid membrane formed from oxidized cholesterol, after addition of a membrane-bound form of the enzyme from rat brain synaptic vesicles to one side of the membrane [114,115]. The addition of ATP to the same compartment in which the enzyme was added results in a 1 to 3 orders of magnitude decrease in the electrical resistance of the membrane, to a level of 10^6 ohm $\cdot$ cm^{-2}, and the generation of an electrical current across the membrane [114,115]. The dependency of this phenomenon on the presence of ATP and Na^+, and its inhibition by ouabain, suggested that it was directly mediated by the enzyme [114,115], or more specifically by its α-subunit [116,117]. Using the alternative substrate, *p*-nitrophenyl phosphate, it was also observed that the enzyme produced an increase in the electrical conductance in the absence of sodium and the presence of potassium ions [116,117].

To attain inhibition by ouabain, the inhibitor has to be added to the opposite side of the membrane from that on which the enzyme and the ATP were added, usually referred to as the *trans* compartment [115]. In other work, however, performed in the absence of ATP, the side of inhibition by ouabain was described as the *cis* compartment (i.e., the side of addition of the enzyme) [118]. Nevertheless, it became clear that the site of inhibition by ouabain was located on the opposite side from which the ATP binding site(s) are located, and the same side on which the K^+ binding sites are located [115,119–121]. In agreement with this proposal, the presence of potassium ion in the system appears to antagonize the inhibitory effect of ouabain [118,120]. In contrast, vanadate appears to inhibit from the same side as that on which the ATP binding site(s) are located [118,120,121]. However, treatment of the Na^+/K^+-ATPase with trypsin from both sides of the membrane abolished the electrical phenomenon [115].

The original observations of the electrical events induced by the enzyme on black lipid membranes were interpreted as the existence of an electrogenic Na^+/K^+ exchange (not a 1 : 1 exchange) catalyzed by the Na^+/K^+-ATPase [114]. However, the failure of Na^+/K^+-ATPase preparations from different origins in reproducing the results obtained with the rat cortical brain tissue preparations [115], cast some doubts on the nature of the observed phenomena. Indeed, some reports indicated that the short-circuit current and the open circuit voltage was observed in response to the simple addition of ATP in the presence as well as in the absence of the reconstituted Na^+/K^+-ATPase [122]. It was suggested that the observed electrical events were due to a particular alignment of ATP molecules on the phospholipid membrane by ions associated at its interphase with the water phase [122]. However, further accumulation of experimental evidence suggested that Na^+/K^+-ATPases isolated from different sources were indeed directly responsible for establishing the ionic currents across the black lipid membrane [116,118–121,123–126].

The incorporation of the Na^+/K^+-ATPase into the lipid bilayer conferred ion-gated channel properties to the planal membrane [118]. The system appears to operate in two states, one of high conductance, sensitive to ouabain and vanadate, and the other of low conductance, and insensitive to the inhibitors, the former observed only when an ion gradient was present [118]. The nature of this gating was described as non-electrical [118]. However, incorporation of the α-subunit of the Na^+/K^+-ATPase demonstrates that the presence of an electrical potential was necessary for sustaining high ATP hydrolytic activity, and the presence of a membrane potential of 100 mV results in the closing of the

ouabain-sensitive, monovalent cation-selective channel [116].

When a small amount of protein was added to the black lipid membrane it was possible to measure single channel conductance of 270 ± 14 pS [118]. In another approach, the enzyme was first reconstituted into liposomes containing only a mean of one pump per vesicle, and later the proteoliposome was fused with a planar black lipid membrane [127]. With this system, the measured single-channel conductance was from 40 to 50 pS; however, it was concluded that such conductance was due to disabled pump molecules [127]. Moreover, the addition of ATP, or the pretreatment of the enzyme with trypsin prior to its reconstitution into the liposomes did not affect the electrical conductance [127].

The addition of ATP to a black lipid membrane made of a mixture of different phospholipids plus cholesterol to which the Na^+/K^+-ATPase had previously been added resulted in a lag before the short-circuit current was initiated [123,124]. This electrical current increased over several hours, reflecting a progressive incorporation of individual pump molecules into the bilayer [123,124].

Flat membrane sheets of 0.2 to 1 μm in diameter containing high density Na^+/K^+-ATPase were bound to a planar lipid bilayer [126,128]. This system produces most probably a fusion of both membranes and could be used to study transmembrane voltage generation using the photolabile ATP-derivative, P^3-1-(2-nitro)phenylethyladenosine 5′-triphosphate (caged-ATP) as a substrate, measuring short time-resolution events of approx. 1 ms, upon irradiation with ultraviolet light [121,125,126,128,129]. The pump current was obtained in the presence of Na^+ or Na^+ plus K^+ in the presence of magnesium, but not with K^+ alone in the presence of magnesium [121]. Analysis of the transient ionic current presents a biphasic behavior [128] and shows that a slow non-electrogenic step was followed by an electrogenic transient [125,129] with a rate constant of 100 s^{-1} at 22° C [125]. Treatment of the enzyme with α-chymotrypsin abolishes the current transient [128], since partial proteolysis blocks the E_1-P/E_2-P transition and it was concluded that this transition is the principal electrogenic step followed by the release of Na^+ to the extracellular side of the membrane [128,129]. When the ATP-binding site(s) of the enzyme were labelled with fluorescein isothiocyanate no electrical current was observed [121]. A stationary pump current was obtained after addition of the Na^+/K^+ exchanger, monensin, plus the K^+ carrier valinomycin [121]. The intrinsic pump current can be evaluated from the voltage signal, and the dependence of the intrinsic pump current on the concentration of sodium ion permits a kinetic analysis supporting the assumption that two sodium binding sites have a high affinity and that a third site of lower affinity is rate limiting [126]. Charge-translocation is associated with early events in the normal transport cycle of the enzyme [128], and it was concluded that Na^+ translocation precedes translocation of K^+ supporting the Post-Alberts reaction scheme for the catalytic cycle of the enzyme [129].

Channel conductance in black lipid membrane also has been induced upon addition of the isolated α-subunit and β-subunit of the enzyme [120], and with polypeptide fragments obtained after its bromocyanate treatment [119]. In the latter case, two fractions, one with cationic conductance and the other with cationic-anionic conductance without selectivities for Na^+ or K^+ was observed [119]. However, a passive conductance pathway in the Na^+/K^+-ATPase molecule highly selective for Na^+ was first observed in pronase-sensitive, acid-soluble fractions of tryptic-digested membrane fractions from the electrical organ of *Electrophorus,* and from bovine kidney membranes, after its reconstitution into a black lipid membrane of oxidized cholesterol [130,131]. It was also suggested that the conductive unit may have an oligomeric nature [130]. This Na^+-ionophoretic activity was subsequently demonstrated in a mixture of intact α plus β subunits of the enzyme at a weight-to-weight ratio of approximately one [131]. This suggested that the ionophoretic activity was induced by the association of $1\alpha + 2\beta$ subunits, since the α or the β subunits individually lack ionophoretic activities [131]. Moreover, it was suggested that the Na^+-dependent ionophore was present in the small (β) subunit, and the role of the large (α) subunit was to induce the opening of Na^+ sites for its ionophoretic properties [131].

II-A.2. Lipid membrane–enzyme interactions

To study further the properties and mechanism of the purified Na^+/K^+-ATPase, its reconstitution in proteoliposomes was a necessary step. Recently, the technology involved in the different reconstitution procedures as well as the experimental techniques necessary for the study of the functions and physicochemical properties of the resulting vesicles have been described [132–137].

A first step in this approach has been the understanding of the role of the lipidic components of the membrane on the Na^+/K^+-ATPase. The activating effect of phospholipids on the ATP hydrolytic activity of the Na^+/K^+-ATPase has been known for some time [138,139]. Using 90% delipidated and highly inactivated enzyme preparations from rabbit kidney and bovine brain, it was established that addition of sonicated vesicles of phosphatidylserine or phosphatidylglycerol produced its reactivation, and confer substantial discrimination of K^+ over Na^+ in terms of the permeability of the reconstituted vesicles [140]. Although removal of the native lipids from the enzyme, and its substitution for phosphatidylserine vesicles result in one of the

best reactivations of the enzyme [141], using a lubrol-solubilized enzyme from rabbit kidney it was demonstrated that phosphatidylserine is not an absolute requirement for its activity [142].

When the enzyme was brought in contact with preformed liposomes, it was found that it binds to positively electrical charged liposomes made of a mixture of phosphatidylcholine, cholesterol and stearylamine, but not to negatively charged liposomes [142]. However, later experiments demonstrated that full recovery of the ATPase activity was obtained when negative charged phosphatidylinositol plus cholesterol was employed in the reconstitution procedure [143]. Also, it was established that the Na^+/K^+-ATPase also binds to formally neutral sphingomyelin liposomes, but not to pure phosphatidylcholine liposomes [142]. However, the interaction of the enzyme with preformed liposomes appears to be very weak, since it was easily extracted from the liposomes with high ionic strength solutions [142], demonstrating that the nature of this protein–lipid interaction does not represent the insertion of the enzyme across the membrane bilayer.

It was also established that to obtain reactivation of the ATP hydrolytic activity of the enzyme, no more than six lipid-binding sites of the 30 known to possess the enzyme could be unoccupied [144]. Moreover, the dissociation equilibrium constant for the lipids was determinated to be in the range from 0.3 to 5 μM [144], and maximum reactivation was obtained with phospholipids containing unsaturated fatty acyl chains conferring high fluidity to the membrane [140,145]. Other authors also concluded that to attain good reassembly of the Na^+/K^+-ATPase with the membrane lipid required the addition of diacylphospholipids with fluid acyl-chains and negatively charged polar heads [146]. Furthermore, by modifying the thickness of the membrane bilayer by using phospholipids of different acyl-chain length [145,147], or by the addition of a discrete amount of *n*-decane, an inverse relationship was demonstrated between enzyme activity and chain length of the saturated fatty acid of different phosphatidylcholine vesicles [145] and maximum activity was obtained with membrane of intermediate thickness [147].

Hybrid reconstitution of delipidated Na^+/K^+-ATPase from bovine brain into vesicles made of lipids extracted from preparations of the crab nerve enzyme reduced the sensitivity of the brain enzyme to inhibition by ouabain [148]. In contrast, hybrid reconstitution of the delipidated enzyme from crab nerve into vesicles made of lipids isolated from the brain enzyme increased the sensitivity of the crab enzyme to ouabain inhibition [148].

The variation of the pumping activity with the composition of the lipid membrane results from the different amounts of enzyme incorporated with the correct orientation into the membrane and to a lesser extent from lipid-dependent variation of the intrinsic turnover rate of the enzyme [149].

II-A.3. Reconstitution in liposomes

The first incorporation of an active Na^+/K^+-ATPase molecule into a liposomal membrane was obtained with the enzyme from bovine brain microsomes solubilized by deoxycholate using a mixture of 96% phosphatidylcholine plus 4% phosphatidylserine, followed by dialysis of the detergent [150]. Incorporation of the enzyme into the phospholipid vesicles was demonstrated by co-migration of the lipids and the ATPase activity by sucrose density gradient centrifugation; however, no measurement of transport activities was conducted in this earlier work [150].

A year later, the Na^+ transport function by a purified and reconstituted enzyme isolated from canine renal medulla [151], and the rectal salt gland of the spiny dogfish shark *Squalus acanthias* [152,153] was demonstrated, using proteoliposomes prepared by the cholate-dialysis method. The ATP-dependent uptake of $^{22}Na^+$ by those vesicles was demonstrated to be against a concentration gradient, increasing the intraliposomal Na^+ concentration from 20 mM to 38.4 mM [152]. However, the low efficiency of the transport function was reflected by the low Na^+/ATP ratio, in the order of 0.3 to 0.4 [151,152]. Moreover, the ATP-dependent $^{42}K^+$ efflux by the reconstituted enzyme was negligible [151].

The low Na^+/ATP ratio was attributed to the presence of non-incorporated Na^+/K^+-ATPase molecules [152]. Accordingly, addition of strophantine to the outside medium increased the Na^+/ATP ratio somewhat [151]. Later, ouabain was used to inhibit the non-reconstituted enzyme [154,155]. The enzyme participating in transport functions has been estimated to be on occasion as low as 20% of the total enzyme in the system [154]. However, these data contrast with another report in which it was estimated that only 5% of the enzyme remains non-reconstituted [156].

In those earlier experiments it was also established that ouabain [151–153] or strophantine [151] inhibits Na^+ transport from the inside of the vesicles, but not from the outside. The inhibition of the transport function of the enzyme by ouabain from the inside of the vesicles [151–153,157–159] is due to the interaction of the inhibitor with ATPase molecules presenting the ATP binding site(s) to the outside of the vesicles, and therefore exposing the K^+ binding sites to the vesicular lumen. However, vanadate inhibits the enzyme from the outside of the vesicles, where the ATP-binding site(s) are located in the enzymes responsible for transport functions since ATP is added to the external media [157,158,160,161].

Proteoliposomes, although capable of entrapping [^{14}C]inulin or [^{14}C]glucose [152], also equilibrate $^{22}Na^+$

and $^{42}K^+$ in the absence of ATP [151], suggesting that they are partially leaky, perhaps accounting for the low Na^+/ATP ratio observed [151,152]. In fact, it was reported that $^{36}Cl^-$ was co-transported with $^{22}Na^+$ during ATP hydrolysis, and it was suggested that the enzyme was capable of direct chloride ion transport, or was equipped with some specific mechanism for the translocation of Cl^- along with the actively pumped sodium ion [151,159]. However, this misinterpretation of the mechanism of operation of the pump was promptly corrected [156,162–165]. Although the passive permeability of Cl^- in those proteoliposomes was from 1.5-times [163] to 10-times [156] higher than the passive permeabilities for Na^+ or K^+, external Na^+, but not external Li^+, stimulates the ATP-dependent K^+ efflux [162], and therefore it was concluded that K^+ transport was mechanistically coupled to Na^+ transport without the direct participation Cl^- in the transport process [156,162–165].

Using a sonication procedure to reconstitute the Na^+/K^+-ATPase from the electric eel, *Electrophorus electricus,* it was established that the rate of the ATP-dependent ouabain-sensitive $^{22}Na^+$ transport was from 10- to 20-times higher than using the cholate-dialysis method, particularly in phosphatidylcholine proteoliposomes [166]. In addition, a freeze-thaw/sonication procedure result also in the formation of proteoliposomes with transport properties one order of magnitude higher than previously described, and it was suggested that cholate could inhibit the Na^+/K^+-ATPase [155]. A small amount of residual diethyl ether remaining in the phospholipid vesicles increased the ATPase activity [155]. In addition, preincubation of the enzyme in the presence of Na^+ appears to promote a conformation inhibiting its reconstitution; however, the presence of K^+ does not have any effect [155].

On the methodological front, a microprocedure for reconstitution of about 200 μg of purified enzyme by the cholate-dialysis method and capable of Na^+ and Rb^+ transport has been developed [167]. Also, an ingenious method to continuously monitor ATP-driven K^+ transport is the use of the $\Delta\psi$-sensitive fluorescent probe indocyanine dye in the presence of valinomycin [149,168–171]. Under those conditions the generated K^+ Nernst potential responds to the concentration of potassium ion [149,168–171].

II-A.4. Cation / ATP stoichiometry

Improvement in the cholate-dialysis method, particularly shorter and more efficient dialysis [162–164], and the preparation of proteoliposomes in the presence of magnesium [162,164], allowed for the first time the observation of ATP-dependent, ouabain-sensitive efflux of $^{42}K^+$ [162,156] or its analog $^{86}Rb^+$ [162–164], in addition to uptake of sodium ion. This efflux of K^+ appears to be dependent on the presence of external magnesium [162]. It was demonstrated that both Na^+ and K^+ were transported against their respective chemical gradients [152,156,160,172,173]. In those proteoliposomes the Na^+/ATP ratio was higher than before, from 1.4 [162] to close to 3 [156,159]. This high Na^+/ATP ratio close to 3 was confirmed by other workers [143,174,175]. However, the K^+/ATP ratio varied from near to 1 [166] to close to 2 [156,159]. It soon became apparent that the Na^+/ATP ratio varied from 0.52 ± 0.04, in the absence of K^+, to 2.9 ± 0.1 in its presence [172,173]. The residual ouabain-sensitive Na^+ uptake (in the absence of added K^+) was not due to contaminating endogenous K^+, since it was not affected by the incorporation of 10 μM KCl to the inside of the proteoliposomes [172,173]. However, the authors concluded that these results could not be explained in terms of an Na^+/Na^+ exchange [172]. The apparent K_m for K^+-independent Na^+ uptake was around 1 mM [176], and the Na^+/ATP ratio decreased upon increase of the extravesicular NaCl concentration [176].

ATP induced the exchange of 2.8–3.0 Na^+ per 1.6–2.5 K^+ in most reconstituted preparations with the enzyme from the rectal gland of *Squalus acanthias,* canine brain gray matter, bovine heart, and lamb or dog kidney [156,162–165]. In some preparations of reconstituted dog kidney enzyme K^+ transport was not observed [159]. However, thereafter an ATP-dependent Na^+/K^+ ratio of approx. 3/0.6 was observed [164,165]. The enzyme of the olfatory nerve axon of the garfish also presents a low Na^+/ATP ratio equal to 1 [175]. Nevertheless, the ATP-dependent $3Na^+/2K^+(Rb^+)$ exchange ($Na^+/K^+(Rb^+)$ ratio of 1.5) was reconfirmed with the enzyme from different origins [154,155,161,177–179] including also the enzyme from dog kidney [178]. ATP-dependent Na^+ uptake presented a sigmoidal dependence on the concentration of Na^+, with a Hill coefficient of 2 or 3 [161]. Cesium ion could substitute for potassium ion, and could be actively extruded from the proteoliposomes with an efficiency of $2Cs^+$/1ATP [178].

Effort has been made to understand some of the factors that affect the observed coupling/uncoupling of Na^+ and K^+ transport to ATP hydrolysis. Cholesterol, in addition to inhibiting the V_{max} of the enzyme reconstituted in phosphatidylserine vesicles [180,181], apparently affects the different catalytic phosphorylated conformational forms of the Na^+/K^+-ATPase [182,183], and the Na^+/K^+/ATP ratio has been reported to vary from 2.8/1.8/1 at low concentrations of cholesterol, to 1.6/0.6/1 at higher concentrations [182]. Surprisingly, the Ca^{2+}/H^+ ionophore A23187 provokes an increase of the Na^+/ATP ratio to 2.8 in proteoliposomes rich in cholesterol [182].

Using proteoliposomes in a medium containing chloride, the ATP-dependent Na^+/Rb^+ ratio was 1.61; however, upon substitution of Cl^- for the impermeable

SO_4^{2-}, the Na^+/Rb^+ ratio decreased somewhat, to 0.94–1.25, suggesting that chloride ion movement compensates for the electrical membrane potential (positive inside) generated by the Na^+–Rb^+ exchange, and therefore improves its stoichiometry [184].

The Na^+/K^+ ratio varies depending on the concentration of Na^+ and K^+ present in the medium [185]. Moreover, the $Na^+/K^+(Rb^+)$ ratio also varies during the time of ATP hydrolysis due to the depletion of internal K^+, which results in a mechanistic uncoupling of potassium ion transport [155,179,185]. After the initial ATP-dependent Na^+–K^+ exchange and exhaustion of the intravesicular pool of K^+, the enzyme catalyzes an ATP-dependent Na^+/Na^+ exchange [179].

A good agreement has been demonstrated between the rates of ATP hydrolysis and transport functions of the enzyme for different concentrations of the monovalent and divalent cations, Na^+, K^+, Mg^{2+} and Ca^{2+} [171]. However, this good correlation was not found when the concentration of H^+ in the medium was modified, since H^+ appears to compete with Na^+ at the cytoplasmic site of the enzyme and also to induce a non-competitive inhibition of transport which is not correlated with protein phosphorylation [171].

The phosphorylation of the Na^+/K^+-ATPase of normal cells (possibly its α-subunit) by the catalytic subunit of the cAMP-dependent protein kinase increases both the rate of $^{22}Na^+$ uptake and the Na^+/ATP ratio [186,187].

Reconstitution experiments also established that the enzymes composed only of the α- plus the β-subunits were responsible for the transport functions, excluding other polypeptides (at least of molecular mass higher than 12 kDa) from these functions [156]. In addition, using reconstitution procedures it was established that the Na^+/K^+-ATPase is an entity distinct from the Na^+/Ca^{2+} exchanger [188] and does not present Ca^{2+}-activated Rb^+ uptake or bumetanide-inhibitable Rb^+ uptake, representing a diuretic-sensitive Na^+/K^+ co-transport [189].

II-A.5. The Na^+–Na^+ exchange

As presented in Fig. 4, in the absence of K^+ the reconstituted enzyme catalyzes an ATP-dependent Na^+/Na^+ exchange [152,182,190–193], with an affinity for ATP of 2.5 μM [191]. However, when K^+ was present, the Na^+–Na^+ exchange was only a small percentage of the total Na^+ transported [152]. The ATP-dependent Na^+–Na^+ exchange or net Na^+ transport in the absence of K^+ is an electrogenic process [173,176,190–192] translocating 2.8 Na^+ inside and 1.3 Na^+ outside per ATP hydrolyzed [191,192], suggesting that in those conditions Na^+ occupied the sites normally occupied by K^+, approaching the mechanistic translocation of 3 Na^+ to the inside per 2 Na^+ extruded to the outside [173,176,182,190], and generating

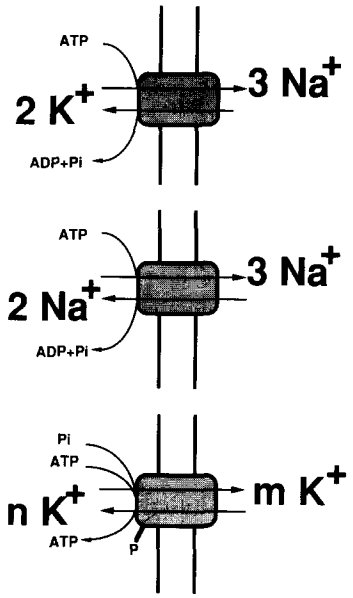

Fig. 4. Different modes of operation of the Na^+/K^+-ATPase. (Top) ATP-dependent Na^+–K^+-exchange. (Middle) ATP-dependent Na^+–Na^+-exchange. (Bottom) (ATP + P_i)-dependent K^+–K^+-exchange.

an electrical membrane potential (positive inside) of +50 mV [176,194], or +60 mV [191,192].

The Na^+–Na^+ exchange was inhibited by ouabain from the inside of the proteoliposomes and also was partially inhibited by oligomycin [191,192]. Thereafter, it was demonstrated that oligomycin reacts with an ADP-sensitive K^+-insensitive phosphorylated form of the enzyme(E_1P) and probably with an ADP-sensitive K^+-sensitive phosphorylated form (E*P) of the enzyme, therefore inhibiting their conversion to an ADP-insensitive K^+-sensitive phosphorylated form (E_2P) and preventing its interaction with K^+ [195]. This basic observation was confirmed with a pump covalently labelled with fluorescein, since oligomycin greatly reduces the rate of fluorescence quenching observed upon addition of the artificial substrate acetylphosphate in the presence of Mg^{2+} and Na^+, thought to represent the conversion of the E_1 form of the enzyme to its E_2P form [196]. In addition, ADP also partially inhibits the Na^+–Na^+ exchange reaction [191], as is the case for the enzyme when working in the presence of potassium ion [170]. In this latter case it appears that ADP reverses the phosphorylation step in the catalytic cycle [170].

In the absence of Na^+ and K^+ in the inside of the proteoliposomes the enzyme catalyzes an ATP-supported $^{22}Na^+$ uptake inhibitable by digitoxigenin, so-called uncoupled Na^+-efflux (when referred to in vivo orientation), that has been demonstrated to be an electrogenic process as measured by oxonol VI, with an Na^+/ATP ratio of 3 : 1 determined by measuring both initial rates of transmembrane potential generation and liberation of inorganic phosphate [197].

In the absence of potassium ion (Na^+ in both sides of the membrane), the ATP-activation curve was hyperbolic with high affinity for ATP; however, the incorporation of 50 μM K^+ to the inside of the vesicles transforms the ATP-activation curve in biphasic [190]. This biphasic behavior also was observed upon the addition of 50 mM or higher concentrations of K^+ to the outside of the proteoliposomes [190]. Activation by Na^+ from the cytoplasmic side of the membrane (inside of the liposomes) shows cooperative interaction among three sites [193]. Binding of Na^+ occurs simultaneously at both sides of the membrane [193].

II-A.6. The K^+–K^+ exchange

When the proteoliposomes were prepared in the absence of Na^+, the reconstituted enzyme also carried out an ouabain-sensitive, ATP- and orthophosphate-stimulated K^+–K^+ exchange [162] (see Fig. 4). This K^+–K^+ exchange was later studied in greater detail using $^{86}Rb^+$ as a K^+ analog [194,198–204]. The requirement of both ATP and P_i for the K^+–K^+ exchange was explained in terms of the stimulatory effect of P_i on the reversal of the dephosphorylation, and the facilitation of the conformational change induced by ATP during the catalytic cycle [198]. The (ATP + P_i)-dependent Rb^+–Rb^+ exchange was from 15% to 20% the rate of the ATP-dependent Na^+/K^+ transport [198,201]. A systematic variation in the concentrations of both ligands, ATP and P_i, permitted the study of the stimulation or inhibition of the rate of Rb^+–Rb^+ exchange, as well as the determination of some kinetic parameters [198,200]. However, the uptake of Rb^+ by inside-out-oriented pumps reconstituted in Na^+-loaded proteoliposomes shows only inhibition by ATP [200]. The concentration of Mg^{2+} also was shown to be an important factor in altering the rate of Rb^+ uptake, and in modifying the response to P_i in the Na^+-loaded proteoliposomes [199,200]. Moreover, the presence of Rb^+ in the outside of the proteoliposomes activates the (ATP + P_i)-stimulated Rb^+–Rb^+ exchange [201]. An Rb^+–Rb^+ exchange also was demonstrated to take place in the absence of ATP and P_i, with a half-saturation for external Rb^+ of approx. 0.6 mM for inside-out-oriented pumps, and 0.2 mM for right-side-out-oriented pumps [199]. In contrast, the slow uptake of $^{86}Rb^+$ into Rb-free proteoliposomes represents a net flux of Rb^+ through right-side-out-oriented pumps, and shows a half-saturation at approx. 0.1 mM external Rb^+ [199].

The presence of Na^+ inside the proteoliposomes inhibits the passive $^{86}Rb^+$ uptake [199]. However, the presence of monovalent cations outside the proteoliposomes, in the sequence $Li^+ > Na^+ > Cs^+ > K^+ > Rb^+$, stimulates $^{86}Rb^+$ uptake [199]. The stimulatory effect of K^+ outside was observed only at low concentrations; at high concentrations, external K^+ inhibits the passive $^{86}Rb^+$ uptake [199]. The presence of magnesium in the external media also inhibits the passive Rb^+–Rb^+ exchange as well as the passive net Rb^+ uptake [199]. Ouabain [199,203] and vanadate [199,200,203] inhibit both the passive [165,203] as well as the (ATP + P_i)-stimulated [200] Rb^+–Rb^+ exchange.

II-A.7. Kinetics and mechanism of transport

The turnover number, expressed as the number of Rb^+ or Na^+ translocated per second and per mol of phosphoenzyme has been measured for the ATP-dependent $^{22}Na^+$–Rb^+ exchange (43 per s), the (ATP + P_i)-stimulated $^{86}Rb^+$–Rb^+ exchange (7 per s), the vanadate (out)-sensitive $^{86}Rb^+$–Rb^+ exchange (0.25 per s), the vanadate (out)-sensitive net $^{86}Rb^+$ uptake (in the absence of ATP and P_i) (0.15 per s), and the vanadate(out)-sensitive $^{86}Rb^+$–Na^+ exchange (0.63 per s) [203]. Other authors describe variable turnover rates for the Na^+/K^+-ATPase from approximately 14 per s [168,169] to 3500 per min [174] and an activation energy for K^+ transport of 115 kJ · mol^{-1} [169].

A mathematical treatment of different vesicle inhomogeneity parameters has been considered in the kinetic analysis of transport functions by the reconstituted Na^+/K^+-ATPase [205]. Using the statistical properties of the proteoliposome population where it is assumed that the number of ATPase molecules per vesicle is given by a Poisson distribution and the extent of the distribution of vesicle radii is estimated, under favorable conditions the intrinsic turnover number of the enzyme and the density of functional pump molecules in the proteoliposome membrane could be calculated [206].

During the catalytic cycle $K^+(Rb^+)$ passes through an occluded state, bound to the E_2 conformation form of the enzyme [200,201]. The functional role of this occluded form of Rb^+ in active transport is to minimize passive cation leak (in the absence of ATP or P_i) through the Na^+/K^+-ATPase and to allow a direct control of the direction of cation movement after physiological ligands such as ATP and inorganic phosphate bind to the enzyme [194,201,203]. Excess of inorganic phosphate has been shown to inhibit the enzyme [170].

Treatment of the Na^+/K^+-ATPase with trypsin or chymotrypsin inactivated the ATP hydrolytic activity and the Na^+/K^+ transport activity to different degrees [202]. However, trypsinization of the enzyme appeared to affect $^{22}Na^+$ transport to a greater extent than $^{86}Rb^+$ transport [207]. It was also demonstrated that this phenomenon was not due to increased passive permeability of the vesicles to either cation [207]. The cleaving of a bond in the α-subunit, on the amino-terminal side of the aspartyl phosphate, inhibits the Rb^+–Rb^+ exchange more than the ATPase activity, but inhibits the Na^+–Na^+ exchange in proportion to the inhibition of the ATPase activity [202]. However, clea-

vage of a bond on the carboxyl-terminal side of the aspartyl phosphate causes only a moderate alteration in the Na^+–Na^+ or Rb^+–Rb^+ exchanges [202].

It was proposed that the $\alpha\beta$-dimer is the minimum functional protein unit of the Na^+/K^+-ATPase both in $C_{12}E_8$ solubilized form and after reconstitution by a freeze-thaw/sonication procedure [208]. Moreover, using irradiation-inactivation analysis it was established that the minimum mass for the ATP-independent Rb^+–Rb^+ exchange was 117 ± 4 kDa, corresponding to the mass of the α-subunit of the enzyme. However, the minimum mass for the (ATP + P_i)-activated Rb^+–Rb^+ exchange was established to be 206 ± 7 kDa, similar to the minimum mass required for the ATP-dependent Na^+–K^+ exchange (201 ± 4 kDa), suggesting that a minimum of an $\alpha\beta$-dimer was required for ATP-dependent transport associated functions by the enzyme [204].

The role of the β-subunit has been also inferred from studies using lectins [154,157]. Wheat-germ agglutinin, and to a decreasing extent concavalin A, lectin type VI from soybean, and lectin from *Tetragonolobus purpureas* inhibit the transport function of the reconstituted Na^+/K^+-ATPase when incorporated to the inside of the vesicles [154,157]. The inhibitory action induced by the wheat-germ agglutinin was more pronounced on the Na^+ transport (70% inhibition) than on the K^+ transport (50% inhibition), suggesting that partial uncoupling between Na^+ and K^+ transport take place [154,157]. Wheat-germ agglutinin bind to the *N*-acetylglucosamine residues of the β-subunits and do not bind to the α-subunit [154,157]. Accordingly, this inhibitory effect could be reversed by ovomucoid (rich in *N*-acetylglucosamine residues) [154], and by β-1,4-di-*N*-acetylglucosamine [157]. In contrast, removal of sialic acid by neuraminidase did not affect Na^+ transport [154,157].

The partial uncoupling of the Na^+–K^+ exchange was also obtained by chemical compounds such as the -SH-alkylating reagent *N*-ethylmaleimide, since ATP-driven Na^+ transport was inhibited by 30%; however, the ATP-driven K^+ transport was unaffected by this inhibitor [160].

The existence of three forms of the phosphorylated intermediate of the enzyme, an ADP-sensitive K^+-insensitive form (E_1P), an ADP-sensitive K^+-sensitive form (E*P), and an ADP-insensitive K^+-sensitive form (E_2P) has been described [182,183,195,209]. The ADP-sensitive K^+-sensitive form appears to be responsible for the binding of the cardiac steroids such as digitoxigenin [183]. In the reconstituted system the membrane induces an accumulation of the ADP-sensitive K^+-insensitive form, and this barrier is counteracted by CCCP or monensin [195]. The transition from the ADP-sensitive K^+-sensitive to the ADP-insensitive K^+-sensitive phosphorylated forms of the enzyme is the rate-limiting step of the pump in the presence of high concentrations of ATP and it appears that K^+ in the cytoplasmic side (inside the proteoliposomes) may control this reaction step by enhancing its rate [209].

The reconstituted Na^+/K^+-ATPase also was capable of utilizing other substrates distinct from ATP [196,210–213] to maintain transport functions, although at lower rates [210,211]. Acetylphosphate in the presence of extravesicular Mg^{2+}, and in the absence of internal K^+, support an ouabain-sensitive Na^+ transport [210]. However, in the presence of internal K^+ the transport of Na^+ promoted by acetylphosphate was very small, and not statistically significant [210,211]. Although acetylphosphate could replace ATP in the catalytic site, this was not the case in the regulatory site [210].

When the medium contained a low Na^+/K^+ ratio (20 mM/50 mM) a correlation was observed between the proton-accepting capacity of different nucleotides (ATP, CTP, ITP, GTP, UTP, N_1-oxy-ATP and N_1-methoxy-ATP) and its efficiency as a substrate to maintain active transport functions [212,213]. The ATP analog N_1-oxy-ATP, possessing proton-accepting ability, was as effective as ATP in sustaining active accumulation of $^{22}Na^+$ by the proteoliposomes [212,213]. However, the ATP analog N_1-methoxy-ATP, the negative charge of which in the position 1 of the purine ring is quenched by a methyl group, was ineffective [212,213]. The proton-accepting properties of the substrate seem to be a necessary condition for the shift from the K-form to the Na-form of the enzyme [213].

Using the MgATP complex analog, chromium(III) ATP, it was demonstrated that the MgATP complex is the true substrate for the Na^+/K^+-ATPase capable to support Na^+ and Rb^+ transport [211]. However, the enzyme hydrolyzing CrATP is quickly inactivated, since the chromo-phospho-enzyme cannot occlude $K^+(Rb^+)$ nor has it the capability to bind $^{86}Rb^+$ at the high-affinity site for K^+ [211]. Also, it has been demonstrated that free Mg^{2+} above a Mg^{2+} : ATP ratio of 1 : 1 inhibits the enzyme [180]. It has been proposed that the effects of Mg^{2+} could be due to modulation of the lipid–membrane/protein interaction [214].

II-A.8. Electrical properties

The electrogenic nature of the ATP-dependent Na^+–K^+ exchange catalyzed by the reconstituted enzyme was first suspected because valinomycin (plus potassium) produce a 20–50% stimulation of the gramicidin-sensitive Na^+ transport [166]. The addition of valinomycin prevents depletion of internal potassium [174]. However, this ionophore alone did not sustain the maximum rate of Na^+ transport [161], but a combination of valinomycin plus FCCP appears to further stimulate the rate of Na^+-transport [161].

Direct measurement of a vanadate and ouabain-sensitive $\Delta\psi$ (positive inside) was attained monitoring the distribution of $[^{14}C]SCN^-$ [157,184]. The electrical

TABLE I

Structural properties of proteoliposomes containing reconstituted ATPases

Structural properties	Reconstituted enzyme							References
	Na^+/K^+-ATPase	Ca^{2+}-ATPase sarcoplasmic reticulum	Ca^{2+}-ATPase plasma membrane	Ca^{2+}-ATPase synaptosomes	H^+-ATPase plasma membrane	H^+/K^+-ATPase gastric mucosa	K^+-ATPase bacteria	
Liposome diameter (nm)	50 to 170							145,150,156,169,174,177, 178,216,220–223
	90 to 100 [a]							169,177,220,222,223
		35						243
		60						275
		30 to 500						276
		100 ± 20						271
		110 ± 70						257,260
		224 to 273						263
		60000 to 300000						277
			50 to 600					340,342
			50 to 100 [q]					340
			200 to 400 [r]					340
				55				370
					95 ± 20			380
							50 to 100	393
Membrane thickness (nm)	5							150
	7							177
		6						238
Internal volume	0.7% of total vol.							
	39–52 µl/mg protein at 3000 PL/enzyme (mol/mol)							169
								262
					0.35 µl/mg PL			381
Enzyme orientation	85% (inside out) [b]							221
	60% (right-side-out)							143
	10% (inside-out) [c]							143
	30% (non-oriented) [c]							143
	30% (inside-out) [d]							143
	10% (non-oriented) [d]							143

	50% (inside-out)				156,178,223–225
	50% (right-side-out)				156,178,223–225
		14 to 16% (inside-in) [c]			250
		40 to 50% (inside-out) [e]			262
		57 to 58% (inside-out) [f]			278
			75% (inside-out) [e,f]		354
			25% (inside-in) [e,f]		354
				70% (inside-out)	376
				30% (inside-in)	376
				60% (inside-out)	378
				40% (inside-in)	378
Size [h] intramembranous particles (nm)	8 to 10, 9 [a]				177,216,220–222,224, 226,227
		7.1 [g]			272
		9 ± 1			276
		8 to 10			238,240,241,271
Size [i] surface particles (nm)	3 to 5				224,226,227
		3 to 5			239–241,273
Number of intramembranous particles per μm^2	3410 ± 270 [j]				226
	390 ± 170 [k]				226
	6100 [l]				227
		4500 to 6000			271
Number ATPase molecules per vesicle		1 [m]			243
		4 to 16 [n]			243
		3 to 5			275
Number of lipid-binding sites of the enzyme	30				144
		22 [o]			288
		32 [p]			288
Dissociation equilibrium constant for lipids (μM)	0.3 to 5				144

PL, phospholipids; [a] average values; [b] measuring sialic acid of β-subunit; [c] low phospholipid/protein ratio; [d] high phospholipid/protein ratio; [e] measured with alamethicin; [f] measured with Triton X-100, [g] peak value, [h] in freeze-fracture electron microscopy; [i] in negative-staining electron microscopy; [j] concave impression; [k] convex impression; [l] concave plus convex impressions; [m] light fraction; [n] heavy fraction; [o] at 37 °C; [p] at 0 °C; [q] at 4 °C; [r] at 23 °C.

membrane potential was generated very quickly upon addition of ATP, as measured by indocyanine dye, a $\Delta\psi$-sensitive probe [169]. The addition of nigericin to the system abolished both the Na^+ and K^+ gradients, but did not prevent [^{14}C]SCN^- distribution [157,184]. The ATP-dependent $\Delta\psi$ (positive inside) was on average +14 mV, and in the presence of nigericin decreased somewhat to +9 mV, suggesting that the overall potential was composed of two components, an electrogenic potential and a diffusion potential [184]. This small $\Delta\psi$, contrasts with the higher $\Delta\psi$ of approx. +50 to +60 mV (positive inside) generated during Na^+–Na^+ exchange [176,191,192,194].

In contrast, the $\Delta\psi$ (positive inside) also was measured with the $\Delta\psi$-sensitive probe oxonol VI [215–217], and values as high as +90 mV [217] or +150 to +200 mV [215] (positive inside) were recorded. After the build-up of the $\Delta\psi$, a quasi-stationary state is reached in which the pump current is compensated by a backflow of charges through passive conductance pathways [215]. The generation of the $\Delta\psi$ was inhibited by vanadate [216].

Using a fluorescein-labelled enzyme reconstituted in proteoliposomes it was established that the transitions $E_1(K)$ to $E_2(K)$ and $E_2(K)$ to $E_1(Na)$ were 2-fold slower and 4- to 6-fold faster, respectively, in the vesicles when compared to the solubilized enzyme [218]. In addition, an imposed cation diffusion potential (positive outside) with K^+ (plus valinomycin) or Li^+ (plus the Li^+ ionophore AS701) does not affect the rate of the transition $E_1(K)$ to $E_2(K)$. Therefore it was concluded that the reversible transition $E_1(2K)$ to $E_2(2K)$ is a voltage-insensitive step [218]. However, the transition, $E_1P(Na)$ to $E_2(P)$ using acetylphosphate as substrate and associated with transport of 3 Na^+ was established to be a voltage-sensitive reaction carrying a net positive charge [196].

The generated $\Delta\psi$ (positive inside) was responsible for the uptake of $^{36}Cl^-$ during ATP-dependent transport of Na^+, and it was also concluded that it was highly unlikely that a protonic gradient plays any role during the operation of the Na^+/K^+-ATPase [184]. Nevertheless, in the absence of Na^+ or K^+ inside, and using 1 mM Na^+ outside, it was demonstrated that $^{36}Cl^-$ uptake was less than the equivalent amount required to compensate the generated $\Delta\psi$, and the reason was traced to the concomitant H^+ efflux that takes place as measured by the pH-sensitive fluorescent probe, 1-hydroxypyrene-3,6,8-trisulfonic acid [176]. It was also demonstrated that this Na^+–H^+ exchange varied depending on the nature of the anions in the media. Therefore it was concluded that H^+ were not directly pumped by the ATPase, but rather were moved in response to the $\Delta\psi$ generated, although direct H^+ transport was not excluded [176]. However, in the absence of Na^+, it was demonstrated that the reconstituted Na^+/K^+-ATPase was capable of generating an ATP-dependent, ouabain-sensitive acidification of the interior of the liposomes, as measured by entrapped fluorescein isocyanate-dextran [219]. The internal pH changed upon addition of ATP from 5.87 to 5.71 [219]. The H^+ uptake was maximal at an external pH of 5.5–5.6, and was inhibited by the addition of Na^+ to the outside [219]. The ΔpH (acid inside) was abolished by the addition of the protonophores X-537A or CCCP; however, it was not affected by the addition of 3 mM tetra-*n*-butylammonium, demonstrating that H^+ movement was not passive, but rather was mechanistically transported in place of Na^+ by the sodium pump [219].

II-A.9. Physicochemical properties of the reconstituted vesicles

The physicochemical properties of the reconstituted Na^+/K^+-ATPase vesicles have been studied. The size of the proteoliposomes containing the Na^+/K^+-ATPase, prepared by a variety of reconstituted procedures, and estimated by a variety of methods were in the range 50–170 nm in diameter [145,150,156,169,174,177,178, 216,220–223], with an average of 90–100 nm in diameter [169,177,220,222,223], and 5 nm [150] or 7 nm [177] membrane thickness. The vesicles containing the enzyme were slightly larger (90–100 nm diameter) than the vesicles of plain lipids (60–70 nm diameter) [169,222]. However, using Sepharose 2B gel filtration it was estimated that the size of the vesicles containing the enzyme was on average 60 nm diameter [156,178]. Moreover, the diameter of the vesicles increased with the average number of molecules of Na^+/K^+-ATPase per vesicle [222].

Also it has been established that using the freeze-thaw/sonication procedure to reconstitute the enzyme, 40% of the vesicles contains ATPase molecules, and 60% was plain liposomes [161]. Using $^{137}Cs^+$ the entrapped volume of the reconstituted Na^+/K^+-ATPase vesicles was also determined; this represented only 0.7% of the total volume [169].

Preparation of proteoliposomes by the removal of the detergent Chaps results in an asymmetrical orientation of the Na^+/K^+-ATPase in the liposomal membrane [174]. In addition, treatment of the glycoprotein moiety (β-subunit) of the Na^+/K^+-ATPase with neuraminidase demonstrated that the enzyme was also asymmetrically oriented in proteoliposomes prepared by removal of the detergent Triton X-100 by a Bio-Beads SM-2 column [221]. In this case, more than 85% of the total sialic acid of the preparation was directed to the outside of the vesicles [221]. Moreover, reconstitution of the enzyme by removal of the detergent $C_{12}E_8$ by polystyrene beads results in the formation of proteoliposomes with 60% of the enzyme with right-side-out orientation, 10% with inside-out orientation, and the rest representing non-oriented molecules [143]. How-

TABLE II

Experimental stoichiometries of reconstituted ATPases

Enzyme	Source	Stoichiometry	Comments	References
		Na^+_{in}/ATP		
Na^+/K^+-ATPase	rectal gland *S. acanthias*	0.3 to 0.4		151,152
	Garfish olfactory nerve axon	1		175
	rectal gland *S. acanthias*	1.4		162
	dog kidney	0.52 ± 0.04	absence of K^+	172,173
	dog kidney	2.9 ± 0.1	presence of K^+	172,173
	dog renal medulla, rectal gland *S. acanthias*, electric organ *E. electricus*, Garfish olfactory nerve axon	close to 3		143,156,159,174,175
	rectal gland *S. acanthias*	3	uncoupled Na^+ flux	197
		$K^+(Rb^+)_{out}$/ATP		
	rectal gland *S. acanthias*	near 1		162
	rectal gland *S. acanthias*, dog renal medulla	close to 2		156,159
		Cs^+_{out}/ATP		
	dog kidney	2		178
		$Na^+_{in}/K^+(Rb^+)_{out}$		
	dog renal outer medulla, lamb renal medulla	3/0.6		164,165
	rectal gland *S. acanthias*, dog renal medulla, dog brain gray matter, lamb kidney medulla	2.8 to 3.0/1.6 to 2.5		156,162–165
	rectal gland *S. acanthias*, dog kidney, pig renal outer medulla, bovine heart, rabbit renal outer medulla	3/2		154,155,161,165,177–179
	electric organ *E. electricus*	1.6/0.6	high cholesterol	182
	electric organ *E. electricus*	2.8/1.8	low cholesterol	182
		Na^+_{in}/Na^+_{out}		
	rectal gland *S. acanthias*	2.8/1.3		191,192

continued on next page

TABLE II (continued)

Enzyme	Source	Stoichiometry	Comments	References
		Ca^{2+}_{in}/ATP		
Ca^{2+}-ATPase	rabbit skeletal muscle	0.16	crude soybean phospholipids	251
sarcoplasmic	rabbit skeletal muscle	0.64	purified soybean phospholipids	251
reticulum	rabbit white skeletal muscle	0.2	palmitoylphosphatidylcholine	296
	rabbit white skeletal muscle	0.75	myristoylphosphatidylcholine	296
	dog heart ventricle	0.69 ± 0.09	Triton X-100 removal method	234
	rabbit skeletal muscle	0.83	Triton X-100 removal method	256
	rabbit skeletal muscle	0.2 to 0.5	cholate or deoxycholate dialysis method presence of oxalate	261,263,268,278
	rabbit white skeletal muscle	0.9 to 1.2	cholate or deoxycholate dialysis method presence of oxalate	248,266,291
	rabbit skeletal muscle	0.8 to 1.2	phosphatidylethanolamine	266,291
	rabbit back and hind leg muscles	0.96	egg yolk phosphatidylcholine /cholesterol (9 : 1)	265
	rabbit skeletal muscle	close to 1	dialysis of $C_{12}E_8$	244
	rabbit skeletal muscle	1.25	dialysis of cholate plus $C_{12}E_8$ method sarcoplasmic reticulum lipids	276
	rabbit skeletal muscle	1.3 to 1.6	dialysis of cholate plus $C_{12}E_8$ method egg yolk phosphatidyl-choline	276
	rabbit skeletal muscle	1.43 to 1.54	freeze-thaw/sonication method plus cholate and removal of detergent with Sephadex G-50	316
	rabbit skeletal muscle	0.3 to 0.7	absence of proteolipids	313,315
	rabbit skeletal muscle	0.7	absence of proteolipids	319
	rabbit skeletal muscle	1.3 to 1.7	presence of proteolipids	313,315
	rabbit back and hind leg muscles	0.5	applied $\Delta\psi + 50$ mV positive inside	265
	rabbit back and hind leg muscles	1.5	applied $\Delta\psi - 100$ mV negative inside	265
	rabbit skeletal muscle	2.2 ± 0.3	stoichiometry that accounts for thermodynamics of the catalytic cycle	258
		H^{+}_{out}/Ca^{2+}_{in}		
	rabbit white skeletal muscle	approx. 1	at low levels of Ca^{2+} accumulation	259
	rabbit white skeletal muscle	lower than 1	at high levels of Ca^{2+} accumulation	259
	rabbit skeletal muscle	1 or 2		275

		Ca^{2+}_{in}/ATP		
Ca^{2+}-ATPase platelets	human platelets, pig platelets	2.1 ± 0.2		374,375
		Ca^{2+}_{in}/ATP		
Ca^{2+}-ATPase synaptosomes	rat brain	0.95		370
Ca^{2+}-ATPase plasma membrane		Ca^{2+}_{in}/ATP		
	human erythrocyte	approx. 0.3	crude preparation presence of oxalate	341,342
	pig erythrocyte	0.4 to 0.79	partially purified enzyme	344
	human erythrocyte	0.94 ± 0.17 to 1.1 ± 0.1		110,111,346,351,355
	human erythrocyte	0.4	85 kDa and 81 kDa tryptic fragments	359
	human erythrocyte	0.60 ± 0.1	phosphatidylcholine absence of calmodulin	354
	human erythrocyte	0.44 ± 0.08	phosphatidylcholine presence of calmodulin	354
	human erythrocyte	0.52 ± 0.03	absence of calmodulin treated with Calpain I	354
	human erythrocyte	0.41 ± 0.05	presence of calmodulin treated with Calpain I	354
	human erythrocyte	1.20 ± 0.17	corrected values absence of calmodulin	354
	human erythrocyte	0.83 ± 0.16	corrected values presence of calmodulin	354
	human erythrocyte	1	assuming a $Ca^{2+}/2H^+$ ratio	350,352
	human erythrocyte	0.9	sickle cell anemia patients	361
	calf heart	1		112,362
	smooth muscle porcine antrum	0.96 ± 0.18 to 1.04 ± 0.15		364,365
	rat liver	0.3		369
		H^+_{out}/ATP		
	human erythrocyte	1.7 ± 0.2 to 2.4 ± 0.3	correcting for scalar H^+ production	111,350
		Ca^{2+}_{in}/H^+_{out}		
	human erythrocyte	1/2		111,350,355
H^+,K^+-ATPase gastric mucosa		H^+_{in}/ATP		
	pig stomach	2.1 ± 0.17		378
		$K^+(Rb^+)_{out}$/ATP		
	pig stomach	1.93 ± 0.24		378

TABLE III

Functional parameters of reconstituted ATPases

Enzyme	Mode operation	$\Delta\psi$ (mV) (positive or negative inside)	Electrical conductance parameters	Minimal mass (kDa)	ΔpH (units)	Kinetic parameters	Comments	References
Na^+/K^+-ATPase	ATP-dependent	+14					absence of nigericin $[^{14}C]SCN^-$ uptake	184
		+9					presence of nigericin $[^{14}C]SCN^-$ uptake	184
		+90					oxonol VI	217
		+150 to +200					oxonol VI	215
	Na^+–Na^+ exchange	+50						176,194
		+60						191,192
	ATP-dependent				0.16			219
			270 ± 50 pS				single channel conductance	118
			40 to 50 pS				disable pumps	127
			100 s^{-1}				rate constant at 22°C	125
			100 mV				close ouabain-sensitive monovalent-cation selective channel	116
	ATP-independent Rb^+–Rb^+ exchange			117 ± 4			radiation inactivation analysis	204
	$(ATP + P_i)$-activated Rb^+–Rb^+ exchange			206 ± 7			radiation inactivation analysis	204
	ATP-dependent Na^+–K^+ exchange			201 ± 4			radiation inactivation analysis	204
	charge movement					14 s^{-1}	turnover rate	168,169
	ATP hydrolysis					3500 min^{-1}	turnover rate	174
	ATP-dependent $^{22}Na^+$–Rb^+ exchange					43 s^{-1}	turnover rate	203

	$(ATP+P_i)$-stimulated $^{86}Rb^+-Rb^+$ exchange				$7\ s^{-1}$	turnover rate	203
	vanadate (out)-sensitive $^{86}Rb^+-Rb^+$ exchange				$0.25\ s^{-1}$	turnover rate	203
	vanadate (out)-sensitive net $^{86}Rb^+$ uptake (absence of ATP and P_i)				$0.15\ s^{-1}$	turnover rate	203
	vanadate (out)-sensitive $^{86}Rb^+/Na^+$ exchange				$0.63\ s^{-1}$	turnover rate	203
					$115\ kJ \cdot mol^{-1}$	activation energy	169
Ca^{2+}-ATPase sarcoplasmic reticulum	ATP-dependent	$+61 \pm 10$				clamping the $\Delta\psi$ with a K^+ diffusion potential	255
		+51				ANS	255
		+30 to +40					322
Ca^{2+}-ATPase plasma membrane					$0.18\ \mu M$ $K_{0.5}(Ca^{2+})$	pigeon erythrocyte	345
H^+-ATPase plasma membrane	ATP-dependent		0.2 pA			*N. crassa* proteoliposomes fused in BLM [a]	400
	ATP-dependent			3.6		*S. pombe*	385
				higher than 5		*S. cerevisiae*	384
	H^+-pumping			105		*N. crassa*	399
				100		*S. pombe*	379
K^+-ATPase bacteria	ATP-dependent	−10 to −15				*S. faecalis*	393

[a] BLM, black lipid membrane.

ever, when the protein/lipid ratio was changed from 1:10 to 1:75, the inside-out-oriented molecules increased to 30% with a parallel decrease in the percentage of non-oriented enzyme molecules [143].

The reconstitution of the Na^+/K^+-ATPase by the cholate-dialysis method [156,178,223–225], or by dialysis of octyl glucoside [178] resulted in an apparent random incorporation of the enzyme into the lipid membrane. Different types of vesicle could be separated by sucrose density gradient centrifugation [178]. Sealed vesicles sedimented to higher density than unsealed vesicles after passive equilibration with the heavy salt CsCl, and vesicles with inside-out-oriented enzyme sedimented at lower density than the ones with right-side-out-oriented enzyme after the trapped Cs^+ was pumped out after incubation with MgATP [178]. The orientation was reconfirmed by access of the ATPase to trypsin, and access of the sialic acid to neuraminidase [178].

The transport functions of both subpopulations of reconstituted and opposite oriented enzymes has been studied by timed asymmetric addition of ATP, Rb^+ and cardiac glycosides to alternative sides of the lipid membrane [223,225].

Freeze-fracture electron microscopy of reconstituted Na^+/K^+-ATPase vesicles reveals intramembranous particles in the range of 8–10 nm, with average diameter of 9 nm [177,216,220–222,224,226,227]. Particle density varied with the protein/lipid ratio of the proteoliposomes [177]. The density of concave impressions on the membrane was estimated to be 3410 ± 270 per μm^2; however, the convex impression density was estimated to be 390 ± 170 per μm^2 [226]. However, other reports show that the combined particle frequency of the two fracture faces of the membrane are 6100 particles per μm^2 [227]. It was also demonstrated that vesicles prepared only with the glycoprotein (β-subunit) do not exhibit intramembranous particles [221]. Therefore, it was proposed that these intramembranous particles represent the catalytic subunit of the enzyme, or the catalytic plus the glycoprotein subunit [221]. Also it was noticed that a 280 kDa $\alpha_2\beta_2$ heterodimer complex could represent the 9 nm intramembranous particles impressions observed in freeze-fracture electron micrography [177,227]. Moreover, it was found that maximum Na^+ and Rb^+ transport was obtained when each vesicle contained on the average more than two of these particles [222]. Negative staining electron microscopy also reveals the presence of 3–5 nm surface particles, representing some protrusion of the enzyme to the outside medium [224,226,227].

The passive ion conductance of the reconstituted Na^+/K^+-ATPase proteoliposomes has been also studied. Preparations of proteoliposomes by the cholate-dialysis method demonstrated that, in the absence of ATP, the Na^+/K^+-ATPase increases $^{22}Na^+$ conductance 15-fold, and $^{42}K^+$ or $^{86}Rb^+$ conductance 40-fold [179,228–230]. This passive conductance, somewhat selective for K^+, was only a fraction of the rates of fluxes mediated by ATP [161,179,228–230]. Although this passive ion flux could be mediated by a channel located in the Na^+/K^+-ATPase molecule, the possibility could not be excluded that the phospholipid region surrounding the ATPase molecule could contribute to the leak [230], in particular, when it has been established that the Na^+/K^+-ATPase induces a leakage of [^{14}C]sucrose-loaded phosphatidylserine liposomes [141]. However, trypsin treatment suggested that proteolysis appears to remove a barrier for passive ion leaks and abolished the K^+/Na^+ discrimination [231]. Also, the treatment of the enzyme with trypsin in the presence of Na^+ resulted in the production of an enzyme that has 30–40% lower rate of ATP-dependent Na^+ transport; however, the transport of Rb^+ appears to be preserved [232,233]. Moreover, passive Na^+ and K^+ transport were identical after trypsinization [232]. The residual active Na^+ transport in the trypsinized preparations was resistant to inhibition by vanadate [232]. Using $^{48}VO_4^{3-}$ it was established, however, that its binding capacity was similar but its dissociation constant changed from 4.5 nM to 32 nM after trypsinization [232]. Also trypsinization of the α-subunit does not modify its interaction with the lipid membrane [233]. Trypsinization was proposed to produce an effective transition from the E_1P conformation to the E_2P conformation, and K^+-dependent dephosphorylation [232]. Moreover, it was concluded that the reduction of the Na^+/K^+ ratio observed after trypsin treatment could be due to interference with the binding of Na^+, or to interference with the conformational change accompanying the translocation of sodium ion [232].

II-B. The Ca^{2+}-ATPase from sarcoplasmic reticulum

The sarcoplasmic reticulum network from skeletal muscle is reponsible for the removal of calcium ion from the cytoplasm after muscle contraction, therefore contributing to muscle relaxation. The sequestration of calcium ion inside the lumen of the sarcoplasmic reticulum generates a steep chemical gradient of calcium across the membrane, approx. 10^{-3} M free Ca^{2+} inside the lumen and 10^{-6} to 10^{-8} M free Ca^{2+} in the myoplasm. The system responsible for the uptake of calcium ion is a Ca^{2+}-ATPase located in the sarcoplasmic reticulum membrane (see Fig. 3, enzyme 5), that accounts for approx. 80% of all the membrane proteins and approx. 50% of the dry weight of the membrane. This enzyme translocates 2 mol of Ca^{2+} per each mole of ATP hydrolysed in native sarcoplasmic reticulum vesicles, forms an acylphosphate intermediate during the catalytic cycle and has a molecular mass of approx. 102–115 kDa. A comparison between the Ca^{2+}-ATPase from the sarcoplasmic reticulum and the

Ca^{2+}-ATPase from the plasma membrane has been recently reviewed [50]. The mechanism of Ca^{2+} transport by the sarcoplasmic reticulum and the molecular properties of the Ca^{2+}-ATPase from rabbit skeletal muscle have been studied in great detail (see for reviews Ref. 64–70), and the enzyme of this origin has been utilized by most workers in reconstitution studies. However, a crude preparation from dog heart ventricle has also been reconstituted [234].

II-B.1. Reconstitution in liposomes

The first attempts to understand the mechanism of Ca^{2+} transport by the isolated Ca^{2+}-ATPase from sarcoplasmic reticulum consisted in the solubilization of the native membranes by deoxycholate, followed by the removal of the detergent, resulting in the reassembly of vesicles containing endogenous lipids [235,236]. These reconstituted sarcoplasmic reticulum vesicles demonstrated that lipids were required for the ATP hydrolytic activity and for the Ca^{2+} transport, although the latter was of very small magnitude, and the ATP hydrolytic activity did not increase with respect to the levels found in native membranes [235,236]. The subsequent purification of the enzyme [237] paved the way for its reconstitution in the absence of exogenously added lipids [238–241].

Calcium transport was not observed in the vesicles made of purified Ca^{2+}-ATPase and the lipids associated to the enzyme [238,242]. This preparation contains only from 25 to 30% of the lipids associated with the enzyme in native sarcoplasmic reticulum [242]. Moreover, in vesicles containing a high number of Ca^{2+}-ATPase molecules per vesicle those vesicles were leaky to Ca^{2+} and ATP-dependent Ca^{2+} uptake was not observed [243]. The efficiency of Ca^{2+} transport increased after separation of the leaky subpopulation of vesicles [243,244].

More competent reconstitution was achieved by the addition of excess exogenous phospholipids [241,242, 245] or endogenous sarcoplasmic reticulum lipids [242,246–248] and removal by dialysis of the detergent cholate [241,242,245], deoxycholate [246–248], $C_{12}E_8$ [249] or a mixture of $C_{12}E_8$ plus cholate [244]. When endogenous sarcoplasmic reticulum lipids were employed, the resulting vesicles had a lipid/protein ratio similar to that of the native membranes [247]. Removal of cholate by the anion-exchange resine Dowex 2-X4 resulted in proteoliposomes of lower coupling efficiency [250]. The reconstitution of the Ca^{2+}-ATPase also was achieved by prolonged sonication in the absence of detergents [245,251,252], by a large dilution of the cholate with a detergent-free media [253], by centrifugation in a discontinuous sucrose gradient containing Tween-80 and preformed small lipid vesicles [254], by a freeze-thaw/sonication procedure [255], by the removal of the detergent Triton X-100 [256] or Triton X-100 plus Triton X-114 [257] or octyl glucoside [258] with a Bio-Bead SM-2 column [256,257,259] or filtration in a Sephadex G-50 column [258], and by a salting-out method consisting in addition of high salt concentration and removal of excess Triton X-114 by hydrophobic interaction chromatography in ω-amino-*n*-octyl derivative of Sepharose 4B [260]. These basic methods of reconstitution [109] have been amply utilized with different modifications by many workers in the field.

ATP-dependent calcium uptake was demonstrated in the vesicles reconstituted by the methods described above [241,242,245–248,251–253,255,256,258,260]. To observe calcium uptake in the proteoliposomes usually requires the presence inside the vesicles or the presence in the outside medium of calcium-chelating agents such as oxalate or inorganic phosphate [234,241,242,245–249,251,253,255,261,262]. However, calcium uptake was also observed in the absence of calcium-chelating agents [241,242,247,248,256,261–263]. The presence of oxalate was particularly important in vesicles of higher permeability such as in the case of dioleoylphosphatidylcholine vesicles [242]. In vesicles prepared by the freeze thaw/sonication method it was demonstrated that the chelating agents were required inside the vesicles and did not have any effect when added to the outside medium [255]. In addition, in proteoliposomes prepared by the cholate-dialysis method it was demonstrated that [^{14}C] oxalate or [^{32}P]P_i as well as [^{3}H]ATP or [^{32}P]ATP were impermeable through the proteoliposomal membrane [261]. Therefore, the success of exogenously added chelating agent to support calcium uptake can be considered as an indicator of partially permeable vesicles.

Reconstitution of a crude preparation of sarcoplasmic reticulum Ca^{2+}-ATPase presents uptake of inorganic phosphate concomitant with Ca^{2+} uptake [264]. In contrast, reconstitution of a pure Ca^{2+}-ATPase does not exhibit phosphate uptake as expected, since phosphate is transported by an independent carrier [264].

The rate of $^{45}Ca^{2+}$ uptake in vesicles prepared by the cholate-dilution method is twice the rate obtained by the sonication procedure, and identical to the rate obtained by the cholate-dialysis method [253]. However, the vesicles prepared by the salting-out method [260] exhibit a lower Ca^{2+} uptake than the vesicles prepared by the freeze-thaw/sonication procedure [255]. Calcium uptake was prevented, or the calcium gradient collapsed, in the presence of the Ca^{2+} ionophores A23187 [245,251,256,259,265,266] or X-537-A [245–247,263]. The temperature during the reconstitution by the detergent-dialysis procedure was reported to be an important parameter to obtain functional proteoliposomes [247,267]. Using endogenous sarcoplasmic reticulum lipids, the reconstitution performed at 20 °C was found to be optimal; however, when the reconstitution was performed at 0–4 °C it failed to produce functional vesicles [247]. In proteoliposomes prepared with exoge-

nous phospholipids it was also found that when the reconstitution was performed at 25°C the rate of ATP hydrolysis was better stimulated by A23187 in contrast to the proteoliposomes prepared at 4°C, particularly when low concentrations of KCl were added to the reconstitution medium [267].

The reconstitution of solubilized crude sarcoplasmic reticulum proteins results in the formation of functional vesicles devoid of the 55 kDa Ca^{2+}-binding protein [246,268], or highly depleted of a 50 kDa [248,259] and 20–26 kDa proteins [248]. Moreover, the addition of extrinsic proteins from the sarcoplasmic reticulum to the reconstituted Ca^{2+}-ATPase vesicles did not increase the affinity to Ca^{2+} binding or the rate of Ca^{2+} transport [241]. The coupling of Ca^{2+} transport to ATP hydrolysis was independent of the presence of calsequestrin [260,267]. However, a 53 kDa glycoprotein was found to increase the coupling in proteoliposomes prepared by the cholate-dialysis method without affecting the permeability of the membrane to calcium [267]. In contrast, other authors found that the same glycoprotein (described as the 55 kDa protein) did not affect Ca^{2+} transport in phosphatidylcholine vesicles prepared with the zwitterionic detergent Chaps and preformed unilamellar vesicles (prepared by the French-press method) in the presence of cholate after filtration in a Sephadex G-50 column [269]. It should be noted that in the earlier work [267], in addition to the 53 kDa glycoprotein and the Ca^{2+}-ATPase, a 160 kDa glycoprotein and unidentified low-molecular-mass proteins were also present in the reconstituted vesicles. Phosphorylation of endogenous phospholamban with exogenously added catalytic subunit of cAMP-dependent protein kinase in crude reconstituted sarcoplasmic reticulum vesicles from heart did not affect Ca^{2+} accumulation by this system and the affinity for Ca^{2+} was very high, suggesting that the reconstituted Ca^{2+} pump was fully activated, in contrast to the case in native sarcoplasmic reticulum vesicles [234].

The reconstituted enzyme in proteoliposomes also could be utilized to understand the physiological process. It has been possible to fuse reconstituted sarcoplasmic reticulum Ca^{2+}-ATPase proteoliposomes in whole erythrocytes and measure $^{45}Ca^{2+}$ uptake upon addition of ATP [270]. This process was inhibited by NEM and *p*-hydroxymercuribenzoate [270]. The erythrocytes were rendered more fragile but there was no induction of lysis upon accumulation of exogenous calcium ion [270].

II-B.2. Physicochemical properties of the reconstituted vesicles

The purified protein, containing some endogenous lipids, readily formed vesicles of 6 nm membrane thickness [238] after the removal of the detergent [238–241]. Freeze-fracture electron microscopy revealed 8–10 nm diameter particles embedded in the membrane [238,240,241,271], although some authors reported smaller particle size with a peak at 7.1 nm [272]. The reconstituted vesicles were thought to be free of the protruding 3 to 5 nm diameter knob-like particles characteristic of the native sarcoplasmic reticulum vesicles [238]. However, its presence was later observed in negatively stained electron micrography [239–241,273]. Using X-ray diffraction (10–15 Å resolution) and neutron beam diffraction, it was confirmed that the Ca^{2+} pump presents its most bulky part outside the vesicles, and a less bulky domain expands within the membrane [274]. It was concluded that the ATPase consists of a hydrophobic globular portion embedded into the membrane and a hydrophilic portion protruding to the outside of the vesicles [240,274]. A count of the number of particles per surface unit gave a value of 45–60 particles per 10^4 nm^2 [271]. The number of particles per vesicles is variable and it was possible to separate a heavy fraction containing from 4–16 Ca^{2+}-ATPase molecules per vesicle from a light fraction containing an average of 1 Ca^{2+}-ATPase molecule per vesicle [243]. The number of particles per unit area in the vesicle was independent of the temperature of reconstitution at 0°C or 35°C [272]. Other authors reported 3–5 molecules of Ca^{2+}-ATPase per vesicle [275].

Using freeze-fracture electron microscopy it was demonstrated that proteoliposomes prepared with excess lipids also contain 9 ± 1 nm particles embedded in the membrane [276]. These particles have been suggested to be formed by two or more molecules of pump protein [268].

The diameter of the reconstituted Ca^{2+}-ATPase proteoliposomes varies with the phospholipids employed from 30 to 500 nm [276] or more uniformly sized from 224 to 273 nm [263], containing an internal volume ranging from 39 to 52 μl per mg protein at a molar ratio of 3000 molecules of phospholipid per molecule of Ca^{2+}-ATPase [262]. Other authors reported values of 35 nm [243], 60 nm [275], 100 ± 20 nm [271] or 110 ± 70 nm diameter [257,260]. The preparation of giant proteoliposomes of 60 to 300 μm diameter has been also reported, which could make its impalement by microelectrodes possible [277]. Functional ATPase molecules were suggested to be mostly, if not totally, oriented in the liposomes as in native sarcoplasmic reticulum fragments, with the ATP catalytic site facing the outside [261]. In agreement with this observation, other authors have also demonstrated that the ATP-permeabilizing agent alamethicin increases the rate of ATP hydrolysis above the stimulation induced by A23187 by only 14–16% [250]. However, other work has determined a bidirectional arrangement of the pump in which only 40–50% (262) or 57–58% [278] of the molecules have the catalytic site facing outside, as determined by solubilization of the enzyme with Triton X-100 [278] or the

A23187 plus alamethicin method [262]. However, using X-ray and neutron diffraction analysis an asymmetric orientation of the enzyme into the bilayer was demonstrated [279].

To study the organization of the reconstituted Ca^{2+}-ATPase in the lipid membrane several approaches have been used. The co-reconstitution in the same vesicles of two different populations of purified enzyme, one covalently labelled with 1,5-IAEDANS and the other covalently labelled with IAF, allow the study of fluorescence energy transfer from the IAEDANS (donor) to the IAF (acceptor) fluorophores [280,281]. It was concluded that the ATPase forms oligomeric structures of sufficiently long lifetime to allow energy transfer from the fluorophores to occur [280]. Moreover, a 10-fold dilution of the lipid phase (egg phosphatidylcholine) had no measurable effect upon energy transfer, suggesting that random collision among ATPase molecules was not the principal cause of the observed interactions [280]. However, this conclusion was later questioned, since some authors indicated that fluorescence energy transfer between labelled polypeptides should not be taken as a reliable evidence in favor of the oligomeric structure of the pump [281]. The electron micrograph of negative stained preparations with image enhancement techniques reveals a pattern of four subunits, suggesting a tetrameric organization of the ATPase [280]. Other authors suggested as well that the particles observed in freeze-fracture electron microscopy appear to consist of two or more molecules of the pump protein [268]. When the Ca^{2+}-ATPase was reconstituted with excess phosphatidylcholine at different ratios it was observed that the phospholipid-to-protein ratio was always similar to that of the native sarcoplasmic reticulum, therefore presenting resistance to dilution as expected if protein-to-protein interactions occur [282]. However, labelling of the protein with fluorescein isothiocyanate suggests that dilution of protein in the reconstituted system induces a decrease in the level of self-quenching by promoting dissociation of the Ca^{2+}-ATPase [283]. Other work also suggests that the Ca^{2+}-ATPase protein molecules are in aggregated state in vesicles of dimyristoylphosphatidylcholine or dipalmitoylphosphatidylcholine [284].

Using freeze-fracture electron microscopy it was found that when the temperature was decreased below the transition temperature for the lipids, the reconstituted enzyme formed patches of high protein content separated by regions of pure lipids [285,286]. In contrast, when the temperature was increased above the transition temperature for the lipids, the Ca^{2+}-ATPase appeared to be randomly distributed in the bilayer [286]. Above the transition temperature in vesicles prepared of dimyristoylphosphatidylcholine vesicles the protein produces relatively small perturbations of the surrounding lipid; however, below the transition temperature the protein aggregates into high protein-lipid patches [287]. However, other authors concluded that the transition in the ATPase activity was not due to change of aggregation state of the Ca^{2+} pump [272]. It has been suggested that vectorial Ca^{2+} transport requires a more complex level of protein structure than that for ATP hydrolysis [278]. However, vesicles containing Ca^{2+}-ATPase monomer are effective in translocating calcium ion [243] and it was concluded that aggregated Ca^{2+}-ATPase molecules contribute to passive Ca^{2+} leak [243]. Radiation inactivation analysis also indicates a dimeric structure not only in the native sarcoplasmic reticulum vesicles but in the reconstituted proteoliposomes as well (see Ref. 278). In contrast to these results, it has been described that protein-to-protein interaction is drastically reduced in a reconstituted system as demonstrated by a decrease of cross-linking with cupric phenantroline [244,276], or calculated on the basis of a random mixing model [288].

The stability of the reconstituted enzyme has been studied. The enzyme decreases its activity by 50% in 24 h, in contrast to 72 h in the case of the native non-reconstituted enzyme [249]. Also, the inactivation of the enzyme by mild acid treatment was reverted 80% upon reconstitution, and it was concluded that the inactive conformation was constrained by lipid–protein interaction [289].

II-B.3. Effects of lipids

Substitution of the native lipids associated to the purified Ca^{2+}-ATPase by lipids of known composition by a lipid titration technique, consisting in the centrifugation of a mixture of the purified enzyme plus the desired lipids in the presence of cholate [290], has increased our understanding on the effect of the lipid bilayer on the ATPase and transport functions of the enzyme.

Calcium transport was stimulated by the presence of cardiolipin in the vesicles; however, the ATP dependence of Ca^{2+} transport was less complete [251,291]. The use of purified phospholipids improves the efficiency of Ca^{2+} transport when compared to crude soybean phospholipids [251]. Initially it was found that both phosphatidylethanolamine and phosphatidylcholine support Ca^{2+} uptake in vesicles prepared by sonication in the absence of detergents, and a mixture of both phospholipids was optimal for obtaining functional vesicles [251]. However, in vesicles prepared by the cholate-dialysis method, functional vesicles could be obtained only with phosphatidylethanolamine but not with phosphatidylcholine [261,291,292] or with a mixture of both phospholipids [292]. Acetylphosphatidylethanolamine could not replace phosphatidylethanolamine in reconstituted Ca^{2+}-ATPase. However, inclusion of suitable amounts of stearylamine or oleylamine during the reconstitution yielded acetylphosphatidyl-

ethanolamine liposomes with a Ca^{2+} transport activity similar to that of pure phosphatidylethanolamine liposomes [293]. An increase in the phosphatidylethanolamine/phosphatidylcholine ratio favors Ca^{2+} uptake [262], consistent with an observed decrease in the passive Ca^{2+} efflux from these vesicles [294]. The increase efficiency of phosphatidylethanolamine was independent of the presence of cholesterol [295]. However, the coupling efficiency increased with cholesterol content and was more pronounced for proteoliposomes containing high phosphatidylethanolamine [295]. It was concluded that the nature of the tightly bound phospholipids to the Ca^{2+}-ATPase were not essential for the pump, since they could be substituted by phosphatidylethanolamine from soybean [291]. In addition, the cholesterol-induced enhancement of Ca^{2+} transport could be due to an increased order of the bilayer [295]. Moreover, it should be emphasized that, since the phospholipid requirement depends on the method of reconstitution employed, the physiological significance of a given lipid for the control of the pump is at best highly questionable.

It was found that the thickness of the phospholipid bilayer affects the ATP hydrolytic activity [296–298] or the Ca^{2+} transport activity [262,296] of the enzyme. Maximum activities were observed in membranes of intermediate thickness [296,298,299]. This was demonstrated not only by varying the length of the acylhydrocarbon chain of the phospholipids but also by incorporating *n*-alkanes in the bilayer [298]. In addition, phospholipids with unsaturated acyl chains favor the function of the enzyme [297,300]. However, phospholipids with saturated acyl chains inhibit the function of the enzyme, particularly at temperatures below the transition temperature for the lipid membrane [297]. A certain degree of membrane fluidity was considered of importance for the function of the enzyme [297].

Electron spin resonance saturation transfer spectroscopy using spin labels selectively attached to different -SH groups of the protein allow us to measure the intramolecular motion of the protein polar head reconstituted in egg-yolk phosphatidylcholine or dipalmitoylphosphatidylcholine vesicles [301]. The reconstituted enzyme was capable of hysteretic behavior in some physicochemical properties such as light scattering and fluorescence when labelled with the same fluorescent probes [271]. Using laser-flash photolysis of a Ca^{2+}-ATPase covalently labelled with eosin and reconstituted in dipalmitoylphosphatidylcholine, it was possible to measure the intrinsic motion of the ATPase molecule, and it was determined that when the temperature reached 28 °C to 30 °C the membrane started to melt and the rotation of the Ca^{2+}-ATPase began, accompanied by a marked increase in the ATPase activity [285]. In addition, infrared spectroscopy data suggest that the reconstitution of the Ca^{2+}-ATPase results in an increase in the conformational and/or motional freedom of the protein, as well as an increase in the unordered region(s) of the enzyme within the lipid bilayer [302].

The inhibition of the enzyme reconstituted in dipalmitoylphosphatidylcholine vesicles has been proposed to be related to an increase in the viscosity of the hydrocarbon region of the membrane, which appears to inhibit a conformation change of the enzyme, leading to Ca^{2+} translocation and the eventual cleavage of the phosphorylated intermediate of the enzyme [303]. The native sarcoplasmic reticulum membranes are less fluid than liposomes prepared from the total membrane lipids [304]. The fluidity of the reconstituted ATPase and the total lipid extract depends on the lipid/protein molar ratio and sharply decreases when this ratio becomes lower than 44, a process that does not appear to depend on the temperature [304]. However, other reports indicate that the activity of the enzyme does not correlate with the fluidity of the membrane [299] or the fluidity of the boundary lipids [284], or with the rotational mobility of the Ca^{2+}-ATPase [284].

The nature of the polar head of the phospholipids also appears to affect the activity of the enzyme [299]. Although phosphatidylethanolamine appears to bind less strongly than phosphatidylcholine, the difference is small [305]. In addition, the binding constants of phosphatidylserine and phosphatidylcholine for the enzyme appear to be similar [305].

The incorporation of the Ca^{2+}-ATPase into the lipid membrane perturbs each monolayer of the two composing the membrane in different manner [279]. Using brominated phospholipids, that quench the intrinsic-fluorescence of the ATPase, it was possible to study the affinity of the phospholipids for the enzyme; however, it was not possible to establish significant differences in selectivity based on the length of the fatty acyl chain of the phospholipids [305].

The motion of the phospholipids forming an annulus around the ATPase molecule appear to be inhibited [288,306]. However, other reports have described only minor perturbations of the phospholipid motion [307]. The number of phospholipid molecules closely associated to the Ca^{2+}-ATPase has been measured with spin-labelled phosphatidylcholine, and depending on the temperature it was established to be 32 molecules at 0 °C and 22 molecules at 37 °C [288]. It has been also reported that the packing of lipids immediately adjacent to the annulus of tight-bound phospholipids appears to be disrupted [306]. However, another report indicated that the Ca^{2+}-ATPase has little effect on the organization of the hydrocarbon chain of the membrane [308]. Moreover, using deuterated phospholipids, no evidence for any ordered boundary lipid in association with the protein was found above the transition temperature, perhaps due to the rough nature of the protein surface [308]. The lipid motion, however, is not coupled to the

Ca^{2+}-ATPase motion, and the boundary phospholipid appears to be rapidly exchangeable for the phospholipids in the bulk of the bilayer [307]. Cholesterol appears to be effectively excluded from the annulus around the ATPase molecule [309,310]. In agreement with this observation, cholesterol appears not to affect the ATPase activity significantly [299]. Lipid domains around the ATPase molecule depend very strongly upon the lipid composition of the vesicles [311], and it appears that no special lipid forms an annulus controlling the enzymatic activity [312].

Reconstitution of the Ca^{2+}-ATPase in liposomes prepared by the cholate-dialysis method using different phospholipids has demonstrated that the enzyme induces an increase in the Ca^{2+} permeability by several orders of magnitude, and a nonspecific increase of the permeability to Na^+, choline$^+$ SO_4^{2-}, Cl^-, acetate$^-$ and sucrose, also [273]. It was suggested that this increase in permeability to Ca^{2+} was not due to a carrier function of the enzyme, but was perhaps a reflection of the formation of some kind of channels in the membrane [273]. The passive equilibration of H^+ and K^+ across the proteoliposome membrane prepared by the freeze-thaw/sonication procedure is slow compared with that of active ATP-dependent Ca^{2+}-uptake [275].

II-B.4. Calcium/ATP stoichiometry

In earlier experiments, linear Ca^{2+} transport was demonstrated up to 2 min [245]. With the determination of the initial rates of Ca^{2+} uptake and ATP hydrolysis it was possible to experimentally establish the stoichiometry of moles of Ca^{2+} translocated per each mole of ATP hydrolysed. The Ca^{2+}/ATP ratio obtained in proteoliposomes consistently yielded values lower than 2 [234,244,248,251,256,261,263,265,268,276,278,291, 296,313–317], in contrast to the value of 2 generally obtained with native sarcoplasmic reticulum vesicles. In addition, the thermodynamics of the catalytic cycle could be totally accounted for by a Ca^{2+}/ATP ratio of 2.2 ± 0.3 [258].

The reconstitution of the enzyme by the sonication procedure in the absence of detergents gave a Ca^{2+}/ATP ratio of approx. 0.16 when crude soybean phospholipids were employed; however, this ratio increased to 0.64 when further purified phospholipids were employed [251]. A Ca^{2+}/ATP ratio of 0.83 was obtained in proteoliposomes with the enzyme from skeletal muscle, prepared by the Triton X-100 removal procedure using a Bio-Bead SM-2 column [256], and 0.69 ± 0.09 in proteoliposomes with the enzyme from heart muscle prepared by the same technique [234]. No preloading of the proteoliposomes with oxalate or phosphate was required to obtain this relatively high Ca^{2+}/ATP ratio [256]. Using a freeze-thaw/sonication procedure, the reported Ca^{2+}/ATP ratio was 0.96 in egg-yolk phosphatidylcholine/cholesterol (9 : 1) vesicles [265], 0.2 in palmitoylphosphatidylcholine vesicles [296] and 0.75 in myristoylphosphatidylcholine vesicles [296]. Dialysis of the detergents $C_{12}E_8$ and cholate yielded proteoliposomes with Ca^{2+}/ATP ratios near to 1 [244]. However, using a freeze-thaw/sonication procedure in the presence of the detergent cholate followed by the removal of the detergent in a Sephadex G-50 column, the reported Ca^{2+}/ATP ratio reached values from 1.43 to 1.54 in the absence of calcium-precipitating agents [316]. These vesicles presented relatively high permeability to ^{42}K and lower permeability to ^{86}Rb [316]. The Ca^{2+}/ATP ratio originally reported in oxalate-loaded proteoliposomes prepared by the cholate or deoxycholate dialysis method was generally low, from 0.2 to 0.5 [261,263,268,278] or at best 0.9 to 1.2 [248,266,291].

The Ca^{2+}/ATP ratio was dependent on the nature of the phospholipids in the proteoliposome membrane [266,291,317]. Increasing methylation or glycosylation of the lipids decreased the Ca^{2+}/ATP ratio [266]. This was particularly higher (approx. 0.8–1.2) in phosphatidylethanolamine vesicles [266,291] and decreased when phosphatidylethanolamine was progressively substituted by phosphatidylcholine [291]. Addition of 1,2-dioleoyl-*sn*-glycerol increased Ca^{2+} transport and the Ca^{2+}/ATP ratio; however, 1,2-dipalmitoyl-*sn*-glycerol did not increase the rate of Ca^{2+} transport or the Ca^{2+}/ATP ratio [317]. This could be related to the destabilization of the bilayer induced by diolein instead of dipalmitin [317]. No Ca^{2+} transport was observed in pure phosphatidylcholine vesicles [291]. Cone-shape lipid molecules appear to stabilize the Ca^{2+}/ATP ratio [266]. The Ca^{2+}/ATP ratio also was highly sensitive to the lipid/protein ratio, being higher at relatively low lipid contents in the vesicles [268]. When the proteoliposomes were prepared by the dialysis of a mixture of cholate plus $C_{12}E_8$, significantly higher Ca^{2+}/ATP ratios were obtained [276], in the order of 1.25 with lipids from sarcoplasmic reticulum, and 1.3 to 1.6 with egg-yolk phosphatidylcholine [276]. The lower efficiency of Ca^{2+} transport by the reconstituted Ca^{2+}-ATPase from the sarcoplasmic reticulum versus the native membrane does not appear to be simply related to the different degree of permeability of these vesicles. Quercetin has been employed to investigate this phenomenon. However, it was demonstrated that this flavonoid partially inhibits Ca^{2+} translocation, particularly at high concentrations (75 μM) [265].

Earlier work had demonstrated that a low-molecular-mass (12 kDa), heat-stable proteolipid enhances several-fold the rate of Ca^{2+} transport and the Ca^{2+}/ATP ratio when added during the reconstitution procedure [313,315]. The increase in the Ca^{2+}/ATP ratio was typically from 0.3–0.7 in the absence of the proteolipid, to 1.3–1.7 in its presence [313–315]. However, the proteolipid appears to work as an ionophore when added after the reconstitution procedure [313–

315]. At least two different species of proteolipids appear to be present in the isolated fractions [315]. The isolated, purified and partially delipidated proteolipids were reported to increase the permeability of egg-yolk phosphatidylcholine vesicles not only to Ca^{2+} but also to Mn^{2+}, Na^+ and Rb^+ [315]. This increase in permeability was not inhibited by *p*-mercuriphenylsulfonic acid, mersalyl or *N*-methylmaleimide [315]. Surprisingly low concentrations of Triton X-100 also appear to substitute for the proteolipids in increasing the Ca^{2+}/ATP ratio [315]. In contrast, it has been demonstrated that a purified proteolipid preparation reduces both nonspecific ion and water permeabilities of artificial planar phospholipid membranes, and does not show any ionophoric effect or specific pore formation for calcium [318]. It has been proposed that the physiological role of the proteolipid in the sarcoplasmic reticulum membranes could be to reduce leakage and therefore to increase the efficiency of the Ca^{2+} pump [318]. However, other authors consider that the proteolipid is not essential to obtain Ca^{2+}/ATP ratios as high as 0.7 [319].

II-B.5. Electrical properties and reversal of the pump

To study the mechanism of Ca^{2+} transport by the reconstituted enzyme, a series of ionophores were used to determine how they might affect the rate of Ca^{2+} uptake. It was found that valinomycin in the presence of potassium increases the rate of calcium ion transport [245,251,255,257,259,262]. Earlier reports indicate that the protonophores FCCP [245] and 1799 [251] were slightly inhibitory for Ca^{2+} transport. However, stimulation of the rate of Ca^{2+} uptake by FCCP has been thereafter reported in the absence [259,262] or presence [262,275] of valinomycin, and PCB^- is accumulated during Ca^{2+} uptake [320]. Moreover, the lipophilic anions tetraphenylboron or picrate also stimulate the rate of Ca^{2+} uptake [255] and PCB^- is accumulated during Ca^{2+} uptake [320]. These observations strongly suggest the electrogenic nature of the Ca^{2+} transport. In agreement with these observations, it was also demonstrated that the imposition of a potassium diffusion potential, negative inside, stimulates the rate of Ca^{2+} uptake, and the imposition of a potassium diffusion potential, positive inside, inhibits the rate of Ca^{2+} uptake [255,265]. The rate of ATP hydrolysis was not significantly affected by the imposed potassium diffusion potential; therefore, the Ca^{2+}/ATP ratio varied with the applied electrical potential, from 0.5 at +50 mV (positive inside) to 1.5 at −100 mV (negative inside), suggesting that the voltage across the membrane influences the coupling between Ca^{2+} transport and ATP hydrolysis [265]. In contrast, lowering the concentration of Ca^{2+} depressed both Ca^{2+} transport and ATP hydrolysis [265]. Co-reconstitution of bacteriorhodopsin and the Ca^{2+}-ATPase in the same liposomes demonstrates that the generation of a $\Delta\psi$ (positive inside) by bacteriorhodopsin upon illumination alters the activity of Ca^{2+} inside the liposomes and therefore the equilibrium between ATP hydrolysis/synthesis coupled to calcium transport [321].

The determination of the membrane potential generated by the Ca^{2+}-ATPase was assessed to be $+61 \pm 10$ mV (positive inside) measured by the clamping of the potential generated by the enzyme with a potassium diffusion potential of opposite polarity, and +51 mV (positive inside) when it was directly measured by the $\Delta\psi$-sensitive probe, ANS [255]. Other determinations range between +30 mV to +40 mV [322] up to +50 mV in a system deprived of interfering ion channels [257].

The electroneutral K^+/H^+ exchanger nigericin also was shown to stimulate the rate of Ca^{2+} uptake in the absence [245] or presence [245,251] of valinomycin. This observation could have some connection with the demonstrated H^+ extrusion [259] and intravesicular alkalinization, as measured by entrapped pyranine [275], that occur in proteoliposomes during ATP hydrolysis. The generated ATP-dependent H^+ gradient was not collapsed by FCCP, although it was collapsed by A23187 and therefore it was not considered by some authors to be a primary H^+ ejection by the enzyme but rather a consequence of Ca^{2+} accumulation [259]. However, the intraliposomal alkalinization was prevented by the presence of CCCP plus valinomycin [275]. In addition, the H^+/Ca^{2+} ratio was approx. 1 at low levels of Ca^{2+} uptake and lower than 1 at high levels of Ca^{2+} uptake; therefore, the overall process still remains electrogenic [259]. Other authors have suggested that 1 or 2 H^+ could be exchanged per Ca^{2+} [275].

In a potassium-free medium, nonactin (plus sodium) also stimulates Ca^{2+} transport [255]. In contrast, the Na^+/H^+ exchanger, monensin, did not affect Ca^{2+} transport in proteoliposomes prepared in the presence of Na^+, in the absence or presence of valinomycin [255]. However, the addition of potassium stimulates the rate of Ca^{2+} uptake in the presence of monensin plus valinomycin [255]. Moreover, the imposition of a potassium gradient in the presence of valinomycin stimulates the ATP-dependent Ca^{2+} transport by the reconstituted enzyme [323]. The effect of potassium does not appear to be specific, since it could also be obtained by a sodium or a choline gradient [323]. The simple addition of potassium to the proteoliposomes also stimulates the rate of Ca^{2+} uptake [262]. However, the coupling of 1 Ca^{2+}/1 K^+ or 1 Ca^{2+}/1 Na^+ exchange was not excluded [255].

The exchange of Ca^{2+} for Mg^{2+} by the enzyme has been ruled out [259]. However, uptake of $^{54}Mn^{2+}$ has been demonstrated by competing with Ca^{2+} [259]. The above-mentioned evidence still leaves open the question of whether or not the sarcoplasmic reticulum Ca^{2+}-

ATPase is mechanistically involved in the translocation of other cation(s) under physiological conditions.

Reconstitution of the enzyme in asolectin proteoliposomes was fused with a black lipid membrane and Ca^{2+} uptake was induced by photolysis of caged-ATP [324]. An ATP-dependent electrical current was measured, demonstrating that the process is electrogenic although the exchange of $1Ca^{2+}/1H^{+}$ was not excluded [324]. The process was dependent on Mg^{2+}, demonstrating that the true substrate was the MgATP complex, and was inhibited by vanadate and ADP [324]. In addition, photolysis of caged-ADP does not produce Ca^{2+} uptake except in the presence of an ATP regenerating system [324].

The reconstituted Ca^{2+}-ATPase is capable of a Ca^{2+}-dependent ^{32}P–ATP exchange reaction [245,261, 325]. Surprisingly, this ^{32}P–ATP exchange was not inhibited by the Ca^{2+}-ionophores A23187 [245,261] or X-537-A [245], nor was it inhibited by the K^{+}/H^{+} exchanger, nigericin [261]. The ^{32}P–ATP exchange also takes place in permeable vesicles unable to accumulate calcium [325], demonstrating that this process was not coupled to vectorial reversal of the pump, but rather to a contaminating scalar reaction. ATP hydrolysis, Ca^{2+} transport as well as $[^{32}P]P_i$–ATP exchange reaction was inhibited by chlorpromazine [245]. Nevertheless, net ATP synthesis by the reversal of the pump was also demonstrated [258,261,278,321]. ATP synthesis follows simple Michaelis-Menten kinetics as was the case for ATP hydrolysis, and the K_m of both processes was independent of the Ca^{2+} gradient, although its rate was proportional to the Ca^{2+} gradient [258]. ATP synthesis driven by a $\Delta\psi$ (positive inside) generated by bacteriorhodopsin under illumination has been demonstrated [321]. Moreover, the synthesis of ATP from ADP plus inorganic phosphate was accompanied by the mobilization of Ca^{2+} in the opposite direction to the translocation occurring during ATP hydrolysis [258].

II-B.6. Ionophoretic properties of the fragmented pump

The fragmented Ca^{2+}-ATPase also has been used to study the conductance pathway of Ca^{2+} through the pump. However, the physiological significance (if any) of those findings is not readily apparent.

Succinylation of the Ca^{2+}-ATPase with succinic anhydride produced a soluble, albeit inactive, form of the enzyme that increased the electrical conductance of black lipid membranes of oxidized cholesterol several hundred fold [326,327]. The modified ATPase increases permeability in the sequence $Ba^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+} > Mn^{2+} > Zn^{2+}$, Na^{+}, K^{+}, Cs^{+}, Li^{+} and Rb^{+} [326]. Zn^{2+} and Na^{+} inhibit the increase in Ca^{2+} conductance [326]. Both mercuric chloride and methylmercuric chloride inhibit the ATP hydrolytic activity of the native enzyme. However, only the former inhibits the Ca^{2+} ionophoretic activity of the succinylated enzyme. Therefore, it was concluded that the site for ATP hydrolysis and that for Ca^{2+} ionophoric activity are separate [327].

Partial trypsinization of a non-succinylated enzyme results in the formation of 55 kDa, 45 kDa, 30 kDa and a 20 kDa fragments, and this mixture retains the Ca^{2+} ionophoretic activity. However, trypsinization of the succinylated-enzyme suppresses the Ca^{2+} ionophoretic activity [326]. The 55 kDa fragment retains ionophoretic activity [319,328,329] However, it was reported that the 45 kDa fragment caused no increase in conductance [319,328,329]. Further purification of the 45 kDa fragment shows a relative non-selective ionophoretic activity in the sequence, $Ba^{2+} \simeq Ca^{2+} \simeq Sr^{2+} \simeq Mg^{2+} \simeq Mn^{2+} \simeq Cl^{-}$ [330]. However, the 55 kDa fragment presents the same $Ba^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+} > Mn^{2+} > Zn^{2+}$ ionophoretic specificity as the succinylated intact enzyme [319,328]. Other authors reported $Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+} > Zn^{2+} > K^{+} > Na^{+}$ for a 40–50 kDa tryptic fragment [331]. Reconstitution of a mixture of the 55 kDa plus 45 kDa fragments in liposomes demonstrates ATP-dependent Ca^{2+} transport activity [332]. Further trypsinization of the 55 kDa fragment results in the formation of a 30 kDa fragment without ionophoretic activity [319,328,329] and a 20 kDa fragment containing the Ca^{2+}-ionophoretic activity [329]. In other reports this fragment was described as having a molecular mass of 25 kDa [319]. The ionic selectivity for the 20 kDa fragment was first described as Ba^{2+}, Ca^{2+}, Sr^{2+}, $Mg^{2+} > Mn^{2+} > Zn^{2+}$ [329]. However, further purification of the 20 kDa fragment removing some minor components of low molecular weight demonstrates that the ionic selectivity was similar ($Ba^{2+} > Ca^{2+} > Sr^{2+} > Mn^{2+}$, Mg^{2+}) [333] or identical ($Ba^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+} > Mn^{2+}$) [334] to that in the intact succinylated-enzyme. Treatment with cholate to remove sodium dodecyl sulfate resulted in the same sequence except for the selectivity for $Mn^{2+} > Mg^{2+}$ [334]. This last reconstitution was made in black lipid membrane made not only of oxidized cholesterol but of a mixture of cholesterol plus phosphatidylcholine without affecting the conductance for calcium [319,333,334].

It was described that further fragmentation of the 20 kDa fragment with cyanogen bromide results in a smaller peptide, perhaps lower than 2 kDa, containing the Ca^{2+} ionophoretic activity [329]. However, other work describing the fragmentation of the 25 kDa fragment by the same reagent indicates that from a mixture of 13 kDa, 7.5 kDa, 3 kDa and 1 kDa fragments, the 13 kDa fragment is the one retaining the Ca^{2+} ionophoretic activity with a selectivity sequence of $Mn^{2+} > Ca^{2+} > Ba^{2+} > Sr^{2+} > Mg^{2+}$ [319]. Moreover, the ionophoretic region of the enzyme was described as being located near its N-terminus [319]. Dithiothreitol affects Ca^{2+} conductance in the succinylated intact enzyme but not in the 55 kDa or the 25 kDa fragments [319].

However, reducing agents inhibit the non-selective ion conductance in the 45 kDa fragment [330]. As in the case of the succinylated enzyme [327,329], the conductance of the 55 kDa, 45 kDa and the 20 kDa fragments is inhibited by mercuric chloride but not by methylmercuric chloride [329,330]. It was concluded that the 45 kDa fragment contains 1 or 2 relatively accessible disulfide bonds essential for transport [330]. However, using the succinylated enzyme it was concluded that sulfhydryl groups are not essential for the Ca^{2+}-ionophoric activity [327].

The active non-modified enzyme has been shown to mediate passive Ca^{2+} efflux in a reconstituted system by dilution of $^{45}Ca^{2+}$-loaded liposomes in a calcium-free medium [294]. The same proteoliposomes present a very small passive Ca^{2+} influx or passive glucose efflux [294]. Passive Ca^{2+} efflux decreases by increasing the concentration of Ca^{2+} or Mg^{2+} outside or by dropping the pH from 7.2 to 6.0 [294]. Moreover, non-hydrolyzable analogs of ATP such as AMP-PCP increase Ca^{2+} efflux. However, inositol trisphosphate had no effect on the rate of Ca^{2+} efflux, either in the presence or absence of external magnesium [294]. A potassium diffusion potential (positive inside) induced by valinomycin does not promote Ca^{2+} efflux, demonstrating that this is not a nonspecific process [294]. Silver ion interacts with sulfhydryl groups in the reconstituted enzyme, inhibiting the ATPase activity and inducing Ca^{2+} release from the vesicles, and it was concluded that the Ca^{2+}-ATPase acts as a pathway for rapid Ca^{2+} release [335]. It has been proposed that the Ca^{2+} efflux observed is directly mediated by the Ca^{2+}-ATPase, and could represent a phenomenon of physiological importance during muscle contraction [294,335].

II-C. The Ca^{2+}-ATPases from plasma membrane

The plasma membrane of most, if not all, cells is equipped with Ca^{2+}-translocating ATPases (see Fig. 3, enzyme 4) responsible for the extrusion of calcium ion from the cytoplasm to the extracellular medium, and contributes to the maintenance of an intracellular free calcium ion concentration in the order of 10^{-6} to 10^{-7} M. The most common of these enzymes has a molecular mass of approx. 140–130 kDa, forms an acylphosphate catalytic intermediate during its catalytic cycle, and is regulated by the Ca^{2+}-binding protein calmodulin. This enzyme has been extensively studied in its membrane-bound form and its solubilized and purified form, particularly in erythrocytes, although the enzyme from many other types of cell has been identified (see for reviews Refs. 51–63). In the liver plasma membrane, however, the molecular properties and regulation of the Ca^{2+}-stimulated ATPases appear to be somewhat different, and perhaps reflect the presence of several enzymes [336–338], some of which do not even seem to be involved in calcium transport [338,339].

II-C.1. The erythrocyte enzyme

The earlier attempts to reconstitute the human erythrocyte Ca^{2+}-transport ATPase were made with crude supernatants resulting from the solubilization of membranes with Triton X-100 [340] or deoxycholate [341,342] without any further purification, or after chromatography in a Sepharose CL-6B column [343]. Alternatively, reconstitution of the ATPase after a 60-fold purification of the enzyme by isoelectrofocusing, resulting in a concomitant decrease of the anion carrier (band III) from the preparations, was also achieved [340]. The reconstitution was performed upon addition of phospholipids and removal of the detergents with a Bio-Bead column [340] or dialysis [341–343]. Separation of the reconstituted enzyme from the free enzyme was made in a Sepharose 4B column [340]. The ATPase activity was strongly stimulated in vesicles of phosphatidylserine [340], and calcium transport was demonstrated in phosphatidylcholine vesicles in the presence of oxalate with a Ca^{2+}/ATP ratio of approx. 0.3 [341,342]. This low ratio may reflect the leakiness of the proteoliposomes and/or the co-reconstitution of other systems capable of recycling the calcium ion, despite the presence of the calcium-chelating agent, oxalate. The presence of the Ca^{2+}/H^{+} exchanger A23187 inhibits the net transport of calcium ion as expected [342]. However, Ca^{2+} transport and ATP hydrolysis were not altered by the addition of calmodulin to the reconstituted enzyme [342]. The diameter of the monolamellar vesicles formed varied from 50 to 600 nm [340,342]. However, temperature was a strong determinant in the size of the vesicles obtained by the Triton X-100 removal procedure, in which at a lower temperature (4° C) the size tends to be smaller – (50–100 nm) – than at higher temperature (23° C) – in the order of 200–400 nm [340].

An enzyme partially purified by mixed micelle gel chromatography from pig erythrocyte was reconstituted in asolectin liposomes by a freeze-thaw/sonication method and removal of the non-ionic detergents, Triton X-100 and Tween-20, by a phenyl-Sepharose 4B column [344]. ATP-dependent $^{45}Ca^{2+}$ uptake was demonstrated in the proteoliposomes with a Ca^{2+}/ATP ratio of 0.4 to 0.79 after removal of the non-reconstituted enzyme by a Sepharose 4B column [344]. Both ATP hydrolysis and rate of Ca^{2+} uptake were stimulated by the endogenous or the exogenously added bovine brain protein activator. However, the Ca^{2+}/ATP ratio did not significantly change by the presence of the activator [344]. The initial rate of ATP hydrolysis did not decline with time as initially expected, although A23187 stimulated this rate by 2.9-fold and prevented calcium uptake. However, the initial maximal Ca^{2+}/ATP ratio declined dramatically

within 1 or 2 min of functioning of the enzyme [344].

A crude preparation of Triton X-100-solubilized pigeon erythrocyte membranes was reconstituted in a mixture of phosphatidylcholine, phosphatidylethanolamine plus cholesterol liposomes by removal of the detergent with the addition of bovine high-density lipoproteins [345]. The reconstituted system was capable of ATP-dependent $^{45}Ca^{2+}$ uptake and was sensitive to A23187, and the Ca^{2+} uptake rate was dependent on the square of the calcium ion concentration, with an apparent $K_{0.5}$ of 0.18 μM [345].

Highly purified Ca^{2+}-ATPase (200- to 360-fold) [110,346,347] from human erythrocytes was obtained by calmodulin-affinity chromatography and reconstituted in phospholipid vesicles [55,59,60] of different composition using a Triton X-100 removal procedure with a Bio-Beads SM-2 column (110,346,348,349], a cholate-dialysis method [111,348,350–354] or a deoxycholate dialysis method near room temperature [347]. The reconstituted enzyme was stimulated 2- to 4-fold by A23187 when the Triton X-100 removal procedure was employed [110,346] and as much as 9- to 11-fold when the cholate-dialysis method was utilized, particularly with asolectin vesicles [111,350,352]. Although the use of soybean commercial grade phosphatidylcholine also yielded tightly coupled vesicles [352], the egg-yolk phosphatidylcholine or other phosphatidylcholine of unspecified origin yield less tighly coupled proteoliposomes, even when the cholate-dialysis method was employed [350,352,354]. However (2- to 3-fold) stimulation of the ATP hydrolytic activity was obtained when the non-protonable Ca^{2+} ionophore Cyclex-2E was utilized [352]. ATP-dependent calcium uptake has been determined with $^{45}Ca^{2+}$ [110,347], the metallochromatic dye Arsenazo III [110,346,354], or by Ca^{2+}-selective electrodes [110,111,346,350–352]. The calcium gradient was collapsed or prevented by the addition of A23187 [110,111,346,349–352,354]. The presence of oxalate inside the proteoliposomes was not an essential requirement to observe efficient calcium uptake [110,346,352, 354]. However, its presence increased the loading capacity of the proteoliposomes [347,350,352].

The rate of ATP hydrolysis by the reconstituted enzyme could be most efficiently determined spectrophotometrically using an ATP regenerating system employing the pyruvate kinase/lactate dehydrogenase coupled assay [111,346,350,352,354], and alternatively by an isotopic/filtration method using [^{32}P]ATP [351], the colorimetric determination of inorganic phosphate released to the medium [352], or following the acidification of the external medium with a pH electrode, due to the dissociation of H^+ occurring during ATP hydrolysis in a low-buffer-capacity medium [111,350]. Both the rate of calcium transport and rate of ATP hydrolysis were inhibited by vanadate [346].

The measurement of the initial rates of calcium transport and ATP hydrolysis by the reconstituted and highly purified human erythrocyte Ca^{2+}-ATPase demonstrates a Ca^{2+}/ATP ratio ranging from 0.94 ± 0.17 to 1.1 ± 0.1 [110,111,346,351,355]. In liposomes presenting a low Ca^{2+}/ATP ratio the experimental stoichiometry could be corrected using the calculated degree of coupling to determine the mechanistic Ca^{2+}/ATP ratio [354]. The Ca^{2+}/ATP ratio appears to decrease during time in poorly coupled liposomes [346], as in the case of proteoliposomes containing the pig erythrocyte enzyme [344]. However, in tightly coupled liposomes the Ca^{2+}/ATP ratio remains approximately at a value of 1 after 5 min of operation of the enzyme [351,355]. The Ca^{2+}/ATP ratio was determined in most cases in the absence of calmodulin, although the authors point out that no differences in this stoichiometry were observed by the presence of this regulator [351] as in the case of the pig erythrocyte enzyme [344]. This point has been re-examined recently using a human erythrocyte Ca^{2+}-ATPase reconstituted in phosphatidylcholine vesicles and a corrected Ca^{2+}/ATP ratio of 1.20 ± 0.17 and 0.83 ± 0.16 in the absence and presence, respectively, of calmodulin has been calculated [354].

The stimulating effect of calmodulin on the activity of the reconstituted enzyme was most readily observed in phosphatidylcholine liposomes [111,346–348,354, 356], but was not observed in phosphatidylserine liposomes where a concomitant increase of the ATPase activity was also observed [111,346,348,356]. When the enzyme was reconstituted in phosphatidylinositol vesicles, calmodulin also failed to stimulate the ATP hydrolytic activity [348]. From these experiments was concluded that acidic phospholipids mimic the stimulatory effect of calmodulin [111,346,348,356].

Stimulation of the Ca^{2+}-ATPase activity and loss of its activation by calmodulin was also obtained by controlled proteolysis with trypsin, resulting in an increase of the V_{max} and decrease of the K_m for calcium ion [111,348,357]. A 90 kDa tryptic fragment of the ATPase conserving the ATP hydrolytic activity and the calmodulin-binding domain was reconstituted in asolectin proteoliposomes, and an ATP-dependent A23187-sensitive calcium transport was demonstrated [357,358]. Using calmodulin in the presence of calcium or vanadate plus magnesium to lock the ATPase in two alternative stereospatial conformations (putative E_1 and E_2 states, respectively), further proteolysis of the 90 kDa fragment was obtained [358,359]. In the presence of calmodulin plus calcium an 85 kDa fragment conserving the capacity of binding calmodulin was formed, and in the presence of vanadate plus magnesium an 81 kDa fragment without the capacity to bind calmodulin was formed, both conserving the ATP hydrolytic activity and the capacity of transporting calcium in proteoliposomes [358,359] with a Ca^{2+}/ATP ratio of approx. 0.4 [359]. The low Ca^{2+}/ATP ratio obtained could be

readily explained by the low stimulatory effect of A23187 on the ATP hydrolytic activity in this study (3.1- to 3.6-fold) not only with the reconstituted fragments but also with the reconstituted intact enzyme, reflecting a high permeability of the vesicles [359].

The Ca^{2+} dependent proteinase calpain I from human erythrocyte also has been shown to transform the reconstituted native Ca^{2+}-ATPase (136 kDa) from the same origin in major active 127 kDa or 124 kDa fragments, depending on the presence or absence respectively of calmodulin during proteolysis [354]. Calpain treatment increased the affinity of the enzyme for the transported Ca^{2+} as well as inducing stimulation of the ATP hydrolytic activity in a similar manner as the induction by calmodulin [354]. The presence of calmodulin during calpain treatment of the Ca^{2+}-ATPase prevented to a great extent the activation of the enzyme by proteolysis [354]. Calpain treatment does not significantly change the experimental Ca^{2+}/ATP ratio, which in those phosphatidylcholine vesicles was from 0.60 ± 0.10 to 0.52 ± 0.03 in the absence of calmodulin and without and with, respectively, calpain treatment; or from 0.44 ± 0.08 to 0.41 ± 0.05 in the presence of calmodulin and without and with, respectively, calpain treatment [354]. The low Ca^{2+}/ATP ratio was due to the relatively high permeability of the phosphatidylcholine vesicles (fold stimulation by A23187 close to 3) [354].

An electroneutral mode of Ca^{2+} transport by this enzyme has been proposed based on the demonstration that the enzyme exchanges Ca^{2+} for H^+ [111,350,352, 355]. Proton countertransport in oxalate-loaded liposomes with high-buffer-capacity internal medium was measured during ATP hydrolysis after correction of the scalar proton production due to dissociation of the inorganic phosphate released to the medium in a low-buffer-capacity external medium [111,350]. The stoichiometric ratio of vectorial H^+/ATP observed varied from 1.7 ± 0.2 to 2.4 ± 0.3 depending on the pH of the medium, and assuming a $2H^+$–Ca^{2+} exchange, a Ca^{2+}/ATP ratio equal to 1 was derived [111,350]. In addition, no movement of the lipophylic anion TPB^- was observed, further supporting the electroneutral nature of the $Ca^{2+}/2H^+$ exchange [111,350]. The rate of ATP hydrolysis was slightly stimulated by K^+ in the presence of valinomycin. However, this stimulation was not observed in the presence of A23187 [350,352] and the initial rate of calcium transport was not significantly affected by valinomycin and/or a potassium ion diffusion potential (positive or negative inside) induced by valinomycin, even in the presence of inhibitors of band III (the anion carrier) that could contaminate the purified Ca^{2+}-ATPase preparations [350]. However, the inhibitors DIDS and NAP-taurine were demonstrated thereafter to directly inhibit the reconstituted Ca^{2+}-ATPase [355,360]. The small stimulatory effect of valinomycin on the ATP hydrolytic activity of the reconstituted enzyme was considered non-significant by some authors [111,350]. However, this stimulation could reflect an electrogenic mode of operation by the enzyme able to operate alternatively in both electroneutral as well as electrogenic modes [352]. Further evidence on the H^+–Ca^{2+} exchange mechanism was provided by the strong stimulation of the rate of ATP hydrolysis by H^+/monovalent-cation exchangers, nigericin or monensin, and by the additive stimulation of the ATPase activity by proton-conducting agents plus the non-protonatable Ca^{2+} ionophore Cyclex-2E [252]. The reconstituted Ca^{2+}-ATPase was excluded to be responsible for a Ca^{2+}-activated K^+ channel activity, since an antibody against the purified Ca^{2+}-ATPase did not inhibit Ca^{2+}-activated Rb^+ influx [353].

Using the ATP-permeabilizing agent alamethicin or the detergent Triton X-100 it has been demonstrated that approx. 75% of the Ca^{2+}-ATPase molecules present their ATP-catalytic site(s) to the external medium and 25% to the lumen of the proteoliposomes, since those agents induce an increase in the rate of ATP hydrolysis of 25% above the level induced by the ionophore A23187 [354].

The transport activity of the Ca^{2+}-ATPase from erythrocytes of sickle cell anemia patients has been demonstrated in a reconstituted system [361]. The enzyme was strongly stimulated by calmodulin in liposomes of 90% phosphatidylcholine plus 10% phosphatidylserine, and was not stimulated in asolectin liposomes [361]. The rate of ATP hydrolysis was increased 6.3 ± 0.76-fold by A23187 as expected, and the Ca^{2+}/ATP ratio was 0.9 by measuring the initial rate of ATP hydrolysis with an ATP-regenerating system coupled assay, and the initial rate of Ca^{2+} transport by Arsenazo III [361]. Therefore, the enzyme from those patients appears to behave in a normal fashion with regard to regulatory and transport properties [361].

II-C.2. The heart enzyme

A Ca^{2+}-ATPase from bovine heart sarcolemma capable of immune cross-reactivity with the erythrocyte enzyme [362] has been purified by calmodulin affinity chromatography, and reconstituted in asolectin vesicles prepared by the cholate-dialysis method [112,362]. The 140 kDa enzyme was strongly stimulated by calmodulin, and the proteoliposomes were capable of ATP-dependent Ca^{2+} uptake with an efficiency of 1 Ca^{2+} transported per each molecule of ATP hydrolyzed, as measured by a Ca^{2+}-electrode and scalar H^+ production technique, respectively [112,362]. A23187 collapsed the calcium gradient and also stimulated the rate of ATP hydrolysis by about 4-fold [112,362]. The purified and reconstituted enzyme was not phosphorylated by a cAMP-dependent protein kinase in the presence of ATP [362]. Hence, the target for the regulatory phosphorylat-

ing system must be component(s) other than the Ca^{2+}-ATPase molecule itself [362].

II-C.3. The smooth muscle enzyme

A crude preparation of smooth muscle porcine antrum was reconstituted by the addition of exogenous phospholipids and was capable of $^{45}Ca^{2+}$ uptake in the absence or presence of oxalate [363]. Thereafter, the Ca^{2+}-ATPase from smooth muscle, porcine antrum [364,365] and bovine aorta [366] was affinity-purified as described for the erythrocyte and sarcolemma enzymes. The purified enzyme of 150–140 kDa in the pig antrum [364] and 135 kDa in bovine aorta [366] was reconstituted by the cholate-dialysis method with different phospholipids [364–366], was stimulated (4- to 7.6-fold) by calmodulin [364–366], particularly in phosphatidylcholine vesicles [364,365], although the stimulation was absent from phosphatidylserine vesicles [364]. Calmodulin also stimulated the rate of calcium transport [365,366]. The efficiency of calcium transport was also from 1.04 ± 0.15 to 0.96 ± 0.18 Ca^{2+} per ATP hydrolyzed [364,365]. The generation of the calcium gradient was prevented or collapsed by A23187, as expected [364–366]. The best-coupled liposomes were obtained with asolectin (6.5- to 8-fold stimulation by A23187) instead of with phosphatidylcholine (1.7- to 3.5-fold stimulation by A23187) using the enzyme of the pig antrum [364,365]. However, the reconstitution of the bovine aorta enzyme in phospholipids from soybean give a poor, 1.8-fold, stimulation by A23187 [366].

II-C.4. The intestinal brush-border enzyme

A crude preparation of plasma membrane Ca^{2+}-ATPase from intestinal brush border was reconstituted in lipid vesicles prepared from the same membranes by a deoxycholate-dialysis method [367]. The preparation exhibited both ATP-dependent and ATP-independent $^{45}Ca^{2+}$ transport activities, the latter at lower level [367]. A low concentration of the local anesthetic, dibucaine, induced a conformational change in the proteoliposomal membrane, after interaction of this compound with Ca^{2+}, resulting in an increase of the passive permeability of the membrane to Ca^{2+} and activation of the ATP-dependent Ca^{2+} pump [367].

II-C.5. The kidney enzyme

A calmodulin-stimulated ATP-dependent $^{45}Ca^{2+}$ uptake was reconstituted from a crude preparation of outer cortex rat kidney in asolectin plus cholesterol proteoliposomes removing the Triton X-100 by a Bio-Beads column [368]. The Ca^{2+} uptake was strongly inhibited by Na^+, indicating the presence of a Na^+/Ca^{2+} exchange in the crude preparation that produces a futile recycling of calcium ion [368].

II-C.6. The liver enzyme

As mentioned previously, the plasma membrane from liver cells appears to contain several ATPases activated by calcium [336–339]. However, some of them do not appear to be involved in Ca^{2+} transport, since ATP-dependent Ca^{2+} transport could not be demonstrated in a reconstituted proteoliposomal system [338]. This enzyme was not inhibited by vanadate [338,339] and could be involved in H^+ extrusion instead [339]. However, a crude [337] or partially purified [369] Ca^{2+}-transport ATPase from rat liver was reconstituted in proteoliposomes by a cholate-dialysis method [337] or removal of the detergent $C_{12}E_8$ in Extractil-Gel D [369]. This enzyme (110–118 kDa) forms a Ca^{2+}-dependent phosphorylated intermediate and is inhibited by vanadate [337]. ATP-dependent and A23187-sensitive $^{45}Ca^{2+}$ transport was demonstrated in the liposomal system in the presence [337,369] or absence [369] of oxalate. However, the initial Ca^{2+}/ATP ratio was only 0.3 due to the partial permeability of the proteoliposomes [369]. Transport-specific fractionation of Ca^{2+}-loaded vesicles was performed [337]. The rate of Ca^{2+} transport was stimulated by a preparation of an activator protein factor distinct from calmodulin containing two polypeptides of 90 kDa and 80 kDa in gel electrophoresis in the presence of sodium dodecyl sulfate [369].

II-D. Miscellaneous Ca^{2+}-ATPases

In this section are included several Ca^{2+}-ATPases of difficult classification, although their homology with either the Ca^{2+}-ATPase from plasma membrane or from the endoplasmic reticulum appears evident. Theses include the Ca^{2+}-ATPases from synaptosomes, vesicular structures derived from brain tissue rich in synapses, and the Ca^{2+}-ATPase isolated from the internal tubular network of platelets.

II-D.1. The synaptosomal enzymes

Calcium transport ATPase(s) from rat brain synaptosomes has been reconstituted in oxalate-loaded asolectin vesicles by a cholate-dialysis method [370,371]. After reconstitution of the crude synaptosomal proteins, the vesicles were subjected to an ATP-dependent calcium-loading procedure, and thereafter the vesicles containing the calcium-oxalate complex were separated by density gradient centrifugation from the rest of the vesicles, resulting in approx. 100-fold purification of the Ca^{2+}-transport system(s) [370]. The proteoliposomes of average size of 55 nm in diameter were capable of efficient ATP-dependent $^{45}Ca^{2+}$ uptake, with a Ca^{2+} uptake/ATP hydrolysis ratio of 0.95 [370]. The reconstituted enzyme was inhibited by flavonoides in the sequence quercitin > morin > routine [371].

The purified Ca^{2+}-transporting proteoliposomes contained two major proteins of 140 kDa and 94 kDa, and both components were believed to be responsible for the Ca^{2+} transport [370]. Although no direct evidence

was presented on the origin of the Ca^{2+} transport system(s), the authors pointed out the analogies in mass of the high- and low-molecular-weight protein components of the reconstituted vesicles, to the plasma membrane, and the sarcoplasmic reticulum type Ca^{2+}-ATPases, respectively [370]. They indicated that further work has to be performed to establish whether or not two distinct calcium transport proteins from separated regions of the nerve terminal were co-reconstituted in the vesicles [370].

From the rat brain synaptosomal vesicles was later purified (80–160-fold) a Ca^{2+}-transport ATPase with a calmodulin-Sepharose 4B affinity chromatography column. The purified enzyme was reconstituted in asolectin or phosphatidylcholine liposomes and ATP-dependent calcium transport was observed that was stimulated 7–9-fold by calmodulin [372]. The reconstituted system was purified by transport-specific fractionation, and it was demonstrated that the main polypeptide is of 140 kDa and forms a phosphorylated intermediate, and it was proposed that its function was to extrude calcium ion from the nerve terminals [372].

A 70-fold purification of Ca^{2+}-ATPase from bovine brain synaptosomes yielded a preparation containing two major polypeptides of 230 kDa and 94 kDa that are immunologically related to each other as demonstrated by immunoprecipitation of Ca^{2+}-transport activity in a reconstituted system [373]. However, this ATPase appears to be distinct from the Ca^{2+}-ATPase from sarcoplasmic reticulum or bovine erythrocyte plasma membrane [373]. It was proposed that this Ca^{2+} pump is specific to nerve tissue and that the 230 kDa and 94 kDa polypeptides are structurally homologous components of the transport activity [373].

II-D.2. The platelet enzyme

The Ca^{2+}-ATPase from the internal tubular membrane system of human platelets has been solubilized by octyl glucoside, purified about 14.8-fold (80% by protein), and reconstituted in crude soybean phospholipid vesicles by dialysis of the detergent [374,375]. The purified ATPase preparation used for reconstitution contained two major polypeptides of 100 kDa and 89 kDa at a variable 5 : 1 to 1 : 1 ratios, and the lower-mass component was thought to be an inactive proteolytic product of the Ca^{2+}-ATPase [374]. Both polypeptides from the purified human platelets cross-react with an antiserum against the purified Ca^{2+}-ATPase from the sarcoplasmic reticulum from rabbit skeletal muscle, suggesting the similarity of both enzymes [374]. The reconstituted ATPase was capable of Ca^{2+} uptake as monitored by the metallochromatic probe Arsenazo III, and the addition of the $Ca^{2+}/2H^+$ exchanger, A23187, resulted in the release of the accumulated calcium [374]. The extent and initial rate of calcium uptake was stimulated in the presence of inorganic phosphate [374]. The kinetic properties of the purified Ca^{2+}-ATPase were similar to those of the enzyme from sarcoplasmic reticulum. The platelets Ca^{2+} pump transported 2.1 ± 0.2 Ca^{2+} per each ATP molecule hydrolyzed, further supporting its analogies to the enzyme from sarcoplasmic reticulum [374,375].

II-E. The H^+/K^+-ATPase from gastric mucosa

The H^+/K^+-ATPase is involved in acid secretion by the parietal cells from the stomach and is thought to exchange H^+ for K^+ in an electroneutral mode (see Fig. 3, enzyme 3) [71–73]. A non-purified preparation of H^+/K^+-ATPase from hog gastric fundus has been reconstituted in proteoliposomes by a freeze-thaw/sonication procedure using a mixture of phospholipids plus cholesterol [376–378]. The proteoliposomes responsible for H^+-transport were separated from the non-reconstituted enzyme in a sucrose gradient, and the polypeptide suggested to be responsible for the transport functions had a molecular mass of 94 kDa [376].

The reconstituted enzyme was capable of ATP-dependent H^+ uptake [376–378] as measured by fluorescence quenching of acridine orange [376] or by a pH electrode in a low-buffer-capacity medium [377,378]. The H^+ uptake induced by ATP hydrolysis was dependent on the presence of K^+ inside the proteoliposomes [376,377]. A direct demonstration of ATP-dependent K^+ transport was obtained by monitoring the efflux of the potassium analogue $^{86}Rb^+$ [376,378]. The stoichiometric ratio of H^+/ATP was 2.1 ± 0.17 and the $(^{86}Rb^+)K^+$/ATP ratio was 1.93 ± 0.24, demonstrating the electroneutral ($2H^+/2K^+$/ATP) nature of the exchange [378]. Consistent with this electroneutral mode of operation were the negative response of the $\Delta\psi$-sensitive probe DOCC in the absence of the H^+-conducting agent TCS [376]. However, in the presence of TCS an H^+ diffusion potential (negative inside) is generated and detected by DOCC. The TCS-dependent diffusion potential was dissipated by addition of CDTA [376]. Moreover, the H^+ gradient was reversed by the combined addition of CCCP plus valinomycin in the presence of potassium [377], and the ATP-dependent $^{86}Rb^+$ efflux was insensitive to protonophores [376].

A passive vanadate-sensitive $^{86}Rb^+$ exchange was also demonstrated in the absence of ATP, indicating that the ATPase was reconstituted asymmetrically into the proteoliposomes (70% *cis*/30% *trans*-vanadate site) [376]. Using a cholate resolubilization method it was demonstrated that 60% of the enzyme was reconstituted in a right-side-out, and 40% in a right-side-in orientation [378]. The passive $^{86}Rb^+$ exchange was inhibited by ATP and stimulated about 2-fold by low concentrations of Mg^{2+} and by 5 mM phosphate [376]. A passive vanadate-sensitive H^+ transport in the absence of ATP was also demonstrated in the proteoliposomes [377].

This passive H^+ transport was dependent and saturated by K^+ inside, suggesting that it was mediated by the H^+/K^+-ATPase [377]. An amiloride-sensitive Na^+/H^+ passive exchange was also observed in the same proteoliposomes. However, this exchange was probably due to the co-reconstitution of a Na^+/H^+ antiporter present in the crude ATPase preparation [377].

II-F. The H^+-ATPase from yeast/fungi plasma membrane

The prominent role of the H^+-ATPase from the yeast/fungi plasma membrane (see Fig. 3, enzyme 2) in ion transport [74] as well as the biochemical properties of this enzyme [75–82,91] have been extensively reviewed.

II-F.1. The yeast enzyme

The first attempts to reconstitute the purified enzyme from the yeast *Schizosaccharomyces pombe* were carried out with preformed sonicated vesicles of L-α-dimyristoylphosphatidylcholine [379,380]. It was demonstrated that the activity of the partially delipidated ATPase dramatically increases in the presence of these vesicles reaching near plateau at one molecule of ATPase per vesicle, suggesting that a monomeric form of the enzyme was sufficient for ATP hydrolysis [379]. In addition, the interaction of the sonicated vesicles (26 ± 4 nm diameter) [380] with the purified ATPase results in spontaneous vesicular fusion at 8–10 °C [379,380] and the formation of larger vesicles containing the ATPase (95 ± 20 nm diameter) [380]. The intravesicular volume was determined to be 0.35 μl/mg phospholipids in proteoliposomes prepared from egg yolk and solubilized plasma membranes containing the ATPase [381]. The reconstituted proteoliposomes containing the enzyme appear to have intact permeability barriers at 30 °C [380], although evidence for H^+ transport was not obtained in this system until later [382], as suggested by a 50% stimulation of the rate of ATP hydrolysis by FCCP and by the formation of a FCCP-sensitive ΔpH (acid inside) as measured by fluorescence quenching of ACMA in the absence of valinomycin [382].

A freeze-thaw/sonication procedure [83,383–386] and a cholate dialysis method [386–389] were used to obtain better reconstitution of the purified enzymes of *Saccharomyces cerevisiae* [83,383,384] and *S. pombe* [385–389]. The reconstituted enzyme was capable of a $[^{32}P]P_i$–ATP exchange reaction partially resistant to addition of CCCP alone but almost totally prevented by the addition of valinomycin (in the presence of potassium) plus CCCP [383,384] or the uncoupler 1799 [384]. The authors concluded that the uncoupler 1799 was more potent than CCCP, and that valinomycin increased the effect of CCCP by forming a ternary complex in the presence of K^+ [384]. Therefore, the early proposal that the enzyme was capable of two types of transport (i.e., electrogenic proton transport, and electroneutral proton-potassium exchange) [383] was reconsidered later in favor of a pure electrogenic proton transport mechanism [384]. However, no direct evidence was presented for the formation of the valinomycin-K^+-CCCP complex under the experimental conditions chosen or that the ionophore 1799 was unable to transport K^+ in addition to H^+.

The electroneutral H^+/K^+ exchanger nigericin was an efficient agent capable of stimulating the rate of ATP hydrolysis by the reconstituted enzyme from yeasts [83,383,385–389], suggesting that generation of a ΔpH was of great importance in the mode of operation of the enzyme [387,388]. However, some authors suggested that nigericin could exchange H^+/K^+ with variable stoichiometry and therefore was capable of collapsing a $\Delta\psi$ [385]. Formation of a ΔpH (acid inside) was directly demonstrated by measurement of fluorescent quenching of ACMA [83,381,384–386] or by a pH electrode in a low-buffer-capacity medium [386,387]. The ΔpH was estimated to have a value of 3.6 pH units [385] or higher than 5 pH units [384]. The first value could be consistent with a H^+/ATP ratio equal to 2 [385] and the second value could accommodate only a H^+/ATP ratio equal to 1 [384]. As expected, ATP-dependent proton pumping was prevented in preilluminated proteoliposomes containing both a yeast plasma membrane ATPase plus bacteriorhodopsin [390], due to the formation of an electrochemical H^+ gradient.

The electrogenicity of the H^+ transport by the yeast plasma membrane ATPase was demonstrated by the increase of the rate of ΔpH formation in the presence of the charge compensating cation K^+ in the presence of valinomycin [381,384–387] or charge-compensating anions [83,381,384,385]. In addition, direct measurement of a FCCP-sensitive $\Delta\psi$ was also done with the probe oxonol V [381].

However, indications for an alternative electroneutral mode of operation of the enzyme were obtained when it was observed that K^+-loaded proteoliposomes exhibit a decrease of the stimulation of the ATPase activity induced by CCCP [388]. Moreover, non-purified plasma membrane ATPase of the yeast *Metschnikovia reukaufii* reconstituted in phospholipid vesicles shows an increase in the rate and extent of ΔpH formation in the presence of potassium ion [391,392]. Potassium ion was required from the inside of the proteoliposomes to increase the extent of the ΔpH formation; however, external K^+ also has some effect [392], perhaps due to its partial entry into the liposomal lumen.

Solubilized plasma membrane vesicles reconstituted in an egg-yolk phospholipid mixture by removal of *n*-octylglucoside were obtained as well in *S. pombe* [381]. In this crude ATPase preparation was demonstrated the formation of an ATP-dependent electro-

chemical proton gradient in which both components $\Delta\psi$ and ΔpH behave in an opposite manner depending on the presence or absence of salts in the medium [381]. The formation of $\Delta\psi$ was maximal in the absence of NO_3^- and ΔpH was maximal in its presence [381]. The electrogenic nature of the system was, however, modified depending on the presence of K^+ in the system. The membrane potential was more efficiently dissipated by NO_3^- in the presence of K^+, than in the presence of Na^+ or Li^+ [381]. Moreover, the formation of a ΔpH induced by NO_3^- was smaller in the presence of K^+ than in the presence of Na^+ or Li^+ [381]. The authors concluded that the results can be best explained if the H^+-ATPase operates as an H^+–alkali cation exchanger with variable stoichiometry [381].

Direct ATP-dependent CCCP-sensitive K^+ translocation by the purified and reconstituted H^+-ATPase of *S. pombe* was demonstrated using a K^+-selective electrode [388,389]. The yeast plasma membrane ATPase proteoliposomes present also an asymmetric passive conductance of opposite directions for H^+ and K^+ in the absence of ATP after the imposition of an electrical gradient of appropriate polarity and magnitude [389]. The K^+ conductance in the absence of ATP was inhibited by vanadate [389]. Moreover, the ATP-dependent K^+ transport did not change in proteoliposomes exhibiting variable H^+ conductance, and the rate of K^+-transport showed a saturation curve with respect to the substrate MgATP [389], demonstrating that although the presence of a $\Delta\psi$ (positive inside) was required for the ATP-dependent K^+ extrusion from the proteoliposomes [388,389], the ATPase was directly involved in K^+ translocation perhaps by a $\Delta\psi$-gated channel of the ATPase [389] (see Fig. 5). High sensitivity to $\Delta\psi$ of a reconstituted K^+-motive ATPase from bacteria has also been demonstrated [393]. Direct coupling of K^+ transport to the hydrolysis of ATP has the advantage of being a more efficient mechanism than a simple electrophoretic carrier for establishing high concentration gradients of K^+ across the plasma membrane of lower eukaryotic cells [394].

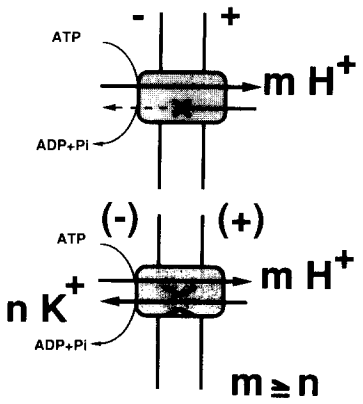

Fig. 5. Different modes of operation of the plasma membrane H^+-ATPase of lower eukaryotic cells. (Top) ATP-dependent electrogenic pure H^+ ejection. The pathway for K^+ translocation is close by a $\Delta\psi$-sensitive gate. (Bottom) ATP-dependent H^+–K^+-exchange. The pathway for K^+ transport and the $\Delta\psi$-sensitive gate is open. The system could be electrogenic or electroneutral depending on the stoichiometry of H^+ and K^+ translocated.

Limited proteolysis – with trypsin, α-chymotrypsin, elastase and carboxypeptidase Y – of the H^+-ATPase of *S. cerevisiae* reconstituted into phosphatidylserine vesicles has been used to study the topology of the enzyme in the membrane [395]. A model was proposed in which the C-terminus of the enzyme is located in the inside of the vesicles and the N-terminus is on the outside, the ATP binding region is on the outside, and there are a minimum of five passes of the polypeptide through the membrane [395].

II-F.2. The fungus enzyme

Reconstitution by a freeze-thaw/sonication method of the purified plasma membrane H^+-ATPase from amoebae of the slime mould *Dictyostelium discoideum* in asolectin liposomes has been reported [396]. Generation of a CCCP-sensitive and vanadate-sensitive ΔpH (acid inside) in the presence of K^+ plus valinomycin was monitored by fluorescence quenching of ACMA, demonstrating the electrogenic nature of the enzyme [396].

The plasma membrane H^+-ATPase of *Neurospora crassa* has been reconstituted with asolectin, using a supernatant of deoxycholate-solubilized membranes and removal of the detergent with Bio-Gel P-10, resulting in proteoliposomes enriched in ATPase (35% of the protein) [397] or using a highly purified enzyme and a freeze-thaw/sonication procedure [398,399]. Generation of a ΔpH (acid inside) was demonstrated by the use of acridine orange [397] or ACMA [398]. The pH gradient was collapsed by the presence of FCCP [397] or nigericin plus potassium [398]. Valinomycin in the presence of potassium increases the rate of formation of the ΔpH [397,398]; however, the effect of valinomycin was not observed in the presence of permeable anions [397]. Formation of an FCCP-sensitive $\Delta\psi$ (positive inside) was also measured with the probe oxonol V [397]. These observations suggest that H^+ transport is electrogenic [397,398]. However, as in the case of the enzyme from yeasts [383,385–388], nigericin strongly stimulates the rate of ATP hydrolysis of the reconstituted fungus enzyme, in this case above the 2.5-fold stimulation observed with CCCP [398]. To explain this apparently surprising result, the authors suggested that this observation will be consistent with $\Delta\psi$ and ΔpH not being equipotent with respect to their effectiveness in reversing the ATPase reaction and ΔpH being more effective than $\Delta\psi$ in this regard [398].

Using reconstituted radiolabelled ATPase it was demonstrated that a functional H^+-ATPase of *N. crassa* has no subunits other than the 105 kDa polypeptide,

excluding polypeptides larger than 2.5 kDa [398], and that a monomeric form of the enzyme can catalyze efficient ATP hydrolysis-driven proton translocation [399], in agreement with the results obtained with the enzyme from yeast [379].

The enzyme of *N. crassa* also has been reconstituted in a planar phospholipid bilayer by previously forming proteoliposomes that later are fused with the planar membrane [400]. In this system it was possible to detect an ATP-dependent vanadate-sensitive current of approx. 0.2 pA, and it was demonstrated that the reconstituted enzyme was able to work against a positive membrane potential [400].

II-G. The H^+-ATPase from higher plant plasma membrane

The extrusion of protons across the plasma membrane of plants cells and the secondary transport of ions and nutrients [89,401–403] is attributed to the function of an electrogenic H^+-ATPase (see Fig. 3, enzyme 2) whose properties have been established in a series of organisms from algae to higher terrestrial plants [79,80,83–90,92].

The plasma membrane H^+-ATPase from different higher plants has been purified to different degrees, and the reconstitution of the enzymes from oat (*Avena sativa*) roots [80,404,405], red beet (*Beta vulgaris*) storage tissue [406–409], suspension-cultured rose (*Rosa damascena*) cells [410], radish (*Raphanus sativum*) seeds [411], tomato (*Lycopersicon esculentum*) roots [412], mung bean (*Phaseolus mungo*) roots [413], and corn (*Zea mays*) roots [414] has been described.

The first successful reconstitution was obtained with the enzyme from oat roots [404,405]. This enzyme was solubilized from KCl plus Triton X-100-striped membranes using the detergent Zwittergent 3-14 plus cholate [404] or lysophosphatidylcholine [405]. The solubilized enzyme was partially (10-fold) [404] or highly (70-fold) [405] purified, and reconstituted in soybean phospholipid vesicles by a freeze-thaw/sonication procedure [404,405]. A similar reconstitution procedure was used with the enzyme of tomato roots [412] and corn roots [414].

The other methods of reconstitution employed with the enzymes from other plants included the cholate-dialysis method [411], the detergent-removal method using Sephadex G-200 [406–409], the detergent-dilution method [413,414] or the simple incubation of the enzyme with preformed sonicated vesicles on ice-cold medium [410]. Soybean phospholipids with different degrees of enrichment in phosphatidylcholine were employed in the reconstitution procedures [404–407,410–414]. In the case of the enzyme from rose cells, however, 10% cholesterol was also added [410], or a mixture of phosphatidylcholine and phosphatidylethanolamine was used as well in the case of corn roots [414].

The qualities of the enzyme preparations used in reconstitution ranged from highly crude [406,407,410, 411,414], partially purified enzyme containing several polypeptides (93, 72, 60, 40, 33 and 24 kDa [404], or 105, 67 and 57 kDa [413]) to highly purified (70% to 80% of the protein) [405,408,412], containing a major polypeptide of 90 kDa [412] or approx. 100 kDa [405,408].

The plasma membrane origin of the reconstituted enzyme was well established in most cases by demonstrating the formation of a ^{32}P-labeled aspartyl intermediate [404,408], and the high sensitivity of the ATP hydrolytic activity [404, 406–408, 410–414], as well as the transport of H^+ [405–408,410–414] to vanadate. The ATPase activity [404,407,408,411,413] and the transport of H^+ [407,411,412] were also sensitive to DCCD. However, oligomycin had a negligible [405,407,408,411] or slight [413] effect on the enzymes used for reconstitution, as well as azide [408], excluding significant contamination by a F_1F_0-type ATPase. Contamination by the tonoplast [406,407,411] or Golgi apparatus [406,410] H^+-ATPases was also excluded, although it was suggested that an ATPase associated with transition vesicles en route from the Golgi apparatus to the plasma membrane could account for some of the ATPase from red beet used for reconstitution [406]. From the preparations of rose cells it was possible to isolate two different H^+-ATPases separated by Sephadex G-150 gel-filtration chromatography [410]. Irradiation of the isolated ATPases by ultraviolet light (290 nm or higher) resulted in the inhibition of the ATP-dependent H^+ pumping by the reconstituted ATPase of higher molecular weight [410]. However, the low-molecular-weight ATPase was resistant to ultraviolet irradiation [410]. The kinetic properties of the enzyme from red beet change after solubilization and reconstitution in phospholipid vesicles, particularly the K_m for ATP and its activation energy [405].

The generation of a ΔpH (acid inside) by the reconstituted plasma membrane ATPase from different plants was monitored either by fluorescence quenching of ACMA [404,405,414], fluorescence quenching of quinacrine [406,410,412,413] and fluorescence quenching [406–408] or absorption [411] of acridine orange. The ΔpH was either collapsed or prevented by nigericin (in the presence of potassium) [404,405,414], gramicidin [404,408,411,414], FCCP [406,407,410–413], CCCP [414], 1799 [404], SW26 [414], the weak base imidazole [404] and ammonium salts [411,414], Valinomycin (in the presence of potassium) stimulated the rate of formation of the ΔpH to a different degree [404,406,407,411, 414], suggesting the electrogenic nature of the H^+ transport, although in some cases ΔpH was generated at significant rate in its absence [404,405,407,410–414], due perhaps to the high permeability of the proteoliposomal membrane to anions, particularly chloride. Nitrate

was also shown to significantly increase the rate of formation of ΔpH [404,406,408] as expected for an electrogenic enzyme. Direct measurement of a $\Delta\psi$ (positive inside) was measured with the reconstituted enzyme from red beet using the $\Delta\psi$-sensitive probe oxonol V [408].

The reconstituted enzymes from oat roots [404], red beet storage tissue [407,408], rose cells [410] and mung bean roots [413] were shown to be stimulated to a great extent by potassium ion in its ATPase activity [404,407,408,413] or H^+ transport function [407,414]. However, since H^+ transport takes place in the absence of potassium, some authors concluded that at least the enzyme of oat roots was not directly involved in potassium ion transport [404]. Nevertheless, other authors noted that the stimulation by K^+ was a key property of the enzyme from red beet, and concluded that further studies should be conducted with the reconstituted enzyme to properly answer whether or not the enzyme is directly involved in K^+ transport [408].

III. Reconstitution of proton-ATPases from the eukaryotic vacuolar system

The dynamic membranal organization of the eukaryotic vacuolar system is provided with a series of similar electrogenic H^+-translocating ATPases, responsible for the intraluminal acidification of the vesicles not only in animal cells (see for reviews Refs. 86,87,415–417) but also in plant cells (see for reviews Ref. 85–91). The localization of the vacuolar type H^+-ATPases are indicated in the lower part of the archetypal cell represented in Fig. 3 (enzymes 6 and 7). These H^+ pumps are distinct from the F_1F_0-ATPases and the plasma membrane H^+-ATPases from yeast/fungus and higher plants discussed previously (see Fig. 6). The vacuolar-type H^+-ATPases are multisubunit complexes of molecular mass in the 300 to > 500 kDa range, constituted perhaps from three to eight different polypeptides depending on origin [25], although the precise subunit compositions are not yet well established in all cases.

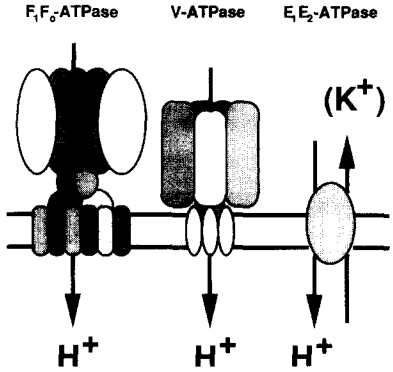

Fig. 6. Different types of H^+-translocating ATPases. (Left) The multi-subunit F_1F_0-ATPases from prokaryotic organisms, chloroplasts or mitochondria translocate H^+ during ATP hydrolysis. (Center) The multi-subunits vacuolar type ATPases from eukaryotic cells translocate H^+ during ATP hydrolysis. (Right) The monomeric E_1E_2-ATPases from plasma membrane of lower eukaryotic cells alternatively translocate H^+ or exchange H^+ for K^+.

It has been suggested that the vacuolar type H^+-ATPases evolved together with the vacuolar system from archaebacteria or proteokaryotes [25]. However, the different members of this group of ATPases may

TABLE IV

Polypeptide composition of reconstituted H^+-ATPases from the eukaryotic vacuolar system

Source of enzyme	Reconstituted polypeptides (kDa)	Comments	References
Chromaffin granules	115 + 72 + 57 + 39 + 17	H^+-pumping	424,425
Clathrin-coated vesicles	116 + 70 + 58 + 40 + 38 + 34 + 33 + 15	H^+-pumping	427,430
	17	H^+-conducting subunit DCCD-sensitive	431
	70 + 58	not able of Ca^{2+}-activated ATP hydrolysis	432
	70 + 58 + 40 + 33	restore Ca^{2+}-activated ATP hydrolysis	432
	116	required to convert Ca^{2+}-stimulated ATP hydrolysis to Mg^{2+}-activated ATP hydrolysis and H^+ pumping	432
Bovine kidney medulla microsomes	70 + 56 + 45 + 42 + 38 + 33 + 31 + 15 + 14 + 12	H^+-pumping	438,439
	70 + 56 + 45 + 42 + 38 + 31 + 15 + 14 + 12	H^+-pumping, form 551 kDa and 523 kDa complexes in non-denaturing gels and 586 kDa high pressure size-exclusion liquid chromatography	437
Bovine kidney cortex enriched in Golgi apparatus	68 + 58 + 40 + 37 + 16	also contains endosome H^+-pumping	440

have diverged into a few subclasses quite early in evolution [25]. Moreover, the progenitor of ATP-synthases appears to be closely related to the present vacuolar H^+-ATPases [26].

The vacuolar H^+-ATPases appear not to form a covalent aspartyl phosphorylated intermediate, since they are resistant to orthovanadate. They are also resistant to oligomycin. However, they appear to be highly sensitive to NEM, NBD-Cl and NO_3^- or SNC^-, and inhibited only at high concentrations of DCCD.

The reconstitution of partial or highly purified vacuolar-type H^+-ATPase from different subcellular structures involved in endo/exocytosis and subcellular storage systems has clarified enormously the mechanism and functioning of this type of ATPases. In some detail will be described the reconstitution of the H^+-ATPases from chromaffin granules, clathrin-coated vesicles, lysosomes and plant tonoplants as well as H^+-ATPases located in liver plasma membrane, kidney medulla, and kidney cortex, the latter apparently localized in the terminal cisterna of the Golgi apparatus.

III-A. The chromaffin granule H^+-ATPase

Solubilization of chromaffin granules isolated from bovine adrenal glands and reconstitution of the crude solubilized proteins with endogenous or exogenously added phospholipids has resulted in the preparation of proteoliposomes containing the H^+-translocating ATPase [418–422] and the catecholamine transport system, capable of sustaining uptake of 5-[^{3}H]hydroxytryptamine [418], [^{14}C]methylamine [420], or [^{3}H]noradrenaline [420] driven by the ΔpH (acid inside) generated by the ATPase. Exogenously added chromaffin granule lipids were better for the reconstitution procedure than soybean phospholipids [419]. The reconstituted ATPase actually was moderately stimulated by the presence of the uncoupler S-13 [419]. However, when only endogenous phospholipids were used in the reconstitution procedure the ATPase actually failed to be stimulated by the uncoupler CCCP [420]. A direct demonstration of the electrogenic H^+ translocation by the ATPase was attained in this system by measuring the generation of a proton gradient (acid inside) with the ΔpH-probe acridine orange [422] and the generation of a membrane electrical potential (positive inside) with the $\Delta\psi$-sensitive probe oxonol V [420]. The $\Delta\psi$ was collapsed by the addition of CCCP or SCN^- [420] and the ΔpH by the addition of FCCP [422]. The solubilized ATPase can be substantially delipidated by ammonium sulfate precipitation, and the resulting delipidated enzyme could be fully reactivated by soybean or extracted chromaffin granule pospholipids [421]. Reconstitution with dipalmitoylphosphatidylcholine or dimyristoylphosphatidylcholine resulted in the generation of an ATPase which showed two activation energies with a change of slope at a temperature almost identical to the gel-to-liquid-crystalline phase transition temperature as measured by fluorescence polarization of the probe diphenylhexatriene [421], suggesting that the ATPase activity could be regulated by the viscosity of the membrane [421].

The reconstituted crude ATPase was also capable of net ATP synthesis by the combined actions of an artificially imposed ΔpH (acid inside) and a K^+ diffusion potential (positive inside) by resuspending proteoliposomes (previously equilibrated in a K^+-free medium at pH 5.0) in a medium containing 80 mM KCl at pH 8.3 in the presence of valinomycin [420]. The same system was capable of a [^{32}P]P_i–ATP exchange reaction [420]. The [^{32}P]P_i–ATP exchange activity was further purified by sucrose gradient centrifugation and reconstituted with exogenous phospholipids by a freeze-thawing method [423]. This [^{32}P]P_i–ATP exchange reaction was inhibited by CCCP [423]. The reconstituted ATPase actually was completely resistant to oligomycin [423]. However, DCCD [419,423] or tributylin tin [423] inhibit both ATPase activity [419,423] and [^{32}P]P_i–ATP exchange [423]. Furthermore, reconstitution of the ATPase solubilized from chromaffin granule membranes treated with sodium bromide to inactivate the mitochondrial F_1F_0-ATPase demonstrated that the generated ΔpH (acid inside) was not due to contamination by the mitochondrial enzyme [422].

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of the semipurified chromaffin granule ATPase used in the reconstitution of the [^{32}P]P_i–ATP exchange reaction demonstrated the presence of three major polypeptides, of 75, 52 and 50 kDa [423]. The authors concluded that the 75 kDa protein was likely to be contaminating dopamine β-hydroxylase and the 52 kDa and 50 kDa proteins the α and β subunits of a F_1-ATPase [423]. However, further purification and reconstitution of the chromaffin granule ATPase has shown it to be composed of five polypeptides, of 115, 72, 57, 39 and 17 kDa [424,425]. In this highly purified system it was demonstrated that endogenous lipids were enough for forming vesicles, and addition of purified phospholipids from *Escherichia coli* or brain had little effect on the system. Moreover, soybean phosphatidylcholine inhibited H^+ uptake [424].

In the absence of co-reconstitution of an anion transport system in these proteoliposomes it was demonstrated that in order to generate a ΔpH (acid inside) the presence of potassium plus valinomycin was required, further demonstrating the electrogenic nature of the transport of H^+ by the ATPase [424]. Moreover, $\Delta\psi$ could be directly measured by the probe oxonol VI [424]. The $\Delta\psi$ was increased in the presence of Cl^- and inhibited by SO_4^{2-} or NO_3^- [424], similarly to the reconstituted lysosomal H^+-ATPase [426] and the clathrin-coated vesicle H^+-ATPase [427]. The generation of a

ΔpH by the purified and reconstituted chromaffin granule H^+-ATPase was strongly inhibited by *N*-ethylmaleimide [424,425]. This inhibition was prevented to different degrees by the presence of AMP, ADP, ATP and AMP-PNP, most efficiently in the presence of Mg^{2+} [425]. It was demonstrated that oxidation of an -SH group in the 72 kDa subunit prevented H^+ pumping, and its subsequent reduction restored H^+ translocation [425]. The ATP-dependent uptake of proton by the purified and reconstituted enzyme was absolutely dependent on the presence of Cl^- or Br^- outside the vesicles [424,428], whereas, sulfate, acetate, formate, nitrate and thiocyanate were inhibitory [424]. However, without external chloride, proton uptake could be observed when Cl^- or sulfate were present inside the vesicles [428]. Moreover, if a $\Delta\psi$ (negative inside) was present, the enzyme was able to form a ΔpH regardless of the anion species present inside or outside the proteoliposomes [428]. The authors suggest that both an internal anion binding site and the $\Delta\psi$ regulate the proton pumping activity of the enzyme [428].

III-B. The clathrin-coated vesicle H^+-ATPase

Initially, non-solubilized material from clathrin-coated vesicles from bovine brain depleted of ATPase by extraction with the detergent $C_{12}E_9$, was reconstituted with crude brain lipids by a cholate dilution method in the presence of the partially purified (100-fold) NEM-sensitive ATPase previously extracted from the vesicles [429]. The reconstituted vesicles were capable to generate a ΔpH (acid inside) as measured by acridine orange absorption, that was prevented by the presence of the uncoupler 1799 [429]. The intact ATPase complex capable of H^+ transport was later purified (200-fold) and reconstituted into proteoliposomes of crude brain lipids [427] or a mixture of pure lipids [430]. The purified preparation contains eight major polypeptides (116, 70, 58, 40, 38, 34, 33 and 15 kDa) five of them of molecular mass similar to that of the purified and reconstituted H^+-ATPase from chromaffin granules [424,425].

The electrogenic nature of the H^+ pump was demonstrated by the requirement of K^+ in the presence of valinomycin for the generation of the ΔpH (acid inside) [427,430] and by the direct measurement of a $\Delta\psi$ (positive inside) with the probe oxonol VI [427]. The reconstituted ATPase activity was stimulated about 3-fold by 1799 plus valinomycin [430], and both, ΔpH [427,430] and $\Delta\psi$ [427] were collapsed by 1799. In addition, the reconstituted enzyme was capable of sustaining a 1799-sensitive $[^{32}P]P_i$–ATP exchange reaction [430].

Although initially it was shown that phosphatidylserine but not other commercial phospholipids tested could replace the brain lipid fraction as an activator of the delipidated ATPase [429], later it was demonstrated that phosphatidylserine was not required for reconstitution of proton pumping [430]. However, cholesterol was required to stabilize the proteoliposomes and to prevent the spontaneous collapse of the pH gradient generated by the ATPase [430]. The resistance of the ΔpH generation [429] and the $[^{32}P]P_i$-ATP exchange reaction [427] to the well-known mitochondrial F_1F_0-ATPase inhibitors, oligomycin and sodium azide, respectively, demonstrated that the preparations were not contaminated by the mitochondrial enzyme. Moreover, the polypeptide pattern of the purified clathrin-coated vesicle H^+-ATPase was different from that of the mitochondrial F_1-ATPase. As in the case of the lysosomal [426] and the chromaffin granule H^+-ATPases [424,425], the reconstituted H^+-ATPase from clathrin-coated vesicles was inhibited by *N*-ethylmaleimide [427,429,430].

The 17 kDa subunit of the clathrin-coated vesicle H^+-ATPase has been isolated and co-reconstituted in liposomes with bacteriorhodopsin or with the intact clathrin-coated vesicle H^+-ATPase, preventing in both cases the generation of a ΔpH in the presence of K^+ plus valinomycin [431]. However, $[^{14}C]$DCCD labeled the 17 kDa subunit and DCCD treatment or trypsin digestion of the 17 kDa subunit restored the ability for generation of an H^+ gradient by the co-reconstituted intact enzyme or by bacteriorhodopsin, demonstrating that the 17 kDa subunit is a constituent of a proton pore of the H^+-translocating ATPase complex [431].

Calcium ion could support ATP hydrolysis but not H^+ translocation by the purified and reconstituted H^+-ATPase [432]. Nevertheless, the Ca^{2+}-stimulated ATPase activity represents a useful partial reaction for examining the subunit composition of the clathrin-coated vesicle H^+-ATPase. It was found that an ATPase subcomplex containing the 70 kDa and 58 kDa subunits was not capable of Ca^{2+}-activated ATP hydrolysis. However, recombination of the 70, 58, 40 and 33 kDa polypeptides restored the Ca^{2+}-activated ATP hydrolysis [432]. It was suggested that the 116 kDa subunit was required to convert Ca^{2+}-stimulated ATP hydrolysis to Mg^{2+}-activated hydrolysis and proton pumping [432].

III-C. The lysosomal H^+-ATPase

The H^+-ATPase purified from isolated rat or mouse liver lysosomes has been reconstituted by detergent dilution methods [426,433,434]. Earlier reconstitutions were performed in asolectin [433,434]. However, it was later demonstrated that better results could be obtained using purified phospholipids from *Escherichia coli,* although the bacterial lipid mixture lacks phosphatidylcholine, phosphatidylinositol, cholesterol and sphingomyelin, components that amount to 80% of the lysosomal membrane lipids [426]. The reconstituted enzyme it was able to generate a ΔpH (acid inside) as measured by fluorescent quenching of quinacrine [433] or acridine

orange [426], and a $\Delta\psi$ (positive inside) as measured by fluorescent quenching of oxonol V [433]. The electrochemical H^+ gradient was collapsed by SF6847 [433] or by FCCP [426]. The electrogenic nature of the H^+ translocated was further established by the requirements of valinomycin (in the presence of internal K^+) for the generation of the ΔpH [426,433]. It was excluded that the enzyme operates as an H^+-anion symporter, a K^+-motive ATPase or an ATP-linked nH^+–K^+ exchanger [426].

To assess the origin of the reconstituted enzyme it was important to exclude the co-reconstitution of other H^+ translocating ATPases with the lysosomal one. It was established that a polyclonal serum against the β subunit of the mitochondrial F_1-ATPase or a monoclonal antibody against the plasma membrane antigen Ly-24 did not bind to the proteoliposomes [426]. In contrast, monoclonal antibodies against the lysosomal proteins LAMP-1 and LAMP-2 strongly bind to the proteoliposomes [426]. However, significant binding was also found with a monoclonal antibody to the putative α_2-macroglobulin receptor, suggesting the presence in the proteoliposomes of endosomes or other vesicular proteins [426].

Generation of ΔpH [426,433] and $\Delta\psi$ [433] was totally inhibited by 100 μM DCCD. However, this concentration of DCCD inhibited only 50% of the reconstituted ATPase activity [433]. This apparent discrepancy could be explained by the increase in the H^+ permeability of the liposomal membrane induced by high concentrations of DCCD (Villalobo, A., unpublished data). The generation of the ΔpH by the reconstituted enzyme was also inhibited by DIDS and NEM, but not by orthovanadate, ouabain or oligomycin [426]. The sensitivity of the lysosomal H^+-ATPase to anions and inhibitors also depended on whether or not the enzyme was in a solubilized form or reconstituted in proteoliposomes [434]. Most significantly, the solubilized lysosomal H^+-ATPase was strongly inhibited by classical F_1F_0-ATPase inhibitors, such as azide or oligomycin [434]. However, the native membrane-bound enzyme or the reconstituted enzyme was apparently not affected by these inhibitors [434].

III-D. The tonoplast / vacuole H^+-ATPases

An anion-sensitive ATPase from corn roots was solubilized from membrane fractions with deoxycholate, and reconstituted by a Sephadex G-200 filtration method without any further purification in soybean phospholipid vesicles containing 40% phosphatidylcholine [435]. The reconstituted enzyme was capable of generating an FCCP-sensitive ΔpH (acid inside) in the presence of Cl^-; however, NO_3^- or SO_4^{2-} was strongly inhibitory [435]. The formation of the ATP-dependent proton gradient was monitored by fluorescence quenching of acridine orange, and was mostly insensitive to oligomycin or orthovanadate. However, it was strongly inhibited at high concentrations of DES [435]. Therefore, the reconstituted enzyme was putatively identified as the tonoplast H^+-pump based on its anion and inhibitor sensitivities [86,88,89]. The preparations, however, were not free of mitochondrial and plasma membrane ATPases [435]. The H^+-conducting properties of DES in the proteoliposomes were not tested, although the fact that 50 μM DES did not inhibit the deoxycholate-solubilized ATPase activity, but strongly affected the generation of the proton gradient [435], suggests that DES would have uncoupling properties. The Zwittergent 3-14-solubilized ATPase was more sensitive to DES (60%); however, the large micelle size and the relative low critical micelle concentration of this detergent prevented its efficient removal by dialysis or gel filtration, and therefore its reconstitution [435].

The ATPase from vacuoles from *Saccharomyces carlsbergensis* was solubilized with Zwittergent TM-314, partially purified by glycerol density gradients and reconstituted in soybean phospholipid liposomes by a freeze-thaw procedure [436]. The purified enzyme was not inhibited by vanadate or azide; however, NO_3^- was strongly inhibitory, as expected for a vacuolar type ATPase [436]. The reconstituted enzyme was stimulated by FCCP and valinomycin or nigericin in the presence of potassium. In addition, Dio-9 also stimulated the ATP hydrolytic activity of the reconstituted enzyme, perhaps due to its capacity of permeabilizing the membrane to anions [436]. Direct determination of the formation of a ΔpH (acid inside) was monitored with the probe ACMA [436]. The formation of the ΔpH was strongly stimulated by valinomycin in the presence of potassium and collapsed by the ionophore FCCP, demonstrating the electrogenic nature of the proton ejection process [436].

III-E. Miscellaneous H^+-ATPases

This section includes some vacuolar type H^+-ATPases the intracellular location of which is not yet totally clarified. They include enzymes from liver origin, probably localized in the plasma membrane (see Fig. 3, enzyme 7), and enzymes from microsomal renal fractions, some of which are thought to be localized in the plasma membrane and/or the Golgi apparatus.

III-E.1. The liver plasma membrane enzyme

A Ca^{2+}- or Mg^{2+}-activated ATPase has been solubilized by lysophosphatidylcholine from cholate-stripped rat liver plasma membrane fractions. It was poorly purified (12.5-fold) and reconstituted in asolectin proteoliposomes by a cholate-dialysis method [339]. The reconstituted enzyme generated an FCCP-sensitive ΔpH (acid inside) as measured by fluorescence quenching of

ACMA in a medium containing chloride ion. However, that was not the case when chloride was substituted by sulfate [339]. The generation of the ΔpH was independent of the presence of valinomycin in the presence of K^+ [339]. The ATPase activity was stimulated by FCCP by only 20% [339], perhaps indicating that the proteoliposomes contain other secondary transport systems. Therefore, the electrogenicity of the system could not be ascertained. Although the isolated ATPase was activated by Mg^{2+} or high concentrations of Ca^{2+}, no evidence for Ca^{2+} transport was presented, consistent with the findings that the reconstitution of other ATPases of the rat liver plasma membrane also fails to show ATP-dependent Ca^{2+} transport [338]. Moreover, the ATPase activity was resistant to oligomycin and vanadate. However, it was partially sensitive to DCCD, NEM and tributylin tin [339], suggesting its analogy to the H^+-ATPases of the vacuolar system [415–417].

III-E.2. The kidney medulla microsomal enzyme

A partially purified (40-fold) H^+-translocating ATPase responsible for urinary acidification was isolated from bovine kidney medulla microsomes and reconstituted in asolectin vesicles by dialysis of the detergent Chaps [437]. The ATPase preparations contained nine polypeptides in denaturating polyacrylamide gel electrophoresis, of 70, 56, 45, 42, 38, 31, 15, 14 and 12 kDa, forming a complex of 586 kDa as determined by high-pressure size-exclusion liquid chromatography, or two bands, of 551 and 523 kDa, in non-denaturating gels [437]. The mitochondrial origin of the isolated ATPase was excluded [437]. The enzyme was capable of generating an ATP-dependent NEM-sensitive $\Delta\psi$ (positive inside), as measured by the probe oxonol V. This $\Delta\psi$ was collapsed by gramicidin D, consistent with electrogenic proton transport by the enzyme [437].

Furthermore, an enzyme preparation from the same origin containing the same number of polypeptides as described above, in addition to a 33 kDa polypeptide, was purified by an immobilized anti H^+-ATPase monoclonal antibody [438,439]. The immunoaffinity-purified enzyme generated an ATP-dependent ΔpH (acid inside) as monitored by acridine orange [438,439] and a $\Delta\psi$ (positive inside) as monitored by oxonol V [438]. The ATPase inhibitor NEM prevented the formation of the ΔpH [438,439] and the $\Delta\psi$ [438].

III-E.3. The kidney cortex microsomal enzyme

Purification and reconstitution of an H^+-ATPase from bovine kidney cortex microsomes enriched in Golgi enzymatic markers, also containing endosomes, has been reported, and it was suggested that this enzyme could be responsible for transepithelial H^+ transport in the proximal tubule [440]. The purified preparation contained five polypeptides, with apparent molecular masses of 68, 58, 40, 37 and 16 kDa, and the ATPase was resistant to oligomycin and vanadate but sensitive to NEM [440]. This enzyme was reconstituted in asolectin proteoliposomes by removal of *n*-octylglucoside by dialysis and was capable of generating a ΔpH (acid inside) as measured by acridine orange [440]. The ΔpH was generated in the presence of valinomycin, suggesting the electrogenic nature of the H^+ transport. This ΔpH was absent in the absence of valinomycin, its formation was prevented by NEM and it was collapsed by the electroneutral H^+–K^+ exchanger, nigericin [440].

IV. Reconstitution of cation transport ATPases from prokaryotic organisms

Prokaryotic cells are provided with a series of cation-translocating systems [441–445]. In some instances ATPases are responsible for Ca^{2+} extrusion (see for reviews Refs. 441, 443), Na^+ extrusion (see for reviews Refs. 441, 444) and K^+ uptake (see for reviews Refs. 441, 444, 445). Fig. 7 represents the bacterial ATPases for the transport of potassium, sodium and calcium ions. Both a Ca^{2+}-transport and a K^+-transport ATPase have been isolated from different *Streptococci* and reconstituted in proteoliposomes, contributing to the understanding of the mode of operation of these cation-transport systems. However, little is known on the reconstitution of the prokaryote Na^+-transport ATPase.

IV-A. The Ca^{2+}-transport ATPase

An ATP-dependent Ca^{2+} transport system has been solubilized with Triton X-100 from *Streptococcus faecium* membrane vesicles and reconstituted without

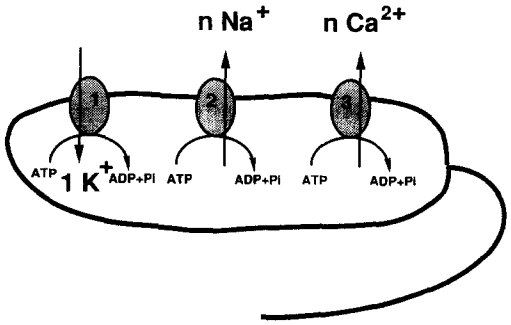

Fig. 7. Prokaryotic cation transport ATPases. (1) Potassium ion transport ATPase. (2) Sodium ion transport ATPase. (3) Calcium ion transport ATPase.

further purification in soybean phospholipids by removal of the excess detergent with Bio-Beads SM-2 [446] or with Amberlite XAD-2 columns [447]. The vanadate-sensitive ATPase activity could be further separated from vanadate-resistant ATPase activity in a 10–30% glycerol gradient before reconstitution [447]. The proteoliposomes were capable of an A23187 sensitive Ca^{2+} uptake strictly dependent on the presence of ATP as monitored with a Ca^{2+}-selective electrode [446] or $^{45}Ca^{2+}$ uptake [447]. The Ca^{2+} transport system was not affected by the presence of DCCD, an inhibitor of the BF_1F_0-ATPase [446,447] and also was insensitive to the presence of protonophores, valinomycin [446,447] or monensin [447] in the presence of potassium ion. However, the Ca^{2+} transport system was strongly inhibited by the presence of vanadate [446,447]. It was also demonstrated that a hydroxylamine-sensitive 60 kDa phosphoprotein is probably responsible for the Ca^{2+} transport, and represents a covalently phosphorylated intermediate of the Ca^{2+}-ATPase, since both vanadate and EGTA were capable of lowering the turnover of the ^{32}P-labelled phosphorylated intermediate.

These preliminary observations were later confirmed by reconstitution of the Ca^{2+}-ATPase in the other three members of the same bacterial genus [448]. The proteoliposomes were formed with phospholipids isolated from *Escherichia coli* or with asolectin by a dilution procedure from octyl glucoside-solubilized membranes [448]. To obtain a 20–30-fold increase in the activity of the ATP-dependent Ca^{2+} transport system, the solubilization was performed in the presence of a series of protein stabilizers such as glycerol, xylitol, sorbitol or glycine; however, ethylene glycol was ineffective [448].

It was excluded that the bacterial calcium transport was the result of the coupling of a secondary Ca^{2+}–H^+ exchange system to proton transport by the BF_1F_0-ATPase, since agents capable of collapsing a ΔpH such as nigericin (in the presence of potassium ion) did not affect the ATP-dependent Ca^{2+} transport [448]. Moreover, as previously described, the system was little affected by DCCD, and valinomycin (plus K^+) or FCCP slightly stimulated the Ca^{2+} transport system rather than inhibiting it [448]. The system was also highly sensitive to micromolar concentrations of vanadate [448]. The Ca^{2+}-transport system was not affected by Na^+, K^+ or phosphate, although SO_4^{2-} appears to have a somewhat inhibitory role of unknown cause [448]. It was also observed that $^{45}Ca^{2+}$-loaded proteoliposomes were capable of a rapid $^{45}Ca^{2+}$ release upon addition of excess unlabeled $CaCl_2$ [448], suggesting that an exit pathway was also present in the non-purified Ca^{2+}-ATPase proteoliposomes. Reconstitution of a highly purified bacterial Ca^{2+}-ATPase has not yet been obtained, but it would be of importance to clarify the nature of this $^{45}Ca^{2+}$ exit from the crude reconstituted preparations.

IV-B. The K^+-transport ATPase

A 190-fold purified K^+-transport ATPase from *Streptococcus faecalis* has been reconstituted by removal of Triton X-100 with an Amberlite XAD-2 column [449,450] or by this procedure followed by resolubilization of the liposomes with 3% octyl glucoside and subsequent dialysis, in order to obtain unilamellar liposomes of 50–100 nm in diameter [393]. For the reconstitution procedure, asolectin [393,449] or a mixture of 3 : 1 phosphatidylcholine/ phosphatidylethanolamine from eggs [450] was used. The purified ATPase consisted of a 78 kDa polypeptide capable of forming a phosphorylated aspartyl intermediate [449,450] and was strongly sensitive to vanadate (50% inhibition attained at 3 μM) [449].

The reconstituted enzyme increased the rate of ATP hydrolysis by 8-fold in the presence of valinomycin plus K^+ [393,449] and further doubled this rate in the presence of Triton X-100 [393,449], indicating, respectively, that the enzyme is responsible for K^+-transport and that it is randomly incorporated into the liposomal membrane [393,449]. However, the ATP hydrolytic activity of the enzyme is not stimulated by potassium ion [449]. The reconstituted ATPase was also stimulated to a lesser degree by the K^+–H^+ exchanger nigericin and it was not stimulated by the Na^+–H^+ exchanger, monensin, or the $Ca^{2+}/2H^+$ exchanger, A23187 [449].

Direct ATP-dependent K^+ transport was demonstrated in $^{42}K^+$-loaded proteoliposomes, or by the use of a K^+-selective electrode, and a K_m for potassium ion equal to 1.4 mM was determined [393]. The potassium efflux from the proteoliposomes was fully inhibited by somewhat higher concentrations of vanadate (100 μM) than in the solubilized system, and by anti-ATPase IgG [393].

The K^+/ATP ratio was initially equal to 1, but soon declined over time when a moderate membrane potential (negative inside) of -10 to -15 mV was generated, indicating that the $\Delta\psi$ regulates the coupling of K^+ transport to ATP hydrolysis [393]. In contrast, when a $\Delta\psi$ (positive inside) was artificially imposed in ferrocyanide-loaded proteoliposomes by adding ferricyanide to the outside and using phenazine methosulfate as an electron carrier (inducing electron transfer from the inside to the outside), this resulted in a transient increase in the rate of ATP-dependent K^+ transport and ATP hydrolysis [393]. Moreover, proteoliposomes loaded with polyelectrolytes, such as Polybuffer 74 or BioLyte 3/10 generate a positive inside Donnan potential and result in an increase in the initial rate of ATP hydrolysis. However, when the membrane surface potential was rendered more negative by the inclusion of dicetyl phosphate in the lipid bilayer or by introducing negative surface charges with the addition of sodium lauryl sulfate, the initial burst of ATP hydrolysis was totally

or almost totally abolished, indicating further that an electrical phenomenon regulates the rate of ATP hydrolysis and K^+ transport [393]. The importance of $\Delta\psi$ in the coupling of K^+ transport to ATP hydrolysis has been also demonstrated with the reconstituted H^+-ATPase from plasma membrane from yeast cells [388,389].

The proteoliposomes were not capable of ATP-dependent $^{22}Na^+$ transport or generation of a ΔpH [393], further supporting the concept that this ATPase is an electrogenic K^+-transport system, tentatively identified as the constitutive Ktr I potassium transport system of *Streptococcus* [393] resembling the Trk system of *Escherichia coli* [445].

IV-C. The Na^+-transport ATPase

A purified ($Na^+ + Mg^{2+}$)-ATPase (composed of five subunits) from the (wall-less prokaryote) micoplasm *Acholeplasma laidlawii* B has been reconstituted in vesicles of the photosensitive phospholipid, arylazidophosphatidylcholine [451] or dimyristoylphosphatidylcholine [452]. The profile of the elution of the reconstituted ATPase through a Sepharose 4B-CL column indicates that all the ATPase molecules are associated with the vesicles and that the Stokes' radii of the proteoliposomes are smaller than those of plain phospholipid vesicles [452]. Reconstitution resulted in enhancement of the ATPase activity [451,452], reduction in the sensitivity of the enzyme to radiation inactivation [451], stabilization against cold inactivation, oxidative degradation and thermal denaturation [452]. Photolysis of the arylazidophosphatidylcholine proteoliposomes resulted in the labelling of the α-subunit of the enzyme when [^{14}C]arylazidophosphatidylcholine was used [451]. This suggests that this subunit must penetrate into or traverse the phospholipid bilayer, supporting the conclusion that this enzyme does not belong to the F_1F_0-ATPase type [451].

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