1

Primary active transport

Introduction

A living cell must do work to maintain the composition of its internal medium different from that of the external. If that cell is growing, work has also to be done to generate the small but necessary osmotic gradient for the inwardly directed influx of water to take place. In a cell with a wall, such as a fungus, the osmotic gradient is much more significant, in order to maintain the necessary internal hydrostatic pressure for turgor (see Chapter 13). Two important loci for the above work are the plasma and the vacuolar membranes. When work is carried out in moving solutes across these two or indeed any other membranes, we can speak of active transport, i.e. the movement of solutes against their electrochemical potential gradient. Of course, there may also be diffusion of solutes across the membrane, albeit almost always, if the solute is polar, aided by the presence of carrier proteins that overcome the activation energy required by such a solute to enter through the lipid portion of the membrane. When diffusion occurs, it must be down the electrochemical potential gradient. However, it needs to be remembered that if the affinity of such a carrier for the solute on one side of the membrane were to be much higher than on the other or the mobility of solute transfer were to be faster in one direction than in the other – both of which changes would depend on the cell doing work – then active transport would occur (Jennings, 1974).

Active transport may be classified as primary or secondary. Primary active transport or translocation is brought about by reactions that involve the exchange of primary bonds between different chemical groups or the donation or acceptance of electrons. These reactions lead to the translocation of a chemical group or solute across the membrane. Thus, chemical energy is used to generate a vectorial process. Secondary

transport or translocation does not involve primary bond exchange between different chemical groups or donation or acceptance of electrons. Thus, the involvement of proteins in secondary active transport is different from that in primary active transport. In the latter process, the protein is acting like an enzyme, catalysing a vectorial rather than a scalar process. Proteins involved in secondary active transport do not function like the classical concept of an enzyme. It is for this reason that they are called 'porters' (Mitchell, 1967). A consequence of the mode of action of porters is that they are able to catalyse vectorial processes in a reversible manner.

The reactions bringing about primary active transport can be described as chemiosmotic (Mitchell, 1979). It is these reactions that ultimately drive secondary active transport or purely osmotic reactions (Figure 1.1). These latter reactions can be described either as uniport, namely the bringing about of exchange diffusion (Ussing, 1947), or sym- or antiport, in which there are coupled flows (Figure 1.2). A uniport will lead only to accumulation of a solute if it is charged and there is an appropriate electrical potential difference across the membrane to provide the driving force for such accumulation. Where there are coupled flows, the flow of one solute, i.e. either protons or sodium (Figure 1.1), down its electrochemical potential gradient can bring about the movement of

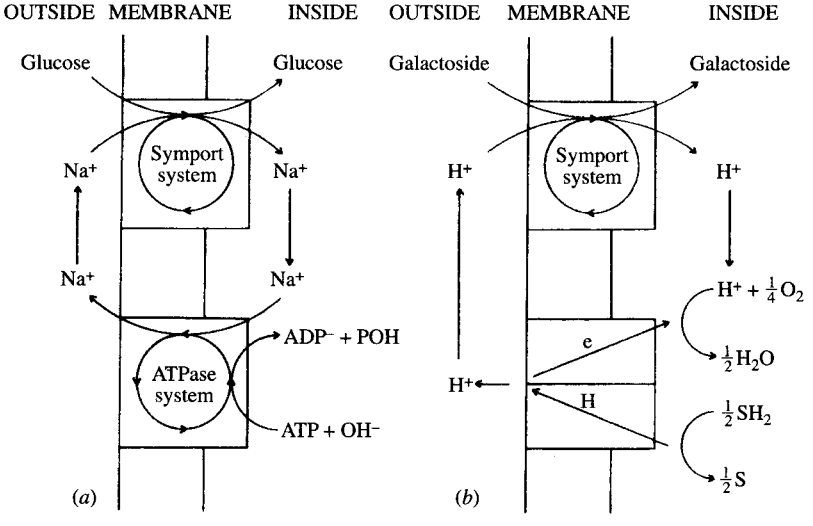

Figure 1.1. Coupling in two well-studied transport systems: (*a*) between the sodium-transporting ATPase and the sodium/glucose symporter in mammalian intestinal mucosa; (*b*) between a proton-motive respiratory chain system and an H^+/galactoside symporter in *Escherichia coli*. S, sulphur. (From Mitchell, 1979.)

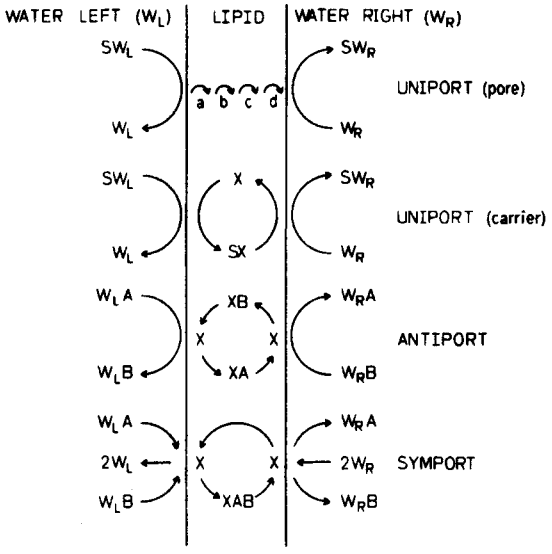

Figure 1.2. Diagrams of porter-catalysed translocation of solutes (S, A and B) across a lipid membrane between aqueous phases in which S, A and B exist as hydrates SW, WA and WB. Left and right aqueous phases are denoted by suffixes L and R. a–d and X represent a chemically specific pore and many carriers. (From Mitchell, 1979.)

another solute against its electrochemical potential gradient. Thus, there is active transport of one solute across the membrane brought about by the free energy in the gradient of the other solute across the membrane. The latter gradient of course is maintained by primary active transport. In considering secondary translocation, movement of charge is important, as well as any ensuing changes in concentrations on either side of the membrane. Figure 6.3 (p. 209) shows possible movements of charge that can occur as a result of secondary translocation.

As is discussed more fully below, primary active transport at the plasma membrane in fungi is predominantly the extrusion of protons using the free energy of hydrolysis of ATP. The evidence for any other primary active transport process is tenuous, a matter that also is considered below. As far as we know, therefore, fungi interact with the external medium almost entirely through a proton economy. There are indications that sodium can accompany solutes such as phosphate (see Chapter 7) in translocation processes but essentially it is protons that cotransport with other solutes requiring entry into or expulsion from a fungus. Secondary active transport is not considered as a particular topic. There is now such a

plethora of secondary active transport processes occurring at the plasma membrane that they are considered as appropriate in the relevant sections of the text dealing with utilisation of different nutrients.

The plasma membrane H^+-ATPase of *Neurospora crassa*

We know more about this particular primary active system than any other because it has been possible to probe it electrically, as well as biochemically and by use of molecular biological techniques. Details about the system have been reviewed elsewhere (Slayman, 1987; Sanders, 1988).

The reason there is greater knowledge about the H^+-ATPase of *Neurospora crassa* than that of other fungi is because this enzyme has been probed more extensively electrically. One cannot stress too strongly the importance of electrical studies for our understanding of active transport of charged atoms or molecules. In the case of primary active transport in *N. crassa*, electrophysiological studies have allowed a kinetic description of the process of proton extrusion, taking into account not only the characteristics of the ATPase *in vitro* and in membrane vesicles but also the membrane electrical field in which the enzyme resides.

The success of electrophysiological studies with *N. crassa* is due to the fact that, within mycelium growing on cellophane overlaying agar, there are hyphae 10–20 μm in diameter that are found 7–9 mm behind the growing margin (Slayman, 1965). The diameter is sufficiently large for insertion of electrodes for measuring the voltage and for either injecting current or measuring pH.

The functioning H^+-ATPase leads to the extrusion of protons electrogenically from the hyphae. Thus, we can speak of the enzyme acting physiologically as a proton pump. If the pump is non-functional, the membrane potential difference is *c.* -25 mV, inside negative (Slayman, 1970; Slayman, Long & Lu, 1973). When the pump is operational, the potential difference under normal physiological conditions is *c.* -270 mV, thus something like -200 mV of potential is generated by pump activity. When a mycelium is in 100 mM potassium chloride in a calcium-free medium, the potential difference is as low as -60 mV (Slayman, 1965); on the other hand, in the absence of chloride (which tends to depolarise the membrane) the potential difference can be as great as -300 mV (Blatt & Slayman, 1983).

The evidence that ATP is the substrate for the pump comes from the equivalence of the decay of the potential with the decay of ATP in the mycelium upon the addition of sodium azide. Both decay in an exponential

manner with maximal rate constants of $0.18\,s^{-1}$ and half-times of 3.7 s. One proton is pumped out of the hyphae for each molecule of ATP hydrolysed (Warncke & Slayman, 1980). This stoichiometry has been confirmed with studies on membrane vesicles (Perlin *et al.*, 1986; see below). With this stoichiometry and knowing the average rate of ATP synthesis (Slayman, 1973), it has been possible to calculate the proportion of ATP so synthesised that is utilised in pump activity (Gradmann *et al.*, 1978). The calculation shows that 38%–52% of the total ATP production is consumed by the pump.

The dissection of pump activity by current–voltage analysis has led to the production of a four-step kinetic model of pump operation (Slayman & Sanders, 1984; Sanders, 1988; Figure 1.3). The fast steps of the process of proton extrusion are the transmembrane movement of the proton and its dissociation from the carrier; the slow steps are the binding of protons and of ATP. Carrier cycling in the direction of efflux is limited by binding of protons on the cytoplasmic side; the model gives a pK_a for the internal binding site of 5.4 (cytoplasmic pH 7.2) and for the external site of 2.9 (external pH *c.* 5.5). This is important physiologically, because it means that the external pH must drop very considerably before there is an effect on pump activity. Thus, the pump can respond to changes in the internal pH without being very much influenced by the external pH (Sanders, 1988).

Under normal conditions of culture (pH 5.8) and with a potential

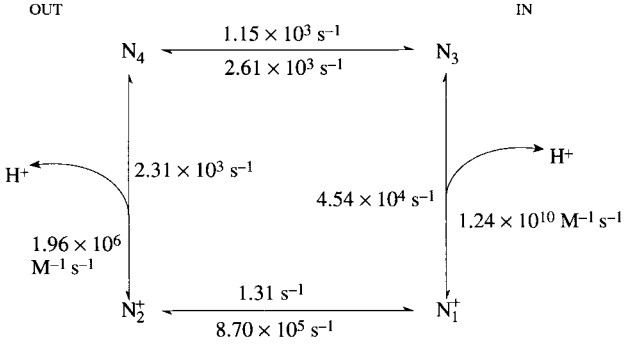

Figure 1.3. Four-step reaction kinetic model for the plasma membrane proton pump of *Neurospora crassa* derived from pH-dependent behaviour of the pump current–voltage relationships. Selective manipulation of the external and internal pH enables specific identification of the H^+-binding reactions. Those reactions that are voltage insensitive and thus not identifiable are lumped in the transition between carrier states 3 and 4. Values for the reaction constants from Slayman & Sanders (1984) are shown. (From Sanders, 1988.)

Table 1.1. *Physical and chemical properties of the different H^+-ATPases in* Neurospora crassa. *Citations in each column indicate references where the relevant information can be located*

	Plasma membrane	Mitochondrial membrane	Vacuolar membrane
Subunit masses (kDa)	104	59, 56, 36, 22, 21, 19, 16, 15, 12, 8	70, 62, 15
pH optimum	6.7	8.3	7.5
K_m (ATP) (mM)	1.8	0.3	0.2
Nucleotide specificity	ATP	ATP > GTP ITP > UTP > CTP	ATP > GTP ITP > UTP > CTP
Divalent cation specificity	Mg, Co > Mn	Mn > Mg > Co	Mg, Mn > Co
Inhibitors	DCCD, vanadate	DCCD, oligomycin, azide	DCCD, SCN^-, NO_3^-
References	Bowman & Slayman (1977) Scarborough (1977) Bowman *et al.* (1978) Bowman, Blasco & Slayman (1981) E. J. Bowman *et al.* (1981) Scarborough & Addison (1984)	Jackl & Sebald (1975) Mainzer & Slayman (1978) Sebald & Hoppe (1981) E. J. Bowman (1983)	Bowman & Bowman (1982) Bowman *et al.* (1986)

DCCD, *N*,*N*′-dicyclohexylcarbodiimide.
From Slayman (1987).

difference across the plasma membrane of −200 mV, the estimated current carried by the pump is 0.123–0.25 A m^{-2} (Gradmann *et al.*, 1978; Sanders, Hansen & Slayman, 1981; Gradmann, Hansen & Slayman, 1982; Sanders, 1988). This current is equivalent to a proton flux of 1.2–2.6 μmol m^{-2} s^{-1} (Slayman & Slayman, 1968). The net loss (net efflux) of hydrogen ions from the hypha under the same conditions is 0.04 μmol m^{-2} s^{-1} (Slayman & Slayman, 1968). This difference is highly likely to be due to the passive influx of protons.

There is now an extensive biochemical and molecular description of the proton pump. The major problem in the isolation of the plasma membrane H^+-ATPase is contamination with those ATPases that reside in other membranes, i.e. mitochondrial and vacuolar. Plasma membranes of *N. crassa* have been prepared in two ways. One way has been to use the cell wall-less mutant (*sl*) (Scarborough, 1975, 1988; Smith & Scarborough, 1984). The membranes are first stabilised against fragmentation and vesiculation with concanavalin A. Membranes thus treated can be purified by low-speed centrifugation and converted to vesicles by removal of the concanavalin A. Of course, the lectin acts not only as a stabilising agent but also as a marker for the plasma membrane. The other method of preparation involves the removal of hyphal walls by snail digestive enzyme, followed by gentle lysis (Bowman, Bowman & Slayman, 1981; Bowman & Bowman, 1986). The plasma membrane vesicles so formed can be separated by low speed or density gradient centrifugation.

The characteristics of the ATPases found in *N. crassa* are given in Table 1.1. It is not appropriate to go into further details than are presented there. However, it is necessary to highlight the different inhibitor sensitivities and other properties of the three ATPases, noting particularly the sensitivity of that of the plasma membrane to vanadate, the high specificity for ATP and the much lower pH optimum compared with that of the mitochondrial ATPase. While such features characterise the plasma membrane ATPase, they do not characterise it as a proton pump. For that to be possible, it has been necessary to study the functioning of the enzyme in plasma membrane vesicles.

The use of such vesicles has been pioneered by Scarborough (1975, 1976, 1980). Vesicles prepared as above contain a significant population of the everted type, namely those where the morphologically inner surface is exposed to the experimental solution. ATP hydrolysis therefore takes place when the compound comes into contact with such vesicles. Fluorescent compounds placed inside the vesicles during their preparation

(Scarborough, 1980) or radiolabelled or fluorescent compounds that distribute across the membrane according to either the pH gradient or the potential difference across it (Scarborough, 1976; Perlin *et al.*, 1984) can be used to determine the pH inside the vesicles and potential difference across their membranes. The temporal changes of internal pH and membrane potential on addition of ATP to the vesicles and the effects of inhibitors such as vanadate on such changes is in keeping with the ATPase acting as an electrogenic pump (Figure 1.4). Calibration of the potential change has been said to give a value of 120 mV (Slayman, 1987). This is much lower than what is observed for intact hyphae (see above); the difference is probably due to the leakiness of the vesicle membrane to protons. Vesicle preparations have been used to determine the apparent

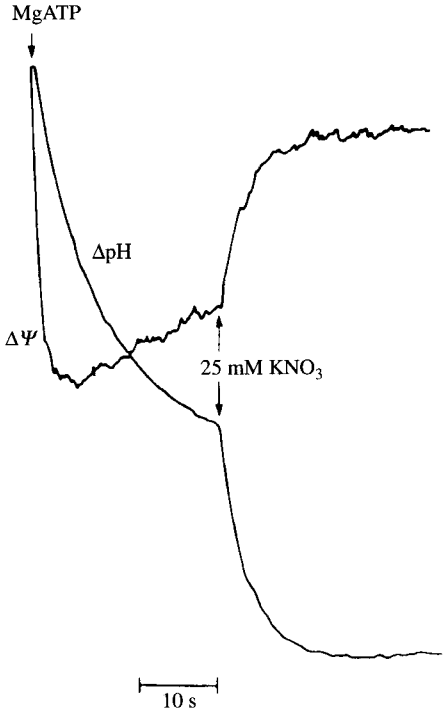

Figure 1.4. Superimposed demonstrations of the creation of a pH difference and a membrane potential in everted vesicles of the plasma membrane of *Neurospora crassa*. ΔpH, pH difference monitored via quenching of acridine orange fluorescence. ΔΨ, membrane potential (inside positive) monitored via quenching of oxonol V fluorescence. Quenching in both instances was initiated with 1.5 mM MgATP. Note that addition of the permeant nitrate ion at 25 mM reverses oxonol quenching and enhances acridine orange quenching. (From Perlin 1984.)

stoichiometry of H^+ translocated per ATP split by the ATPase; values close to unity were obtained, in keeping with values obtained by electrophysiological measurements (Perlin *et al.*, 1986).

A picture of the molecular structure of the H^+-ATPase in relation to its functioning is now starting to emerge. The structural gene for the enzyme has been cloned and sequenced (Addison, 1986; Hager *et al.*, 1986). The single polypeptide coded by the gene contains 920 amino acid residues and has a molecular mass of 100 kDa, confirming the similar value (105 kDa) found by gel electrophoresis studies (Dame & Scarborough, 1980). A hydropathy plot has allowed some idea of the transmembrane orientation of the polypeptide. The relationship of the structure of the polypeptide to function has been probed further either by incubation of isolated plasma membrane vesicles with trypsin (Dame & Scarborough, 1980; Addison & Scarborough, 1982; Hennessey & Scarborough, 1990; Scarborough & Hennessey, 1990; Hennessey & Scarborough, 1991; Figure 1.5) or by site-specific agents such as *N*-ethylmaleimide (for sulphydryl groups) (Brooker & Slayman, 1982, 1983; Davenport & Slayman, 1988; Chang & Slayman, 1990), phenylglyoxyl or 2,3-butanedione (for arginine) (Kasher *et al.*, 1986), *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline for carboxyl groups (Addison & Scarborough, 1986; Table 1.2). As a result of such studies we are obtaining a clearer view of the residues that are essential for catalytic activity. In particular, we now have a view of the transmembrane topography of the polypeptide and of those portions associated with the cytoplasm (Rao, Hennessey & Scarborough, 1991; Figure 1.6).

An important question for which there is an uncertain answer concerns the number of polypeptides constituting the functional proton

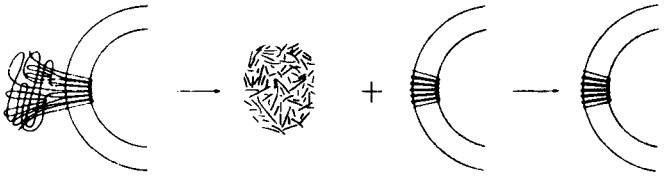

Figure 1.5. A diagram of the experimental approach to defining the membrane-embedded regions of the H^+-ATPase of *Neurospora crassa*. Vesicles with the enzyme molecules oriented predominantly with their cytoplasmic surface facing outwards are treated with trypsin. That part of the polypeptide molecule outside the membrane is cleaved, leaving that inside the membrane untouched. The peptides released by trypsin and those remaining in the membrane can be analysed separately. (From Rao *et al.*, 1991.)

Table 1.2. *Use of group-specific reagents to define functionally important residues of the plasma membrane H^+-ATPase of* Neurospora crassa

Residue	Inhibitor	Protection by ATP	Reference
Lys474	Fluorescein isothiocyanate	Yes	Pardo & Slayman (1988)
Cys532	*N*-ethylmaleimide	Yes	Pardo & Slayman (1989)
Cys545	*N*-ethylmaleimide	No	Pardo & Slayman (1989)
Arg	Phenylglyoxal, butanedione	Yes	Di Pietro & Goffeau (1985) Kasher *et al.* (1986)
Glu129	*N*,*N*′-dicyclohexylcarbodiimide	No	Sussman *et al.* (1987)
Glu/Asp	*N*-ethoxycarbonyl-2-ethoxy-1,2,-dihydroquinoline	No[a]	Addison & Scarborough (1986)

[a] Protection is seen in the presence of MgATP + vanadate (Addison & Scarborough, 1986).
From Nakamoto & Slayman (1989).