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Review

Functional principles of solute transport systems: concepts and perspectives [1]

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Contents

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[1] This paper is dedicated to Prof. Martin Klingenberg on the occasion of his 65th birthday.

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1. Introduction

Carrier proteins catalyze the translocation of solutes across biological membranes and thus provide a central function indispensable for every living cell. The significance of membrane transport is not simply to supply the cell with nutrients and other essential substrates but also to remove metabolic waste products. Besides this, membrane transport plays a role in other aspects of cell function, e.g., energy transduction, cell motility and information processing.

In the past, most investigations focused on characterizing the kinetic and energetic properties of particular carrier systems, each of which is responsible for some essential function within the metabolic network of the cell. As a result, the general impression was that of an enormous diversity of mechanisms and strategies in the way nature has solved the cells demand for catalyzing solute translocation. As in other fields, the impact of the techniques of molecular biology then significantly changed the strategies for solving questions of membrane transport. Consequently, major efforts are currently concentrated on structural aspects of the various carrier proteins. The first primary structures of carrier proteins were determined little more than a decade ago [1,2]. Nowadays a large amount of data on the amino acid sequence of various prokaryotic and eukaryotic carrier proteins is available, in many cases the transmembrane topology of these hydrophobic polypeptides has also been determined. Moreover, for several carriers not only is the 'original' structure known, but many amino acids of particular interest have been altered by random and site-specific mutagenesis, the most elaborate example being the β-galactoside:H^+ symport carrier of *E. coli* [3–5]. The most fascinating result of these investigations into the structural aspects of carrier proteins has been the recognition that most, if not all of these carrier systems, irrespective whether of prokaryotic or eukaryotic origin, have a common structural design, as expressed in the arrangement of transmembrane domains [6–8]. This finding immediately led to two conclusions. First, the common design of carrier systems should possibly be related to a common evolutionary origin, and second, it seems reasonable to assume that a surprisingly similar structural concept will be correlated to an equally similar general principle of function. The latter concept then obviously encouraged the suggestion of a consensus function [9] by membrane transporters in analogy to the impressive arguments for a consensus structure [6–8,10–12]. Thus, in studies trying to relate structural and functional properties, it was in turn recognized that the observed enormous diversity of transport systems could basically be explained by the modification of a few common principles. The variation of these principles could be explained (a) by the need for specificity for the structural demands of particular substrates, (b) by the particular energetic requirements under which a particular solute is transported, and (c) by the need for mechanisms to couple various kinds of energy input to the vectorial reaction of solute translocation. The latter requirement was frequently found to be solved with the help of additional peripheral domains.

Theoretical concepts for understanding carrier mechanism were first developed with the aid of detailed kinetic measurements. These led to concepts such as the 'simple' or 'complex' pore and carrier [13–17], as well as to methods and criteria for testing their significance [18–22]. In parallel, progress has been made in the elucidation of carrier mechanisms by using biophysical methods and interpretations. Although these concepts have already contributed significantly to understanding of the transport mechanism at times when it was impossible to work on the molecular level, valuable concepts with respect to energetic principles of membrane transport have especially been obtained in recent years (see 3.1). This development was stimulated by directly comparing the functional concept of enzymes and carriers. In fact, carriers have a lot in common with 'normal' enzymes, and direct correlation in energetic and kinetic terms shows that the basic principle of their catalytic cycle is closely related: (a) binding of substrate, (b) chemical transformation (enzyme) or translocation (carrier), and (c) product release. The main difference is the scalar (chemical) reaction in the case of enzymes, which is vectorial for carrier proteins, as well as the fact that the reactants of carriers are located in two separate compartments. As will be outlined later in more detail, this comparative approach has led to significant progress in understanding the energetics of carrier-catalyzed transport.

Obviously, there is encouraging progress with several aspects of the elucidation of membrane transport processes, from the structural point of view, from new strategies of functional analysis and from refined concepts of transport energetics. In contrast to many recent reviews, which have focused mainly on the structural aspect, the purpose of this paper is to describe the current state of correlation between the concepts of carrier mechanism and molecular data on their function. In the first sections, different aspects of carrier catalysis and conceptual principles of carrier mechanism will be outlined. In the following parts the discussion will concentrate on whether and how these concepts can be correlated to events on the molecular level for various functional principles. In this respect, three major questions will be addressed using representative examples of carrier proteins.

(1) How can the pathway of a substrate through the carrier protein be rationalized in molecular terms?

(2) What is the current state of knowledge concerning the mechanism(s) of coupling in carrier catalysis with respect to the different types of transport mechanisms, i.e., primary and secondary transport?
(3) Is the conceptual differentiation between carrier and channel still valid or have the concepts merged? In view of the unifying concepts for membrane transport which have been put forward based on structural and evolutionary relationships, this question may also be formulated to ask whether unifying concepts also exist for membrane transport function.

These topics will be discussed by using selected examples where the correlation of mechanistic concepts and molecular data is at an advanced state of elucidation. Even in view of this restriction, it was obviously necessary to arbitrarily select examples out of a variety of systems in a comparable state of characterization, e.g., in the case of P-type ion ATPases only the sarcoplasmic Ca^{2+}-ATPase is discussed, or in the case of secondary sugar carriers, where the intestinal Na-glucose carrier and the bacterial secondary sugar carriers (lactose and melibiose carrier) have been chosen.

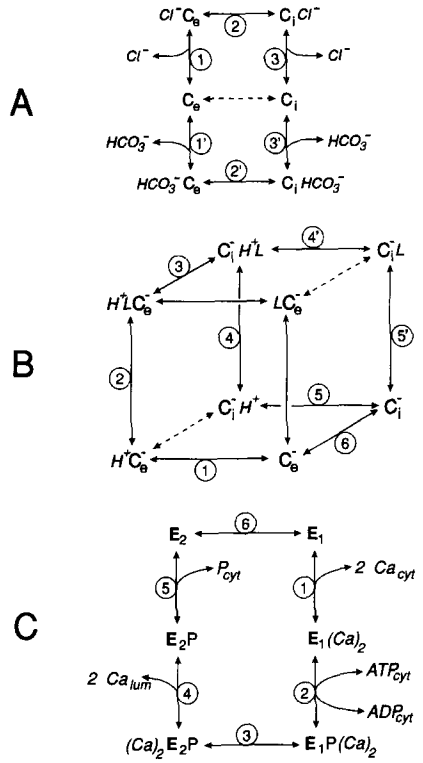

Fig. 1. Kinetic schemes for the anion carrier from erythrocytes (A), the β-galactoside/H^+ symport carrier from *E. coli* (lactose permease, B) and the Ca^{2+}-ATPase from sarcoplasmic reticulum (C). For these three examples, a minimum number of catalytic steps, labeled with consecutive numbers is shown. Broken lines indicate energetically or kinetically 'forbidden' transitions. (A) The complete heterologous exchange reaction of the red blood cell anion carrier, i.e., of a transporter with ping-pong-type kinetics, includes two identical half-reactions carrying two different substrate molecules. (B) Since in the case of the *E. coli* lactose permease, data indicating the specified binding order of the two substrates are available only for the external reactions (first H^+, then sugar [111,120]), a three-dimensional scheme is shown in order to provide the two possible reaction orders at the inside. The front part represents the transitions with outward-facing binding site (C_e), whereas the back part shows the catalytic steps with inward-facing binding site (C_i). (C) The simplified scheme of the Ca^{2+}-ATPase [27,475] includes the two major conformational states (E_1 and E_2) and the reactions at the cytosolic side (ATPase phosphorylation and dephosphorylation, Ca^{2+} binding), as well as that at the lumenal side (Ca^{2+} release into the sarcoplasmic reticulum, dephosphorylation).

2. Interpretation of carrier mechanism

In the course of many years, various experimental and conceptual strategies have been applied for understanding how a carrier protein works. Each approach has its own value and emphasizes a particular aspect of carrier catalysis. On the other hand, it seems to some extent confusing that a common process may be correctly described by greatly differing concepts.

The first and most widely used approach is the description by kinetic schemes. Fig. 1 exemplifies this concept for two secondary carrier proteins, the anion carrier from erythrocytes and the β-galactoside carrier from *E. coli* (lac-permease) and for a primary carrier, the Ca^{2+}-ATPase from sarcoplasmic reticulum. The examples of Fig. 1 will be discussed in detail below (4.2, 4.3, 6.1). This description is formally similar to the well known King-Altman schemes used for enzymatic reactions [23]. The carrier protein, represented as C or E (for enzyme, Ca^{2+}-ATPase), may exist in different states. They differ with respect to number and type of bound ligands (Cl^-, HCO_3^-, lactose, H^+, ATP, ADP, Ca^{2+} and P_i), with respect to covalent modification (e.g., acyl-P_i), and with respect to the conformational state of the protein (C_e and C_i, or E_1 and E_2, respectively). The connecting arrows indicate the probability of a reaction from one state to the other. Some connections are shown by broken lines which means that these pathways are kinetically or energetically 'forbidden'. In other words, the probability of a reaction along these particular pathways is extremely low. Charges may not only be attributed to substrates, but also to the carrier protein, i.e., to the substrate binding site itself. The advantage of this schemes lies in the fact that a formal kinetic description can be derived directly from it, including respective charge movements. The disadvantage is mainly psychological: it is tempting to take the indicated carrier 'movement' literally, whereas only a description of a conformational change is intended. Furthermore, it suggests that those, and only those, states shown in the scheme really exist, whereas the process may be much more dynamic including various substates.

A different description of carrier processes, which is also of great advantage, is much less frequently used. In Fig. 2 the energy profile for the mechanism of the adenine nucleotide carrier is shown (first part of the catalytic cycle) [24]. This diagram describes the free energy change in different steps of the catalytic cycle, the construction principle of a carrier thus becomes understandable in energetic terms. Moreover, the reason for a particular reaction being rate-limiting or even 'forbidden' (e.g., 'reorientation' of the unloaded carrier) becomes clear because of the presence of a high energy barrier for this reaction.

The energy profile of a carrier-catalyzed reaction has also been described differently [25–27]. In Fig. 3 the energy barrier model for the Ca^{2+}-ATPase (P-type

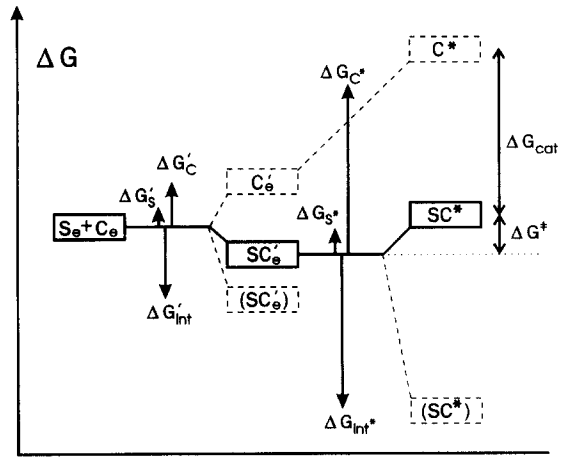

Fig. 2. Energy profile of a secondary carrier. This type of diagram was developed for the mitochondrial ADP/ATP carrier [24], based on energetic principles derived for primary transport systems [36,44]. For simplicity, only half of a translocation reaction of a secondary carrier is shown, i.e., altogether a quarter of the full catalytic cycle (cf. Fig. 1A). The arrows represent free energy contributions of conformational changes and binding interactions of both substrate (S) and carrier protein (C). In the first transition from the ground state with externally exposed binding site, both substrate (S_e) and carrier (C_e) must be converted into the binding state (C'_e, S'_e, for clarity, S'_e is not shown in the figure) by input of free energy ($\Delta G'_C$, $\Delta G'_S$). In contrast to enzymes, where the substrate S is changed during the reaction, $\Delta G'_C$ clearly dominates over $\Delta G'_S$. The negative free energy of binding interaction ($\Delta G'_{int}$) leads to the energy level of the carrier-substrate complex in the binding state (SC'_e). The high negative free binding energy of the interaction of carrier and substrate in the transition state (ΔG^*_{int}) is not realized because of the large positive conformational energy necessary to bring the carrier protein into its transition state (ΔG^*_C). The states indicated by boxes with broken lines are not expressed in this mechanism. The 'energy trap' of the (SC*) complex is avoided by the transfer of binding energy into conformational energy. The high free energy level of the transition state of the unloaded carrier (C*) indicates that in this type of carrier (coupled antiport) a transition using the unloaded carrier species is forbidden (cf. Fig. 1A). The utilization of binding energy for conformational changes of the carrier protein in both the binding and the transition steps (expressed as the 'catalytic energy' ΔG_{cat}) leads to a relatively smooth energy profile (small $\Delta G^{\ddagger}$) of the transport reaction.

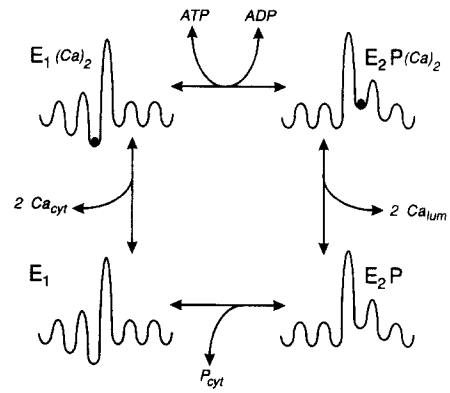

Fig. 3. Energy barrier diagram of a P-type ATPase (Ca^{2+}-ATPase) (modified from [27] with permission). In a multistep reaction, the substrate (black circle) moves to the internal binding site, further movement (transport reaction) is inhibited by the high energy barrier. Input of free energy from the chemical reaction (ATP binding and phosphorylation of the ATPase) is expressed in a conformational change of the protein thus moving the energy barrier to the other side of the substrate. The substrate dissociates along several intermediates and the unloaded carrier protein switches back to the original conformational state correlated with dephosphorylation of the ATPase.

ATPase) is shown. This scheme emphasizes the presence of multiple substates of the carrier, or multiple binding sites within the path of the solute. It furthermore indicates the putative 'movement' of the critical energy barrier which is essential for carrier function in preventing 'forbidden' reactions. This description is well-suited for explaining the differences between pores, channels and carriers, as well as for properly explaining experimental observations like leaks, slips and 'tunneling'.

Schematic models with an artist's impression are often helpful when trying to rationalize the molecular events or to emphasize a particular topological concept. As an example, in Fig. 4 the 'single-center gated pore model' as developed for the mitochondrial ADP/ATP carrier is shown [24,28,29]. The advantage of these models is inherently also their drawback. They lead to the impression that we in fact have a conception of the conformational changes occurring during transport catalysis, which is definitely not the case for any single carrier. However, the different steps (binding, translocation and release), as well as the presence of two ground states in which the binding site is exposed to different sides, are represented in a neat way.

This type of model may be refined when either distinct functional domains can be determined or when data concerning the 3D-structure are available. Obviously, this is easier if the protein is constructed of distinguishable domains (e.g., Figs. 9–11). True 3D structures on the atomic level have not been obtained for any carrier protein. At least two cases, however, are remarkable: the structure of bacteriorhodopsin as re-

fined by electron diffraction [30], and the X-ray structure of the outer membrane channel of *Rhodobacter capsulatus* [31,32] and *E. coli* [33].

It is obvious that every particular type of model has its own advantages and drawbacks. Although, for example, kinetics may in fact provide a correct description and even prediction of the macroscopic movements of substrates, inhibitors etc., it cannot be used to prove any molecular model of carrier function. On the other hand, even a correct 3D structure of a carrier protein will not, at least not immediately, give us an idea of the dynamic behavior of conformational states and substates nor will it give a simple answer to challenging questions about coupling and stoichiometry (Section 6).

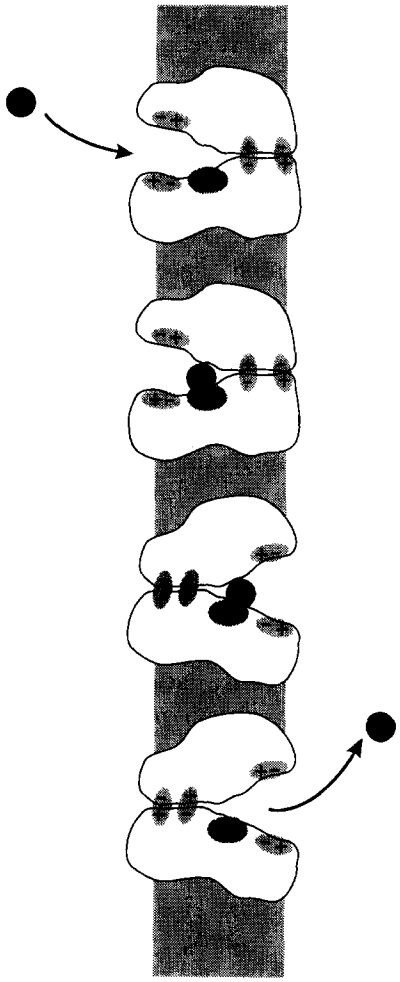

Fig. 4. General model of conformational changes related to the transport reaction of an anion carrier protein. The model includes putative charge interactions and represents a modified version of a scheme developed for the mitochondrial ADP/ATP carrier and designated as 'single center gated pore mechanism' [24,28,29]. Electrostatic interactions provide 'gating' mechanisms at the two sides of the membrane, alternatively 'closing' either the external or the internal access channel. Charge compensation is accomplished by interaction of the amino acid side chains in positions neighboring either along or across the access channel. It should be emphasized that the upper and the lower protein 'parts' in the scheme clearly do not mean two identical subunits, but simply represent an intersection across the substrate pathway.

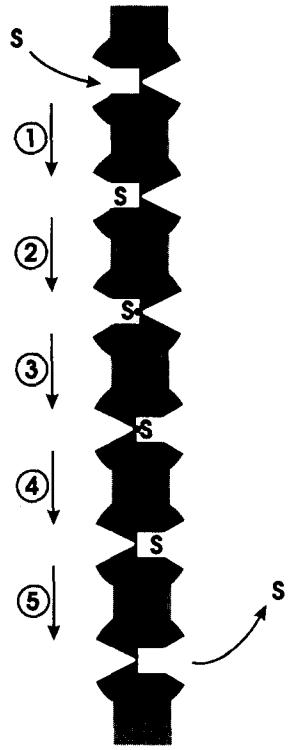

Fig. 5. Schematic model of the five basic reactions in carrier catalysis. (1) Diffusion into the access channel at side 1, (2) binding, (3) translocation, (4) dissociation of the substrate, and (5) diffusion out of the access channel at side 2.

3. Carrier catalysis: general background

3.1. The concept of carrier catalysis

The basic concept of carrier catalysis has been described in detail in numerous reviews both from the energetic and from the kinetic point of view [16,25–27,34–44]. Obviously, the aim of carrier catalysis is a vectorial reaction, i.e., solute movement from one compartment to the other. The overall reaction includes several steps (Fig. 5): (1) diffusion of the solute to the binding site, (2) binding of the solute, (3) translocation step, (4) dissociation of solute and carrier protein, and (5) diffusion of the solute away from the binding site. Steps (1) and (5) are not unimportant especially for channel-type proteins [45–48], because of solute interaction with the surface of the carrier and the phospholipid membrane. Furthermore, the binding 'vestibule' may geometrically restrict the solute's movement. These steps, however, are not part of the intrinsic carrier function and will be neglected here.

The translocation step as the central reaction in carrier catalysis involves a major conformational change (reorientation) of the carrier protein (or its binding sites), thus representing the essential 'switch' in the transport reaction. Since models involving rotational movement of the carrier have been discarded long ago for basic energetic reasons, the most simple rationale for this conformational change is a model based on alternating access of the solute to the binding site at the protein [37,49,50]. Most of the numerous models proposed for the translocation event are based on this concept (see also Fig. 4). A variation of the general model postulates the partial movement of the binding site in the conformational change which leads to an alternate access from the two sides of the membrane.

It is important for the further discussion to realize that, after translocation and solute release (Fig. 5), the carrier is in a conformational state different from that at the beginning [24,51]. Thus the reaction shown in Fig. 5 represents only half the catalytic cycle, and completion of the cycle needs 'return' reactions. The type of return reaction defines whether the carrier will catalyze net transport (return in the unloaded state) or exchange (return in the substrate-loaded state). This fundamental property of carrier proteins, i.e., the fact that the catalytic cycle involves two ground states with different conformations, is the basis not only for the vectoriality of carrier catalysis itself, but also for various mechanisms of energy coupling (see below). Moreover, this is a main difference between enzyme and carrier catalysis, since an enzyme is essentially in the ground state after release of the product(s) [36,52].

Since knowledge about the contribution of substrate-protein interactions to carrier function is important for the topics discussed below, a current view of carrier catalysis in energetic terms will be briefly outlined. A basic principle is the utilization of binding energy in carrier catalysis [24,36,44]. The free energy profile for carrier-catalyzed reactions was shown in Fig. 2. The intrinsic binding energy (favorable carrier-substrate interaction) is not expressed, but is 'stored' as conformational energy in the carrier protein, thus lowering the energy of the transition state. This leads to a smooth energy profile of the reaction [24,36,44], and thus to reasonable transport rates. The substrate only fits poorly to the active site in both ground states [24]. Thus, the energy realized in the binding step is small. Strong binding would be disadvantageous since it would trap the substrate in an unproductive state. The substrate, on the other hand, strongly binds to the carrier in the transition state. The active site will therefore be very 'substrate-like' in the transition state, in contrast to the two ground states (outward or inward facing, respectively). By this mechanism, the intrinsic binding energy is utilized to stabilize the transition state [24,44,53]. Consequently, good substrates are discriminated from poor ones in general not by differential binding to the ground state, but by interaction in the transition state. Thus, carrier proteins often express their specificity for different substrates by different transport rates (V_{max}) and not by different half-saturation constants (K_m or K_t) [53,54]. Another consequence is the fact that, in contrast to enzymes, good transport inhibitors will often be not closely related to the respective transport solute, since they should fit the ground state of the carrier protein thus leading to an energetic trap.

The energy profiles for primary carrier mechanisms are significantly more complicated; they have been extensively reviewed [16,27,36,44,55,56]. The concept of utilizing binding energy, which was in fact first described for that type of reaction [36,44], is the central aspect. The basic principles will be briefly outlined by one of the best studied examples, i.e., ATPase systems. These types of transport system are coupled to ATP synthesis or hydrolysis, i.e., to a chemical reaction with a large difference in free energy which is practically irreversible in solution. In order to render this reaction reversible, it is essential that this difference in free energy is not realized for the nucleotide bound to the enzyme. The difference between the large free energy of ATP hydrolysis in solution and that at the active site of the carrier/enzyme (the interaction energy) is caused by strong binding of ATP, but not of the products ADP and phosphate. It is essential that intermediates with very low or very high free energy are avoided, otherways the reaction will not occur at reasonable rates. Similar considerations are valid for ATPases which form acyl-phosphate intermediates during catalysis. Again, the formation of an acyl phosphate is highly endergonic in solution. Phosphate, however, is tightly bound to the Ca^{2+}-ATPase, leading to an equilibrium constant close to unity for the synthesis (and hydrolysis) of an acyl-phosphate from the free carrier protein and free phosphate.

In primary systems like those mentioned above, the situation is of course largely different from secondary systems. The conformational changes of the 'carrier domain' of primary systems and the change in affinity of solute binding are, in contrast to secondary systems, induced by the input of energy (from light or ATP) other than the binding energy of the transported solute. Nevertheless, the basic mechanistical concept with respect to the function of the 'carrier domain' of these primary systems is identical to that of the other carriers. Taking this restriction into account, the above mentioned example of primary carriers is well suited to emphasize that one central question in carrier catalysis is the coupling of one (exergonic) reaction to another (endergonic) one. In many types of carrier systems, most obvious in the case of the primary type, the flux of a certain solute is coupled to some kind of 'energy

input'. The carrier is therefore able to concentrate solutes on one side of the membrane, a process essential for all living cells. As discussed above, this energy input may be provided by an exergonic chemical (or photochemical) reaction or by the electrochemical potential of another solute, or by the membrane potential. It is obvious that different mechanisms for coupling must exist, with respect to whether chemical reactions are involved or whether simply two fluxes of solutes are coupled together. Whenever one of the reactions proceeds without the concomitant functioning of the other, the transport system will be uncoupled. These events will be discussed in detail in Section 6.

3.2. Classification of transport processes

Transport processes can be classified according to structural, kinetic, or energetic aspects. The concept introduced by Mitchell [57], based on the utilization of energy sources for transport, is now widely accepted.

(I) In primary transport the vectorial reaction of solute translocation is directly coupled to some kind of chemical or photochemical reaction. Primary transport systems thus directly convert light or chemical energy into electrochemical energy, i.e., the electrochemical potential of a given solute. This class of transport systems is relatively heterogeneous: photosynthetic electron transport and the ion pumps bacteriorhodopsin and halorhodopsin are light-driven, the respiratory electron chains utilize redox energy, several different systems are driven by the chemical energy of ATP, finally some sodium pumps utilize the chemical energy of a decarboxylation reaction.

(II) The only driving force in secondary transport is the electrochemical energy of a given solute. This energy is utilized to drive the uphill transport of another solute, i.e., against its own concentration gradient. This is achieved, depending on the direction of the electrochemical gradients, either by cotransport (symport) or by countertransport (antiport) of the driving and the driven ion. Because of the similarity in energy dependence and mechanism, also carrier-mediated unidirectional transport simply driven by its own electrochemical gradient is classified as secondary transport (uniport). Since symport and antiport may lead to uphill transport of the driven ion, they have been classified as 'secondary active'. However, this designation should not be used since the process is not intrinsically active. On the other hand, secondary uniport is often named 'facilitated diffusion'. This name is also misleading because a carrier catalyzed transport event is different in principle from the mechanism of diffusion.

(III) Group translocation differs from all other mechanisms by the fact that the transported solute becomes chemically modified during translocation. The only characterized systems catalyzing group translocation are the phosphoenolpyruvate : sugar phosphotransferase systems (PTS) in bacteria. From the energetic point of view, they are closely related to primary transport.

(IV) For comparison and discrimination, transport mediated by channels will also be mentioned to some extent. It is not always easy to discriminate between the different mechanisms (carriers, channels, pores) by which the flux of solutes through membranes is mediated (see Section 5).

In thermodynamic terms, a given carrier system under steady-state conditions should equilibrate the chemical/electrochemical potential of the driving force with that of the driven solute. In most primary transport systems the Gibbs free energy of the energy source is highly negative. This should lead to high electrochemical potentials and concentration gradients of the transported solute. Because of inherent leaks and slips (see 6.4 and 6.5) in the membrane as well as the carrier protein itself, the driving forces are often far from being in equilibrium with the electrochemical potential of the driven ion. In secondary transport, on the other hand, the driving forces are in general much smaller. The driving force of secondary transport consists of two components, the chemical and the electrical part. The former includes the chemical gradient of the solute ($\Delta \mu_{solute}/F$) and of the coupling ion ($\Delta \mu_{ion}/F$), which in the case of H^+ coupled secondary transport means $Z\,\Delta pH$ (F represents the Faraday constant and $Z = 2.3\ RT/F$). The latter, the electrical part, includes the contribution of the membrane potential if solute and/or coupling ion are charged. The different driving forces, as listed above, must of course be multiplied according to the transport stoichiometry and the number of individual charges. Solutes with different charges, both in sign and number, as well as different numbers of solute molecules per carrier protein are transported. Since these variations have to be considered for uniport, symport and antiport, the list of possible contributions to various driving forces is immense. Energy coupling in secondary transport in general and selected aspects of this field have been extensively treated in many reviews and books [16,35,41,42,58–63].

3.3. Structural basis

Major efforts in investigating carrier systems are currently focused on structural aspects. Consequently, this has been extensively described in many reviews [e.g., 7,8,10–12,64–67]. In order to appropriately connect the functional aspects to knowledge of carrier structure, a brief outline of the current view will be given here. In recent years, a common motif for many, perhaps all, transport systems has been revealed (see Section 1). Irrespective of the wide variety in function

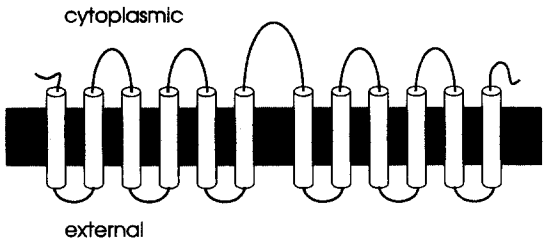

Fig. 6. A general model of a carrier protein of the 12-transmembrane-helix family [6,7].

and irrespective of whether the membraneous part of the carrier systems consists of a dimer (e.g., mitochondrial carrier proteins, some binding protein dependent systems, PTS systems), a monomer (e.g., most secondary carriers), or even only a domain within a complex protein structure (some PTS systems, P-type ATPases), protein structures responsible for membrane transport in general seem to be constructed of an array of about 12 (6 + 6) transmembrane segments (Fig. 6). This structural paradigm is best conserved in the large class of secondary carriers [7,8,10,12,64,68,69] which show similar structural properties, irrespective of whether they function as uniporters (facilitators), as symport, or as antiport systems. Within this class of transporters, based on sophisticated sequence comparison, further structural superfamilies have recently been identified [8,12,65–67,70].

Two general elements seem to be involved in this common structural theme [6,7]: the motif of about 12 transmembrane segments and the dimeric construction (true dimer or two intrinsically distinguishable halves of the protein). The latter motif has further been corroborated by experiments where carrier proteins have been cut into predicted segments by genetic methods [71–74]. When expressed properly, the individual halves obviously combine within the membrane into functional units. It has been argued that an oligimeric (dimeric) structure might in principle be advantageous for the catalytic function in transport [75,76]. Although many carriers actually function as monomers [7,77], these monomers may have a particular structure which corresponds to the action as a functional dimer (Fig. 6).

It should be noted that, besides this large family, there are further common structural principles in proteins which mediate transport. These principles may be closely related, e.g., the pattern observed for eukaryotic plasma membrane ion channels showing again multiples of (about) six transmembrane segments [7,12,78,79], or the related structural concept as detected in some receptors and channels [7,70]. On the other hand, completely different motifs are also found, e.g., the barrel structure formed of multiple β-sheets which is the structural principle of pore proteins from the outer membrane of bacteria or mitochondria [31,33,80,81].

4. Tracing the solute: the pathway through the carrier

Whatever the molecular mechanism of transport may be, the solute has to cross the membrane barrier. With the exception of some small (e.g., gases, ethanol, water) or hydrophobic (e.g., alkanes, uncouplers) substances, this process necessarily involves proteins. In principle, two possible pathways for a solute can be imagined: either through the proteinaceous part of the carrier/ channel or along the protein/lipid interface. Although the latter pathway has been discussed for particular cases [82], there is no indication that it is a common mode of solute flux. This alternative will not be considered here.

Numerous models describing the movement of a substrate through a carrier protein, as well as conceptions for operational parts to be involved in the vectorial reaction (gates, selectivity filters, binding sites, intrinsic channels, energy barriers etc.), have been suggested. The question is whether this reflects a variety of possible mechanisms or simply the lack of knowledge of the true mechanism. The current view of the construction principles of such solute pathways and their individual elements will be discussed in this section with selected examples.

4.1. A primary carrier with defined solute pathway: bacteriorhodopsin and halorhodopsin

The two retinal proteins from halobacteria, bacteriorhodopsin (BR) and halorhodopsin (HR), are light-driven proton and chloride pumps, respectively [83–89]. The retinal moiety is bound to a lysine residue (lysine-216 in BR) via a protonated Schiff base. The 26 kDa proteins contain 7 transmembrane helices and belong to the above mentioned structural family [90]. A model of the three-dimensional structure has recently been obtained by electron diffraction [30]. The seven helices are arranged in a circular manner, four of them mainly contributing to the formation of a transmembrane channel. The Schiff base is located approximately in the middle of this pathway, at an equal distance from the two surfaces. It separates the transmembrane channel into an extracellular and an intracellular part (see Fig. 7). The primary structure of HR is very similar to that of BR, which is also true of its topology in the membrane [83,84,88,91]. The physiological significance of these ion pumps is either generation of an electrochemical proton potential by proton ejection (BR), or osmotic balancing by inward pumping of chloride ions (HR).

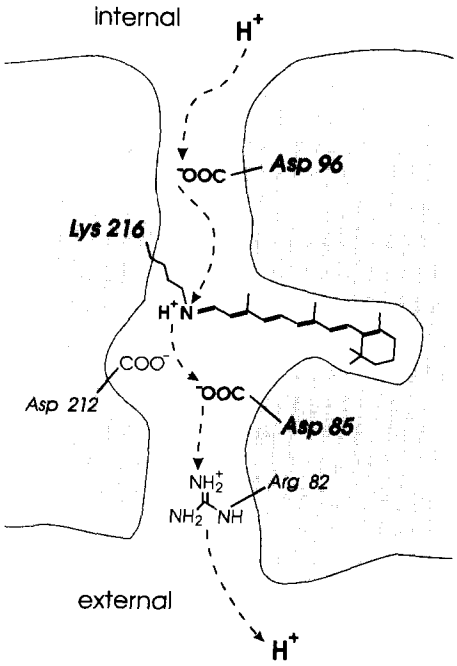

Fig. 7. Model of the substrate (H^+) pathway across the bacteriorhodopsin molecule (modified from [89] with permission). The putative H^+-pathway is indicated by the broken line. The retinal molecule, fixed to lysine 216 by a Schiff base, is shown in the middle of the pathway. The residues asp96, asp85 and arg82, are all located in helix C, whereas lys 216 and glu212 belong to helix G. The reactions involving the catalytic cycle are described in the text.

Upon photon absorption, the retinal moiety (protonated Schiff base) isomerizes around the double bond at C13/C14. The photoisomerization is thermally reversible, during reisomerization the chromoprotein undergoes several distinct steps (photocycle). In BR, the photoisomerization leads to the deprotonation of the Schiff base, followed by reprotonation and reisomerization. Obviously, the simple deprotonation/reprotonation cycle alone would not lead to the vectorial movement of protons. In order to function as a carrier, additional construction principles had to be developed (see below). The release of protons should take place only at the extracellular side and, vice versa, uptake is allowed only from the cytoplasmic side. In BR, this key concept is realized by a crucial 'switch' determining the vectoriality of the proton movement. The molecular explanation for this 'switch' is the rearrangement correlated to the *cis-trans* isomerization. Upon isomerization of the retinal, not only is the nitrogen of the Schiff base and the retinal moved, but the carbon chain of lysine-216 and the complete protein moiety also undergo conformational changes [88,89,92–94].

Although a defined spatial rearrangement correlated to the protonation/deprotonation event has thus been established, this construction is still not sufficient to provide vectorial proton movement. It is essential that the protons released after photoisomerization take a different pathway (to the extracellular side) as compared to that of the protons accepted (from the cytosol) in the thermal reisomerization. This fundamental principle of carrier function has been convincingly demonstrated for BR by recent investigations, based on biochemical and biophysical data from many laboratories including mutational studies [88,89,94,95]. In accordance with the location of the retinal halfway through the protein moiety, the proton pathway is divided into two halves. That part of the channel which connects the Schiff base to the cytosol (donor side) contains an important aspartate residue (asp-96) which delivers a proton to the deprotonated Schiff base. Also in the extracellular part of the channel (acceptor side) important residues (asp-85, possibly also arg-82 and asp-212) have been determined, asp 85 being the primary acceptor of the proton released from the Schiff base [96–102]. The probability of back reactions (H^+ donation to asp-85 or H^+ acceptance from asp-96) must be kept very low by an additional step within the reaction cycle. This is provided by the fact that the Schiff base changes its pK value dramatically in the catalytic cycle [93,103,104] from below 3.5 (donating H^+ to asp-85) to above 10 (accepting H^+ from asp-96). This 'switch' step is correlated to major conformational changes in the retinal and the protein moiety of BR [88,92–94].

Although the events occurring during the reaction cycle of BR are most conveniently described by starting with absorption of the photon, they can be equally well ordered in a sequence of events by analogy to other carrier mechanisms as follows [88] (Fig. 7). (1) Binding step: a proton enters the entry access pathway (moving along so far undefined residues) and binds to asp-96 (high pK). (2) Translocation step: (2a) the retinal captures a photon and undergoes trans to cis isomerization. (2b) The Schiff base protonates asp-85 in the exit pathway. (2c) The Schiff base changes from low to high pK. (2d) The Schiff base becomes reprotonated from asp-96 of the entry pathway. (2e) The Schiff base reisomerizes to the trans state. (3) Release step: the proton delivered to asp-85 (low pK) moves along other residues, possibly arg-82, and is released to the extracellular side.

In this scheme, several essential steps of this primary pump could possibly serve as a paradigm for other carriers, at least for primary transport. First, there are defined entry and exit pathways for the transported molecule. Second, the process is triggered by a crucial switch of the 'substrate (proton) binding site', correlated to a major conformational change of the protein. In this step, the solute does not move significantly within the protein. Third, besides the two steps of solute transfer to and from the intrinsic binding site (the Schiff base), the crucial third step consists of a change in the binding properties of this site (which

in the case of BR is irreversible), accompanied by a conformational change of the protein. In fact, BR is the only case where the molecular mechanism of a carrier protein has been elucidated to this extent. This was mainly possible due to the fact that various catalytic steps are correlated to spectral changes, by which particular events as measured by biochemical and biophysical methods could be correlated both spatially and in time.

A fascinating extension of this concept has recently been provided by comparative studies of BR with the second ion pump in halobacteria, i.e., halorhodopsin (HR). Besides many similarities (see above), the chloride pump HR mainly differs from BR in lacking reversible protonation and in having specific anion binding sites with changing affinity instead of proton binding sites. It transports chloride in the inward direction, in contrast to the movement of protons in BR. Interesting variations have now been introduced. If BR is altered by site-directed mutagenesis in lacking both the proton donor (asp-96) and acceptor (asp-85) [88,105], the molecular mechanism becomes similar to that of HR. On the other hand, by a particular experimental design involving the addition of hydrazoic acid as an artificial proton donor and specific radiation conditions, HR transports protons in the opposite direction to that observed in BR [88,106]. This demonstrates that the construction of a molecular machine converting light energy into vectorial ion movement is in principle a multipurpose device which can be modified for very different uses (ejection of protons vs. active uptake of chloride) by minor structural alterations.

4.2. The lactose carrier from E. coli and related systems: combination of kinetics and molecular biology

Whenever the structure-function relationship of secondary carriers is discussed, a particular example is inevitably recalled: the β-galactoside:H^+ symport carrier from *E. coli*, also called lactose permease (LacY). LacY is well-studied with respect to functional properties [3–5,107–116] and it is, together with the H^+ pump bacteriorhodopsin, undoubtedly the carrier where the most extensive mutational analysis has been carried out. It is thus an example well-suited for discussing the benefits and limitations of mutational analysis to resolve carrier function.

LacY catalyzes transport of a variety of galactosides together with one proton. The model of the secondary structure and membrane topology is based on data from many different methods including sequence prediction, spectroscopy, proteolysis, chemical modification, and immunological studies [for review see 5,117]. It has recently been refined by a series of phoA-fusions, confirming the motif of 12 transmembrane segments [118].

A simplified kinetic model of LacY is shown in Fig. 1B. The binding sequence at the outside (first H^+, second lactose) has been established, whereas it is not known at the cytoplasmic side [111,119,120]. The ternary carrier-lactose-H^+ complex and the unloaded carrier isomerize in the translocation steps. Three different modes of lactose transport are commonly used for functional characterization: H^+-coupled lactose uptake against a lactose gradient, H^+-coupled lactose efflux along the lactose gradient (zero trans experiment), and equilibrium exchange (labeled lactose outside against unlabeled lactose inside). In both uptake and efflux the complete catalytic cycle of the carrier (steps 1–6 in Fig. 1B) is involved, whereas for equilibrium exchange only steps 2–4 are needed. The membrane potential affects only uptake and efflux, but not exchange. It was thus concluded that only reorientation of the unloaded carrier is influenced by the membrane potential. Consequently, reorientation of the unloaded carrier is supposed to translocate one negative charge. The influence of H^+-binding and pH-gradient is complicated. Based on the pH-dependence of and solvent deuterium effects on lactose transport, it was concluded that H^+-binding/dissociation steps may be rate-limiting in the absence of electrical potential and pH-gradient [111]. In fact, the situation is even more complicated since it has been shown that there are two kinetically distinguishable substrate binding sites [121,122], a catalytically active one with low and a regulatory one with high affinity. Consequently, biphasic transport kinetics have been measured [111,115, 123,124]. Although the inhibition of efflux by an increase in external H^+-concentration could simply be explained by kinetic trans inhibition [111,112], additional (regulatory) sites may also be involved in this case.

This picture should be kept in mind when summarizing the studies aimed at localizing substrate binding sites and identifying the pathway of the substrate(s). Several groups have contributed to this work and different strategies have been employed. On the one hand, mutants have been selected showing altered substrate recognition [125–128] or resistance to toxic substrate analogs [129]. On the other hand, more than 200 site-directed mutants have been constructed [5,130].

The mutational approach was triggered by former results on the effect of chemical modification of particular amino acids on carrier function. In spite of the fact that LacY is inhibited by SH-reagents in a substrate-protectable manner [131,132], LacY is still active when each of its 8 cysteines has been changed [133]. The same holds true for the replacement of proline residues, although proline is supposed to have an important influence on the secondary structure of proteins [134–136]. On the other hand, replacement of his and glu had severe effects on LacY function. Based on

these studies, three neighboring amino acids, i.e., his-322, glu-325, and arg-302, have been interpreted in terms of an H^+ charge-relay mechanism, a motif which was discovered in serine type proteases [137,138]. By analogy to this, arg and glu are supposed to transfer protons to the his residue [139–144]. Defects in particular transport modes of LacY as observed in mutants of the putative charge-relay system were interpreted in the following way. In his-322 or arg-302 mutants all transport modes involving protonation or deprotonation are blocked, thus step 1 in the cycle obviously does not function (Fig. 1B). When glu-325 is replaced, deprotonation in step 4 is defective. Similar effects on proton coupling and transport function by mutations of histidine residues have been observed in related carrier proteins, e.g., the lactose carrier from *Streptococcus thermophilus* [62,145] or the tetracycline carrier from *E. coli* [146]. There is a body of evidence, however, which questions the significance of these findings, both in general for this motif as a model of a proton pathway in carriers and in particular for LacY. Carriers of the same subfamily [8], e.g., the sucrose:H^+ symporter from *E. coli*, lack this motif. In the melibiose carrier of *E. coli* (MelB), which uses H^+, Na^+, or Li^+ as coupling ions (see 6.5), no such histidine has been found. Only one of the histidines in MelB proved to be important, however, since replacement with arginine changed the expression and stability of MelB but not its catalytic activity [147,148]. Further evidence against the arg/glu/his motif being the proton pathway in LacY came from mutants where his-322 has been replaced by tyrosine [149,150] or by aparagine [151]. In these mutants, both binding and translocation of H^+ coupled to galactosides can be observed. Furthermore, a double mutant with the substitution of ala-177 and his-322 catalyzes proton-coupled lactose influx [152]. So far, the only conclusion which may be drawn is that this histidine seems to be at least important for sugar recognition and/or accumulation.

The function of MelB furthermore raises another problem, i.e., that of the equivalence of protons to other monovalent cations. This question will be discussed in more detail in connection with the F_1F_o ATPase (4.4). It is clear that in thermodynamic terms there is no significant difference between the movement of H^+ and Na^+, and cation binding sites can accomplish the binding of both ions. In fact, several observations argue for H^+ and Na^+ being transported on the same pathway in MelB. (i) H^+ and Na^+ mutually compete in transport coupled to melibiose and (ii) mutants exist in which the alteration of a single amino acid changes the specificity for the coupling ion [153–157]. It is obvious, however, that a charge-relay system cannot function in the same way in transporting Na^+-ions.

Fortunately, identification of the sugar binding site

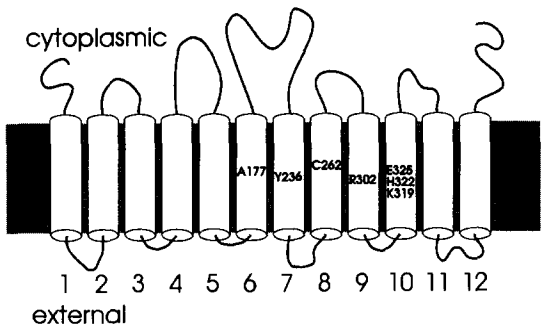

Fig. 8. Structural model of the lactose permease from *E. coli*, based on refs. [161,163,164]. The position of those amino acids mentioned in the text in connection with the substrate pathway (Section 4.2) or the mechanism of energy coupling (6.5) are indicated in the model.

seems to be somewhat clearer. A vast number of single, double, and even triple mutants of LacY with altered substrate specificity have been obtained or constructed. Most importantly, the amino acids which seemed to be important for substrate specificity were restricted to a few (only about 8) positions. A change in sugar specificity, however, does not necessarily mean that the amino acid residue responsible for this change must be located in the substrate binding site (cf. discussion in 6.5). A conformational effect on the binding site could lead to the same result. Nevertheless, the low number of mutations causing specific changes and the fact that most of these mutations affect amino acids in a relatively limited area (core area of putative helices VII to X, see Fig. 8), argues in favor of these residues being directly involved in substrate recognition. By using similar strategies, amino acid residues responsible for substrate recognition in MelB have also been mapped [153–160]. Interestingly, also in this case a very limited number of mutations affect substrate binding and/or transport in a specific way. In locating these residues in the topological model of the melibiose carrier, however, no similarity to the pattern obtained for LacY is observed [62,154].

Recently, the results on the amino acids important in sugar recognition have been combined with investigations on the spatial arrangement of the 12 transmembrane helices, e.g., by defining salt-bridges between charged residues [161–164]. A suggestive picture was thus derived [62,127,163–165]. The pathway for the sugar molecule (and probably also for the proton) seems to be localized in a region between helices V (or VI) to X (Fig. 8). More or less all residues detected as essential in substrate recognition in fact face this common 'pathway'.

4.3. The anion carrier of erythrocytes: identification of functional substructures by chemical modification

In primary carrier systems, different steps in the catalytic cycle can often be correlated to the function

of defined substructures in different subunits (e.g., F_1F_o ATPase, see 4.4) or subdomains (e.g., sarcoplasmic Ca^{2+}-ATPase, see 6.1). For secondary carriers, the erythrocyte anion carrier is an example where this correlation has reached a significant level. This has been achieved by the extensive use of noncovalent and covalent binding inhibitors and effectors, in fact more than 200 interacting reagents are known [166–169]. Although significantly improved recently [170], the structural characterization and definition of structure-function relations are nevertheless still on a relatively low level in molecular terms in comparison to well-known examples of bacterial carriers (see 4.2). In view of the enormous amount of contributions to the understanding of this particular carrier protein from many different groups, mainly review articles are cited here with respect to the basic aspects.

The anion carrier, also called band 3 protein after its identification in SDS-gels, catalyzes the exchange of Cl^- and HCO_3^- across the red blood cell membrane, thus improving the CO_2-carrying capacity of the blood. The glycoprotein with a molecular mass of 95 kDa is very abundant in the red cell and exists in different states of aggregation [167,171,172]. Its catalytic activity is extremely high, much higher than that of the other carriers mentioned in this review [166,167]. The anion carrier transports a surprisingly large number of different anions with widely varying rates, from small monovalent anions (halides, about $5 \cdot 10^4$ Cl^- ions per second at 37°C), to divalent anions (e.g., sulfate, about 10^3 times slower) and bulky organic anions (about 10^4 times slower) [167]. The tentative secondary structure of the hydrophobic transport domain [167,173,174] consists of 14 transmembrane helices [175,176]. It functions in an electrically silent 1 : 1 exchange of monovalent anions in ping-pong-type kinetics (Fig. 1A) [177–179]. This mechanism implies one single binding site alternatively exposed to the two sides of the membrane [180,181]. Consequently, if the concentration of anions is different on both sides or if different substrates (with different transfer rates) are present, a recruitment of binding sites is observed. Recruitment means the asymmetric distribution of outward- and inward-facing binding sites, i.e., the binding sites will accumulate at the side where the substrate concentration is lower or where the substrate with the lower transfer rate is present. This situation is especially useful for the application of chemical modification to characterize different conformations of the protein.

Various sites and particular amino acids have been identified and correlated to specific substructures by the application of various reversible and irreversible inhibitors and modifiers. About 14 different interacting sites including the substrate binding site have been listed [166]. The most intensively investigated site is that of stilbene disulfonate derivatives. It is accessible only from the extracellular side and can be recruited to the outside. All available data indicate that the outward-facing anion binding site is contained in the stilbene disulfonate binding site. There are a number of chemically different inhibitors of the anion carrier which bind in a mutually exclusive manner with stilbene disulfonates [166]. The different size of these inhibitors and different location of interacting groups provided a detailed picture of the outward-facing substrate binding site. Two different lysine and several arginine residues are directly involved in substrate binding and in translocation [166,167]. In addition to specific interaction, the cluster of positively charged groups leads to changes in the electrostatic field and local pH, thereby attracting anions.

A long list of amino acids has been defined as important for anion binding or transport or related to the binding site by conformational interaction [166,167,182–184]. In addition, site-directed mutagenesis has recently been successfully employed to explore the substrate pathway. As with the lactose carrier in *E. coli* (4.2), specific functional properties could in general not be attributed to particular amino acids [185], since direct functional and conformational effects are not easy to separate. Nevertheless, lysine residues in the putative hydrophilic access channel have been identified by mutational analysis [185,186]. These residues not only define the access pathway of the substrate anions, but at least one of them is linked to the substrate binding site by conformational interaction. This demonstrates the complications which frequently arise in such modification studies, i.e., that the susceptibility to chemical modification of one site depends on the conformational state (in this case occupancy with substrate) of a second site.

Two examples will be mentioned to exemplify the usefulness of the chemical approach in identifying residues with particular importance for catalytic steps. The interaction of stilbene disulfonates and pyridoxalphosphate (two substrates of the anion carrier transported extremely slowly) with certain external lysine residues triggers a conformational change in the carrier protein. By this rearrangement, the labeled ligand, previously bound from the outside, now becomes accessible to reagents from the internal side [183,187]. This study again demonstrates a common carrier mechanism [183]. After opening the external hydrophilic access channel and fast electrostatic interaction with residues in the channel, the substrate becomes bound to the active site which is recruited to the outside. Binding then triggers the conformational change ('switch' of the intrinsic binding site), which in turn opens the inner and closes the outer hydrophilic access channel [183,187].

An equally interesting picture was obtained by chemical modification of a particular glutamate residue

[188–190]. As mentioned above, the anion carrier also accepts sulfate. For the translocation of divalent anions, this glutamate residue must be protonated, consequently, protonation of the same group inhibits transport of monovalent anions (see also 6.6). After the reaction of a carboxyl group with the specific reagent (Woodward K) and subsequent reduction to the corresponding alcohol, the carrier behaves as if this glutamate were permanently protonated, i.e., monovalent anion transport is inhibited whereas that of divalent anions is accelerated. However, sulfate influx is no longer coupled to proton flux. On the other hand, sulfate interaction with the internal binding site can also be altered by modifying the carboxyl group from the outside. Taken together, these results demonstrate that this particular carboxyl group is within the central binding site of the anion carrier, i.e., it represents at least part of the permeability barrier which can be contacted from the external and from the internal side in the alternative conformations of the protein.

These investigations on the erythrocyte anion carrier are valuable arguments for a decision between two possible models of the permeability barrier in the pathway of the substrate [167]. On the one hand, the 'thick barrier' model postulates a 'zipper-like' array of pair charges which are thought to be broken and reformed during the passage of the substrate through the transport pathway. On the other hand, the 'thin-barrier' model postulates that the major portion of the transport pathway allows free access of the substrate to the thin permeability barrier which changes its position during translocation. The latter model exactly reflects the situation described for bacteriorhodopsin (see 4.1). Also in the case of the anion carrier, the majority of the results, some of them mentioned above, tend towards the direction of a thin barrier.

As mentioned above, the anion carrier accepts very different substrates. Interestingly, the substrate specificity is mainly expressed in transport rates which differ by more than six orders of magnitude [183]. Thus the anion carrier is a classic example of the statement that the basis for selectivity is not simply the size of the substrates passing through the access channel or their interaction with so-called 'selectivity filters', but presumably their ability to stabilize the transition state of the carrier by the utilization of binding energy [36,191]. The different ability to form the appropriate transition state is expressed in different transport rates according to the different ability to transform the carrier from an immobile into a mobile state.

Another important feature of carrier processes, which has been extensively studied in the case of the anion carrier, is the occurrence of reactions other than that of the 'normal' catalytic cycle of a carrier, namely leaks, slips and tunneling events [192–194]. The importance of slippage in carrier mechanism is discussed in more detail in Sections 6.4 and 6.5.

4.4. Are protons special substrates?: The F_1F_o ATPase, the uncoupling protein and other H^+-conducting carriers

Whether protons are special transport substrates differing from other ions is an old question in carrier biochemistry. In fact, the abundance of H^+ binding sites in proteins and the versatility of possible modes by which protons can pass through a protein represent a special problem in ion translocation [37]. This has led to various hypotheses on the mechanism of H^+ translocation, including the so-called 'proton wire' of proton conductance channels [195,196]. Proton transport is involved in many transport systems, and one of the examples where questions concerning the mechanism of proton translocation has been discussed extensively is the F_1F_o ATPase. This discussion is, of course, related to the discrimination of carriers and channels which will be treated in Section 5.

The F_1F_o ATPase is a multisubunit complex consisting of the hydrophilic F_1 part and the membrane-embedded F_o part [197–202]. The F_1 part contains several nucleotide binding sites with strong cooperativity. Mitochondrial and chloroplast enzymes, due to kinetic reasons, mainly function in the direction of ATP synthesis, whereas bacterial enzymes in general catalyze both directions equally well [203]. Several mechanisms have been suggested for ATP synthesis, the most widely accepted being the 'binding change' or 'alternating site' model with three interacting catalytic sites [204–206]. According to the principles of utilization of binding energy (Section 3), ADP and P_i bind strongly to the enzyme. Whereas the formation of ATP at the binding site does not involve a significant change in free energy, the main energy-consuming step is the release of bound ATP [204,207]. The detailed mechanism of ATP synthesis is not fully understood, and this is even more true of the mechanism of energy transduction from the electrochemical H^+ gradient to force the release of ATP from the enzyme. The crucial point of interest here is the question of how these protons are transported through the F_o part. This also refers to the old problem of direct vs. indirect coupling. Direct coupling means that the protons transported through F_o are (directly) involved in the chemical reaction of ATP synthesis, as originally suggested [208]. A mechanism of indirect coupling, on the other hand, assumes that H^+ translocation induces a conformational change in the whole enzyme including the F_1 part thereby providing the catalytic energy for the chemical reaction. Whereas most experimental evidence supports an indirect coupling, i.e., by long-range conformational changes [201,205,209–211], the mechanism of proton translocation through F_o is still under debate.

The F_o part of the F_1F_o ATPase typically consists of three subunits a, b and c with a stoichiometry of $1:1:10 \pm 1$ (*E. coli* ATPase) [212]. Covalent reaction of dicyclohexylcarbodiimide (DCCD) with one crucial carboxylic side chain (asp or glu) in subunit c blocks proton translocation through F_o and coupled ATP synthesis/hydrolysis in F_1. Since direct coupling is unlikely, the proton pathway does not necessarily need to traverse F1. The observed coupling stoichiometry of F_1F_o ATPases is 3 H^+/ATP. The maximum rate of ATP synthesis by the chloroplast enzyme is about 100–1000 s^{-1} [213]. The proton transfer rates which are observed for F_o, either after stripping off F_1 from F_1F_o, or after isolation and reconstitution, vary widely. In the *E. coli* enzyme, rates of 6–20 $H^+ \cdot s^{-1} \cdot F_o^{-1}$ [214] were observed, which are comparable to those measured in the mitochondrial ATPase. On the other hand, evidence has been provided that after removal of F_1, the remaining F_o part of the chloroplast ATPase acts as a proton channel, with translocation rates of up to 10^6 $H^+ \cdot s^{-1} \cdot F_o^{-1}$ [215–217].

In spite of attractive models of an H^+ conductive pathway through F_o and the observations of channel-type H^+ flux in F_o isolated from chloroplasts, there is now convincing evidence that protons are translocated through F_o by a carrier-type mechanism. It can be shown both with respect to activity and selectivity that a channel-type 'water wire', like that observed in gramicidin cannot account for proton translocation in F_o [218]. Several observations argue further for a carrier-type mechanism. Under particular conditions, a slip-type of H^+ translocation (i.e., proton transport uncoupled from ATP synthesis/hydrolysis) can be induced in F_1F_o ATPases [reviewed in 219], this mode is indicative of carrier mechanisms. Additionally, proton binding groups with very high cooperativity have been detected [62,220]. This finding can be interpreted in terms of Mitchell's concept of a proton well within F_o [34]. It may be possible that the above mentioned findings indicating a channel-type function of the F_o subunit of the chloroplast ATPase were caused by the isolation procedure leading to a portion of severely 'distorted' F_o with altered conduction properties.

Possibly the most convincing evidence for a carrier-type mechanism comes from studies with the F_1F_o-type ATPase from *Propiogenium modestum* [221–223]. This bacterium establishes a primary Na^+-gradient by decarboxylation coupled to Na^+ transport (see also Section 7). ATP synthesis is then catalyzed by an Na^+-dependent F_1F_o-type ATPase. The Na^+ binding site is located in the F_o part of the enzyme as shown by the construction of hybrids from the *E. coli* F_1 and *P. modestum* F_o, which act as an Na^+ pump [224]. It has furthermore been shown by in vivo complementation of an *E. coli* deletion mutant that the 'normal' F_1F_o ATPases coupled to protons and the *P. modestum* ATPase are obviously very similar [225]. The striking point is now that at low Na^+ concentrations (< 1 mM) the *P. modestum* ATPase functions as an H^+ pump [226]. Thus F_o can translocate both Na^+ and H^+, which is much easier to reconcile when considering hydronium ions (H_3O^+) instead of protons since the size of hydronium ions is very similar to Na^+ [227]. These findings argue against some of the models mentioned above, since Na^+ cannot be transported in conductive mechanisms as has been discussed for protons. Recent results have confirmed a carrier-type mechanism for the *P. modestum* ATPase. It was shown that F_o behaves asymmetrically with respect to ion translocation, that different driving forces (membrane potential and Na^+-gradient) are not equivalent and, most importantly, that F_o-catalyzes Na^+ counterflow [223,228]. This is generally accepted proof of a carrier mechanism. Taken together, these results exclude a channel-type mechanism for ion (Na^+ and presumably H_3O^+) translocation in the *P. modestum* ATPase and most probably also in the closely related F_1F_o-type ATPases from other sources. This, of course, does not rule out the presence of putative access channels to the respective ion binding sites in the interior of F_o, however, also in these channels ions must be transported in a way which can accommodate for both H^+ and Na^+.

Several examples have been mentioned where H^+ binding sites have been identified in carrier proteins, e.g., bacteriorhodopsin, the erythrocyte anion carrier and also the lactose permease and related transporters. Finally, the presence of fixed binding sites for protons instead of a conductive pathway will be briefly exemplified by another type of proton translocator, the uncoupling protein (UCP) from brown fat mitochondria. UCP converts the electrochemical proton potential generated by mitochondria in brown adipose tissue into heat by recycling the ejected protons [229–233]. In spite of the different ions to be transported, UCP is clearly a member of the structural family of mitochondrial anion carriers (see 6.5) [234]. H^+-translocation by UCP is regulated by various parameters including pH and the inhibitory action of purine nucleotides [229,235–238]. In addition to protons, UCP was also found to transport Cl^- [229,230,239–241], which was taken as an indication that the actual transported substrate is the OH^- ion [230].

A striking property of H^+ transport by UCP is the activation by free fatty acids. Since the discovery of this dependence [236,238], several hypotheses have been put forward to explain the effect of free fatty acids and related hydrophobic anions on UCP activity, including a postulated role as transport substrates [240,242]. An interesting alternative model has been recently presented [243]. It is based on observations with respect to modulation of the pH-dependence of H^+ translocation by fatty acids and the lack of this modulation in the

case of Cl^--transport. The model furthermore overcomes the problem of the extremely low substrate concentration (below 1 μM!). The carboxyl groups of specifically bound fatty acids are taken to function as 'prosthetic' groups, donating and accepting protons from a central H^+ binding site with alternative access from the exterior and the interior. This model would again also mean 'classic' carrier-type transport for protons. It furthermore resembles the functional model derived for bacteriorhodopsin including proton donor and acceptor sites. The obvious difference between these two systems is the presence of an active, light-driven 'switch' in the retinal and in the protein moiety of bacteriorhodopsin, whereas this conformational change is passively triggered by occupation of the respective binding sites in the UCP.

4.5. Diversity of translocation pathways from protons to ATP: The mitochondrial carrier family

The mitochondrial carriers, mainly catalyzing the transport of anions between the mitochondrial matrix and the cytosol of eukaryotic cells, form a family characterized by a close structural relationship [234,244–247]. They function as dimers, consisting of two identical subunits with 6 transmembrane segments each. Although thus adding up to the 'canonical' 12 transmembrane segment motif family (see Section 3), structural arguments have been put forward indicating that the mitochondrial carriers in fact represent a family of transporters of independent evolutionary origin [66]. Among other data especially the tripartite structural motif, which is unique for this carrier family, argues for this interpretation. Besides the availability of a detailed structural characterization, especially the adenine nucleotide carrier and to some extent also the proton carrier (UCP, see 4.4) are among the most extensively studied secondary carriers in terms of function and regulation [231,232,248–253].

Several properties of these carriers are noteworthy with respect to transport function and the substrate pathway. In spite of a similar topological arrangement of transmembrane segments and hydrophilic loops, this family of carriers can handle the most extreme types of substrates: from one of the largest solute molecules to be transported (ATP) to the smallest (protons). It is thus of major interest to elucidate the substrate pathway within this family of carrier proteins, to gain insight into the possible variations of a common theme for adapting protein structure to very different functional requirements (see below).

Also in another respect, these proteins seem to be interesting candidates for elucidating carrier mechanisms. Kinetic studies in reconstituted systems have shown that these carriers in general function in a simultaneous (or alternatively called 'sequential') type of kinetic mechanism. By analogy to enzymes, this means for carriers that the second substrate binds before the first is released, i.e., a ternary complex is formed during transport. Consequently, two substrate binding sites are assumed, one on each membrane side. This does not only hold true for typical antiport carriers like the aspartate/glutamate or the oxoglutarate carrier [254,255], but also for transporters catalyzing unidirectional substrate flux like the carnitine carrier [256] and the phosphate carrier [257]. Thus the function of the latter could in fact be explained as P_i/OH^- antiport [257a]. The presence of two active sites leads to a model which involves two substrate pathways within the dimer, i.e., one pathway per monomer with 6 transmembrane segments. It should be noted that this model is in contradiction to the assumption of only one transmembrane pathway per 12 transmembrane segments for other carriers, e.g., in the lactose carrier [163] or the anion carrier in erythrocytes [170]. However, the functional pattern of the mitochondrial carrier family is not homogeneous. The carnitine carrier functions in a ping-pong-type mechanism [258]. The situation is even more complicated in the case of the ADP/ATP carrier. Whereas there are kinetic results indicating a simultaneous mechanism [259], the data on site recruitment clearly argue for a ping-pong mechanism [28,252]. On the other hand, the mitochondrial carrier family is not the only example where different mechanisms have been found among closely related translocators. Whereas the erythrocyte anion carrier unequivocally functions according to a ping-pong mechanism (4.3), an anion transporter in HL60 cells of the same carrier family has been identified as carrying out transport by a simultaneous type of mechanism [260].

The presence of two substrate pathways has gained more probability in view of recent experiments with the yeast mitochondrial ADP/ATP carrier. Functionally inactive site-directed mutants were subjected to selection for spontaneous suppression. Two important results were thus obtained. When projected in the helical wheel arrangement, the observed mutated and also the charged residues within the hydrophobic segments of the protein fall within one half of each putative transmembrane helix [261–263]. Consequently, these residues are thought to be involved in helix contacts and/or lining the sides of the substrate (nucleotide) pathway. If true, this leads to a channel made up of the six transmembrane segments of one monomer of the ADP/ATP carrier. When this arrangement is applied to other members of the mitochondrial carrier family, a consistent pattern of the distribution of side chains is observed, in which more or less all charged residues face the inside of the putative substrate pathway. Interestingly, the mutants which suppress the effect of mutations in an arginine triplet at the matrix side of the

protein were all found at the opposite, i.e., cytosolic, side of the ADP/ATP carrier. Thus the two spatially widely separated regions are thought to be conformationally coupled during the catalytic function of the ADP/ATP carrier [263].

It should be recalled that the uncoupling protein of brown fat mitochondria, although closely resembling the ADP/ATP carrier in structure and topology (see 4.4), presumably uses a different fine structure of the substrate pathway. This has already been discussed above (4.4).

5. Channel versus carrier: merely a semantic problem or a concept which is still valid?

The discrimination between channel-type and carrier-type mechanisms as catalytic principles of solute movement across membranes is an everlasting conceptual attempt to characterize the specific properties of particular transport processes. Besides the well known fundamental examples of channel-type and carrier-type ion translocation mediated by the oligopeptides gramicidin and valinomycin, respectively, there is some confusion with respect to these concepts in the case of carrier proteins. In fact, although numerous unequivocal examples of each type of vectorial process exist, e.g., ion channels of eukaryotic cells on the one hand and strictly coupled exchange systems like the mitochondrial ADP/ATP carrier on the other, discrimination between the different mechanisms is not always that easy. Even the simplest pore is not solely a water-filled hole giving unrestricted passage to solutes of a given size. More sophisticated types of channels can formally be treated as enzymes, as can carriers [47,264]. Channels modify the rate of a (vectorial) reaction, similar to the action of enzymes. The only formal distinction which remains is the fundamental difference that the solute binding site of a carrier protein is, at a given time, either accessible from one side of the membrane or from the other, but not from both, whereas the binding site(s) of a channel are accessible from both sides of the membrane at the same time. Other properties often used for discriminations between these two principles are ambiguous to some extent. This holds true for translocation rates, which are in general significantly higher for channels, as well as for the energy barrier (activation energy), which is normally significantly higher for carriers.

Currently there is a strong tendency to mix the concepts of channel- and carrier-mediated solute flux. This view was induced by the growing evidence of basic structural similarities between many of these proteins, irrespective of whether they function as channels or as carriers [9,265–267]. Evolutionary lines have been constructed, starting from a simple pore, via simple uniporters (facilitators) and more complex secondary carriers, up to the sophisticated structures of primary carrier systems [12,265]. Mixing of the concepts was also induced by more theoretical considerations, where a unifying kinetic model for channels and carriers was suggested [25]. The basic concept, however, was probably not to converge these principles, but to show how they can *formally* be treated similarly to some extent, based on particular common functional principles. The tendency to combine these concepts was further stimulated by experimental observations and conclusions. The channel-type function of carrier proteins was observed under physiological conditions [268,269], or after modification [249,270–272]. Moreover, different functional principles were found within carrier families of related structure [267], the best example being the ABC transporter family (see 6.2). In this class of transporters very diverse mechanisms are represented, ranging from primary transport systems, e.g., bacterial binding protein-dependent transport (active solute transport coupled to ATP hydrolysis), up to ion channels, e.g., the cystic fibrosis transmembrane conductance regulator (CFTR acting as Cl^- channel) [267,273–275]. Taken together, mainly the observed similarity in structural elements triggered the idea of a functional convergence, which consequently led to the assumption of an equivalence of carrier-type and channel-type mechanisms.

There is also another view by which these observations may be explained. Channels and carriers obviously catalyze the same vectorial reaction. They must, to some extent, fulfil the same requirements: controlled flux across a basically impermeable physical barrier. Thus it is not surprising that similar functional principles are used in both constructions, e.g., binding sites, gates, sensors for ion gradients and voltage. It is obvious that not only carriers need specific binding sites for transported solutes, but such sites may also be detectable in channels which are often highly specific for particular transport ligands [81,276–279]. Both carriers and channels sense the membrane potential, as described here for carriers like the F_1F_o ATPase (4.4) or the Na-glucose cotransporter (6.4). This is of course also true not only of typical eukaryotic ion channels [79,280,281], but also of bacterial porins [81,278,282] or the mitochondrial outer membrane channel (VDAC) [283–285]. However, the principles of voltage sensing may possibly be rather different. There is evidence that in channel proteins mobile loop structures changing the permeability of the channel pathways may be responsible for this function [79,281], whereas in carriers, potential-dependent binding pockets (ion wells) or potential triggered mobility of barrier domains may explain voltage sensing (see Sections 4.4 and 6.4). On the other hand, the analysis of bacteriorhodopsin has shown that carrier proteins may also have typical channel

elements responsible for the the substrate pathway through the protein.

Obviously, the old conceptual distinction which keeps channels and carriers clearly separated is not correct. Not only the description of a channel as a simple 'water-filled hole' must be abandoned, but also the carrier concept [34,57] of a mobile element moving around the substrate molecule does not fit the present data. Elements necessary for catalyzing the controlled flux of substrates are found in either member of the two different principles. It should be emphasized, however, that these elements may be used differently to some extent. Most carriers can clearly be discriminated from channels by some functional properties. Although uniporters (facilitators) have been put into close functional vicinity to channels, they (and more or less all other carriers) show the distinctive kinetic property of a counterexchange maximum, which channels never do. The function of coupling, whether to another vectorial or to a chemical reaction, is clearly restricted to carrier systems. In conclusion, there is in fact convincing evidence that a close relationship between carriers and channels in terms of evolution and structural principles in fact exists. There is furthermore evidence that several functional principles, originally thought to be restricted to channels (e.g., channel-type substrate pathways through the protein moiety, broad substrate specificity) or to carriers (e.g., conformational change, specific binding sites), are found in members of both classes. There is, however, no doubt that the intrinsic construction principle of channels and carriers is to a significant extent different and thus the distinction is not only semantic. This restricts to some extent the usefulness of unifying concepts for the understanding of the function of those proteins which mediate solute flux through the membrane barrier.

Another aspect should be pointed out here where some caution in interpreting solute translocation mechanisms is necessary. A simple uniporter (facilitator) or even a true 'pore' may accomplish carrier-type properties, i.e., specificity, directionality, energy dependence etc., by close functional coupling to a subsequent enzyme. This has first been supposed for the glycerol facilitator in *E. coli* [286] and was recently studied in more detail [287]. In this case, the direct interaction between a simple uniporter and a kinase creates complex kinetic and regulatory properties of the combined system. In this respect, the functional similarity between this combination and a PTS system (see 6.3) should be emphasized [286]. Another, even more striking example of this kind has been described recently for the interaction of the mitochondrial outer membrane pore (VDAC or mitochondrial porin, see above, this section). By close functional interaction of this channel protein with kinases (e.g., the cytosolic hexokinase), unidirectional, selective and tightly regulated transport properties of the combined system are generated [288–290].

6. Mechanism of coupling and voltage sensing

As stated for solute transport in general, and the same holds true for coupling in particular, the mechanism of free energy coupling has not been elucidated in detail for any single carrier system. Although the principles of energy transduction in coupled solute transport mechanisms have been described in reasonable concepts (see Section 3), the situation becomes somewhat confused when these principles are applied to different kinds of carrier mechanisms in molecular terms. At first sight, it seems to be a completely different situation whether a vectorial reaction is coupled to an exergonic chemical reaction (primary transport) or to the translocation of another solute (secondary transport). Moreover, these considerations are currently the major focus of attention because of attempts to establish unifying concepts for carrier catalysis (see 3.3 and 7). In this section, recent developments in understanding the structural and functional basis of free energy coupling in primary and in secondary transport will be outlined by a few representative examples.

6.1. Free energy coupling in primary transport: the Ca^{2+}-ATPase from sarcoplasmic reticulum

The investigation of energy transduction is particularly attractive in primary transport systems since the two aspects, i.e., the chemical and the vectorial reaction, in general take place in different subunits or subdomains of the respective carrier protein. Thus they can be studied independently to some extent. Of the many systems which have been elucidated in detail, only a few can be discussed here. F-type ATPases have already been mentioned (Section 4.5). Besides the sarcoplasmic Ca^{2+}-ATPase, which will be discussed in the following, detailed information is also available for other P-type ATPases, e.g., Na^+,K^+-ATPase [291–294], K^+,H^+-ATPase [295,296] or plasma membrane H^+-ATPases [297,298]. Further examples of primary systems mentioned in this review are ABC-ATPases (6.2) and phosphotransferase systems (6.3).

The ATPase in the membrane of Ca^{2+}-storage organelles of muscle cells is essential for muscle function in pumping Ca^{2+} from the cytoplasm into the lumen of the sarcoplasmic vesicle. The single subunit protein consists of about 1000 amino acids (in various species), and can be divided into several domains (Fig. 9) [299–301]. Some of the subdomains have been attributed to particular functions of the protein, e.g., ATP binding and phosphorylation (P-type ATPases are reversibly phosphorylated during the catalytic cycle). The hy-

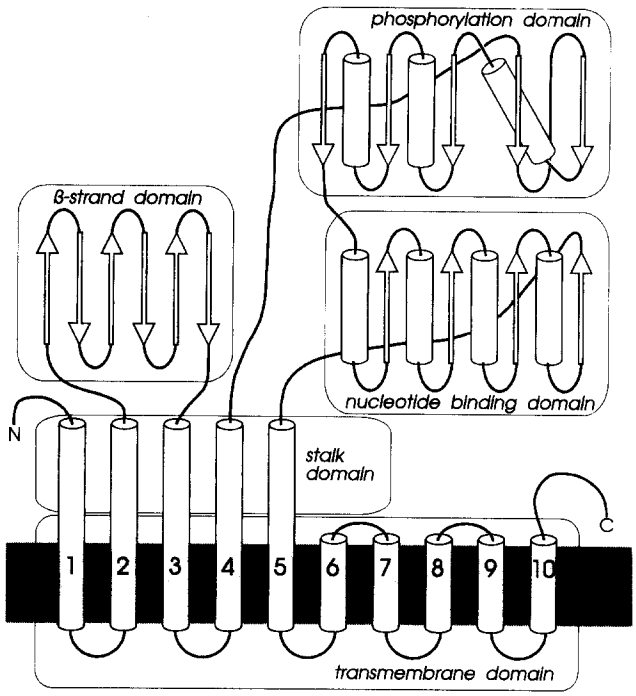

Fig. 9. Structural model of the sarcoplasmic Ca^{2+}-ATPase, modified from [304] with permission.

drophobic domain, consisting of ten putative transmembrane helices, is the Ca^{2+}-translocating subdomain.

The most interesting functional aspect of the Ca^{2+}-ATPase is the coupling between ATP hydrolysis or synthesis (chemical reaction) and the movement of 2 Ca^{2+} ions (vectorial reaction). From the basic energetic concept (3.1) it became clear that there is no particular catalytic step in which the free energy is transferred [302,303]. Coupling is mediated by changes in the catalytic specificity at the nucleotide binding site, as well as changes in the affinity for calcium at one side or the other of the membrane domain. As generally assumed for P-type ATPases, the reversible reaction cycle of the Ca^{2+}-ATPase is characterized by two putative conformational states of the protein, namely E_1 and E_2 (Fig. 1C). In state E_1, the Ca^{2+}-ATPase binds two Ca^{2+} ions at the cytoplasmic side as well as ATP with high affinity at the nucleotide binding site. After transfer of the terminal phosphate of ATP to an aspartyl residue of the phosphorylation domain, the Ca^{2+}-ATPase changes into the E_2 conformation by which the Ca^{2+} binding site reorients to the lumenal side and releases Ca^{2+} due to strongly reduced binding affinity. Consecutively, the Ca^{2+}-ATPase becomes dephosphorylated and the enzyme switches back to the E_1 conformation. Thus the state of the nucleotide binding/phosphorylation domain (chemical reaction) is controlled by the state of the translocation domain (vectorial reaction) and vice versa [300–304]. Whereas the Ca^{2+}-ATPase becomes phosphorylated by ATP in the presence of bound Ca^{2+}, in its absence the enzyme is phosphorylated by inorganic phosphate. On the other hand, the Ca^{2+} binding site of the phosphorylated enzyme is exposed to the lumenal side of the membrane, whereas in the free enzyme the Ca^{2+} binding site is exposed to the cytoplasmic side. This strict mutual correlation of changing specificity in the chemical reaction and orientation of the two functional domains in the vectorial reaction provides the mechanistic basis for the coupling principle [44,302].

The question is now how coupling of the chemical and the vectorial reaction is brought about by structural elements of the Ca^{2+}-ATPase. The existence of different conformations is supported by various observations, i.e., difference in the intrinsic tryptophan fluorescence, in chemical reactivity and in sensitivity to tryptic cleavage [305]. It should be emphasized that in practice there are many subtle conformational changes within the catalytic cycle and not simply two major ones from E_2 to E_1 [55,302]. Thus, since conformational changes are presumably involved in most of the individual steps shown in Fig. 1C, the E_1-E_2 model in its simple version is somewhat misleading.

Two major strategies are currently used for further elucidating the coupling mechanism of the Ca^{2+}-ATPase. On the one hand, the detailed examination of individual catalytic steps by using presteady-state kinetics [55,302,306–308]. As mentioned above, these results indicate that the chemical and the vectorial reactions are coupled by conformational events which are triggered by binding and dissociation of the non-covalent (Ca, ATP) and covalent (P_i) ligands of the enzyme [302]. On the other hand, extensive site-specific mutagenesis of the Ca^{2+}-ATPase and expression in COS-1 cells [309] has brought new insight into the mechanism of coupling.

The ATP and the Ca^{2+} binding sites are widely separated. Thus mutations in different parts of the enzyme not only define the location of individual events, but also help to elucidate the 'cross-talk' by long-distance transmission of conformational changes between individual subdomains. As usual, a large number of mutations had little effect on function. Several mutants, however, in which catalytic activity was lost, still retained their competence for partial reactions [300,304]. The following mutations were characterized. Some affected Ca^{2+} binding or affinity [310,311], others ATP binding or phosphorylation of the enzyme [310]. Finally, a third type affected conformational changes of the Ca^{2+}-ATPase [312]. The first class of mutants was subdivided into those which lead to reduced transport rate due to reduction of binding affinity and those in which Ca^{2+} transport was lost because of the lack of triggering functions of Ca^{2+} binding on the catalytic cycle, presumably since Ca^{2+} binding was

destroyed. Since all these mutants were located near the center of the transmembrane segments 4, 5, 6, and 8 (Fig. 9), this domain has been putatively identified as the Ca^{2+} binding and translocation site. Mutants which affect ATP binding or phosphorylation of the enzyme [309,313,314] could be located in the respective domains (Fig. 9). Most interestingly, a number of mutants were identified which, although unable to transport Ca^{2+}, retained interaction with ATP, phosphate, and Ca^{2+}. Consequently, those mutants were identified as being unable to undergo the correct conformational changes which lead to coupling of the partial reactions in the catalytic cycle. Those mutations were found not only in the Ca^{2+} translocation domain and near the catalytic center, but, in contrast to the other mutant classes, also in the connection between the nucleotide binding and the stalk domain, in the β-strand and in the transmembrane domain.

In summary, the effect of the various mutations indicated that the different functional state of the two domains of the Ca^{2+}-ATPase involved either in Ca^{2+} translocation (binding/dissociation of Ca^{2+} at the cytosolic or at the lumenal face) or in ATP processing (binding/release of ATP or P_i) are strictly correlated by conformational changes of the domains topologically connecting the distant catalytic centers. The change in accessibility of the Ca^{2+} binding sites to either the cytosolic (high affinity) or the lumenal face (low affinity) is correlated to a change in binding affinity by three orders of magnitude. The free energy of ATP hydrolysis is utilized to accumulate Ca^{2+} at the lumenal side, and is thus transformed into a steep electrochemical gradient of Ca^{2+} across the sarcoplasmic membrane.

6.2. *Irreversibility in primary transport: the bacterial binding protein-dependent systems*

Bacterial binding protein-dependent transport systems (BPD systems) belong to a large superfamily of carriers present both in prokaryotes and eukaryotes [65,273,274,315–321]. Transporters of this class have been called 'traffic ATPases', because of the wide range of substrates transported in either direction, frequently coupled to ATP hydrolysis. An alternative term is 'ABC transporters', based on the presence of a conserved *A*TP-binding *c*assette [274,319,322], a structural motif which is invariably associated with these proteins. Members of this family are related to a multitude of biological processes both in bacteria (e.g., solute uptake, antibiotic resistance, cell development) and in eukaryotes (e.g., multidrug resistance, peptide and Cl^--transport) [274,321]. ABC transporters are related to each other in sequence and structural organization and probably have a common evolutionary origin [65,316,323]. In this review, some functional properties of the BPD systems, a subclass of this carrier family, will be discussed.

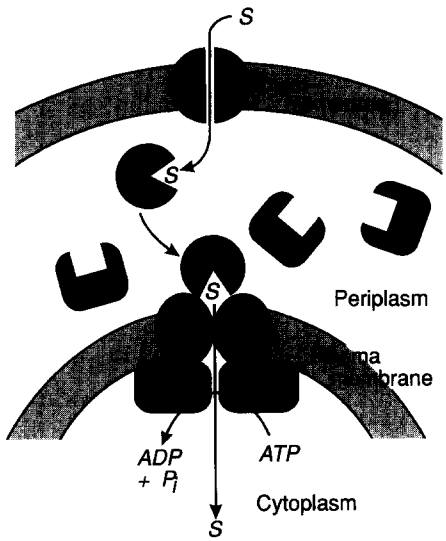

Fig. 10. Scheme of the binding protein dependent maltose transport system of *E. coli*. The functions of the different subunits located in the plasma membrane, in the periplasm and in the outer membrane are explained in the text (B, LamB; E, MalE; F, MalF; G, MalG; K, MalK).

In *E. coli*, more than 20 binding BPD transport systems are known so far. These carriers are widespread in Gram-negative bacteria, and their occurrence in Gram-positive bacteria has also been documented [324,325, for review see 65,274,318]. Well-characterized examples are the uptake systems for maltose [315,326–328], histidine [329,330], and oligopeptides [331–333]. BPD systems of Gram-negative bacteria are typically composed of four distinct membrane-associated domains, two being highly hydrophobic, whereas the two other domains are presumably only peripherically associated at the cytoplasmic face (Fig. 10). The characteristic feature of these high-affinity transport systems is the presence of a soluble substrate-binding protein in the periplasm (MalE for the maltose, HisJ for the histidine system). Some systems, e.g., that of maltose, additionally involve a specific protein which mediates the passive permeation of the substrate through the outer membrane. The hydrophobic proteins (MalF, MalG and HisQ, HisM, respectively) span the membrane by about six putative segments each, i.e., 12 transmembrane segments altogether. The peripheral components (a dimer of MalK or HisJ, respectively) carry two sequence motifs indicative of nucleotide binding [318,322,334,329].

A basic functional scheme of the maltose transport system is shown in Fig. 10. After permeation through the maltose-specific porin (LamB), the substrate is tightly bound by MalE, which thereby dramatically changes its conformation, i.e., the bilobed, two-domain structure switches from an 'open' to and 'closed' state

[335–338]. The MalE-maltose complex interacts with the membrane-bound subunits (at least with MalF and MalG), upon this interaction the substrate is released from MalE and translocated along an unidentified pathway by a conformational change of the membraneous domains. The translocation event, possibly the 'return' of the carrier site in the catalytic cycle, is coupled to ATP hydrolysis [339–343]. After a decade-long controversy, it has now been clearly shown for histidine transport by using a reconstituted system [343–347] that translocation is coupled to ATP hydrolysis by the peripheral component (HisP or MalK). However, both the mechanism of translocation and its coupling to ATP hydrolysis are still unknown.

With respect to structure/function correlation in energy coupling the BPD systems are both particularly fascinating and highly complex. Transport reactions are in general paradigms of reversible processes. This is not only true of secondary processes, also of primary transport systems, e.g., P-type ATPases, reversibility is provided by particular functional properties of the reaction cycle (see Section 3). BPD systems show unprecedented behavior with respect to these properties. The vectorial reaction is not only practically irreversible [315,348,349], but properties typical of carrier proteins like exchange diffusion or countertransport are not observed either.

The membraneous domains of BPD systems catalyze substrate translocation only if substrate-loaded binding protein is available externally [329]. On the other hand, mutants have been obtained which mediate translocation even in the absence of binding protein. The mutations were located either in the hydrophobic components (MalF or MalG) [350] or in the peripheric nucleotide binding protein (HisP) [351]. Interestingly, mutants of the maltose system, lacking the binding protein component, were found not only to transport substrate in the absence of binding protein, but also to constantly hydrolyze ATP, even in the absence of maltose and binding protein. The rates of ATP hydrolysis were related to the rates of maltose transport [327]. These findings have been interpreted to indicate that interaction of the liganded binding protein at the periplasmic side by conformational coupling transmits a signal to the nucleotide binding domain (MalK, HisP), thus controlling ATP hydrolysis [327,351]. The consequence of ATP hydrolysis would be the induction of conformational changes in the membrane domains which lead to solute translocation, similar to the common model of transport (see Section 3). These putative conformational changes have not been proven so far. Since the binding, hydrolysis and release of ATP, ADP and P_i, respectively, have not yet clearly been correlated to particular steps within the reaction cycle, this functional model is still very similar to the first one proposed 10 years ago [315]. Although details of the coupling mechanism still remain unclear, these studies clearly show that the membraneous components are basically able to catalyze the translocation step, and that the catalytic activity of the nucleotide-binding cytoplasmic domain is controlled by interaction of the liganded periplasmic binding protein component with the membraneous domains [327]. Evidence has even been presented that this interaction involves a direct contact between the soluble binding protein and a membrane-spanning loop of the cytoplasmic domain [352–354].

A clue to the still unsolved problems of the unidirectionality of BPD systems and their peculiar kinetic properties may be provided comparing these systems with other members of the superfamily of ABC transporters. Only in BPD systems are such unprecedentedly high solute gradients of more than 10^5 observed as well as practically unidirectional uptake [315,355]. These systems differ from all other ABC transporters in the additional presence of binding proteins. Thus it is an obvious conclusion to correlate particular functions of this additional subunit, i.e., the binding protein component of these systems, with their extraordinary properties. Consequently, this led to several hypotheses explaining the function of the soluble binding proteins by dramatically increasing the apparent substrate affinity of the BPD system [356] or the actual substrate concentration in the binding pocket [329,353]. The latter hypothesis, for example, is based on the assumption that upon interaction of the binding protein-substrate complex with the membraneous subunits, a pocket, occluded from the surroundings, is created. Within this pocket, the single substrate molecule is (formally) present at extremely high concentration which consequently leads to flux of the substrate to the cytosol along a pathway through the membrane-spanning subunits. ATP hydrolysis is then assumed to change the conformation and thus the affinity of the binding protein via interaction with the membraneous subunits. These hypotheses are difficult to prove experimentally, and kinetic arguments against their validity have been put forward [357].

An interesting concept has been published recently, which, mainly based on kinetic evidence, seems to explain the observed peculiar properties of BPD systems [357,358]. The kinetic analysis depends on the same basic model as mentioned above [315]. An essential assumption is that exclusively the liganded form of the binding protein interacts with the carrier. Although this seems to be generally accepted [328,329], there are also conflicting data [359]. The kinetic model for BPD transport is derived by combining (i) a separate cycle of binding/release of the substrate to/from the binding protein correlated with conformational changes of the binding protein with (ii) a conventional carrier scheme involving the membrane-embedded subunits (carrier)

and the substrate delivered from the binding protein. It correctly describes the main kinetic features of BPD systems, i.e., strong asymmetry (unidirectionality) and lack of exchange transport and countertransport. In this model, the asymmetry imposed by the binding protein leads to the function of an inwardly directed valve, thus preventing the reverse reaction which would mean loss of substrate [357]. The input of free energy is necessary to change the affinity of the membraneous domains for the transport substrate, thus converting the inward-facing binding site from high-affinity to low-affinity, similar to the mechanism of the Ca^{2+}-ATPase (6.1). It is not yet fully clear whether this model in fact correctly describes the complete function of BPD systems. However, it is instructive by demonstrating that the puzzling properties of BPD systems can in principle be explained by a combination of simple and well-known functional principles and do not need any exclusive mechanistic constructions.

6.3. Group translocation by phosphotransferase systems: an exception?

Solute transport catalyzed by the mechanism of group translocation is in general treated as an exceptional case among transport mechanisms and has thus been classified separately. This mechanism is peculiar in coupling the vectorial process to chemical modification of the transported solute (see below), i.e., it is the only transport mechanism known in which the transported solute becomes chemically changed. Although it was formerly postulated as a fundamental principle in chemiosmosis [360], in fact the only known example is phosphoenolpyruvate-dependent phosphotransferase (PTS) systems found solely in bacteria. A large variety of these systems catalyze the uptake of many different sugars and sugar alcohols coupled to phosphorylation of the transport substrate [361–366]. PTS consist of two water-soluble proteins common for all systems, enzyme I (EI) and histidine protein (HPr), and a series of substrate-specific enzymes II (EII). The EII subunits are further characterized by three subdomains (EIIA–EIIC), which are differently organized in the various PTS, either fused together or separate subunits [reviewed in 366–371]. EIIc, the membrane-integrated subdomain, consists of most probably 6 (possibly 8) transmembrane helices [366,367,372,373]. Since the PTS are organized as homodimers [reviewed in 365,366], the membraneous part again seems to reflect the common pattern of about 12 transmembrane segments.

Fig. 11 describes the sequence of phosphate transfer reactions starting from PEP, via phosphorylated histidine and/or cysteine residues of EI, HPr, EIIA, and EIIB, finally leading to phosphorylation of the transport substrate. This sequence of events can be described by the following equations, exemplified for the glucose-PTS in *E. coli*.

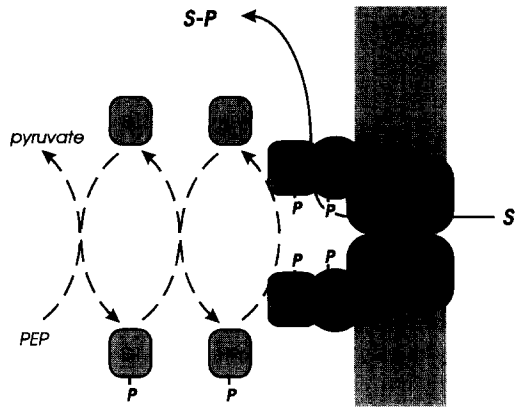

Fig. 11. Scheme of a phosphotransferase transport system (e.g., mannitol transport system). The different subunits are designated according to the nomenclature described in [370]. The functions of the different subunits and domains are explained in the text. The phosphotransfer reactions from PEP to the different subunits and domains are shown by broken lines, substrate translocation and phosphorylation by a solid line.

$$\text{PEP} + \text{enzyme-I} \rightleftharpoons \text{enzyme-I} \sim \text{P} + \text{pyruvate} \quad (1)$$

$$\text{enzyme-I} \sim \text{P} + \text{HPr} \rightleftharpoons \text{HPr} \sim \text{P} + \text{enzyme-I} \quad (2)$$

$$\text{HPr} \sim \text{P} + \text{enzyme-IIA}^{\text{Glc}} \rightleftharpoons \text{enzyme-IIA}^{\text{Glc}} \sim \text{P} + \text{HPr} \quad (3)$$

$$\text{enzyme-IIA}^{\text{Glc}} \sim \text{P} + \text{enzyme-IIB}^{\text{Glc}} \rightleftharpoons \text{enzyme-IIB}^{\text{Glc}} \sim \text{P} + \text{enzyme-IIA}^{\text{Glc}} \quad (4)$$

$$\text{enzyme-IIB}^{\text{Glc}} \sim \text{P} + \text{glucose}_{\text{out}} \rightleftharpoons \text{glucose-6-P}_{\text{in}} + \text{enzyme-IIB}^{\text{Glc}} \quad (5)$$

The original idea of PTS function assumed that the phosphorylation reaction is the actual 'driving' step. The two partial reactions (vectorial and chemical) should be tightly coupled in order to guarantee proper functioning of the system effectively transferring free energy. However, this is not the case. It has already been argued on basic energetic grounds that the phosphorylation step cannot be directly related to translocation in mechanistic terms [51]. It would be difficult to imagine that the intrinsic binding energy could provide sufficient catalytic energy for both conformational change of the protein (decreasing the energy barrier for translocation) and conformational distortion of the bound substrate (decreasing the energy barrier for the chemical reaction).

It has clearly been shown, at least in the case of the mannitol PTS from *E. coli* and the glucose PTS from *S. typhimurium*, that the two partial reactions are actually not simultaneous events and can be completely uncoupled under certain conditions. By studying mannitol binding, transport and phosphorylation in vesicles

with both orientations (inside out and right side out) [374–376], as well as in a liposomal system [377], it has been shown that phosphorylation and transport of mannitol are separate steps. On the one hand, the EII complex catalyzes phosphorylation of mannitol from the cytoplasmic space without concomitant transport [377,378] and on the other hand, unphosphorylated EII, in fact the membrane-spanning EIIC subdomain alone, catalyzes the facilitated diffusion of mannitol at a slow rate [375,377]. This part of the PTS thus functions as a 'simple carrier', i.e., exposing a single binding site alternatively to either side of the membrane [374,376,377]. This finding is corroborated by the observation that under certain conditions (inducer expulsion [379–381]), in contrast to its 'normal' function, EII mediates the efflux of mannitol from bacterial cells. Although the phosphorylation state of the EIIA subunit does not affect substrate binding to the 'carrier' domain EIIC, interaction of these domains becomes obvious by the fact that phosphorylation of EII enhances the translocation rate by several orders of magnitude [375,377].

These kinetic data on the function of the mannitol PTS are confirmed by mutant studies of the PTS for both mannitol and glucose. Mutants of EII^{Mtl} have been obtained which are functionally uncoupled, i.e., they catalyze phosphorylation without concomitant transport [382]. Similarly, in mutants of EII^{Glc} [372,378,383–385] facilitated diffusion uncoupled from glucose phosphorylation was observed.

Although the structures of the hydrophilic EII domains are known in detail [reviewed in 366] the same does not hold true for the membraneous part EIIC. Based on data obtained with fusion techniques (phoA/lacZ fusion), a topological arrangement of EIIC has been proposed [365,367,386] which include a bundle of at least 5 transmembrane helices. The position of amino acids changed in various mutants suggests that at least part of the substrate binding site is located in hydrophilic loops [386,387]. However, in view of increasing evidence that hydrophilic loops may be backfolded into the region of transmembrane segments, this does not prove a particular location of the binding site.

Taken together, these data suggest the following model of the catalytic cycle of transport by the EII subunit of PTS (cf. Fig. 11 and the equations above) [366,388,389]. (a) The substrate binds from the periplasm to EIIC with high affinity. (b) If EIIB is phosphorylated, EIIC translocates the substrate to the cytoplasmic face by a mechanism identical to that known from 'typical' secondary uniporters. (c) The substrate becomes phosphorylated by the phosphorylated EIIB domain and is released into the cytoplasm. Besides the lack of understanding of the mechanism of solute translocation in general, two particular aspects of this cycle are clearly not sufficiently understood. On the one hand, it has been shown that in the case of the mannitol PTS, the substrate can, with equal probability, in step (c) first be released and then rebind with subsequent phosphorylation. This means that the two partial reactions (vectorial and chemical) are completely uncoupled at least on the substrate level. As mentioned above, this is in obvious contrast to the former understanding of group translocation. On the other hand, translocation seems to be tightly coupled to phosphorylation on the protein level, i.e., in the EIIB domain. This is indicated by findings of a close conformational 'cross-talk' between EIIB and EIIC with respect to phosphorylation state and transport properties. EIIC-coupled translocation is only possible at reasonable rates if EIIB is phosphorylated. All mutations in which effective substrate translocation is uncoupled from phosphorylation, concomitantly lead to a drastic decrease of substrate affinity at the periplasmic face of EIIC.

The question of how coupling can be described mechanistically in PTS largely remains open. The former view of tight coupling of the vectorial and the chemical process on the substrate level is definitely incorrect. Another explanation analogous to the function of the F_1F_0 ATPase which has been put forward for binding protein dependent systems as well (6.2), namely that the chemical reaction (dephosphorylation of EIIB) drastically lowers the binding affinity for the substrate thus being the essential step for its release, is presumably not correct. This is indicated by the data (see above) showing an equal probability of substrate release without concomitant phosphorylation. It may be suggested that there is in fact a conformational coupling between the hydrophilic subdomain EIIB and the carrier domain EIIC in that the height of the energy barrier for reorientation of the substrate loaded carrier is controlled by the phosphorylation state of EIIB. The subsequent phosphorylation of the substrate may then in fact not contribute significantly to energetic coupling of the translocation event, irrespective of the fact that it is essential to trap the substrate within the cell due to phosphorylation. In other sugar transport systems, e.g., the glycerol carrier of *E. coli*, this function is carried out by soluble kinases [286,287]. Thus substrate phosphorylation may only be integrated into the transport enzyme for evolutionary, but not mechanistic reasons.

6.4. Free energy coupling in secondary transport: the Na^+/glucose cotransporter as studied by voltage-clamp techniques

The Na^+/glucose cotransporter from the intestinal brush border is a member of a large family of Na^+-coupled cotransport systems present in many procaryotic

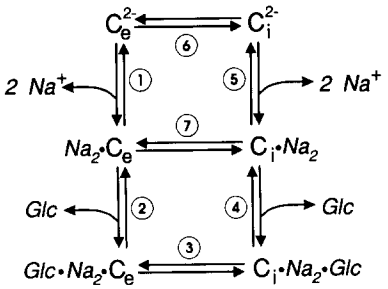

Fig. 12. Simplified kinetic scheme of the Na^+/glucose cotransporter from the small intestine, modified from [403]. Abbreviations as described in Fig. 1 and in the text.

and eucaryotic cells [390,391]. Since its cotransport function was correctly described more than 30 years ago [392], it has become one of the best studied transport systems in terms of kinetic properties as well as modulation by membrane potential and cotransport ion (sodium) [40,390,391,393–397]. The Na^+/glucose carrier is functionally asymmetric [398], quantification of the effect of membrane potential on the kinetic parameters suggested one or two negative charges at the mobile part ('gate') of the carrier protein [395,399]. The analysis further strongly suggested Na^+/glucose stoichiometry of 2 [398,400,401]. The kinetic mechanism proved to be of the simultaneous type including a ternary complex (see Fig. 12) [393,398].

A remarkable breakthrough in the analysis of this carrier and coupled cotransporters in general was made by two steps. First, the protein was expressed in a functionally active form in oocytes in an extremely high concentration, approximately 10^5 carrier molecules per μm^2 [402,403]. This massive overexpression made it possible to use electrophysiological techniques, i.e., steady-state currents across the plasma membrane were measured in response to experimentally varied voltage by the application of two microelectrodes (monitoring the internal and external space, respectively) under voltage-clamp conditions [403–405]. The advantage of this technique lies in the fact that the movement of charges can be followed directly, which in principle holds true for any single step within the catalytic cycle of a carrier protein. In kinetic methods, on the other hand, the effect of charges and electric potential on steps other than the translocation step is in general difficult to analyze. Kinetic values such as so-called 'transport affinities' (K_m values of the transported solutes) are very complex terms which include a whole set of different parameters [35,406,407]. Electrophysiological methods have been applied to many carrier processes including some Na^+-solute cotransport systems in particular [e.g., 179,398,408–412]. Rigorous application of this technique including presteady-state kinetics led to a detailed description of the mechanism of Na^+-binding and Na^+-coupled cotransport by the Na^+-glucose carrier of small intestine [403,405].

When massively overexpressed in *X. laevis* oocytes, the Na^+/glucose cotransporter can generate significant currents which make it possible to measure Na^+/glucose kinetics on a single oocyte [404]. The inward steady-state currents under voltage-clamped conditions were recorded as a function of the type and the concentration of added substrate, of cotransport ion (Na^+), and inhibitor phlorizin at the cis side over a wide range of membrane potentials (+50 to −200 mV). On the one hand, previous data obtained by 'classic' carrier kinetics, e.g., the cotransport stoichiometry of 2 Na^+/glucose and the basic electrogenicity of the process, were confirmed. On the other hand, new information led to a detailed functional description of this cotransport system. Although the voltage dependence of the overall transport had been characterized before [40,398,399], voltage-clamp experiments allowed the discrimination of voltage dependence on particular binding and translocation steps. Furthermore, the slippage mode of the Na^+/glucose carrier could be determined by measurements of Na^+ current in the absence of substrate (sugar).

These data can be rationalized by a kinetic model of a 6 state ordered process (Fig. 12) [403]. For the sake of clarity, this model is still significantly simplified, e.g., an ordered binding of Na^+ ions prior to sugar was chosen. This means that sugar binding (step 2) occurs only after association of the carrier (C^{2-}) with two sodium ions ($C \cdot Na_2$, step 1). Nevertheless, if all the measured kinetic data from various experiments were fitted into this model, one single numerical solution for the respective rate constants related to the individual kinetic steps, as indicated in Fig. 12, was obtained. As expected, reorientation of the unloaded carrier (step 6) is the main voltage-dependent step. However, external Na^+ binding (step 1) also proved to be influenced by voltage, which can be explained by assuming an ion (Na^+)-well effect (see 4.4) [48,398,413]. The distribution of voltage dependence on Na^+ binding (step 1, 30% of total membrane potential) and empty carrier reorientation (step 6, 70% of total potential) can be explained by assuming that the Na^+ ions sense 30% of the voltage when moving to their external binding site from the cytosol, whereas the charges (charged carrier C^{2-}) moving in the translocation step sense 70% of the membrane potential. Binding of Na^+ at the internal side (step 5) is voltage-independent. Under different conditions with respect to voltage and (internal and external) Na^+-concentrations, various different binding or translocation steps within the transport cycle become rate-limiting. Furthermore, the slippage pathway (step 7, conformational change of the Na^+-loaded binary complex) could be quantified. Although experimentally measurable, this reaction is in fact very slow

in comparison to the translocation rate of the unloaded (step 6) and the fully loaded carrier (step 3).

All measurements as reported above were made under steady-state conditions. Further, even more exciting data were obtained by measuring presteady-state kinetics, i.e., following current transients in the millisecond time range after the application of transmembrane voltage [403,414]. These observations were made in the absence of substrate (sugar). It can thus be assumed that the observed transient currents arise from the reorientation of the empty carrier (Fig. 12, step 6) in response to the applied potential. Therefore, the postulated charge movement during conformational change of the empty carrier could be directly quantified.

In summary, the elucidation of the individual steps within the reaction cycle of the Na^+/glucose carrier provided a detailed picture of the functional principles of a cotransporter. Principles which had seemed to be only kinetic formalisms so far (e.g., partial influence of voltage on particular steps), or had been mainly discussed as hypothetical possibilities (e.g., ion-well effects in secondary transport [48,413]) could be directly demonstrated. Thus the coupling of substrate and cosubstrate flux, as well as the modulating influence of membrane potential on these coupling principles, could be exemplified. Investigation of this kind of charge movement originating from conformational rearrangement within the carrier protein is, of course, not restricted to electrogenic transport processes such as the Na^+/glucose carrier. The influence of applied potential has also been successfully studied with electrically silent transport systems like the erythrocyte anion carrier [169,415].

6.5. Free energy coupling in secondary transport: remarks on the lactose and melibiose permease of E. coli

The β-galactoside:proton symport carrier or lactose permease (LacY) from *E. coli* and related secondary carriers have been discussed in Section 4.2 with respect to substrate pathway and binding site(s). The studies using selected mutants as well as site-directed mutagenesis also provided a variety of carrier proteins with known amino acid substitutions in which the energy barrier of certain steps in the catalytic cycle is dramatically changed. These mutants are ideal candidates for analyzing the molecular basis for coupling of the proton-motive force to the chemical potential of the substrate by a secondary carrier protein.

The physiological function of LacY depends on the fact that certain intermediates in the transport cycle (Fig. 1B) are not 'mobile', i.e., a conformational change leading to solute translocation is forbidden. In 'uncoupled' mutants which transport sugar without the concomitant translocation of protons, reorientation of the binary carrier-substrate (CS) complex must be assumed. Vice versa, in those mutants which translocate protons without sugar, the binary carrier-H^+ (CH) complex must be mobile. The question is now which amino acid substitutions can be correlated to the loss of the energy barrier normally preventing these reactions, and which structural consequences may be related to these mutations.

It has been pointed out recently [116] that the introduction of (site-directed) single mutations does in general not lead to severe disruption of the transport function [5]. This indicates that replacement of individual amino acids does not perturb the protein's structure (and function) significantly. But even when specific functional changes could be attributed to the substitution of a particular amino acid, the outcome is often not unambiguous but may lead to 'mutational double effects' [116,416]. Because of the cyclic nature of carrier catalysis and the requirement of thermokinetic balancing [417], a change in the (kinetic) properties of one particular catalytic step necessarily leads to alterations in other steps, too. Thus, an observed correlation of changes in sugar recognition with alterations of cation specificity does not necessarily mean that the respective binding sites for sugar and cation are closely related or even overlapping, since these correlations may be due to the functional interdependence within the catalytic transport cycle [116].

The results on uncoupled mutants should be considered with these reservations in mind. Several mutants of LacY have been found in which sugar transport is not coupled to proton movement, e.g., a his322tyr or his322phe substitution [149,418,419] as well as cys262gly [420]. Substitution of his322, a residue the importance of which has already been discussed above (4.2), by tyrosine led to surprising properties: the efflux of melibiose is still coupled to protons whereas this is not observed for lactose [419]. Substitution of the same residue with phenylalanine slowed down the transport rate significantly (as in the case of the his322tyr mutant), however, uncoupled lactose transport was not observed. Since the functional change in LacY obviously depended both on the type of amino acid in position 322, as well as on the kind of sugar transported, it may be concluded that the mutation does not affect an H^+-binding site, but is instead involved in sugar binding and concomitantly in the structural linkage to the energetic barrier normally preventing a conformational change without a bound proton. This interpretation should not regarded as unlikely, because there is also evidence of the unchanged LacY transporting sugar without protons at very high sugar concentrations [421].

On the other hand, mutants of LacY are available which catalyze uncoupled H^+-transport (e.g., substitutions of ala177 or tyr236 [152,416,422,423]). Of particu-

lar interest is an ala177val/lys319asn double mutant, because these substitutions led to a combined effect of an altered sugar recognition [127], together with sugar transport uncoupled from protons as well as H^+-transport without sugar translocation [152,416,422]. Second site revertants for this mutation were found where again both the efficiency of the H^+-slippage pathway and the sugar binding were affected [423].

The bacterial melibiose carrier (MelB) represents another well-suited candidate for these studies (see 4.2). The peculiar property of this carrier is its variable specificity for the symport cation, which may be H^+, Na^+, or Li^+, as a function of the type of the transported sugar (α-, β-galactosides or monosaccharides) and as a function of the organism studied [424–426]. Besides the interesting situation of a varying ion selectivity, MelB is also an exception to the accepted basic kinetic model of a symport carrier in another respect. Whereas carrier reorientation is in general assumed to be the rate-limiting step of the catalytic cycle, it has been shown for MelB that release of the symport cation at the cytosolic face may in fact be rate-limiting [427,428]. The rate of release is significantly different for the three transport co-ions and is furthermore influenced by the membrane potential. Thus, this situation is exactly the opposite to that discussed for membrane potential effects on Na^+-coupled sugar transport (6.4). This peculiar property with respect to cotransport specificity makes MelB an interesting object for studying coupling mechanisms in secondary symport. Consequently, by specific selection procedures and site-directed mutagenesis, mutants have been obtained which show altered substrate and co-ion specificity [153,157–159,429]. Mutations of this kind were found to be widely distributed throughout the carrier protein. As observed for many other cation transporting carriers (e.g., bacteriorhodopsin, several ATPases, lactose permease), strategic anionic (asp) residues were identified which are essential for cation binding [148,155, 430,431]. As a common motif of most of these mutants with altered substrate specificity, changes in the recognition of one substrate were usually accompanied by an alteration of interaction with the second ligand too. These observations were interpreted as indicating functional vicinity or a least cooperative interaction of the binding sites for the two ligands within the carrier protein. In view of the considerations discussed above with respect to LacY [116] where a similar situation was observed, however, this kind of interpretation should be regarded with some reservations.

In summary, these studies show that both recognition and transport of the two symport substrates cannot be changed independently. Whenever binding or translocation of one of them is affected, it is functionally linked to a change in the corresponding parameters of the other. Obviously, this is simply another meaning of coupling in cotransport. The binding site is constructed in such a way that only after binding of the two substrates can enough intrinsic binding energy be utilized to lower the energy barrier of the conformational change necessary for translocation. In the mutants, due to structural changes by the substitution of one or two amino acids, this energy barrier is now also significantly diminished thus allowing the conformational transition of the binary complexes. However, a simple structural or functional explanation of this kinetic and energetic description is not at hand [116].

In comparison to this, coupling in antiport systems seems to be somewhat easier to understand (see 4.3 and 4.5). In the case of a ping-pong type of mechanism, coupling is brought about simply by connecting the steps of the catalytic cycle (Fig. 1A). In simultaneous mechanisms, the situation is basically similar to that described above for cotransport, only taking into account the fact that the two ligands are located at opposite sides of the carrier. Finally, it should be mentioned that coupling in primary systems such as the Ca^{2+}-ATPase has to some extent a different meaning (see 6.1). In this case, the two reactions (chemical and vectorial) can be changed independently by mutations. In secondary systems like LacY, the two vectorial reactions seem to involve topologically interrelated domains and thus cannot be fully separated by mutations.

6.6. Stoichiometry in secondary transport: the glucose-6-phosphate / phosphate antiporter from E. coli

On the one hand, transport stoichiometry is an important quality of all kinds of carriers, and has thus been thoroughly studied in many transport systems, especially in the presence of a complex stoichiometric situation, e.g., in the case of the eukaryotic $Na^+,K^+,2Cl^-$ cotransport system [432–434] or of neurotransmitter transporters [435,436]. In secondary transport (symport or antiport) the stoichiometry is intrinsically correlated to the problem of flux coupling. The discussion about this question has been nurtured by experimental observations of apparently variable stoichiometries in different carrier systems, e.g., in the case of sugar transport in algae [437] or in the lactose permease of *E. coli* [438]. It is, however, now generally accepted that the sugar/H^+ stoichiometry of the lactose permease is a fixed 1 : 1 ratio and, with the exception of possible regulatory effects of the second sugar binding site (see 4.2) [121,122], experimental problems of indirect measurements have been made responsible for the observed changes in stoichiometry.

On the other hand, there are clear examples of variable stoichiometry in another sense, i.e., carrier systems which change their coupling stoichiometry in order to save constant stoichiometry of charge transfer in transport. The best known examples of this kind of

mechanism are the so-called phosphate-linked transport systems [42,439,440], in particular the sugar phosphate/phosphate antiporter (UhpT) from *Lactococcus lactis* and *E. coli* [77,441,442], where variable stoichiometry has been determined from measurements of the respective net fluxes [441,443]. Due to the complexity of this system, its function has long been misinterpreted in *E. coli*. After re-examination of the corresponding system in *L. lactis* [444,445], its function can now be understood as follows: (i) the antiport reaction (phosphate/sugar phosphate) is always electroneutral, (ii) the UhpT protein is specific for monovalent phosphate, and (iii) the active site accepts two negative charges, irrespective of whether they originate from one divalent sugar anion or from two monovalent anions.

$$G6P^{2-} \rightleftharpoons 2\ P_i^- \quad (6)$$

$$2\ HG6P^{1-} \rightleftharpoons 2\ P_i^- \quad (7)$$

$$2\ HG6P^{1-} \rightleftharpoons G6P^{2-} \quad (8a)$$

$$G6P^{2-} + H^+ \rightleftharpoons \quad (8b)$$

The stoichiometry (phosphate (P_i)/sugar phosphate (G-6-*P*)) will then vary between 2:1 (e.g., at pH 7.0, where the sugar phosphate is divalent, see eq. (6)) and 1:1 (e.g., at pH 5.2, where the sugar phosphate is monovalent, eq. (7)). Thus, under certain circumstances, this system may easily be misinterpreted, e.g., as a simple 2 H + /sugar phosphate symporter in the presence of a pH-gradient, i.e., reaction (8a) which is a correct mechanism of this carrier is formally equivalent to reaction (8b) [77]. Whereas it is clear that this protein is also included among the 12 transmembrane segment family [446], the interesting construction of its binding site is not known so far.

The particular functional properties of this type of system, i.e., to conserve the mode of charge transfer in the translocation step, can also be observed in other systems. One example has already been mentioned above (4.3). The anion carrier of the red blood cell, which in its physiological mode exchanges two monovalent anions (Cl^- and HCO_3^-), was shown to bind an additional H^+ to a specific glutamate residue, if occasionally the divalent SO_4^{2-} was accepted as transport ligand, in order to keep the overall number of charges translocated to -1 [188–190]. It is important to note, however, that this construction should not be taken as a general principle. A well known counterexample is given by some mitochondrial carrier proteins, e.g., the ATP/ADP- or the aspartate/glutamate carrier. When changing the substrate species they freely change the electric nature of the transport reaction from electroneutral to electrogenic.

7. A unifying hypothesis of carrier function: a reasonable concept?

The concept of a closely similar structural organization has proven to be useful in understanding the relationships and common evolutionary origin of the families and superfamilies of carrier proteins (see Section 3). Thus interrelation not only between carriers of a similar type, e.g., sugar transporters [10,64] or 'facilitators' [8], but also between very different transport systems [7,8,12,70,267,371,447] has been deduced. On the other hand, this principle may sometimes be overemphasized, therefore possibly concealing converging evolution from different origins, e.g., possibly in the case of the mitochondrial carrier protein family (see 4.7). Undoubtedly, a unifying concept has been very fruitful for analyzing structural interrelations. The question, however, arises of whether this concept is applicable to membrane transport in a wider sense, i.e., do proteins catalyzing the transfer of solutes across the hydrophobic barrier of the membrane fall into a common functional family? Obviously suggestions in this direction [9,12,265] were stimulated by the growing evidence of structural interrelations.

Similar questions have already arisen in Section 5, where the usefulness of a discrimination between carriers and channels was discussed. Basically similar conclusions can be drawn here with respect to a broader view. Since the basic principle of all these 'molecular machines' is the catalysis of a vectorial movement of solutes across the phospholipid membrane, it is easily conceivable that there must be some common principle reflecting this function. At one step during catalytic action, the solute must move through the protein (or, possibly in some cases, along the protein/lipid interface) which necessarily requires the existence of binding sites (gates) and translocation pathways somewhere within the protein. In that sense, it may be futile to draw detailed conclusions from the observation of basic functional similarity between a protein, specialized in the unspecific passage of solutes, e.g., some bacterial porins, and a carrier protein highly specialized in both substrate specificity and energetic coupling, e.g., the Ca^{2+}-ATPase. If there are in fact common structural principles based on a common evolutionary origin, they will possibly be concealed by the sheer necessity of catalyzing the same vectorial event. Thus, in this very broad sense, the question is to some extent semantic and may, in contrast to the structural approach, possibly not help in discovering evolutionary relations between different classes of translocator proteins.

In a more specific way, however, the application of unifying concepts may in fact be useful to understand principles of organization and of functional strategies used by the cell to catalyze solute movement. In view

of the enormous diversity which is observed with respect to the transport substrates handled, the mechanisms used, to the type of energy coupled, and the coupling principles developed, the question of an underlying general principle is in fact still valid. This general principle has already been formulated in Section 2. It was expressed in the basic principle of substrate binding site(s), accessible from different sides of the membrane at different steps within the catalytic cycle, thus mediating vectorial movement of the substrate without a continuous channel open at any time. In addition to this, and basically independent of this general function of a carrier protein, we observe mechanisms by which free energy is coupled to solute movement. This may be described by certain rules, which are of course based on structural principles and mechanistic constraints, i.e., that 'mobilization' of the energy barrier is only possible under defined configurations of the binding site, dependent on the number and type of the binding ligands. On the other hand, the vectorial reaction of a (primary) carrier may be coupled to chemical reactions by transformation of the free energy of high energy compounds first into the conformational energy of a domain of the respective carrier protein and finally to the specific translocator domain by conformational coupling. The question is now whether the diverse types of carriers are all organized according to common principles or whether there are different classes of carrier proteins, as summarized in Section 3 and discussed in Sections 4 and 6 with some representative examples, which are only classed together because of similar requirements for catalyzing a vectorial reaction.

Common functional principles will easily be accepted in the case of the family of secondary carriers. However, let us recall some examples of specialized primary carriers. Very diverse systems such as bacteriorhodopsin, phosphotransferase systems, and binding protein dependent systems, just a few years ago were thought to differ in their basic construction principles providing specialized 'molecular machines' for particular purposes. As discussed in connection with these particular examples, their detailed functional analysis clearly showed that in fact also these individual systems follow the common principle in their functional design. This statement, however, must be taken with some caution. First, there may be real exceptions to the general scheme. When discussing exceptions, the focus is not so much on cases represented for example by the cystic fibrosis transmembrane receptor protein (CFTR). CFTR, although a member of the structural family of ABC transporters, obviously functions as a channel using ATP hydrolysis to some purpose (regulation?) other than driving the reaction [266,448–450]. It is easily conceivable that functional units, e.g., the solute pathway of a primary carrier of the ABC family or its ATP processing site, could have been evolutionarily modified to an ATP-regulated channel without the invention of new functional principles.

The above mentioned reservation refers more to mechanisms which cannot easily be classified into the general scheme so far. An important example is the decarboxylation-driven Na^+ transport in some bacteria, which has not been treated here. The discovery of this novel type of primary transport mechanism [451–453] emphasizes the possibility of still unknown principles of transport. The structure and function of this type of carrier has been elucidated to a significant extent in the last few years [454–457]. It turned out that, at least with respect to function, its mode of action is also comparable to the basic principles of transport mechanisms [457]. A further prominent example is the Ton- and the related Tol-system in the periplasm of Gram-negative bacteria. Although it is well documented that the TolB-protein couples the free energy of the electrochemical potential at the plasma membrane to transport proteins of the outer membrane, in order to 'energize' uptake of particular solutes (e.g., iron complexes) into the periplasm, its mechanism is still largely unknown and obviously does not fit into the general functional principles [458–465]. Furthermore, several carrier systems exist which exhibit a surprising unspecificity in handling extremely diverse solutes, e.g., the renal organic anion and cation exporters [433,466] or the eukaryotic multidrug resistance protein (MDR) [82,273,274,467]. If the suggested hypotheses are true, i.e., if in this case solutes interact with the carrier from the lipid phase [82], this would not only mean a different type of ligand-carrier interaction but possibly also a different type of translocation reaction. Another possible exception may be found in the transport of larger molecules, i.e., peptides and proteins, across lipid bilayer membranes. Although at least energetically showing some similarity to well-known mechanisms of transport of small solutes [468,469], its true mechanism(s) still remain unknown and may provide surprising results [470–474].

The reservations concerning a simple general theme, which have been put forward above with respect to possible 'new' mechanisms, should also be extended in another direction. A basic unifying concept should presumably only be applied to the general principles of functional construction and not to the same extent to details on the amino acid level. Although investigated thoroughly, examples of common motifs of amino acids or clusters of residues indicative of handling particular substrates (e.g., the ABC motif) or carrying out particular functions (e.g., voltage sensing) are rare. This may be taken as an indication that common functional principles, as outlined in this review, can be carried out using relatively flexible construction principles on the molecular level.

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