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SECONDARY SOLUTE TRANSPORT IN BACTERIA

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Review

Secondary solute transport in bacteria

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Key words: Secondary solute transport; Solute translocation; Transport protein; Carrier mechanism; Bacterium

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Abbreviations: melibiose, 6-*O*- α -galactopyranosyl-D-glucose; α -NPG, *p*-nitrophenyl- α -D-galactopyranoside; methyl- α -Gal, methyl α -D-galactopyranoside; TMG, methyl β -D-thiogalactopyranoside; lactose, 4-*O*- β -galactopyranosyl-D-glucose; n.d., not determined.

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I. General introduction

Bacteria can be subdivided on the basis of the structure of the cell envelope into Gram-negative and Gram-positive. The cell envelope of Gram-negative bacteria is composed of a cytoplasmic membrane, a peptidoglycan layer, and an outer membrane that contains lipopolysaccharides (LPS) at its outer surface [1]. Gram-positive bacteria lack the outer membrane (and LPS), but the peptidoglycan layer is usually much thicker. Many eubacteria and archaea are also surrounded by a proteinaceous crystalline surface layer (S-layer) which then forms the outermost component of the cell envelope [2]. The outer membrane of Gram-negative bacteria functions as a molecular sieve through which molecules with a molecular mass ≥ 600 –1000 Da cannot penetrate, and the hydrophilic LPS layer may form a barrier for lipophilic compounds [1,3]. However, the cytoplasmic membrane is responsible for the major screening of the cytoplasm from the environment, and determines to a large extent the entrance and exit of compounds to and from the cytoplasm.

The cytoplasmic membrane contains specific carrier molecules or transport proteins that allow the selective uptake and excretion of solutes. In addition to its role in solute transport, the cytoplasmic membrane plays a crucial role in the maintenance of the energy status of the cell [4], the regulation of the intracellular milieu [5], the turgor pressure [6] and other membrane-linked energy transducing processes. As will be discussed below many of the cellular homeostatic mechanisms are directly linked to solute transport, since the uptake or excretion of molecules requires in most cases metabolic energy and the transport processes can be affected by the energy status of the cell [7], the intracellular pH [8] and/or the medium osmolarity [9]. Major components of the cytoplasmic membrane are the lipid molecules which expose their polar head groups to the water phases and their apolar fatty acid tails to each other, thereby creating a barrier for most solutes. The lipid bilayer forms a matrix in which energy-transducing enzymes/carrier proteins are embedded and by which specific solute concentration gradients can be gener-

ated and maintained. The diversity of systems which can translocate solutes across the bacterial cytoplasmic membrane has recently been reviewed [10].

Bacterial transporters usually utilize primary (light or chemical energy) or secondary energy (the free energy present in specific ion gradients or substrate-product gradients, see below), or chemically modify the substrate as part of the translocation process; a few catalyze facilitated diffusion. On the basis of these differences, three types of bacterial transport system can be discriminated. (i) Primary transport systems convert light or chemical energy into electrochemical energy, i.e., solute or ion-concentration gradients; to this class of transport systems belong the electron transfer chains (utilizing redox energy), the photosynthetic cyclic electron transfer chains (utilizing light energy), the light-driven ion pumps bacterio- and halorhodopsin, the sodium ion transporting decarboxylases, and the ATP-driven transport systems. (ii) Secondary transport systems utilize the electrochemical energy of one solute to drive the translocation of another solute; in some cases secondary transport occurs without coupling ion (solute). (iii) Group translocation systems couple the translocation of a solute to the chemical modification of the solute resulting in the release of a modified solute at the other side of the membrane; the only group translocation systems found in bacteria are the phosphoenolpyruvate:sugar phosphotransferase systems (PTS).

In this review, only secondary transport processes are discussed, with emphasis on the diversity of the transport mechanisms, and the structural and functional properties of the protein molecules. This includes: (i) biochemical and biophysical analyses of purified carrier proteins, and their functional reconstitution into proteoliposomes; (ii) topographic information about carrier molecules obtained from antibody mapping, proteolytic cleavage studies, sequence comparisons and hydropathy analyses, gene fusion studies, analyses of second-site revertants, and studies employing photoactivatable probes and amino acid residue specific reagents; (iii) information obtained from 'selected' and 'site-directed' mutants; and (iv) the regulation of activity of the secondary carrier molecules.

Although a number of reviews on secondary transport proteins have appeared in the past few years, many of these writings are restricted to discussing properties of a single (type of) carrier molecule [11–15], while others focus on structural information and evolutionary relationships between polypeptides on basis of sequence similarities [16–19] or specific aspects of carrier functions [20,21]. The present paper serves as a comprehensive review on the diversity of secondary carrier mechanisms in the bacterial kingdom. Only a limited number of secondary solute transporters have been analyzed in great detail to date (see below), and these mechanisms serve to illustrate the current state of art of secondary solute transport.

The lactose transport protein (LacY) of *Escherichia coli* is the most extensively studied bacterial transport system, and a great deal of our knowledge about secondary solute transport has been inferred from this galactoside-proton symporter. Other secondary transporters, about which significant information is available, that will be discussed in more detail in this review include: the melibiose carrier protein (MelB) of *E. coli*, the sodium-coupled proline carrier protein (PutP) of *E. coli*, the sodium-proton antiporter (NhaA) of *E. coli*, the transposon Tn10-encoded metal-tetracycline/proton antiporter (TetA), the sugar phosphate/phosphate antiporter (UhpT) of *E. coli* (and *Lactococcus lactis*), and the chimeric galactoside transport protein (LacS) of *Streptococcus thermophilus*.

II. Secondary transport mechanisms

In secondary transport, the free energy for accumulation of a solute is supplied by (electro)chemical gradients of other solutes (including ions). As a result, energy of the (electro)chemical gradient of one solute is converted into the energy of the (electro)chemical gradient of another solute. Three general categories of secondary transport systems can be distinguished in bacteria (Fig. 1) [22,23]. When solute transport is mediated by a carrier protein and the movement of the solute is independent of any coupling ion (solute), the transport is indicated as **uniport**. When a transport protein mediates the coupled movement of two (or more) solutes in the same direction, the transport process is indicated as **symport**, or cotransport. In this type of transport the (electro-)chemical gradient of one solute (usually proton or sodium ion) is used to drive the uphill transport of the other solute. Since the translocation reaction catalyzed by secondary transport systems is reversible, the solute concentration gradient may, in special cases, drive the movement of the proton (or sodium ion). In fermentative bacteria, that have to excrete large amounts of metabolic end-products (e.g., lactate, succinate), the end-product gradients may exceed the electrochemical proton (or sodium ion) gradi-

Fig. 1. Different types of secondary transport in bacteria. The driving force on solute A is indicated for each transport mechanism. The driving forces are expressed in mV; F , Faraday constant; $Z = 2.3RT/F$.

ent and, as a result, efflux of these solutes in symport with protons (or sodium ions) will generate metabolic energy [24]. **Antiport** (or countertransport) refers to the coupled movements of solutes in opposing directions.

Since the solutes transported by secondary transport systems can be neutral, negatively or positively charged, and different numbers of solutes may be co- or countertransported, the driving forces on these processes may differ considerably. Below 10 examples of such different secondary transport processes are given and the energetic consequences are discussed (Fig. 1). In the transport mechanisms 3 to 9 the transport of a solute (A) is coupled to the movement of proton(s). It should be realized that in the last decade more and more transport systems have been identified that use sodium ions rather than protons as coupling ion [25]. In those systems the chemical sodium gradient ($\Delta \mu_{Na}/F$ or $Z\Delta pNa$, in which F represents the Faraday constant; $Z = 2.3RT/F$) instead of the chemical proton gradient ($\Delta \mu_H/F$ or $Z\Delta pH$) provides a driving force *. Transport systems in which protons and sodium ions

* $Z\Delta pNa$ and $Z\Delta pH$ are expressed in mV. Throughout the manuscript the chemical gradient of protons (or sodium ions) is mostly referred to as ΔpH (or ΔpNa) instead of $Z\Delta pH$ (or $Z\Delta pNa$).

are cotransported with a solute have also been described, and in these systems $Z\Delta\text{pH}$, $Z\Delta\text{pNa}$ and membrane potential ($\Delta\Psi$) may act as driving forces. The different forces for the various modes of secondary transport of a solute A are indicated in Fig. 1.

II-A. Electrogenic solute uniport (Fig. 1-2)

This type of transport can be expected for uptake of cationic substrates (A^+) or efflux of anionic molecules (B^-). The driving force of electrogenic solute uniport is supplied by the $\Delta\mu_A/F$ (or $Z\Delta\text{pA}$) plus m times the $\Delta\Psi$, in which m represents the number of positive charges of the solute A. Examples are lysine and arginine uptake in *Bacillus stearothermophilus* [26], and low-affinity K^+ uptake in *R. capsulatus* [27]. Although solid evidence is lacking, it is also believed that Ca^{2+} enters the bacterial cell by means of an electrophoretic uniporter [28]. As far as we are aware, exit uniport of negatively charged solutes has not been described for bacteria.

II-B. Electroneutral solute uniport (Fig. 1-1)

The driving force of this process is supplied by the chemical gradient of the solute ($\Delta\mu_A/F$ or $Z\Delta\text{pA}$). The best-known example of this type of uniport is the glycerol facilitator (GlpF) of *E. coli* [29]. Glycerol uptake is followed by glycerol kinase catalyzed conversion to *sn*-glycerol 3-phosphate [30]. This allows downhill influx of glycerol that is sufficiently fast for an efficient metabolism. Since glycerol is highly membrane permeable active accumulation of the solute could lead to passive outward diffusion of glycerol down its concentration gradient thereby creating a futile cycle. The GlpF protein has been described as a cytoplasmic membrane 'channel' which also facilitates the passage of polyhydric alcohols such as ribitol and erythritol as well as unrelated small molecules like urea and glycine [31]. The postulated facilitator function of GlpF has recently been questioned, because passive diffusion of glycerol across the cytoplasmic membrane is not rate-limiting for phosphorylation in a GlpF⁻ mutant [32]. On the basis of these observations it has been suggested that GlpF acts as an activator of glycerol kinase (GlpK), although the role of GlpF in facilitating transport has not been refuted. Another example of electroneutral uniport is the glucose transport system of *Zymomonas mobilis* [33,34]. Despite these examples, solute uniport in bacteria has not been well documented, but may also occur less frequently than in eukaryotic cells [35].

II-C. Electrogenic solute-cation symport (Fig. 1-3, 1-4, 1-6, 1-7)

In experiments to date, solute uptake in bacteria often occurs in symport with a proton or sodium ion.

Since the solute can be neutral, anionic or cationic, the $\Delta\Psi$ component of the proton (or sodium) motive force will contribute differently to the driving forces of these processes. For uptake of a neutral solute the electrical ($\Delta\Psi$) and chemical ($\Delta\mu_H/F = Z\Delta\text{pH}$ or $\Delta\mu_{Na}/F = Z\Delta\text{pNa}$) component of the cation (proton or sodium) motive force each contribute once to the total driving force (Fig. 1-3), whereas for a cationic solute with m charges the $\Delta\Psi$ contributes $(m + 1)$ times and ΔpH (or ΔpNa) 1 time (Fig. 1-4). *Electrogenic uptake of neutral solutes* in bacteria has been shown for sugars and amino acids [11,16,36-39]. *Electrogenic cationic solute-proton (or sodium ion) symport* has been observed for the uptake of arginine and lysine in a number of bacteria [11,40-42]. For *electrogenic anion-proton (or sodium ion) symport* two extremes exist, i.e., the $\Delta\Psi$ may oppose or contribute to the driving force (Fig. 1-6 and Fig. 1-7). The first case (Fig. 1-6) has been shown for the uptake of malate in *Lactococcus lactis* which is transported as the dianionic species in symport with a proton (or transported as monoanionic malate, malateH^-). Under conditions of equal $\Delta\Psi$ and ΔpH no accumulation of malate is observed, however, upon dissipation of the $\Delta\Psi$, uphill transport can be detected [43]. Since a $\Delta\Psi$ (inside negative) exerts a counterforce on the uptake of malate ($\text{malate}^{2-}/\text{H}^+$ symport or malateH^- uniport), dissipation of this $\Delta\Psi$ results in an increase of the net force on the uptake process despite a lowering of the total protonmotive force (Δp ; $\Delta p = \Delta \bar{\mu}_H + /F = \Delta\Psi - Z\Delta\text{pH}$, expressed in mV). The second case (Fig. 1-7) involves the uptake of citrate in *Klebsiella pneumoniae* which is transported as the dianionic species in symport with three protons, and to which the $\Delta\Psi$ and ΔpH contribute as driving forces [42]. The ΔpH component of the Δp has a far greater influence on the uptake of citrate than the $\Delta\Psi$ since more protons than net charges are translocated. *Electrogenic anionic solute-cation symport* has been observed in various bacterial species for the transport of phosphate, glutamate, aspartate, mono- and dicarboxylic acids, and tricarboxylic acid cycle intermediates [11,36,39,43-46].

Solute-cation symport has been found in all bacteria examined, although the cation preference may differ from system to system and species to species. *E. coli* uses both protons and sodium ions as coupling ions for the uptake of sugars, amino acids and other nutrients [e.g., lactose- H^+ (LacY), galactose- H^+ (GalP), arabinose- H^+ (AraE), xylose- H^+ (XylE), proline- H^+ (ProP), proline- Na^+ (PutP), serine- Na^+ , glutamate- Na^+ (GltS), glutamate- H^+ (GltP), α -ketoglutarate- H^+ (KgtP), pantothenate- Na^+ (PanF)] [11,16,46-51]. Unique examples are the melibiose carrier (MelB) of *E. coli* and the alanine carrier protein (Acp) of thermophilic bacterium PS3 which can choose among protons and sodium ions for the uphill movement of solutes

[13,52,53], and these may actually represent intermediate stages in the evolution from H^+ to Na^+ energetics or *vice versa*. MelB of *K. pneumoniae* can utilize either H^+ or Li^+ as coupling ion [54]. In *Lactococcus lactis* the symport systems studied so far use protons as coupling ion [8,39]. For *Bacillus stearothermophilus* electrogenic uptake of glutamate with a proton plus sodium ion has been described [26,55]. Sodium ion is the predominant cation for solute transport in the examined alkaliphilic, halophilic, thermophilic and marine bacteria. In general, bacteria that live in extreme environments, in which it is difficult to maintain a high Δp , predominantly use Na^+ as coupling ion [56–58]. An organism that may exclusively use sodium as coupling ion for secondary transport is *Clostridium feravidus* [42,59]. A survey of sodium-coupled transport in bacteria has recently been given [25].

Several reports dealing with the bioenergetics of solute transport have not considered transport with high affinity for Na^+ ($K_M < 10 \mu M$)*. Since contaminating concentrations of Na^+ in buffers often exceed $50 \mu M$ stimulation of transport upon addition of Na^+ may not be observed [26,60], and sodium coupling may not be revealed. A feature of several sodium-coupled carriers is the inhibition of transport by high concentrations of sodium ions ($> 10 mM$) [42,48,60]. Since similar inhibitions have been observed for the uptake of glycine, aspartate, phenylalanine and cysteine in *E. coli* [61], it has been suggested that transport of these amino acids may also be coupled to sodium ions rather than to protons [48]. Inhibition of sodium-dependent transport by high concentrations of sodium ions may result from the inhibition of the net release of the sodium ion(s) on the inner surface of the membrane. Although some sodium-coupled transport systems have apparent transport affinity constants (K_M^{app}) for Na^+ in the μM olar range (proline and serine in *E. coli*, glutamate in *B. stearothermophilus*), in other cases the K_M^{app} values are in the millimolar range, e.g., citrate transport in *K. pneumoniae* [62], amino acid transport in *Streptococcus bovis* and *C. feravidus* [38,42], and sodium

coupling in these systems is much more easily recognized. Finally, glutamate uptake by *E. coli* has been characterized as glutamate- H^+ - Na^+ symport and detailed kinetic analyses of ligand binding and transport have been performed [63,64]. More recently, it has been shown that the observed glutamate- H^+ - Na^+ symport is the result of two activities (transport systems), i.e., GltS-mediated glutamate- Na^+ symport [65] and GltP-mediated glutamate- H^+ symport [50]. A similar situation holds for proline transport in *E. coli* and *S. typhimurium* where the transport activities have been resolved to PutP-mediated proline- Na^+ and ProP-mediated proline- H^+ symport activities [60,66,67]. The possibility that bacteria often possess more than one mechanism to transport a solute has to be taken into consideration when studies are performed under relatively undefined conditions, i.e., when purified proteins, defined host strains and/or cloned genes are not available.

II-D. Electroneutral solute-cation symport (Fig. 1–5)

With anionic solutes the charge of the solute may be equal to the number of protons (or sodium ions) symported. In those cases, only the chemical gradients of the species transported supply the energy for accumulation. Although conclusive evidence is lacking, transport of lactate in *E. faecalis* [68] and *p*-toluenesulfonate in *Comamonas testosteroni* [69] most likely occurs as electroneutral solute-cation symport. It should be stressed that lactate, unlike *p*-toluenesulfonate, is a weak acid that may accumulate to equilibrium with the ΔpH independent of the presence of a carrier protein which complicates the analysis of this type of transport. The same holds true for the analysis of transport of acetate, benzoate and other weak acids that have been reported to be carrier-mediated.

II-E. Electrogenic solute / cation antiport (Fig. 1–8)

Antiport systems, in general, are well suited for the excretion of undesired solutes (products) from the cytoplasm since solute efflux is directly linked to proton (or sodium ion) influx [70]. Excretion of a solute against its concentration gradient can be achieved via such mechanism. Well-known proton antiporters in bacteria are the excretion systems for Na^+ and Ca^{2+} ions [28]. Other transport proteins that (most likely) belong to this class of excretion systems are the Ca^{2+}/Na^+ antiporters in *Halobacterium halobium* and *Bacillus* sp. A-007 [71,72], and many of the bacterial (multi)drug and antibiotic resistance secondary carrier proteins [10,14]. Lysine efflux by *Corynebacterium glutamicum* has been proposed to occur as lysine- OH^- symport [73] which is energetically similar to lysine/ H^+ antiport.

* Secondary transport processes often involve two (or more)-substrate reactions, e.g., symport of solute and cation(s). In the steady state kinetics of a ordered two-substrate reaction, the 'apparent' Michaelis constant (K_M^{app}) of one substrate depends on the concentration of the other. Thus for solute-cation symport, the 'true' K_M for the solute is only obtained at saturating levels of the cation and *vice versa*. A distinction between 'true' and 'apparent' can also be made for the determination of the dissociation constants (K_D and K_D^{app}) in equilibrium binding measurements. The discrimination between 'true' and 'apparent' Michaelis (K_M and K_M^{app}) and dissociation (K_D and K_D^{app}) constants will be made throughout the manuscript. It should be noted that, for most transport reactions examined, only K_M^{app} (and K_D^{app}) have been determined.

II-F. Electroneutral solute / cation antiport (Fig. 1-9)

Electroneutral cation linked antiport has been observed for K^+/H^+ in *E. coli* and *Vibrio alginolyticus* [74-77]. The K^+/H^+ antiporter may play a role in the regulation of the intracellular pH [28]. Electroneutral K^+/NH_4^+ (or methylammonium) exchange may also be included in this class of transport mechanisms [78]. The metal-tetracycline efflux system encoded by transposon Tn10 in *E. coli* catalyzes an electroneutral metal-tetracycline/ H^+ antiport (see also below) [79,80].

II-G. Precursor / product antiport (exchange) (Fig. 1-10)

This class of antiport systems catalyzes the uptake of a solute (precursor, substrate) in a coupled exchange with another solute (product). The exchanged solutes can be anions such as sugar-phosphate/phosphate [81], oxalate/formate [82], malate/lactate [20,43], ATP/ADP [83], or cations such as arginine/ornithine [84], agmatine/putrescine [85], putrescine/ornithine [86], or neutral solutes such as lactose/galactose [20]. In these exchange processes, in general, the substrate concentration gradient is directed inwards while the product concentration gradient is directed outwards. Both forces thus work together which allows a high rate of exchange. The Δp or one of its components can affect the translocation process through (de)protonation of the substrate(s) and/or through the differential charge of the individual substrates, e.g., sugar-phosphate/phosphate [87], malate/lactate [43], oxalate/formate [82], and lysine/alanine exchange [88]. Some systems catalyze precursor/product exchange or solute- H^+ symport, depending on the concentrations of the solutes and protons on either side of the membrane, the dissociation constants (K_D) for these molecules and the magnitude of the membrane potential, e.g., lactose/galactose exchange in *S. thermophilus* [89], arginine/ornithine exchange in *Pseudomonas aeruginosa* [90], malate/lactate exchange in *L. lactis* [43]. Under physiological conditions – in vivo – the exchange reaction will normally dominate over the symport reaction [20]. In addition to the excretion of galactose by lactose metabolizing *S. thermophilus* (Gal^-) cells, excretion of monosaccharides is frequently observed during growth on disaccharides, e.g., lactose utilization in *E. coli* (Gal^+) [91]; sucrose and lactose utilization in various lactic acid bacteria [20]. Evidence has also been presented for fumarate/succinate exchange under conditions of fumarate respiration in *E. coli* [92]. Whether this system is a true antiporter that only catalyzes an exchange reaction or whether it can choose among alternative substrates, i.e., protons (sodium ions) or succinate, for fumarate uptake is unknown. Most precursor/product exchanges in bacteria have only been reported very re-

Fig. 2. Schematic representation of metabolic energy conservation by malolactic fermentation. MH^- , mono-anionic malate; LH, lactic acid. Malate uptake is shown as MH^-/LH exchange and MH^- uniport [43]. Exit of lactic acid can occur via the exchange reaction or by passive diffusion across the cytoplasmic membrane.

cently suggesting that it may occur even more frequently than recognized so far [15,20,28,70,93].

For bacteria that generate limited amounts of metabolic energy from their catabolism (e.g., fermentative bacteria), the exchange processes are very attractive since no metabolic energy is needed for the translocation of precursor (substrate) and/or product. An example is the arginine deiminase pathway in which the conversion of arginine to ornithine only yields one ATP [94]. Some exchange processes actually contribute to the production of metabolic energy. Examples are oxalate/formate exchange (oxalate fermentation) in *Oxalobacter formigenes* [82], malate/lactate exchange (malolactic fermentation) in *L. lactis* [43], histidine/histamine exchange in *Lactobacillus buchneri* [95], and aspartate/alanine exchange in *Lactobacillus* sp. (Abe, K., personal communication). In these processes, the exchange reaction results in net inward movement of a negative charge or outward movement of a positive charge, leading to the generation of an electrical potential ($\Delta\Psi$, inside negative) (Fig. 2). Furthermore, one proton is consumed intracellularly in the conversion of substrate to product and this leads to an increase of

the internal pH (or generation of a pH gradient (ΔpH)). The resulting protonmotive force (Δp) can be used to drive energy requiring processes such as ATP-synthesis as has been demonstrated for malolactic fermentation [43] (Fig. 2). In addition to the exchange reaction, the malate-lactate transporter also catalyzes uniport of monoanionic malate (MH^-) or symport of dianionic malate (M^{2-}) together with a proton (see subsection II-C). Also in this process, malate uptake contributes to $\Delta\psi$ formation, and, when lactic acid diffuses out either carrier-mediated or passively, the overall process is energetically equivalent to the exchange mechanism (Fig. 2).

The primary sequences of the arginine/ornithine transporter (ArcD) of *P. aeruginosa* [96] and the lysine/alanine transporter (LysI) of *Corynebacterium glutamicum* [97] display a high degree of similarity. On the basis of the homology between ArcD, LysI and a putative membrane protein (CadB), the latter protein has been proposed to be a lysine/cadaverine exchanger [98]. Lysine and cadaverine carry one and two net positive charges, respectively, and lysine/cadaverine exchange is therefore expected to be counteracted by the membrane potential (inside negative). Since the decarboxylation of lysine, which results in cadaverine and carbon dioxide, also consumes a proton (intracellularly), this system may actually be analogous to oxalate/formate exchange-oxalate decarboxylation in *Oxalobacter formigenes* [82], malate/lactate exchange-malolactic fermentation in lactic acid bacteria [43], and histidine/histamine exchange-histidine decarboxylation in *L. buchneri* [95], and thus be involved in metabolic energy generation.

III. Nature of the coupling ion and contribution of proton dissociation/association of the solute to the driving force of transport

The observations that some transport systems use Na^+ and H^+ as coupling ion (melibiose carrier of *E. coli* [13], the alanine carrier of the thermophilic bacterium PS3 [53], F_0F_1 -ATPase of *P. modestum* [99]) have led to the proposal that H_3O^+ (steric analog of Na^+) rather than H^+ could be the translocated species [100]. In this view, proton translocation does not take place via a hydrogen-bonded chain of (de)protonable groups ('proton wire'), but cations (H_3O^+ or Na^+) interact with the enzyme through coordination to oxygen and/or nitrogen atoms, which mimics the binding and translocation of the solute. Thus, the translocated proton is covalently linked to a water molecule rather than bound to an amino acid residue. Both extremes for proton translocation can, in principle, be combined by assuming that initially an H_3O^+ molecule interacts with the protein, followed by transfer of the proton

from the complexed H_3O^+ to a protonatable group of the transporter. A mechanism for proton (cation) translocation which involves hydronium (cation) coordination can explain competition between protons and sodium ions. Also an evolution from protonics to sodium ionics can be more easily envisaged when protons and sodium ions use a similar translocation mechanism. The high degree of similarity in the primary structures (approximately 60% amino acid identity) of the glutamate- H^+ - Na^+ symport system (GltT) of *B. stearothermophilus* and the glutamate- H^+ symport system (GltP) of *E. coli* fits well in this view [101]. Other examples of homologous proteins with differences in coupling ion are the citrate- H^+ symporter (CitP) of *L. lactis* and the citrate- H^+ - Na^+ symporter (CitS) of *K. pneumoniae* [62], and the melibiose carriers (MelB) of *E. coli*, *S. typhimurium* and *K. pneumoniae* and the galactoside- H^+ symporters (LacS) of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* [54,102,103, 104,105].

In the analyses of the driving forces of secondary transport processes only the forces to which the carrier proteins respond have been considered (Section II; see also Fig. 1). The driving force on transport of an acid or base across the membrane will also be affected by the proton dissociation/association on the in- and outside. The particular acid or base will accumulate to equilibrium with the driving force to which the carrier mechanism responds as any non-protonatable molecule, but the total acid or base concentrations internally and externally will also be affected by the pH gradient (difference in acid/base equilibria). The different buffering of the transported particle on either side of the membrane in the presence of a ΔpH will lead to additional accumulation of the substrate which can be expressed as an additional driving force that takes the form:

$$\text{extra driving force} = n \cdot \Delta\text{pH} - \sum_{i=1}^p \log \left(\frac{[\text{H}^+]_{\text{out}} + K_A^i}{[\text{H}^+]_{\text{in}} + K_A^i} \right)$$

in which p represents the total number of protonatable groups, which is transported as the n -fold protonated species ($n \leq p$); Σ denotes the summation over all protonatable groups, with K_A^i as the acid dissociation constant of group number i . When the proton concentrations internally and externally are negligibly small compared to the K_A^i , e.g., when both internal and external pH are at least one unit above all $\text{p}K_A^i$, then the extra driving force is simply $n \cdot \Delta\text{pH}$. The system then behaves as if the protons on the transported species are part of the carrier mechanism. Conversely, for a base, when the $\text{p}K_A^i$ values are above the internal and external pH, the extra driving force will become $(n - p) \cdot \Delta\text{pH}$.

Fig. 3. Secondary structure model of a carrier protein based on features generally observed in this type of membrane proteins. α -helical transmembrane segments are indicated as cylinders. A surplus of basic relative to acid residues in hydrophilic cytoplasmic loops as well as the localization of amino- and carboxy-termini are shown; the distribution of the charges is based on the amino acid sequence and topographic studies of the LacY protein.

IV. Structure of secondary transport proteins

IV-A. Primary sequence and secondary structure

From the known primary structures of secondary transport proteins it is evident that these systems have less structural complexity than, for instance, the ATPases or the phosphoenolpyruvate:sugar phosphotrans-

ferase systems [10]. The molecular masses of most of these proteins fall in the range 40–55 kDa [16]. Hydrophathy analyses of the primary sequences has indicated that the secondary carriers consist of a hydrophobic polypeptide which traverses the cytoplasmic membrane in a zig-zag manner. It has been proposed that the generic secondary structure of the carrier proteins consists of 2 times 6 (or 5) transmembrane

Fig. 4. Amphipathic nature of transmembrane α -helix II of the LacS-MelB family of homologous transport proteins. LacS, lactose carrier proteins of *S. thermophilus* and *L. bulgaricus*; MelB, melibiose carrier proteins of *E. coli*; and GusB, the glucuronide carrier protein of *E. coli*. The LacS-MelB family also includes MelB of *S. typhimurium* and *K. pneumoniae*; XylP, xylose transport protein of *Lactobacillus pentosus*; and XynC, open reading frame from *B. subtilis* (not shown). The diagonal line shows the division between the hydrophobic and hydrophilic halves of the helices.

α -helices that are separated by a relatively large cytoplasmic loop [16,106,107] (Fig. 3). An α -helical configuration of LacY is supported by circular dichroic studies [108], Laser Raman spectroscopy [109] and Fourier transform infrared spectroscopy [11]; however, using the same data set, partial β -sheet structures have also been suggested [110]. A 12-helix motif of LacY [111], MelB [112], TetA [113], UhpT [114], and several other transport proteins is supported by *phoA* fusion analyses [115]. For LacY and TetA, the presence of 12 transmembrane segments is further supported by limited proteolysis, immunological analyses and chemical modification studies [11,113].

On the basis of hydrophathy prediction methods and various topographic studies, secondary structure models have been built. Common features of most of these models are (Fig. 3): (i) Twelve hydrophobic domains in α -helical configuration traverse the membrane in a zig-zag fashion. (ii) The hydrophobic domains exhibit a mean length of about 20 amino acids, which is sufficient to span the membrane bilayer. (iii) Usually a few of the hydrophobic domains are amphipathic helices (Fig. 4). (iv) The hydrophobic membrane spanning domains are connected by hydrophilic loops. (v) The hydrophilic loops on the cytoplasmic side of the membrane are usually longer than those on the outside [111–114]. (vi) A relatively large cytoplasmic loop separates the helices I–VI from helices VII–XII. (vii) A surplus of positively charged amino acid residues (Arg, Lys and His) is found in cytoplasmic loops (the ‘positive inside’ rule [116,117]). (viii) Only a few charged residues are present in membrane spanning segments, and these may be tolerated in these hydrophobic regions by forming salt bridges that are energetically more favourable than the presence of the ‘free’ polar side-chains (see subsection IV-B) or by forming part of a hydrophilic pocket that interacts with the ligand(s) (see Section VII). (ix) In most cases the amino- and carboxy-termini are on the cytoplasmic side of the membrane. (x) Certain sequence motifs (see below) are duplicated in the amino- and carboxy-terminal halves of the proteins.

Prokaryotic secondary carrier proteins which differ significantly from the majority of the proteins analyzed so far are the efflux systems shown (proposed) to be involved in multiple drug resistance in *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Agrobacterium tumefaciens* [118–121]. These proteins are composed of approximately 100 amino acids, instead of the 400–500 of the other carrier proteins, and span the membrane most likely four times. Also the rhamnose carrier proteins (RhaT) of *E. coli* and *S. typhimurium* form exceptions to the model presented in Fig. 3. These proteins are predicted to have 10 transmembrane segments with the amino- and carboxy-termini on the outside (Ref. 122; Henderson,

P.J.F., personal communication). The glycerol facilitator (GlpF) of *E. coli* is composed of 277 amino acids for which hydropathy plots reveal six putative membrane spanning segments [123,124]. Other deviating and interesting structures form the lactose transport proteins (LacS) of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* [102,103]. These proteins are composed of a carrier domain with most likely 12 transmembrane α -helices [125], and a carboxy terminal hydrophilic domain of approximately 180 amino acids that is homologous to the IIA protein(s) (domains) of the phosphoenolpyruvate: sugar phosphotransferase systems (PTS) (see Section VIII).

By analyzing the primary sequences of many transport proteins from prokaryotic and eukaryotic origin, that differ in substrate specificity, direction of transport, coupling ion and mechanism of transport, similarities have been detected suggestive of a common ancestral gene for all of these proteins [18]. In addition, various highly conserved sequence motifs have been observed, such as the ‘R/K-X-G-R-R/K’ or the extended ‘G-X-X-X-D-R/K-X-G-R-R/K’ motif in cytoplasmic loops of several transport proteins that is predicted to form a β -turn structure (see below) [16,126,127]. These motifs have been observed in the polypeptides despite the fact that homology searches places several of these transporters in different families. Similarities in the sequences of the amino- and carboxy-terminal halves of various secondary transport proteins suggests that the 12 α -helical polypeptides may have been formed from internal sequence duplications.

The role of the ‘G-X-X-X-D-R/K-X-G-R-R/K’ motif in the tetracycline antiporter protein (TetA) of *E. coli* has been analyzed in detail following mutagenesis of each of the residues in the motif present in interhelix loop 2–3 [127]. The motif G₆₂-K-M-S-D₆₆-R-F-G₆₉-R₇₀-R is also present in a somewhat modified form in interhelix loop 8–9. Significant reduction or complete loss of transport activity is observed upon substitution of Gly-62, Asp-66, Gly-69 and Arg-70, whereas expression of the mutant proteins is unaffected. Defects following substitution of Gly-62 and Gly-69 corresponded with steric hindrance of the substituents in the putative β -turn structure of this region. The requirement of a positive charge at position 70 follows from the observations that substitutions other than R70K are totally devoid of activity [127]. Similarly, position 66 requires a negative charge since transport activity is completely lost upon substitution to Asn, whereas the D66E mutant retains activity at 10% of the wild-type level [128,129]. Mutagenesis of interhelix loop 8–9, i.e., G₂₆₆-R-I-A-T-K₂₇₁-W-G₂₇₃-E₂₇₄-K₂₇₅, suggests that this region may have a similar structural role in TetA as the interhelix loop 2–3 [130]. In contrast to the negative charge at position 66 and the

positive charge at position 70 in interhelix loop 2–3, none of the residues in interhelix loop 8–9 are essential for transport activity. On basis of these experiments it has been proposed that loop 2–3 forms an ‘active’ leaflet in TetA, while loop 8–9 forms the ‘silent’ counterpart in the second half of the molecule [130].

The role of the interhelix loops of LacY have been analyzed upon disruption by insertion of two or six contiguous histidine residues [131]. The results indicate that disruption of the hydrophilic domains 2–3, 8–9 and 9–10 markedly affects transport, whereas disruption of the other interhelix loops is essentially without effect. Interhelix loops 2–3 and 8–9 correspond to cytoplasmic regions between putative α -helices II–III and VIII–IX, respectively (Fig. 3). It is important to stress that in LacY, interhelix loop 2–3 comprises the sequence G-L-L-S-D-K-L-G-L-R-K, whereas a similar but more diffuse motif is present in interhelix loop 8–9. These results and those on the homologous sequences in interhelix loops 2–3 and 8–9 of TetA indicate that the conserved R/KXGRR/K (or GXXXXR/KXGRR/K) motifs [16,126,127,130] are structurally and to some extent functionally important in the secondary carrier proteins. Interhelix loop 9–10 of LacY corresponds with a periplasmic region between α -helices IX–X which may be close to some ‘translocation-site’ residues (see Section VII).

³Although prolyl residues are thought to play an important role in the structure and function of many proteins [132], and despite the fact that proline residues are relatively abundant in the transmembrane segments of polytopic membrane proteins [133,134], site-directed mutagenesis of these residues does not indicate an essential role for the prolines in the lactose carrier protein of *E. coli*. Neither *cis*/*trans* isomerization of the peptide bond preceding proline nor possible structural discontinuities, ‘kinks’, in the transmembrane α -helical domains due to the presence of proline residues appear to be important for membrane insertion, stability and activity of LacY [135,136].

IV-B. Tertiary structure

Until the first transport protein has been crystallized, or perhaps structural information has been obtained from two-dimensional or multi-dimensional Nuclear Magnetic Resonance spectroscopy, information about the three-dimensional structures can only be gathered from indirect methods. In principle, it should be possible to determine the structure of parts of the transport proteins (a few transmembrane segments) by two-dimensional or multidimensional NMR [137], provided these segments can be expressed and purified to homogeneity in sufficient quantities and retain their native configuration. Structure information on overlap-

ping parts of these proteins, from which possibly significant information on the three-dimensional structure of the entire protein can be obtained, could lead to an enormous advancement of our understanding of secondary solute transport mechanisms.

Proteins have been constructed in which the individual tryptophan residues have been replaced by phenylalanine and in which single tryptophans have been engineered at selected positions [138]. Information about the environment around single tryptophan residues can be obtained from the fluorescence emission spectrum and from fluorescence quenching by using quenchers of different polarity and size. Similarly, single cysteine containing proteins labelled with sulphhydryl specific spectroscopic probes can be used for examining static and dynamic aspects of carrier structures and functions [139–141]. In addition, fluorescence energy transfer between single tryptophans and fluorescent labeled single cysteines can provide information about distances between residues within the protein and reveal interactions of neighboring α -helices [142]. Various groups have directed their research in this direction and significant results are soon to be expected.

Important information about the interaction of specific domains of LacY has recently been obtained from the analyses of second-site revertants. King et al. [143] were the first to isolate second-site revertants of Asp-237 (D237) and Lys-358 (K358) in the lactose carrier of *E. coli*. Mutagenesis of each of these residues results in a defect in transport. Second-site revertants of D237N had a mutation that converted Lys-358 to Gln-358, whereas revertants of K358T contained a neutral amino acid (Asn, Gly or Tyr) at position 237. The second-site revertants exhibit galactoside accumulation and have activities that are 30–60% the rate of the wild-type [143]. From these studies it is concluded that Asp-237 (helix VII) and Lys-358 (helix XI) are closely juxtaposed in the three-dimensional structure, and possibly form a charge-neutralizing salt bridge. From similar studies Asp-240 (helix VII) and Lys-319 (helix X) are thought to interact with each other in the three-dimensional structure of LacY [144,145]. Whether Asp-237, Asp-240, Lys-319 and Lys-358 or the salt bridges per se are required for transport activity or that perhaps the salt bridges are folding intermediates that play a role in the maturation of LacY is unclear. However, some evidence has been presented that indicates that at least the interaction between Asp-237 and Lys-358 is also present in the mature protein. A single cysteine mutant with Cys at position 237, which is virtually inactive, is restored to full activity upon carboxymethylation with iodoacetic acid thereby compensating the positive charge (Lys) at position 358 [146]. Iodoacetamide which is uncharged does not restore the activity of the D237C mutant. By allowing lipophilic and lipophobic sulphhydryl

reagents to react with single cysteines at position 237 and 358, and analysis of the inactivation of the lactose transport protein, indications have obtained that the residues at these positions are within the membrane bilayer but close to the membrane/water interface at the outer surface of the membrane [146].

Finally, it should be stressed that each of the substitutions for Asp-237, Asp-240, Lys-319 and Lys-358 also result in marked changes in sugar recognition [144]. Several mutants isolated on the basis of altered sugar specificity have amino acid substitutions at or nearby one of these charged residues, indicating that the possible salt bridges form part of the sugar recognition site (see below).

IV-C. Quaternary structure

The association state of the secondary transporters in the membrane is often not known. Over the years evidence has been presented which indicates that the subunit structure of LacY is either monomeric or dimeric [11]. In case of LacY a monomeric state in the membrane is supported by rotational diffusion measurements [141], by freeze-fracture electron microscopy of purified LacY reconstituted into proteoliposomes, and by a linear dependence of the initial rate of transport with protein/phospholipid in the range at which statistically 0 to 3 molecules of LacY are present per (proteo)liposome [147]. Evidence for a LacY protein that functions as dimer has been obtained from radiation inactivation studies [148]. These studies indicated that LacY protein dimerizes in the presence of Δp , a conclusion that is not supported by other experiments that address the oligomeric state of the protein in the membrane. Taking the various data as a whole, the monomeric state of LacY is most likely sufficient for complete function of the transport protein [11]. A monomeric state in the membrane of UhpT is supported by gel permeation chromatography of the detergent solubilized protein and the relationship between activity and varying ratios of protein per proteoliposome [149].

Some mitochondrial and chloroplast antiporters are thought to have 6 (or 7) transmembrane segments. However, there is good evidence that these proteins function in the dimeric state which would also make a total of 12 transmembrane α -helices as the 'functional unit' [107]. On the basis of the quaternary structures of LacY, UhpT and the transporters of the eukaryotic organelles, it is tempting to speculate that the bacterial drug efflux systems, that are composed of most likely four transmembrane spanning segments, are actually functioning in the trimeric state. Along the same line of reasoning, the GlpF protein with 6 putative transmembrane α -helices may function as a dimer.

IV-D. Assembly and membrane insertion

Primarily based on work with the LacY protein, evidence is accumulating that the transmembrane α -helical segments together with adjacent amino-terminal cytoplasmic regions form structurally stable domains that independently contain the information necessary for insertion into membrane [150,151]. This suggests that the individual domains can organize themselves in the two-dimensional plane of the membrane to form an active carrier protein. Support for this proposal follows from the reconstitution of functional LacY protein from the simultaneous expression of amino- and carboxy-terminal halves of the polypeptide, and from pairs of genetically engineered carrier molecules from which different helical domains have been deleted [152,153]. Expression of the individual fragments yields polypeptides that are relatively unstable and exhibit no transport activity.

Amino- and carboxy-terminal deletions in the LacY and MelB proteins have been made to assess the role of these regions in the proper insertion of the proteins into the membrane and correct assembly to functional transporters. The first 22 amino acids of LacY, corresponding with the N-terminal half of the first putative transmembrane α -helix, are not essential for either membrane insertion or transport activity [154]. Sequential deletions and amino acid substitutions at the carboxy-terminus indicate that the residues of transmembrane α -helix XII are required for proper folding and protection against proteolytic degradation [155–157]. A similar conclusion is drawn from the analyses of carboxy-terminal deletions of the melibiose carrier protein (MelB) of *E. coli* [158]. The carboxy-terminal IIA domain of the lactose transport protein (LacS) of *S. thermophilus* can be removed without effect on transport activity. Deletions that extend into twelfth putative transmembrane α -helix of LacS are totally devoid of activity and the protein cannot be detected in the membrane anymore (Poolman, B., unpublished results).

The function of the hydrophobic segments of the polytopic (multiple crossings of the cytoplasmic membrane) membrane proteins is to span the membrane while the cytoplasmic hydrophilic loops with the positively charged residues provide anchors which determine the orientation of the transmembrane segments. It has been suggested that hydrophobic proteins are prevented from aggregation in the cytoplasm *in vivo* by a mechanism which avoids the release of nascent polypeptides from the ribosome until the membrane insertion process has been initiated [159]. The mechanism, however, by which polytopic membrane proteins insert into the cytoplasmic membrane is far from understood. It has been proposed that membrane proteins containing periplasmic regions (loops) longer than 60–70 amino acids are dependent on the Sec secretion machinery, or

at least these portions of the polypeptides, while smaller periplasmic domains do not require Sec [160]. If this is true, it would mean that most (or perhaps all) secondary transport proteins insert into the cytoplasmic membrane and assemble independent of Sec.

With the exception of the M13 phage coat protein, integral proteins of the cytoplasmic membrane are made without cleavable signal sequences. The membrane targeting sequences for such proteins are present in internal uncleaved segments [151,161,162]. Most likely multiple export signals are present throughout the membrane proteins [151,163]. Although experimental data point to a Sec-dependence for membrane insertion of polytopic membrane proteins, the involvement of individual Sec-components (or a subset of the 6 Sec proteins SecA, SecB, SecD, SecE, SecF and SecY) and the energy requirements of the process are far from clear [164].

In a study of the in vitro membrane assembly of the LacY protein it has been shown that the protein aggregates in the absence of membranes, but that it can integrate posttranslationally into membranes in an enzymatically active conformation independent of a Δp [159,165]. Membrane insertion independent of Δp in an in vitro assay has also been suggested for the IICBA protein (MtlA) of the mannitol PTS of *E. coli* [166].

On the basis of in vivo labeling, cell fractionation and assessment of integral membrane anchoring in wild-type and *secY* mutant strains, it has been suggested that the LacY protein requires functional SecY for integration into the membrane [167]. On the other hand, membrane insertion of LacY independent of SecA and SecY has also been reported [168]. Employing a cell-free protein synthesis-membrane insertion system, it has been suggested that the MtlA protein inserts into the membrane independent of SecA and SecB, but that the integration requires SecY [166]. The MalF component of the binding protein dependent maltose transport system has been shown to insert into the membrane when SecA and SecD functions are impaired, either by mutation or by inhibition of SecA

with azide [169]. Overall, these studies indicate that polytopic membrane (transport) proteins do require some components (SecY/SecE?) of the Sec-translocation machinery, in contrast to, for instance, M13 procoat which inserts Sec-independent. Other Sec components such as SecA, SecB and SecD, necessary for secretion of periplasmic and outer membrane proteins, may not always be essential. By recalling that the *E. coli* leader peptidase can switch from a Sec-dependent to a Sec-independent membrane insertion mechanism simply by adding a few positively charged lysine residues to the amino-terminal region [170], one could envisage that differences in requirement for Sec components may exist among the complex polytopic membrane proteins.

V. Catalytic properties

Table I summarizes the basic features of some secondary transport proteins that are discussed in more detail below. Properties related to the substrate and/or ion specificities as well as data on the kinetic mechanisms of transport are treated in this section.

The secondary transport systems described catalyze two-substrate reactions, i.e., symport or antiport of substrate and co-/counter substrate (Table I), and for most of these systems kinetic parameters have been reported. The steady state kinetics of these two-substrate reactions are described by the Michaelis constants (K_M) for the substrate and co-/counter substrate, provided the kinetic parameters are determined at saturating levels of the other substrate. The kinetic information of these secondary transporters serves as basis to which the properties of the corresponding mutants can be compared (Section VII). Unfortunately, kinetic parameters of transport (and binding) of the mutant proteins are most often presented in terms of apparent affinity constants (K_M^{app} , K_I^{app} and K_D^{app} , i.e., the parameters are inferred from conditions where, for instance, the second substrate is not saturating, which limits the information that is obtained from the

TABLE I

Characteristics of some secondary transport proteins

Transporter	Substrates	Co-/counter substrate	Mechanism	Additional Properties
LacY	Galactosides	H ⁺	Symport	Regulated by IIA
MelB	Galactosides	H ⁺ , Na ⁺ , Li ⁺	Symport	Regulated by IIA
UhpT	Sugar-Phosphate	Phosphate	Antiport	Variable stoichiometry
OxPT	Oxalate	Formate	Antiport	Indirect H ⁺ pump
PutP	Proline	Na ⁺ , Li ⁺	Symport	
TetA	Metal-tetracycline	H ⁺	Antiport	Electroneutral
LacS	Galactosides	H ⁺	Symport	C-terminal IIA domain
				Lac/gal exchange
NhaA	Na ⁺ (Li ⁺)	H ⁺	Antiport	Electrogenic

'kinetic' analysis. Conditions under which all substrates are saturating may be difficult to achieve, especially for proton-linked transport systems, since relatively minor variations in the pH may not only affect the kinetic performance of the enzyme through changes in proton concentration but may also modulate the activity through conformational changes of the protein etc. In principle, 'true' K_M and K_D can be extrapolated from measurements in a limited range of substrate (e.g., proton) concentrations [16], but this type of analysis has not frequently been carried out.

The maximal rate of transport (V_{max}) is defined as the product of k_{cat} (turnover number) and enzyme (transporter) concentration. Since the kinetic analysis of most transporters has been carried out with crude membrane preparations or intact cells, the enzyme concentrations are not always known and 'activities' are expressed as V_{max} . In many cases, however, antibodies are available to estimate the relative amounts (expression) of the proteins, and these data together with the V_{max} values have been used to evaluate changes in k_{cat} (Section VII).

A careful and complete kinetic analysis of wild-type and mutant transport proteins is perhaps best appreciated if one considers the non-random association of mutational double-effects. As pointed out by King and Wilson [21], single mutations often affect more than one step in the transport cycle (e.g., substrate recognition, cation recognition or substrate-cation coupling) and these effects may only be revealed by an appropriate kinetic analysis. Although the catalytic efficiency of enzymes is best expressed quantitatively as the k_{cat}/K_M ratio [170a], information regarding substrate specificities is often only available as K_M^{app} , K_I^{app} or K_D^{app} values or relative affinities (ratio of apparent affinity constants).

V-A. Lactose transport protein (LacY) of *E. coli*

The lactose transport protein of *E. coli* catalyzes the uptake of a variety of α - and β -galactosides in symport with 1 proton. The sugars transported include mono-, di- and trisaccharides; the K_I^{app} for the trisaccharide raffinose is, however, 100-fold higher than the K_I^{app} (or K_M^{app}) for lactose and melibiose [171]. LacY has a lower K_I^{app} for disaccharides with an α -linkage (e.g., melibiose) than for those with a β -linkage (e.g., lactose). By using a variety of sugars (sugar analogs) as competitive inhibitors of lactose transport, the importance of the -OH groups along the galactose ring and the optimal size of the aglycone have been determined [171]. The contributions to the relative affinities are OH-3 > OH-4 > OH-6 > OH-2 > OH-1 for the galactose moiety, and hexose (= disaccharide), benzene ring > methyl group > no aglycone (= monosaccharide) $\gg$

Fig. 5. Kinetic scheme showing a minimal number of transitions for carrier-mediated proton-linked solute symport. For proton and ligand association and dissociation both proton first, ligand last and ligand first, proton last are depicted.

disaccharide (= trisaccharide) > trisaccharide (= tetrasaccharide) for the aglycone moiety.

Analysis of *p*-nitrophenyl α -D-galactopyranoside (α NPG) binding to LacY has shown the presence of two kinetically distinguishable substrate binding sites, with K_D values of about 16 μ M and 1.6 mM, respectively [172]. Only the second NPG binding site is implicated in the catalytic cycle of LacY, whereas the first one (K_D = 16 μ M) is assumed to play a regulatory role. Biphasic kinetics has also been observed for the exchange and lactose- H^+ symport reactions of LacY, in which case the high- and low-affinity states may correspond with the catalytic and regulatory site, respectively [173]. The analysis of the substrate specificity of LacY described in the previous paragraph does not take into account the possibility of two sugar binding sites.

The steps minimally involved in carrier-mediated solute-proton symport are shown in Fig. 5. The scheme shows binding of ligand (L) and proton (H^+) on one surface of the membrane, followed by isomerization of the ternary carrier-ligand-proton (CLH) complex to a form on the other surface of the membrane. The ligand and proton are released in the *trans* compartment, and the unloaded carrier (C) isomerizes to the initial state. In principle, binding and release of ligand and proton can be random; however, in case of LacY there is evidence that efflux occurs by an ordered mechanism involving release of lactose first (step 2') and proton last (step 1') [174-177]. Thus, efflux proceeds as (5' $\rightarrow$ 4' (or 5 $\rightarrow$ 4) $\rightarrow$ 3 $\rightarrow$ 2' $\rightarrow$ 1' $\rightarrow$ 6 (Fig. 5). The order of association/dissociation of ligand and proton on the inner surface has not been specified. The maximal rate of lactose efflux increases sigmoidally with increasing pH. Since exit of labeled lactose is independent of pH

when an equimolar concentration of unlabeled lactose is present in the outside medium (equilibrium exchange), it is assumed that either deprotonation on the external surface (step 1') or the isomerization step 6 (return of the unloaded carrier) is rate-limiting for efflux. In case of exchange, dissociation of (^{14}C)lactose on the outer surface is followed by rebinding of (^{12}C)lactose rather than by release of the proton (step 1'), and the translocation reaction may proceed via steps $2' \rightarrow 3 \rightarrow 4'$ (or 4 followed by 5). Thus, under conditions of saturating concentrations of lactose on the outside, lactose exits via the forward and backward steps of the reaction scheme as indicated by $4'$ (or $5 \rightarrow 4 \rightarrow 3 \rightarrow 2' \rightarrow 3 \rightarrow 4'$ (or $4 \rightarrow 5$) (Fig. 5), without net movement of protons. Further mechanistic studies have indicated that lactose efflux down a concentration gradient is affected by $\Delta\Psi$ (inhibited by $\Delta\Psi$, inside negative; stimulated by $\Delta\Psi$, inside positive) whereas exchange is not. On basis of these and other experiments it has been concluded that the membrane potential acts on the isomerization of the unloaded carrier (step 6). Moreover, since a solvent deuterium isotope effect is observed for efflux and facilitated influx, but not for $\Delta\Psi$ -driven uptake [177], it has been suggested that transport solely driven by the concentration gradient is largely rate-limited by the deprotonation steps, i.e., step 1' for efflux and step 5' (or 4, depending on the order of association/dissociation on the inner surface) for influx. In the presence of a proton gradient (Δp) the rate-limitation is distributed over steps not involving the release of the proton [11]. As observed in the galactoside binding studies, there may be more than one proton association/dissociation reaction taking place during facilitated influx and efflux. By analyzing the effects of varying the pH of the external and internal medium on lactose influx and efflux, inhibition by *trans* protons with competitive and non-competitive contributions has been observed [178,179]. This indicates that in addition to catalytic protonation sites, i.e., those that attribute to the association/dissociation of the symported proton, other protonatable groups are affected. Since the charge on the protein will vary with pH, this may have led to conformational and/or activity changes. In general, such 'non-catalytic' effects are likely to take place in physiological ranges of pH, which complicates the kinetic analysis of H^+ -linked transporters.

V-B. Melibiose transport protein (MelB) of *E. coli*

The substrate specificity of the melibiose and lactose transport proteins of *E. coli* largely overlap, but the relative affinities of both galactoside transporters for different sugars vary. However, both carrier proteins differ significantly with regard to the specificity for the coupling ion. The specificity for the symported

TABLE II

Cation selectivity of the melibiose transport proteins of *Escherichia coli*, *Salmonella typhimurium* and *Klebsiella pneumoniae*

Substrates	<i>E. coli</i> ¹	<i>S. typhimurium</i> ²	<i>K. pneumoniae</i> ³
α -Galactosides:			
Melibiose	H^+ , Na^+ , Li^+	Na^+ , Li^+	H^+
α -NPG	H^+ , Na^+ , Li^+	n.d.	n.d.
Methyl- α -gal	n.d.	Na^+ , Li^+	n.d.
β -Galactosides:			
TMG	Na^+ , Li^+ (H^+) ⁴	Na^+ , Li^+	H^+ , Li^+
Lactose	Na^+ , Li^+	n.d.	Li^+
Monosaccharides:			
D-galactose	H^+ , Na^+ , (Li^+) ⁵	n.d.	n.d.
L-arabinose	Na^+ , (Li^+) ⁵	n.d.	n.d.

¹ Data from Tsuchiya and Wilson [301]; Wilson and Wilson [52]; Leblanc et al., [13].

² Data from Niiya et al., [330].

³ Data from Hama and Wilson [54].

⁴ (H^+), weak coupling of TMG coupled proton uptake has recently been reported [54].

⁵ (Li^+), Li^+ -coupling has not directly been tested, but is likely to take place.

cation of MelB is determined by the configuration of the transported sugar (Table II), whereas galactoside transport by LacY is always coupled to protons. The primary structures of the MelB proteins from *E. coli* and *S. typhimurium* are 85% identical [105], whereas the amino acid identity between MelB from *E. coli* and *K. pneumoniae* is 78% [54]. Despite the high identity scores between these homologous proteins, their preferred cation couplings to sugar uptake differ significantly (Table II).

Since mechanistic studies on MelB-mediated transport have been performed mainly on the melibiose carrier protein of *E. coli*, properties of MelB refer to the *E. coli* protein unless indicated otherwise.

The galactoside analog *p*-nitrophenyl- α -D-galactopyranoside (α NPG) is transported via MelB in symport with either Na^+ or H^+ and is competitively replaced by the physiological substrate melibiose. α NPG has frequently been used to monitor the sugar binding activity of MelB since the carrier protein has a relatively low K_D for the sugar analog [180,181]. The apparent dissociation constants (K_D^{app}) for α NPG decrease with increasing Na^+ and Li^+ concentrations without effect on the maximal number of binding sites. The activation of α NPG binding by these ions is inhibited by increasing H^+ concentrations, suggesting that Na^+ (or Li^+) and H^+ compete for a common cation binding site. The true $K_D(\text{NPG})$ in the presence of H^+ is 20-fold higher than in the presence of Na^+ , indicating a less efficient activation of sugar binding by protons. This is also reflected in higher K_M^{app} values for H^+ coupled transport reactions [182]. The competitive behaviour of the cations in the sugar binding assays is

Fig. 6. Relative rates of facilitated diffusion reactions catalyzed by the melibiose transport protein of *E. coli*. The relative rates of influx and efflux down a concentration gradient and exchange are shown for transport of melibiose coupled to H⁺, Na⁺ and Li⁺. The flux of Na⁺ during Na⁺-coupled melibiose influx, efflux and exchange is also depicted. Data are taken from Bassilana et al. [182–184].

consistent with the inhibition of sugar induced proton uptake by sodium ions.

The facilitated diffusion reactions catalyzed by MelB, for simplicity only cation-linked melibiose transport is discussed, are characterized by the following (Fig. 6) [183,184]: (i) The $V_{\max}$ of melibiose efflux down a concentration gradient exceeds the $V_{\max}$ of influx down a concentration gradient irrespective of the coupling ion, indicating that the carrier functions asymmetrically. (ii) Efflux and influx of melibiose are accompanied stoichiometrically with movement of sodium ions, whereas exchange exit of melibiose is not accompanied with net Na⁺ flux (Fig. 6). This suggests that sugar and cation release on the inside are sequential and in the order sugar first and cation last, and that exchange proceeds without dissociation/association of Na⁺. (iii) Na⁺ and Li⁺ coupled influx of labeled sugar is stimulated by the presence of sugar in the *trans* compartment, i.e., the $V_{\max}$ of exchange and counterflow exceed the $V_{\max}$ of influx. (iv) The $V_{\max}$ of H⁺ coupled influx down a concentration gradient is much greater than that of Na⁺ (or Li⁺) coupled influx, indicating that protons dissociate faster than Na⁺ (or Li⁺). In fact, the $V_{\max}$ of H⁺ coupled influx is similar to the $V_{\max}$ of exchange in the presence of H⁺, indicating that cation (proton) release is not rate-limiting under these conditions. The kinetic parameters of the different facilitated diffusion reactions suggest that Na⁺ release on the inner surface of the membrane is a major rate-limiting step. (v) The $V_{\max}$ of exchange in the presence of H⁺ and Na⁺ is faster than in the presence of Li⁺, indicating that the rate of sugar release varies

with the coupling cation or that the rate of translocation of the ternary complex is dependent on the transported cation. If one assumes that the rate of release of sugar in the inner compartment is determined by the stability of the ternary Carrier-Substrate-Cation (CSC[#]) complex, to which both substrate and cation binding will contribute, then the stability of CSC[#] increases in the order H⁺ < Na⁺ < Li⁺, and thus the rates of influx and exchange decrease in this order.

Finally, Na⁺ (or Li⁺) and H⁺ coupled influx are affected differently by the $\Delta\psi$. A $\Delta\psi$ (inside negative) decreases the V_M^{app} for H⁺ coupled uptake, but specifically increases the $V_{\max}$ for Na⁺ (or Li⁺) coupled transport [182]. The results can be explained by assuming that $\Delta\psi$ affects the stability of the CSC[#] complex, which results in an increase of the rates of dissociation of the cations for Na⁺ and Li⁺ coupled uptake ($V_{\max}$ effect) in the presence of $\Delta\psi$. By assuming that sugar influx during H⁺ coupled transport is rate-limited by the dissociation of the sugar, and not by the release of the H⁺, acceleration of the H⁺ dissociation by $\Delta\psi$ may have no effect on the $V_{\max}$. Rather the shift in equilibrium towards the unloaded carrier form could allow a more frequent reorientation of the unloaded carrier to the outer surface of the membrane, which is reflected by an increased apparent affinity for the sugar in the presence of a $\Delta\psi$ (inside negative).

V-C. Lactose transport protein (LacS) of S. thermophilus

As far as the substrate specificity of LacS has been analyzed, the sugar preference is similar to that of LacY and MelB. The relative affinity of LacS for the sugars differs somewhat, i.e., the apparent affinity constants for transport (K_M^{app}) increase in the order galactose < TMG < melibiose < lactose < raffinose (Poolman et al., 1992a; unpublished results). Apparently, the smaller the aglycone moiety attached to galactose the higher the affinity. The $V_{\max}/K_M^{\text{app}}$ increases in the order galactose < TMG < melibiose < lactose, suggesting that the LacS protein has the highest catalytic efficiency for lactose.

The kinetic scheme shown in Fig. 5 is appropriate to describe the reactions taking place during LacS-mediated transport. An apparent difference between LacS and LacY is the pH dependence of the exchange reaction catalyzed by LacS, whereas LacY-mediated exchange has been reported to be independent of pH between 5.5 to 10.5 [177]. The rate of exchange increases sigmoidally upto pH 7 with a pK_A of about 6; above pH 7 the exchange rate decreases with a pK_A of about 8 [89]. Raising the internal pH relative to the external pH affects the exchange reaction differently at low and high pH values. A ΔpH (interior alkaline) stimulates exchange at pH 6 and below, has virtually no effect at pH 7, and inhibits at pH 8 and above. These

results have been evaluated in terms of random and ordered association/dissociation of galactoside and proton on the inner surface of the membrane, and are consistent with a mechanism in which the binding of ligand occurs first and proton last (Fig. 5, step 5 $\rightarrow$ 4). Similar to the LacY protein, the rate of LacS-mediated efflux is decreased by $\Delta\Psi$ (inside negative), whereas the exchange reaction is unaffected, indicating that the unloaded carrier (C) carries a net negative charge and that during exchange the carrier recycles in the protonated form (CSH).

The exchange reaction in particular is relevant for the function of LacS in vivo. *S. thermophilus* is only able to utilize the glucose moiety of lactose and excretes the galactose part stoichiometrically into the medium [20]. By deleting the *lacS* gene from the chromosome, it has been shown that lactose uptake and galactose excretion are facilitated by LacS (Knol, J., Mollet, B. and Poolman, B., unpublished results), and kinetic data indicate that this transport occurs predominantly as lactose/galactose exchange.

V-D. Proline transport protein (PutP) of *E. coli*

The analysis of the energy coupling mechanism of the PutP (proline porter I) system of *E. coli* and *S. typhimurium* has long been complicated by a multiplicity of transport systems with overlapping specificities, and the presence of concentrations of Na^+ exceeding the $K_M(\text{Na}^+)$ in buffers considered to be devoid of Na^+ [185–187]. Now, it has been well established that PutP mediates proline uptake in symport with either Na^+ or Li^+ [47,60,188]. The PutP protein of *E. coli* has a relatively high affinity for proline ($K_D \sim 3 \mu\text{M}$ and $K_M < 1 \mu\text{M}$; in the presence of saturating Na^+); the K_D^{app} and K_M^{app} for Na^+ (in the presence of $1 \mu\text{M}$ proline) are 10 mM and $\sim 30 \mu\text{M}$, respectively [189]. The PutP protein is highly specific for proline, in contrast to the proton linked proline transporter ProP (proline porter II). The ProP protein transports proline ($K_M^{\text{app}} > 100 \mu\text{M}$), but also glycine betaine [190], and this system participates in the osmotic stress response of *E. coli* [191]. An interesting property of ProP is the reversible activation by a hyperosmotic shift which allows cells to respond rapidly ($t_{1/2} \sim 1 \text{ min}$) to changes in the medium osmolarity [66]. The activation of ProP by an osmotic upshift has been demonstrated in intact cells and membrane vesicles. Analysis of the primary sequence of ProP has revealed the presence of an α -helical coiled coil at the carboxy-terminus of the protein (191a). The unusual features of this carboxy-terminal extension suggest a mechanism for the (osmo)regulation of the ProP activity. A third, binding protein-dependent, proline/glycine betaine transport system (proline porter III, *proU* locus) is induced

about 400-fold upon an osmotic upshift giving rise to a slow genetically regulated osmotic response [192].

V-E. Metal-tetracycline / H^+ antiporter (TetA) of *E. coli*

Tetracycline resistance in *E. coli* and related Gram-negative bacteria is mediated by members of a family of related *tet* genes [193]. Resistance to tetracycline occurs by three known mechanisms: (i) energy-dependent efflux of tetracycline from the cell, (ii) ribosome protection, and (iii) inactivation of tetracycline. Of the tetracycline efflux systems, the one encoded by transposon Tn10 (class B) has been characterized at the molecular level of transport. Transposon-Tn10-mediated tetracycline resistance protein (TetA) confers a high tetracycline resistance to cells by excreting the antibiotic. Although tetracycline is largely anionic at neutral pH, the excretion of tetracycline occurs by means of an electroneutral exchange with a proton [194]. It has been shown that exit of tetracycline actually occurs as a chelate with a divalent cation which satisfies the observed electroneutral exchange with a single proton [79,80]. The TetA protein, thus, facilitates the exchange of the monocationic chelation complex metal-tetracycline for 1 H^+ . The degree of stimulation of tetracycline transport by divalent cations showed the following order: $\text{Co}^{2+} > \text{Mn}^{2+} > \text{Mg}^{2+} > \text{Cd}^{2+} > \text{Ca}^{2+}$, and parallels the values for the dissociation constants of tetracycline-metal chelate complexes [79]. Cotransport of tetracycline and $^{60}\text{Co}^{2+}$ has been demonstrated in inside-out membrane vesicles of *E. coli* bearing the TetA protein.

The TetA protein is homologous to a large number of transport proteins, including various sugar- H^+ symporters, the mammalian (facilitated diffusion) glucose transporters and anion- H^+ symporters [18], as well as a number of bacterial multidrug and antibiotic efflux proteins [18,194a,194b]. Similar to the TetA proteins, the multidrug and antibiotic efflux systems excrete their substrates most likely by means of H^+ -linked antiport.

V-F. Na^+/H^+ antiporter (NhaA) of *E. coli*

Growing cells maintain an inwardly directed sodium concentration gradient and a relatively constant intracellular pH at around neutrality. For this purpose bacterial cells possess Na^+ extrusion systems of which the Na^+/H^+ antiporter (NhaA) of *E. coli* is relatively best characterized. NhaA catalyzes the electrogenic exchange of one sodium ion for two protons [195]. Although the antiporter has been proposed to be electroneutral below a certain pH and electrogenic above that pH value, it is quite clear that this apparent change in stoichiometry is the result of the presence of a second Na^+/H^+ antiporter system and the low activ-

ity of NhaA below pH 7 [196–199]. The other antiporter in *E. coli* that specifically exchanges Na^+ (or Li^+) for H^+ is NhaB [200,201]. NhaB was identified as the residual antiporter activity, specific to Na^+ and Li^+ , after *nhaA* had been deleted from the chromosome [200]. The activity of NhaB is independent of pH [200,202], but the actual Na^+/H^+ stoichiometry of the exchange reaction is unknown at present. Since the variations in the apparent Na^+/H^+ stoichiometry in membrane vesicles and cells, expressing both NhaA and NhaB [203,204], parallel the pH dependence of NhaA [196,197,199], an electroneutral exchange by NhaB would be most consistent with the observed stoichiometries.

V-G. Sugar-phosphate / phosphate antiporter (UhpT) of E. coli

The discovery of phosphate/sugar 6-phosphate antiport in *L. lactis* [15,205] has led to a re-evaluation and re-examination of similar systems in other bacteria. For instance, the hexose 6-phosphate, glycerol 3-phosphate and 3-phosphoglycerate transporters of a number of bacteria have been demonstrated to be anion-linked antiporters [15,28,206]. The uptake of sugar phosphates (e.g., glucose 6-phosphate) by *E. coli* has initially been described as sugar phosphate- H^+ symport [207,208]. In these studies the transport of glucose 6-phosphate was analyzed in membrane vesicles that were prepared in the presence of phosphate, and most likely exhibited sugar 6-phosphate/phosphate exchange. These studies were further complicated by the presence of a Δp -driven phosphate transport system [45] which, altogether, resulted in an incorrect analysis of the transport mechanism. The hexose 6-phosphate (UhpT) and glycerol 3-phosphate (GlpT) antiporters of *E. coli*, and the 3-phosphoglycerate (PgtP) antiporter of *Salmonella typhimurium* show a high degree of similarity in the primary structure of the proteins [209].

The features of the sugar 6-phosphate/phosphate antiporter (UhpT) of *E. coli* are the following [15,210,211]: (i) The system catalyzes homologous and heterologous exchange of phosphate and sugar 6-phosphates. (ii) Substrate specificity studies on UhpT have shown that arsenate can replace phosphate, and that the K_M^{app} (or K_I^{app}) for the organic sugar phosphates are in the order: 2-deoxyglucose 6-phosphate < mannose 6-phosphate < glucose 6-phosphate, fructose 6-phosphate < glucosamine 6-phosphate $\ll$ others. (iii). The V_{max} of homologous phosphate exchange is approximately 5-fold faster than the V_{max} of heterologous exchange. (iv) The system favours monovalent phosphate and selects randomly among the available mono- and divalent sugar 6-phosphates. (vi) The exchange is electroneutral under all conditions. To maintain elec-

troneutrality during heterologous exchange, the antiport system translocates phosphate/sugar 6-phosphate with a pH-dependent variable stoichiometry. At pH 7.0 (0.9 pH units above the pK_2 of glucose 6-phosphate) the carrier catalyzes exchange of two molecules of monovalent phosphate for one molecule of divalent glucose 6-phosphate, whereas at pH 5.2 (0.9 pH units below the pK_2 of glucose 6-phosphate) the exchange corresponds with one molecule of monovalent phosphate for one molecule of monovalent glucose 6-phosphate. This aspect, however, has best been documented for the phosphate/sugar 6-phosphate antiporter of *L. lactis* [87], but is most likely also true for UhpT. (vii) UhpT requires a rather high ionic strength (0.3 M KCl) for maximal activity, a phenomenon that has not yet been explained.

VI. Purification and reconstitution

VI-A. Reconstitution and lipid requirement

An often applied method for the reconstitution of transport proteins is based on *n*-octyl β -D-glucopyranoside (octyl glucoside) solubilization of membrane vesicles and removal of detergent by dilution or dialysis [212,213]. The method has been refined by adding phospholipids [214] and stabilants (osmolytes, often glycerol) [215] during the solubilization. The method can be improved further by using a defined lipid composition during the solubilization step [216]. The presence of stabilants and (specific) phospholipids may protect the carrier proteins against denaturation by preventing delipidation. Furthermore, high concentrations of stabilants may be favourable for the exposure of the relatively hydrophobic surfaces of the membrane proteins which in turn may stabilize the native conformation of the protein.

Although alternative protocols have been devised [217,218], the basic method outlined above has been most successful for the reconstitution of several secondary transport proteins, i.e., the lactose transport protein (LacY), the melibiose transport protein (MelB), the arabinose transport protein (AraE), the proline carrier protein (PutP), the anion-linked antiporters (UhpT, GlpT, PgtP), and the galactose transport protein (GalP) of *E. coli* [188,210,214,215,219,220], the hexose phosphate/phosphate antiporter of *L. lactis* [221], the arginine/ornithine antiporter of *L. lactis* [84], the lactose transport protein (LacS) of *S. thermophilus* [89], the branched amino acid carriers of *L. lactis* [216] and *P. aeruginosa* [37], the alanine carrier proteins of thermophilic bacterium PS3 [222] and *Nitrosomonas europaea* [223], and the oxalate/formate antiporter (OxlT) of *O. formigenes* [224].

The phospholipid requirements for functional reconstitution have been determined for a number of

these carrier proteins. The branched amino acid carrier protein of *L. lactis* requires the presence of acidic phospholipids during the solubilization step, e.g., phosphatidylglycerol (PG), cardiolipin, phosphatidylserine (PS) or phosphatidylinositol (PI), whereas aminophospholipids, e.g., phosphatidylethanolamine (PE) or PS (or glycolipids), have to be present in the proteoliposomes for maximal activity [216,225]. The lipid requirement in the solubilization step appears more pronounced for Δp -driven uptake than for leucine counterflow activity. A similar observation has been made for the LacY protein of *E. coli* in which case the Δp -driven uptake requires a high concentration of PE whereas the counterflow activity is hardly affected by the lipid environment [226]. Full activity of the lactose carrier is obtained with PE or PS but not with PG or PC [227]. A comparable phospholipid requirement has been shown for the melibiose transport protein of *E. coli* [219]. The requirement for aminophospholipids of the leucine carrier of *L. lactis*, the lactose and melibiose carrier proteins of *E. coli* suggests that hydrogen bonds between the polar head groups of the lipids and the carrier molecules keep the proteins in their active conformation. Unlike these transporters, the leucine carrier of *P. aeruginosa* is activated by PE or PG, but not by PC [228]. For the leucine carrier proteins of *L. lactis* and *P. aeruginosa* maximal activities have been observed with phospholipids with an acyl chain length of about 18, indicating that also the bilayer thickness is contributing to important lipid-protein interactions [229,230]. Mismatches between the thickness of the hydrophobic α -helices of the membrane proteins and the lipid bilayer are energetically unfavourable (exposure of hydrophobic residues to the water phase). Such mismatches can be minimized by conformational changes in the membrane proteins and/or by changes in the packing of the lipid bilayer, which in turn may have affected the activity of the leucine transporters.

The effect of the degree of unsaturation of the phospholipid acyl chains has been analyzed for leucine transport in hybrid membranes of *L. lactis* [231]. Although the transport rate decreased with increasing number of double bonds, the effect has been attributed to an increased passive permeability of the membranes for leucine rather than to an effect on the carrier protein.

VI-B. Purification

Although several transport proteins have been solubilized and functionally reconstituted into proteoliposomes only LacY, PutP, NhaA of *E. coli*, OxlT of *O. formigenes* and the alanine carrier protein of thermophilic bacterium PS3 have been purified to a high degree of homogeneity and, subsequently, incorpo-

rated into liposomes. In each case, successful isolation and purification has been achieved following significant (over)expression of the proteins, i.e., to at least 5% of the total membrane protein. A number of *E. coli* sugar transport proteins, the arabinose (AraE), xylose (XylE), fucose (FucP) and galactose (GalP) proton-symporters, have been overexpressed to levels that vary between 5–50% of the cytoplasmic membrane protein (Refs. 16,232; Henderson, P.J.F., unpublished results), which should enable purification and further characterization of the proteins.

The *E. coli* LacY protein has been purified to > 95% purity by differential solubilization and ion-exchange chromatography [233,234]. Additional chromatography steps improved the purity of LacY [235–237], and various detergents and phospholipids have been used to stabilize the activity of the protein [179]. Functional characterization of the purified lactose transport protein has shown that a single polypeptide with an apparent molecular mass of 33 kilodaltons is sufficient to catalyze galactoside- H^+ symport and all other modes of facilitated diffusion reported in studies with membrane vesicles or intact cells [177,179,234,235,238]. Moreover, the apparent affinity constants for transport and the turnover numbers of Δp -driven transport, facilitated diffusion, efflux and counterflow are similar for proteoliposomes reconstituted with purified LacY and membrane vesicles [11,239]. Finally, purified LacY reconstituted in dimyristoylphosphatidylcholine liposomes exhibits counterflow activity only at temperatures above the lipid phase transition temperature [240], indicating that the bilayer lipids must be in the fluid state.

The *E. coli* proline carrier protein PutP has been purified as a bifunctionally active membrane-bound fusion protein [241,242]. By fusing *putP* to *lacZ* (β -galactosidase gene) via a collagen linker the fusion protein could be hydrolyzed by collagenase treatment, following solubilization from membrane vesicles and adsorption to anti- β -galactosidase IgG Sepharose [242]. In this way, the proline carrier has been purified to > 95%. Purified PutP catalyzes electrogenic Na^+ -proline symport with kinetic parameters similar to those observed in membrane vesicles [242].

The Na^+/H^+ antiporter (NhaA) of *E. coli* has been purified by hydroxylapatite and anion exchange chromatography [199]. The purified and reconstituted protein exhibits electrogenic Na^+/H^+ exchange both at acidic and alkaline pH values. Sodium efflux from these proteoliposomes increases more than 1000-fold when the pH is increased from 6.5 to 8.5. This property discriminates NhaA from NhaB which catalyzes Na^+/H^+ exchange independent of pH [200]. The purified NhaA protein has a turnover number of about $1500\ s^{-1}$ for downhill Na^+ efflux [199] which is approximately two orders of magnitude higher than efflux (or

exchange) mediated by LacY under optimal conditions [11].

The oxalate/formate antiporter (OxlT) of *O. formigenes* has been purified by sequential use of anion and cation exchange chromatography [243]. The detergent solubilized protein appears to be highly labile in the unliganded form [224]. The inactivation of OxlT can be prevented by including high concentrations of substrate (10 mM potassium-oxalate) in the buffers, used during the solubilization and chromatography steps. In fact, the thermal lability of unliganded OxlT has been used to determine substrate dissociation constants by analyzing the inactivation of OxlT in the absence and presence of varying concentrations of substrate, and by assuming that unliganded and liganded OxlT are at equilibrium [224]. Similar to NhaA, OxlT also catalyzes exchange with a turnover number of at least 1000 s^{-1} . Since the K_D for the OxlT-oxalate complex is $20\text{ }\mu\text{M}$, the k_{cat}/K_D ratio becomes $5 \cdot 10^7\text{ M}^{-1}\text{s}^{-1}$ which is close to the limits of a diffusion limited process (about $10^8 - 10^9\text{ M}^{-1}\text{s}^{-1}$). Purified and reconstituted OxlT catalyzes in addition to homologous exchange of oxalate and formate also heterologous oxalate/formate exchange. This latter process is electrogenic, as has been shown in proteoliposomes obtained from crude detergent-solubilized membrane extracts [82].

The alanine carrier protein (Acp) of the thermophilic bacterium PS3 has been purified through ion-exchange and hydroxylapatite chromatography [53,222]. The purified and reconstituted protein couples alanine uptake to the cotransport of either proton or sodium ions, similar to that reported for galactoside uptake by the melibiose carrier protein of *E. coli* [244].

Almost without exception, the (purified) secondary carrier proteins migrate in sodium dodecyl sulfate-polyacrylamide gels with an apparent molecular mass that is less than predicted from the calculated molecular mass on basis of the DNA sequence. The discrepancy between the calculated and the estimated molecular masses is probably due to the abnormally high binding of sodium dodecyl sulfate to these hydrophobic proteins.

VII. Functional domains and catalytic residues

To identify residues that are important for catalytic function of secondary transport proteins extensive random and site-directed mutagenesis has been performed on the LacY, MelB, PutP, NhaA, TetA and GalP proteins of *E. coli* and the LacS protein of *S. thermophilus*. Only those residues that have been shown to be important for catalytic and/or regulatory function will be discussed. The kinetic characterization of the mutant proteins is often restricted to the determination of K_M^{app} (or K_D^{app}), while expression levels have been estimated with the aid of Western analysis or by deter-

mining the concentrations of binding sites in equilibrium binding measurements (see also the beginning of Section V). Although many mutations have not been characterized in terms of changes in k_{cat}/K_M , useful information can often be inferred from the mutants when clear cut catalytic effects are observed (e.g., uncoupling of transport) or when mutants have been selected on basis of a novel property.

VII-A. Histidine residues

It is conceivable that residues involved in proton translocation or pH sensing (regulation) undergo (de)protonation in the physiological pH range. Since histidine in solution has a pK_A of about 6, histidine residues are likely to have important functions in carrier proteins. Each of the histidines of LacY, MelB, LacS, TetA and NhaA has been replaced by other residues and the catalytic activities of the mutant proteins have been analyzed. In each of these proteins a single histidine appears important, but none is essential for proton (or cation) translocation. A great deal of work has been directed towards the role of His-322 in LacY [11,12]. This residue has been implicated in lactose-coupled H^+ translocation and forms part of a putative proton relay [245–249]. The proton or charge relay mechanism assumes a role for His-322, Glu-325 (which should be on the same side of α -helix X as His-322), and Arg-302 (putative helix IX) in proton translocation, which is based on analogies with proton transfer via Asp, His and Ser in serine-type proteinases [250]. In this view, Glu-325 and His-322 are ion-paired and interact with Arg-302; Glu-325 and Arg-302 would polarize the imidazole group of His-322 which enhances its capacity to act as a proton shuttle. His-322 is poised to accept a proton from Glu-325 and subsequent transfer of this proton to Arg-302, another residue or the medium would lead to net proton translocation. A role for Arg-302 in proton translocation comes from the analysis of LacY(Arg-302) mutants which display properties similar to those of LacY(His-322) mutants [11,248]. More recently, the role of His-322 in proton translocation has been questioned, since the requirement for an ionizable histidine residue at position 322 in LacY is not always applicable to galactoside accumulation and galactoside-dependent proton transport [251–254]. Similarly, some substitutions of Arg-302, i.e., R302S and R302H, result in mutant proteins that do accumulate galactosides, albeit to lower levels, and do exhibit sugar-dependent proton transport [255]. It seems likely that Arg-302 and His-322 play an auxiliary role in proton translocation, e.g., via modulating the pK_A of a nearby residue (Glu-325?), since galactoside accumulation and sugar-dependent proton uptake is highly compromised in some of the mutants and totally absent in others. The degree of

uncoupling of sugar transport from H^+ transport not only varies with the substitution made, but also with the sugar used in the transport assay [251,252] and other parameters such as the pH of the assay medium [254]. The role of Arg-302 and His-322 as well as nearby residues in sugar specificity will be discussed further in a separate section below.

LacS and MelB are homologous proteins with about 25% identity in their primary structures [102] and belong to a family which also includes MelB of *Klebsiella pneumoniae* [54] and *S. typhimurium* [105], the glucuronide transporter (GusB) of *E. coli* (Liang, W.-S., Jefferson, R. and Henderson, P.J.F., unpublished results), and the xylose transporter (XylP) of *Lactobacillus pentosus* (Leer, R., Martena, J. and Poolman, B., unpublished results). LacY is not homologous to members of this family of transporters; however, a stretch of about 20 amino acids around His-322 in LacY can be identified in LacS (Fig. 7). Conserved residues include a histidine (322 in LacY, 376 in LacS; arrow 3), a lysine (319 in LacY, 373 in LacS; arrow 2) and a glutamic acid (325 in LacY, 379 in LacS; arrow 4). K319 in LacY has been proposed to interact with D240 (see subsection IV-B), and preliminary experiments indicate that a basic residue at position 373 in LacS is also required for activity (Poolman, B., unpublished results). The role of the conserved glutamic acid will be discussed below. Of the 11 histidine residues present in LacS, only the His-376 substitution in the carrier domain of the protein significantly affects transport [125]. In fact, the LacS(His-376) substitutions resemble those of LacY(His-322): (i) active accumulation of galactosides is highly compromised, but can be detected under appropriate conditions; (ii) the K_M^{app} for galactosides is

altered. The histidine residue corresponding to His-376 in LacS and His-322 in LacY is not conserved in MelB (Fig. 7). Experiments in which the histidine residues of MelB were replaced with arginine have indicated that only His-94 is important, and that mutations at this position alter the expression and stability of the protein but not the catalytic activity [256,257].

A conclusion similar to the one reached for the role of His-322 in LacY and His-376 in LacS can be made for His-257 in the tetracycline antiporter protein (TetA) of *E. coli* [258]. Some of the TetA(H257) mutants do have tetracycline transport activity, but without coupling to proton transport, whereas others retain significant proton translocation coupled to tetracycline transport [258].

Recent experiments in which the histidine residues of the Na^+/H^+ antiporter (NhaA) of *E. coli* were mutated to arginines have shown that none of the histidines is essential for antiport activity. However, H226R shifts the pH dependence of the antiporter to lower pH values and reversibly inactivates the protein above pH 7.5 to about 10% of wild-type activity [259]. It is proposed that His-226 is part of the pH sensor that regulates the activity of the Na^+/H^+ antiporter.

VII-B. Glutamic acid and aspartic acid residues

A residue important for the putative H^+ relay of the LacY protein of *E. coli* is glutamic acid-325. This residue may directly participate in galactoside-coupled proton translocation since uphill transport and other translocation reactions presumed to involve (de)protonation steps are completely defective [247,260]. Glutamic acid-325 is conserved in LacS and MelB (Fig. 7,

Conserved Residues in Carrier Domain of LacS and other Sugar Transport Proteins

			↓1	↓2	↓3	↓4	
LacY _{EC}	311-339	S A L E V V I L K T L H M F E V P F L L V G C F K Y I T S					
LacY _{KP}	316-344	S A V E V V I L K M L H M F E I P F L L V G T F K Y I S S					
RafB _{EC}	314-342	T M T E V V I L K M L H A L E V P F L L V G A F K Y I T G					
		# : # : # : # : # :					
LacS _{ST}	365-393	D S V E Y G Q W K T G H R D E S L T L S V R P L I D K L G					
LacS _{LB}	358-386	D A V E Y G Q L K T G H R D E A L T L S V R P L I D K L G					
		# # : # # : # # : # # :					
MelB _{EC}	347-375	D I V D Y G E Y K L H V R C E S I A Y S V Q T M V V K G G					
MelB _{SY}	351-379	D T V D Y G E F K L N I R C E S I A Y S V Q T M V V K G G					
MelB _{KP}	351-379	D T V D Y G E Y T M N I R C E S I A Y S V Q T L V V K A G					
GusB _{EC}	345-373	D T V E Y G E Y L T G V R I E G L T Y S L F S F T R K C G					
XylP _{LP}	367-395	D S V D Y G E W K N G V R A E G I V T S F S S F S A K F G					

Fig. 7. Conserved residues in carrier domain of LacS and other secondary transport proteins. LacY, lactose transport protein; RafB, raffinose transport protein; LacS, MelB, GusB and XylP are the same as indicated in the legend to Fig. 4. The subscripts ec, kp, st, lb, sy and lp refer to *E. coli*, *K. pneumoniae*, *S. thermophilus*, *L. bulgaricus*, *S. typhimurium* and *L. pentosus*, respectively (data from Refs. 125,261). Arrow 1 corresponds to a conserved negatively charged residue in each of the proteins; arrow 2 indicates a Lys corresponding with the putative 'salt-bridge' residue Lys-319 of LacY; arrows 3 and 4 correspond to the putative proton relay residues His-322 and Glu-325 in LacY.

arrow 4; positions 379 and 361, respectively). Replacing Glu-379 in LacS by a neutral amino acid, i.e., E379A and E379Q, results in defective uphill transport and loss of galactoside induced proton uptake similar as observed for the corresponding mutations in LacY [261]. LacS and LacY with aspartic acid rather than glutamic acid at positions 379 and 325, respectively, are partly uncoupled and catalyze transport with about 20% of the activity of the wild-type. Notice that the side-chain of aspartic acid is one methylene carbon (about 1.5 Å) shorter than that of glutamic acid. Efflux down a concentration gradient does occur in LacS (E379A and E379Q), but is independent of pH, whereas efflux in the corresponding LacY mutants is virtually blocked. Despite these and other differences between LacS(E379A/Q) and LacY(E325A/Q), the data indicate that Glu-379 in LacS and Glu-325 in LacY may have a similar role in the translocation process and participate directly in the proton transport. For both galactoside transporters mutagenesis of the glutamic acid only moderately affects the apparent affinity constants for sugar binding and transport. Importantly, the expression levels of the proteins are not significantly affected by the mutations.

Although the glutamic acid residue equivalent to LacY(E325) and LacS(E379) is also present in MelB, i.e., Glu-361 (Fig. 7), substitutions at this position have effects distinct from those in LacY and LacS. MelB E361G and E361D substitutions impair galactoside uptake, but H⁺, Na⁺ (or Li⁺)-coupled transport does occur albeit with k_{cat} that is more than 10-fold reduced [13]. The activity of the MelB E361A mutant is dependent on the temperature at which the cells have been grown and transport has been assayed. The E361A mutant catalyzes transport with rates that decrease 5-fold upon shifting the temperature from 25 to 37°C, while the activity of the wild-type protein increases 4-fold under identical conditions. The results obtained with the Glu-361 mutants indicate that the negatively-charged glutamic acid is not required for coupled transport, but that the residue may stabilize the conformation of the protein. This result is somewhat surprising since the sequence similarity between MelB and LacS, and the relative conservation of the stretch of about 20 amino acid residues in which the Glu-361 resides, would suggest a similar catalytic function.

Various aspartic acid residues are found in putative transmembrane segments of MelB, i.e., Asp-31 (helix I), Asp-51 and Asp-55 (helix II) and Asp-120 (helix IV) [262]. The acidic residues at position 51 and 55 are highly conserved in the MelB-LacS family, whereas aspartic acids in LacS, GusB and XylP can also be found at positions equivalent to Asp-31 in MelB (Poolman, B., unpublished results). Asp-120 is conserved in LacS but not in GusB and XylP. The conservation of the acidic residues in helix II as well as the conserva-

tion of an arginine (Arg-48 in MelB) makes the helix highly amphipathic in each of the homologous proteins (Fig. 4; the hydrophobic moments (μ_s) of α -helices II (18-residue segments) in LacS, MelB and GusB are 0.42, 0.42 and 0.38, respectively) [263]. Since the substrates of MelB, LacS, GusB and XylP are hydrophilic, part of the interior of the carrier molecules is also expected to be hydrophilic in order to facilitate transport. In particular, the polar faces of amphipathic helices, such as helix II in the MelB-LacS family, could interact with the solutes and/or cation(s), whereas the apolar faces could interact with the bilayer. To analyze the role of these acidic residues in the catalysis of MelB-mediated galactoside transport, each of the aspartic acids has been replaced by cysteine, asparagine, glutamate or another residue [262, 264–266]. The results indicate that neutral substitutions at MelB positions 51, 55 and 120 lead to (i) loss of Na⁺-linked thiomethyl β -D-galactoside (TMG) transport, and (ii) binding of α - and β -galactosides independent of sodium ions [262]. With most of these mutations the amount of MelB protein in the membrane is reduced relative to membranes carrying the wild-type protein *. Since the transport rates have been normalized on basis of the expression levels, the performance of wild-type and mutant proteins can be compared directly.

Loss of sodium-dependent binding and transport is also observed for MelB(D31C), but not for MelB(D31N). Furthermore, while mutations at position 55 and 120 not only affect Na⁺, but also H⁺-dependent transport, MelB(D51C) still catalyzes small but significant H⁺-linked melibiose transport. These and other features of the mutant MelB proteins suggest that the aspartic acid residues may be at or near the cationic binding site and may participate in the coordination of the coupling cation. Quantitative analysis of the effects of H⁺ and Na⁺ (or Li⁺) on *p*-nitrophenyl α -D-galactopyranoside (α NPG) binding indicates that these cations compete for the same cationic binding site on MelB [181]. Such a competition is perhaps more easily envisaged if H₃O⁺ rather than H⁺ is the translocated species [100]. By analogy with the interactions of H₃O⁺ with a tetracarboxylic 18-crown-6 ligand and Na⁺ with dicyclohexyl 18-crown-6, of which the structures were derived from crystallographic studies [267], the properties of the individual Asp mutants have been discussed in terms of complexation of H₃O⁺ and Na⁺ with the aspartate residues in α -helices I, II and IV [266]. Since the interactions are likely to be different for H₃O⁺ and Na⁺ [267], differences with respect to cationic coupling of the various

* The carrier protein concentrations of right-side out membrane vesicles have been estimated from the maximal number of [³H]NPG binding sites [262].

mutants such as observed with MelB(D51C) can be expected.

As indicated above, the acidic residues at MelB positions 51 and 55 are conserved in LacS, which is known to transport galactosides exclusively with protons [89]; the equivalent residues in LacS are Glu-67 and Asp-71. Substitution of Glu-67 by a neutral amino acid (E67Q) results in a conditionally uncoupled phenotype, i.e., galactoside accumulation is reduced to about 10% at 37°C but normal at 25°C, whereas equilibrium exchange is comparable to that of the wild-type at both temperatures (Knol, J., Fekkes, P. and Poolman, B., unpublished data). The LacS(D71N) mutant is devoid of any activity, but the nature of the defect still has to be established (either k_{cat} or the expression level or both are reduced). These observations are consistent with those made for the equivalent substitutions in MelB. The conservation of the four acidic residues in LacS and MelB, the importance of these residues in cationic coupling of MelB-mediated transport in *E. coli*, and the differences in cation selectivity of both transport proteins, suggest that minor differences in the three-dimensional structures of MelB and LacS rather than specific interactions with a limited number of residues may determine the cation specificity. This conclusion is supported by recent observations that the aspartic acids in putative α -helices I, II and IV are totally conserved in the MelB proteins of *E. coli*, *S. typhimurium* and *K. pneumoniae* [54,105], whereas the cation selectivity of the transporters differs (Table II).

In addition to helix X, containing His-322 and Glu-325, α -helix VIII of LacY also has amphipathic properties. The apolar face of helix VIII contains amino acid residues that can be mutated without much effect on lactose transport activity. These residues are on one side of the α -helix, a stripe opposite Glu-269 [268]. It is proposed that the mutable region has low information content and that the corresponding residues interact with the membrane phospholipids. The negative charge at position 269 in LacY seems essential for galactoside accumulation and lactose-dependent proton uptake.

The transposon Tn10 TetA protein contains four charged residues in putative transmembrane segments, one of which is His-257 the histidine that is thought to play a role in metal-tetracycline coupled proton transport (see '*histidine residues*'). The remaining three residues in the transmembrane regions are Asp-15, Asp-84 and Asp-285. Substitution of Asp-15 and Asp-84 for neutral amino acids affects the K_M^{app} of tetracycline transport which is consistent with the proposal that these residues form part of the substrate binding site [269]. Replacing Asp-15 in another set of experiments indicated that either a large k_{cat} or a large K_M^{app} effect can be observed, depending on the substituent [270]. With each mutation, however, the $k_{\text{cat}}/K_M^{\text{app}}$ is con-

stant and reduced approximately 10-fold relative to the wild-type protein, indicating that Asp-15 is important for the catalytic efficiency. All Asp-285 substitutions made have lost their proton translocation activity [269], and this residue may have a similar and essential role in H^+ translocation like Glu-325 in LacY and Glu-379 in LacS (see above). Asp-365 which is located in the last periplasmic loop of TetA has been suggested to play a role in maintaining β -turn structure. The role of Asp-66 which forms part of the conserved G-X-X-X-X-R-X-G-R-R of TetA is discussed in Section IV.

VII-C. Cysteine residues

The identification of LacY by labeling with *N*-ethylmaleimide (NEM) [271], and subsequent studies with other specific sulfhydryl modifying and oxidizing reagents suggested a role for cysteines in the catalytic mechanism of LacY. Specifically, it has been suggested that in LacY sulfhydryl-disulfide interchange occurs either as intermediate of the respiratory chain [272] or as proton carrier that is affected by Δp -mediated redox changes [273,274]. However, characterization of LacY mutated at each of the 8 cysteinyl residues in the molecule has demonstrated that none of the cysteines is essential for transport [275]. Even the cysteine-less LacY protein displays significant lactose- H^+ symport activity [276]. From the analysis of the single cysteine mutants, it has become clear that Cys-148 and Cys-154, both located in putative helix V, react with sulfhydryl oxidizing reagents. Cys-148 reacts with various sulfhydryl specific reagents, including alkylating compounds like NEM, in a substrate protectable manner, and the location of this residue has been postulated at or near a galactoside binding site [277]. The LacY(C148S) mutant is slowly inactivated by NEM in a reaction that cannot be prevented by substrate (TDG, β -D-galactosyl 1-thio- β -D-galactopyranoside) [278].

Interestingly, the LacY(C148S) mutant displays monophasic (non-equilibrium exchange) kinetics with only the high affinity state for lactose, whereas the wild-type protein exhibits biphasic kinetics with both a high and low K_M^{app} [279]. The maximal rates of efflux and exchange of the C148S mutant are about 5-fold reduced relative to the wild-type LacY protein, and high affinity binding of NPG is no longer detected in LacY(C148S). These results are consistent with the notion that the site with low affinity for lactose (and high affinity for NPG) does not participate in the catalytic cycle of the transport protein but does play a regulatory role [172,173]. Apparently, this regulatory site disappears, at least in kinetic terms, upon substitution of Cys-148. With the LacY(Cys-154) mutants the biphasic kinetics is retained, but the K_D^{app} 's for NPG decrease with increasing hydrophobicity of the substituting amino acid, i.e., the K_D^{app} increases in the order

Val < Gly < Cys < Ser. The data suggest that position 154 in LacY affects NPG binding by forming part of the pocket that interacts with the nitrophenyl group of the galactoside analog. This also indicates that changes in affinity for NPG not necessarily reflect changes in affinity for 'natural' sugars like lactose which limits the use of the galactoside analog in analyzing, for instance, substrate specificity mutants.

The lactose transport protein (LacS) of *S. thermophilus* contains a single cysteine residue (Cys-320) which can be substituted without effect on transport activity (B. Poolman, unpublished results). The wild-type LacS protein is inhibited by the lipophilic sulfhydryl reagent *p*-chloromercuribenzoic acid (pCMB), but not by the hydrophilic analog *p*-chloromercuribenzenesulfonic acid (pCMBS), suggesting that Cys-320 is not accessible from the outside of the membrane. This observation is in accordance with topographic studies on LacS [125].

Each of three cysteinyl residues of the galactose-proton symporter (GalP) of *E. coli* has been substituted in order to identify the substrate protectable *N*-ethylmaleimide reactive residue (McDonald, T.P., Shatwell, K.P. and Henderson, P.J.F., unpublished results). Cys-374 in GalP appears responsible for reaction with NEM, but neither this residue nor the other cysteines are important for transport activity. Substitution of Cys-281 and Cys-344 with serine residues in the sodium dependent proline carrier protein (PutP) of *E. coli* have shown that both residues contribute to the NEM sensitivity of the transporter [280]. Since proline protects the carrier from inactivation by NEM, these two cysteines are thought to be close to the substrate binding site. Using membrane permeable and impermeable sulfhydryl specific reagents, and right-side-out and inside-out membrane vesicles, redox-sensitive (sulfhydryl) groups have been identified in the proline carrier protein at the outer and inner surface of the membrane [281]. These different locations correspond most likely with the locations (accessibilities to the reagents) of Cys-281 and Cys-344. Neither C281S nor C344S substitutions affect the proline uptake activity or the substrate specificity of PutP [280].

VII-D. Other residues

As indicated in Section IV, proline residues in LacY have been mutated in order to assess whether the unique properties of the prolyl peptide bond, such as *cis-trans* isomerizations or the tendency to disrupt α -helices, play a role in transport. However, such specific effects have not been observed with the LacY Pro mutants [135,136]. The results on the proline substitutions indicate that with the exception of Pro-28, which displays some defects upon substitution (see also below), these residues are not important for the transport function. The activity of the Pro mutants is principally

determined by the hydrophobicity and/or the size of the side-chain of the substituent [136].

The intrinsic fluorescence of tryptophan residues and the possibility to use this property to obtain information about the structure of a protein has led to the engineering of a Trp-less LacY protein [138]. The mutant protein, devoid of all the tryptophan residues, retains more than 70% of the activity of the wild-type protein, indicating that tryptophans have no essential role in the function and structure of LacY. By analyzing various substitutions for Trp-33, this residue has, however, been implicated in sugar recognition [282]. Each of the 14 tyrosine residues of LacY have been replaced with phenylalanine, and the effects of the mutations have been analyzed [283]. Although substitution of some of the residues (Tyr-26, Tyr-336 and Tyr-382) affects the overall performance of the protein, the Tyr-236 substitution has the most significant effects. LacY(Y236F) catalyzes lactose exchange with a rate 40% the wild-type but does not catalyze uphill transport or efflux down a concentration gradient [283]. Substitutions of Tyr-236 have also been isolated as sugar specificity mutants (see below).

Mutations in putative Helix I of the LacY protein have been analyzed with respect to the ability of the mutant proteins to bind and translocate galactosides [284]. The mutants G24E and P28S bind normal amounts of NPG with K_D^{app} values somewhat lower than that of wild-type LacY, but transport with less than 5% the wild-type activity. The G24R mutant and two double mutants G24R/P28S and G24E/P28S also bind normal amounts of NPG and do accumulate galactosides to measurable levels; however, the rate of influx is about 0.01% of the wild-type. Not only influx but also efflux down a concentration gradient and exchange are greatly reduced. From these data and the accessibility of the galactoside binding site in right-side-out and inside out membrane vesicles, it is concluded that the mutations affect the isomerization of the ternary carrier-substrate-proton (CSH) complex [284]. Recent studies in which varying numbers of amino acids have been deleted from the amino-terminus of LacY have indicated that the first 22 amino acids, corresponding with the N-terminal half of the first putative transmembrane α -helix are not essential for transport [154]. This suggests that the effect of the G24R and G24E (and P28S) mutations on transport is due to disruption of the protein structure upon introduction of bulky charged residues in a transmembrane segment rather than to a specific requirement for Gly at this position.

VII-E. Cation and substrate specificity mutants

Using random mutagenesis techniques in combination with selection for specific phenotypes, rather than employing site-directed mutagenesis, a variety of cation

and/or substrate specificity mutations have been isolated in LacY, MelB and PutP. Instead of discussing each of these mutants, which often have only partially been characterized, the positions of the residues that affect the cation selectivity and/or substrate recognition of LacY and MelB will be summarized (Fig. 8A,B).

Also included in the figure are the site-directed mutants that have turned out to be sugar and/or cation specificity mutations. The criteria for assigning a role in substrate and/or cation recognition to a specific residue are based on *in vivo* selection for amino acid substitution(s) at that particular position, and in case of

Fig. 8. Secondary structure model of LacY (A) and MelB (B) showing the positions of amino acids that have been selected on basis of an altered substrate specificity or identified as such following analysis of site-directed mutants (closed circles). Many of these also have an altered cation recognition (see text). A. The secondary structure model of LacY is based on the version of King et al., [143]. Glu-269 (helix VIII) and Glu-325 (helix X) of LacY are shown as open circles, whereas Gly-24 and Pro-28 (helix I), that have been implicated in sugar translocation, i.e., isomerization of the ternary CSH complex (Fig. 5; Overath et al., [284]), are indicated as open squares. The closed square in α -helix V corresponds to Cys-148. Closed circles correspond to Ala-177 (helix VI), Tyr-236 (helix VII), Gly-262 (helix VIII), Thr-266 (helix VIII), Arg-302 (helix IX), Ile-303 (helix IX), Ser-306 (helix IX), Lys-319 (helix X), His-322 (helix X) and Ala-389 (helix XII) (see various references in Section VII). The putative salt-bridges between Asp-237 and Lys-358 and Asp-240 and Lys-319 are shown as dashed lines. The arrows indicate sites of interaction with IIA.B. The secondary structure model of MelB is based on the one of Botfield et al. [112]. The amino acid substitutions causing alterations in sugar and/or cation specificity have been taken from Refs. 302,306,307 (Open and closed circles). Closed circles indicate residues that have been selected on basis of altered sugar specificity, but with the exception of I61V (helix II) the substitutions also affect the cation selectivity. Open circles indicate residues that have been selected on basis of altered cation selectivity, but may also have an altered sugar recognition. The aspartic acid residues (Asp-31, Asp-51, Asp-55 and Asp-120) in putative membrane spanning segments I, II and IV and Glu-361 in the interhelix loop 10–11, are shown as open squares. The arrows indicate sites of interaction with IIA; the open arrow has been postulated on the basis of sequence similarity between the central loop of MelB and part of MalK (see text).

site-directed mutants a significant (at least 3-fold) change in the apparent affinity constants K_D^{app} or K_M^{app} for natural sugars (true K_D and K_M values are often not available). Since K_D values for high-affinity binding of α NPG to LacY most likely reflect changes in the second 'regulatory' sugar binding site, rather than diagnosing the 'catalytic' sugar binding site (see subsection VII-C), amino acid substitutions causing changes in K_D for NPG are not shown in Fig. 8A.

The LacY protein. Strategies for isolating LacY mutants with interesting phenotypes have largely been based on selection for resistance to inhibition by toxic sugar analogs (e.g., cellobiose, TDG) and the ability to grow on sugars that are not substrates of the wild-type protein (e.g., maltose, sucrose) [285–288]. Single mutations which cause an increased ability to transport maltose (an α -glucoside; enhanced recognition for maltose, although some mutants also transport faster) are at positions Ala-177, Tyr-236, Thr-266, Ser-306, and Ala-389 [285,288–291]. Mutations at position 177 have also been isolated upon selection for growth on sucrose [287]. Almost without exception the mutants that have acquired the ability to transport maltose are highly resistant to TDG (β -galactoside) and cellobiose (β -glucoside), and display diminished recognition for β -galactosides such as lactose and TMG [285]. Furthermore, most of the mutants are highly defective in accumulating galactosides against a concentration gradient whereas downhill uptake and counterflow activities can be normal [289]. The position 236 mutants are defective in uphill and downhill transport, although galactoside- H^+ symport does occur with a stoichiometry of about 1.

Some of the sugar specificity mutants have been subjected to a second and third round of mutagenesis, and selection on appropriate sugars has been made, e.g., selection for growth on maltose of a mutant that was previously isolated for its TDG-resistant phenotype and vice versa. In this way double mutants have been isolated with mutations at positions 177/236, 177/303, 177/306, 177/319 and 177/322 306/236, and using double mutants as parent strains the following triple mutants have been obtained A177V/S306T/Y236N, A177V/K319N/Y236N, A177V/K319N/Y236H and A177V/K319N/I303F [285,286,292]. Interestingly, the mutable positions remain restricted to a few, i.e., Ala-177, Tyr-236, T266, Ile-303, Ser-306, Lys-319, His-322 and Ala-389, suggesting that relatively few residues confer specificity to LacY protein-sugar complex.

The defect in accumulation against a concentration gradient of most of the mutants varies with the actual replacement that has been made [251,252,285] and the conditions at which transport is assayed [251,254], indicating that the residues are not obligatory involved in proton translocation. Most likely the mutations that

confer an altered sugar specificity to LacY form part of the substrate binding site or indirectly affect the conformation of this domain. The chemical properties of specific residue(s), e.g., alteration in $\text{p}K_A$, may also be affected by these mutations. Interestingly, only 8 of such mutations have been isolated even upon repeated selection and differences in the selection protocols. Several of the mutants have alterations at or nearby residues that have been implicated in the energy transduction mechanism (Fig. 8A), i.e., His-322 (one of the 'proton relay' residues) [252,253,293], Lys-319 which is on the same face of putative helix X as His-322 and Glu-325 [294], Ile-303 and Ser-306 which are close to Arg-302 (one of the 'proton relay' residues) [248,255]. Finally, mutations at more than 200 positions in LacY have been isolated at this moment (Kaback, H.R., personal communication) of which only a few are essential for galactoside- H^+ symport (Glu-325, and perhaps Glu-269), and a limited number of residues affect the sugar recognition and/or the translocation process in a more or less specific manner (Fig. 8A).

It is worth emphasizing that, whereas several of the sugar specificity mutants described facilitate sugar uniport, others mediate proton uniport under the appropriate conditions [255,292,296], i.e., these mutations allow isomerizations of the binary carrier-substrate (CS) or carrier-proton (CH) complexes (Fig. 5). The isomerization steps of the wild-type LacY protein involve the reorientation of the unloaded carrier (C) and the reorientation of the ternary carrier-substrate-proton (CSH) complex. The isomerizations involving CS and CH are (normally) not observed in wild-type carrier proteins although the decreased accumulation levels at relatively high external substrate concentrations, detected even with hydrophilic substrates like lactose and melibiose [89,295], indicate that uncoupled transport may not only be restricted to certain mutants. The LacY(A177V) and LacY(A177V/K319N) mutants catalyze uncoupled transport of H^+ which in case of the single mutant is largely diminished by galactosides, whereas galactosides enhance the level of H^+ leakage in the double mutant [292,296]. The LacY(A177V) mutant is markedly defective in accumulating substrates against a concentration gradient, but retains a normal galactoside- H^+ stoichiometry [289,297]. The double mutant facilitates galactoside transport with or without H^+ [298] and it is assumed that in addition to the H^+ leak that is observed in LacY(A177V) a second leak pathway exists which involves galactoside uptake in symport with a proton followed by exit of the sugar without proton [296]. Apparently, the net H^+ leak via this second pathway is so large that the addition of non-metabolizable galactosides results in a further diminution of the ΔpH across the cytoplasmic membrane rather than in a restoration of the pH gradient as observed with LacY(A177V). The H^+ leak in

LacY(A177V/K319N) is associated with a dramatic inhibition of growth in the presence of TDG which is not observed with LacY(A177V) [296]. Other mutants in which proton leaks have been observed include His, Asn, Phe and Ser substitutions at Tyr-236 [298], and the triple mutants LacY(A177V/K319N/Y236N), LacY(A177V/K319N/Y236H) and LacY(A177V/K319N/I303F). Of the triple mutants, the former two exhibit a higher H^+ leak than LacY(A177V/K319N), whereas the leak is lower in the mutant that has an additional mutation at position 303 [292].

A mutant designated *lacY*⁵⁴⁻⁵¹, is defective in the accumulation of sugars against a concentration gradient, but exhibits elevated downhill transport of several sugars [299]. This mutant has quite recently been characterized genetically [300]. The mutant *lacY* gene has been isolated and shown to contain a single base substitution which converts Gly-262 into Cys. In addition to an apparent uncoupling of transport, the G262C substitution results in a dramatically increased sensitivity to sulfhydryl reagents. Gly-262 is located near T266 in putative α -helix VIII and may be close or part of the sugar recognition domain of LacY (Fig. 8A).

The MelB protein. Strategies for isolating MelB mutants defective in cationic coupling have taken advantage from the fact that melibiose transport is inhibited by high concentrations of Li^+ , which results in inhibition of growth when melibiose is the sole carbon and energy source [301]. Li^+ -resistant mutants have lost the ability to transport with H^+ , but retain the ability to couple galactoside uptake to Na^+ [302]. The following substitutions in MelB have been identified: P142S (interhelix loop 4-5), L232F (interhelix loop 6-7), A236T and A236V (helix VII) (Fig. 8B). In another set of experiments Li^+ -dependent mutants have been isolated [303], and these mutants exhibit Na^+ - and Li^+ -melibiose cotransport but cannot accept H^+ as coupling ion [303,304,305]. Alterations in substrate specificity are also observed in these mutants [304]. In five independently isolated Li^+ -dependent MelB mutants Pro-122 (helix IV) was replaced with Ser [306]. Interestingly, in the Li^+ -dependent mutants a second mutation has been found which increases the $Na^+(Li^+)/H^+$ exchange activity about 10-fold and the apparent affinity constant for Li^+ by a factor of 6 [303]. Apparently, upon growth on melibiose mineral medium the large amount of Li^+ entering the cell via the Li^+ -dependent MelB mutant protein becomes toxic unless it can be extruded at a high rate via NhaA (or NhaB). The substitutions that confer altered cation specificity to MelB occur at four positions, i.e., Pro-122, Pro-142, Leu-232 and Ala-236 (Fig. 8B). Of these residues only Leu-232 is conserved in the LacS protein, whereas Pro-122 has a glycine residue at the equivalent position in LacS [102].

Melibiose carrier mutants have also been isolated

upon growth on melibiose in the presence of an excess of the non-metabolizable galactoside TMG (methyl β -D-thiogalactopyranoside) [307]. These mutants exhibit impaired TMG recognition properties, and all but one (I61V), out of 70 mutants examined, have acquired Li^+ -resistance. That changes in sugar specificity are so frequently accompanied with alterations in cation selectivity and vice versa suggests that both substrates interact cooperatively with the carrier protein. Amino acid substitutions of the 70 mutants occurred at 18 unique positions within the protein and are clustered in three distinct regions, i.e., the amino-terminal end of α -helix I, α -helix IV, and the cytoplasmic loop that has been proposed to connect helices X and XI (Fig. 8B); other substitutions are located at position 61 (I61V, helix II) and 236 (A236T and A236V, amino-terminal end of helix VII). Mutations at and near the 236 position have also been isolated by selection for Li^+ resistance (see previous paragraph).

The PutP protein. A few mutations in the sodium-dependent proline carrier protein (PutP) of *E. coli* that result in altered cation coupling have been mapped. In one case, the resulting mutant, PutP(R257C), has no activity for proline uptake, but does bind proline with altered sodium ion and proton dependencies of binding affinities [308]. Arg-257 is located in the loop connecting transmembrane α -helices VI and VII. Two mutations causing a lowered affinity for Na^+ of proline transport and binding have been identified as G22E (α -helix I) and C141Y (α -helix III) [189]. Similarly, it has been reported that cation specificity mutations in *putP* of *Salmonella typhimurium* map in the amino- and carboxy-termini of the corresponding protein [309]. Based on these findings and observations that replacement of either Cys-281 or Cys-344 causes complete resistance to NEM [280], whereas the wild-type PutP protein is inhibited in a substrate protectable manner, a model has been proposed in which Gly-22 (helix I), Cys-141 (helix III), Arg-257, Cys-281 (helix VII) and Cys-344 (helix VIII) are in close contact and form at least part of the cation-binding site [189]. On the basis of some similarity between Na^+ -solute symporters, a consensus sequence motif ($-Gly- -Ala- -Leu- -GlyArg$), corresponding with Gly₃₂₈-Ala₃₆₆-Leu₃₇₁-Gly₃₇₅-Arg₃₇₆ in PutP, has been implicated in sodium binding [65]. A similar motif has also been detected in the sodium-proton-glutamate symport systems of *B. stearothermophilus* and *B. caldovenax* [101], the proton-dependent glutamate uptake system (GltP) of *E. coli* [50], but, for instance, not in the MelB protein of *E. coli* [310] and the alanine carrier protein of thermophilic bacterium PS3 [311].

VII-F. Conclusions

Using the data set of the LacY and MelB mutants that have an altered sugar recognition and/or cation

coupling, it can be concluded that only a limited number of mutations affect the ligand binding properties of these carrier molecules. If the secondary structure model of LacY is correct (based on 143), the amino acid residues important for sugar recognition/cation coupling are all located in a rather narrow area in the middle of the transmembrane α -helices (Fig. 8A). Since the repeat of an ideal α -helix is 3.6 residues per turn and the rise per residue along the helix axis is 1.5 Å, the largest distance between these residues in a single helix, i.e., Lys-319 to Glu-325 and Gly-262 to Glu-269 would approximately be 9 and 10.5 Å. One of the sugar specificity residues (Lys-319) and three others (Asp-237, Asp-240 and Lys-358) in close vicinity may form two salt-bridges in the protein which brings the α -helices VII, X and XI in close contact with each other. Furthermore, the residues in α -helices VII, VIII, IX and X, including those that form the salt-bridges, are on one face of the helix. Since most of these amino acids have been identified as residues that affect the substrate specificity of LacY, they are likely to interact with the sugar, which suggests that the corresponding regions of the α -helices face each other (Fig. 9). Cys-148 has been placed outside of the putative substrate binding site because substitutions at position 148 cause loss of the putative regulatory sugar binding site rather than having an effect on the catalytic one [172,173,279]. Substitutions of Cys-148 slow down active transport, but do not affect the coupling reaction. The substrate protectable labeling of Cys-148 with NEM is perhaps

due to conformational changes upon binding of galactoside which may bury the cysteine.

Highly refined structures of the liganded forms of the arabinose, galactose and ribose binding proteins of enteric bacteria have revealed that the atomic interactions between the proteins and their substrates primarily consist of hydrogen bonds (for a review, see Refs. 312,313). The interactions are formed between the hydroxyl groups of the sugars and polar amino acids with planar side-chains with at least two functional groups capable of forming hydrogen bonds, e.g., Asn, Asp, Glu, Arg, His. As a result a complex network of hydrogen bonds confers specificity and affinity to the protein-substrate complex. Similar observations have been made for the lactose binding site of the *E. coli* heat-labile enterotoxin [314]. The lactose interacts with the enterotoxin mainly through its galactose moiety. A similar conclusion can be drawn from the analysis of the structural specificity of the LacY protein of *E. coli* towards sugars [171]. Other features of sugar-binding protein interactions indicate that the carboxylate side-chains of Asp and Glu are important in binding anomers and epimers, and that aromatic residues in the binding sites stack on the sugar ring. Most of the residues implicated in the binding of galactosides by LacY (see Fig. 9) meet the criteria of residues that are likely to interact with carbohydrates.

As shown by the constraints imposed by the salt-bridges between α -helices VII-X and VII-XI in LacY (Fig. 9), a few more salt-bridges, that can possibly be

Fig. 9. Structure model of LacY showing a top view of the α -helices and the residues that have been implicated in sugar recognition and cation coupling, as well as the positions of the salt-bridges between helices VII-X and VII-XI. The shaded area of α -helix VIII indicates the region of low information content that is probably in contact with the phospholipids [268]. Cytoplasmic and periplasmic loops are shown as dashed and solid lines, respectively. For further details see legend to Fig. 8A.

created by engineering charged residues at appropriate positions in transmembrane segments and selection for appropriate second-site revertants, could further improve the structure model. The narrow area in which the sugar specificity/proton coupling residues are positioned in the secondary structure model of LacY (Fig. 8A) suggests that the protein barrier that needs to be traversed by the substrate and proton is actually much thinner than the full thickness of the protein. The implications of such a model are that perhaps only a limited number of residues are directly participating in sugar and proton translocation, which is in line with a single substrate binding site alternately exposed to the outer and inner surface of the membrane [12,173,315]. It is worth emphasizing that so far only Glu-325 appears absolutely essential for proton coupled translocations.

The residues that constitute the putative sugar binding site of LacY include Lys-319 ('salt-bridge') and the residues His-322 and Glu-325 that are important for the coupled transport of galactosides and protons. A similar region has been identified in the lactose transport protein (LacS) of some lactic acid bacteria (Fig. 7), and the properties of these residues in LacS resemble those in LacY. This would suggest that these residues in LacS are located in a similar transmembrane segment. However, the region around Lys-373, His-376 and Glu-325 in LacS is much more hydrophilic and predicted to be in a cytoplasmic loop between α -helices X and XI [125]. Since most of the amino acid substitutions that confer an altered sugar specificity/cation selectivity to MelB, homologue of LacS, reside in a similar loop between α -helices X and XI (Fig. 8B), we propose that in LacS and MelB this region is located near the head groups of the lipid bilayer or perhaps forms part of the interior of the protein in order to fulfil its catalytic function.

When mutants, selected on the basis of an altered sugar specificity, are also affected in cation recognition and *vice versa* one could conclude that the sugar and cation binding sites overlap physically. On the other hand, King and Wilson [21] have pointed out, on thermodynamic grounds, that mutations in a substrate binding site should not only affect sugar binding, but also have a secondary effect. Kinetic effects of a particular substitution reflect an alteration of rate constants of a rate-limiting step, which is related to the Gibbs free energy differences (ΔG) between stable intermediates and the unstable transition states separating the intermediates of such a step. Since the sum of all Gibbs free energy changes around the translocation must equal zero, an increase in ΔG of one step should be associated with a similar decrease in ΔG of one or more other steps which could be observed as a secondary effect. Therefore, the high coincidence of mutational double-effects in LacY and MelB involving the

sugar and cation may not reflect real structural and/or energetic linkages between the sugar and cation binding sites [21].

VIII. Interaction with other proteins

Transport of lactose, melibiose and maltose, as well as the activity of glycerol kinase, is regulated in *E. coli* and *S. typhimurium* not only at the level of gene expression (catabolite repression) but also at the level of the protein (catabolite inhibition or inducer exclusion) [316,317]. The dual regulation allows an instantaneous response of the organism to the presence or absence of a specific sugar (inducer exclusion) and a slow response which involves switching on/off the expression of certain genes (catabolite repression). The phosphoenolpyruvate:sugar transferase system (PTS) is involved in both processes, and the regulation is mediated by the phosphorylation state of the phosphoryl transfer protein (IIA, previously enzyme III or III^{Glc}) [316,318,319]. The result of this regulation is that when *E. coli* or *S. typhimurium* grows in the presence of either lactose, melibiose, maltose or glycerol *plus* glucose, diauxic growth is observed with glucose being utilized first in each case.

In the present review only regulation by catabolite inhibition or inducer exclusion will be discussed which involves the mechanism that controls allosterically a number of transport proteins (enzymes). The inducer exclusion mechanism affects secondary transporters such as LacY and MelB [320–322], the binding protein-dependent ATP-driven transport system for maltose [323] and the cytoplasmic enzyme glycerol kinase [324], whereas various other (secondary) sugar transport systems such as GalP and XylE are not under control. In membrane vesicles, and purified LacY protein reconstituted into proteoliposomes, IIA binds stoichiometrically to LacY only in the non-phosphorylated form [320,321]. The binding of IIA to the lactose carrier is enhanced by the presence of galactosides, and, accordingly, binding of IIA causes a decrease in the K_D^{app} for galactosides (α NPG). Most importantly, however, binding of IIA to the lactose carrier causes inhibition of galactoside transport, and this inhibition has been interpreted as the molecular basis for inducer exclusion. In a similar manner IIA affects glycerol kinase, i.e., it binds to and inhibits purified glycerol kinase only in the presence of glycerol [324], and most likely also the melibiose and maltose transport proteins.

How does inducer exclusion occur *in vivo*? The PTS catalyzes phosphoryl transfer from phosphoenolpyruvate (PEP) to sugars (e.g., glucose) via a number of energy coupling proteins. Under conditions that the cell is metabolizing at a high rate a PTS sugar, IIA ~ P (phosphorylated by HPr ~ P) is rapidly dephospho-

rylated as a consequence of phosphoryl transfer to the sugar. Under these conditions IIA is largely in the non-phosphorylated form [325] which inhibits LacY and other transporters as indicated above. In the absence of PTS sugars IIA remains phosphorylated by HPr ~ P since the phosphoryl group cannot be transferred to a sugar, which relieves inhibition of LacY, MelB, MalK (peripheral inner membrane protein of the maltose transport system) or glycerol kinase. Since interaction of IIA with LacY or one of the other systems requires the presence of substrate, IIA does not have to bind to each of these systems at the same time, which prevents escape from inducer exclusion by depletion of IIA. Furthermore, in the absence of appropriate substrate (inducer) the level of expression of the transport systems will be low.

Mutations have been isolated that render LacY of *E. coli* [326,327], MelB of *S. typhimurium* [322], and MalK of *E. coli* [323,328] resistant to inducer exclusion, i.e., to inhibition by PTS sugars. These mutations correspond to T7I, M11I, A198V and S209I in LacY (Fig. 8A, arrows), and D438Y, R441S and I445N in *S. typhimurium* MelB (Fig. 8B, arrows). The Asp-438, Arg-441 and Ile-445 are conserved in the MelB proteins of *E. coli*, *S. typhimurium* and *K. pneumoniae* [54,105], and this region in each of the MelB proteins can be expected to interact with IIA. This region also exhibits some sequence similarity with a portion of MalK in which substitutions of amino acid residues result in a PTS resistant phenotype [322]. Two other amino acid substitutions in MalK of PTS-resistant mutants have been identified in a region that shows some similarity to a part of the central loop of MelB (Fig. 8B, open arrow), suggesting that, in addition to the carboxy terminus, also the large cytoplasmic loop between α -helices VI and VII interacts with IIA. Finally, sequence homology between the central loop of LacY and part of MalK, containing mutations that lead to resistance to PTS sugars, has been detected [328].

The lactose transport proteins (LacS) of *S. thermophilus* and *L. bulgaricus* differ from other secondary transport proteins by having a domain homologous to IIA of various PTS attached to the carboxy-terminal end of the carrier domain [102]. The IIA domain has several structural features in common with the corresponding PTS proteins and can be phosphorylated in the presence of PEP, I (enzyme I) and H (HPr) proteins [125]. It most likely serves a regulatory role in the LacS carrier-domain mediated translocation of galactosides.

Galactoside (TMG) transport has been studied in the heterofermentative lactobacilli *Lactobacillus brevis* and *Lactobacillus buchneri* [329]. In the presence of arginine as exogenous energy source, TMG is accumulated by these cells most likely via a galactoside- H^+ symport mechanism. Addition of glucose results in a

rapid efflux of TMG, and it has been suggested that glucose converts the ion-linked transporter into an uniport mechanism [329]. Since addition of glucose will affect the phosphorylation state of the energy coupling proteins of the PTS, it is conceivable that (de)phosphorylation of a domain similar to IIA in LacS mediates the apparent conversion in transport mechanism. If a similar mechanism is indeed operative in *S. thermophilus* (and *L. bulgaricus*), it would require phosphorylation of the IIA domain of LacS to affect the catalytic mechanism since the non-phosphorylated protein is competent in catalyzing all modes of facilitated diffusion, i.e., galactoside- H^+ uptake and efflux as well as exchange, in membrane vesicles and proteoliposomes [89,125]. Moreover, the IIA domain can be removed without affecting the facilitated diffusion properties of LacS (Poolman, B., unpublished results).

IX. Concluding remarks

Most of the secondary ion-solute or solute-solute coupling mechanisms, that can be thought of, have been found in bacteria (see Fig. 1 for a summary of this diversity). Although not each of these secondary transporters has been studied to the same extent, several systems that differ in direction of transport, ion- or solute coupling, and/or regulation of transport have been studied in great detail (Table I). The genes have been isolated, the primary sequences have been determined, methods have been applied to obtain information about the secondary structure, and in a number of cases the proteins have been isolated and purified to homogeneity. Analysis of the primary structures of a large number of proteins (probably more than 100 sequences of secondary transport proteins are available to date) has revealed a striking similarity in the overall structures of these transporters, suggesting that the proteins may function via similar mechanisms. Furthermore, although sequence comparisons may place transport proteins into distinct families, some cross-relatedness between individual members of different families is often observed which is indicative of a common ancestral gene for many (or perhaps all) of these proteins.

Despite significant information about the primary and secondary structures of the secondary transport proteins, virtually nothing is known about the tertiary structures. To obtain structure/function information of the transport proteins, mutant proteins have been isolated either on basis of sequence homology, properties of certain amino acids in relation to their location in the putative secondary structure, or by selection for a specific phenotype. Most of these are LacY or MelB mutants, and the results indicate that only a limited number of amino acid substitutions significantly affect the sugar recognition and/or cationic coupling of these

transporters (see Fig. 8A, B). Apparently, a large number of substitutions are permitted which do not affect the solute binding and/or the translocation process not even via long distance changes in the overall structure, and most likely many of these amino acid residues face the membrane bilayer. It is worth emphasizing that, so far, of the large collection of LacY mutants available only a single residue, i.e., Glu-325, appears essential for all H^+ coupled transport reactions. Glu-376 in LacS and Asp-285 in TetA may have (a) similar role(s) in the corresponding transporters. Other positions have been implicated in energy transduction by LacY; however, the degrees at which H^+ coupled transport is affected depend on the substitution, the substrate used and/or the specific reaction conditions.

The LacY, PutP, NhaA proteins of *E. coli*, OxlT of *O. formigenes* and the alanine carrier protein of thermophilic bacterium PS3 have been purified to homogeneity and characterized upon functional incorporation into liposomes. These studies have established that a single polypeptide is responsible for the translocation process, and have demonstrated rigorously the mechanism of energy coupling and substrate specificity of these systems without possible interference of additional (transport) proteins. So far, in each case where appropriate and defined host strains have been available the studies on the purified and reconstituted proteins have not revealed mechanistic properties that had not (or could not have) been detected in membrane vesicles or proteoliposomes obtained from crude membrane detergent extracts. The purified proteins can be used for advanced biochemical and spectroscopical analysis, especially when combined with the construction of appropriate mutants, but this type of study is only beginning to emerge. Strikingly, only limited information is available on the interaction of the transport proteins with the lipid bilayer. The lipid requirement has been analyzed in a few cases, but these studies mainly involve proteoliposomes prepared from crude membrane protein extracts rather than employing purified proteins. Finally, one aspect of secondary transporters, or even polytopic membrane proteins in general, that is largely not understood is the mechanism by which the polypeptides insert into the cytoplasmic membranes and assemble into functional proteins.

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References

- 1 Nikaido, H. and Vaara, M. (1985) *Microbiol. Rev.* 49, 1–32.
- 2 Sleytr, U.B. and Messner, P. (1988) *J. Bacteriol.* 170, 2891–2897.
- 3 Nikaido, H. (1992) *Mol. Microbiol.* 6, 435–442.
- 4 Hellingwerf, K.J. and Konings, W.N. (1985) *Adv. Microb. Physiol.* 26, 125–154.
- 5 Booth, I. (1985) *Microbiol. Rev.* 49, 359–378.
- 6 Higgins, C.F. and Booth, I. (1987) In *Homeostatic mechanisms in microorganisms* (Whittenbury, R., Gould, G.W., Banks, J.G. and Board, R.G., eds.), pp. 29–40, Bath University Press, Bath.
- 7 Bakker, E.P. (1988) in *The roots of Modern Biochemistry* (Kleinkauf, K., Von Döhren, S. and Jaenicke, A., eds.), pp. 699–705, Walter de Gruyter, Berlin.
- 8 Poolman, B., Driessen, A.J.M. and Konings, W.N. (1987) *Microbiol. Rev.* 51, 498–508.
- 9 Higgins, C.F., Cairney, J., Stirling, D.A., Sutherland, L. and Booth, I.R. (1987) *Trends Biochem. Sci.* 12, 339–344.
- 10 Poolman, B., Molenaar, D. and Konings, W.N. (1993) in *Handbook of Biomembranes*, Vol. 2 (Shinitzky, M., ed.), pp. 1–50, Balaban Publishers, Rehovot.
- 11 Kaback, H.R. (1990) *The Bacteria*, Vol. XII, pp. 151–202.
- 12 Kaback, H.R. (1992) *Int. Rev. Cytol.* 137A, 97–125.
- 13 Leblanc, G., Pourcher, T., Bassilana, M. and Deckert, M. (1991) in *Ion-coupled sugar transport in microorganisms* (Page, M. and Henderson, P.J.F., eds.), CRC Uniscience, Sery.
- 14 Levy, S.B. (1992) *Antimicrob. Agents Chemother.* 36, 695–703.
- 15 Maloney, P.C., Ambudkar, S.V., Anantharam, V., Sonna, L.A. and Varadhachary, A. (1990) *Microbiol. Rev.* 54, 1–17.
- 16 Henderson, P.J.F. (1990) *J. Bioenerg. Biomem.* 22, 525–569.
- 17 Henderson, P.J.F. (1991) *Curr. Opin. Struct. Biol.* 1, 590–601.
- 18 Griffith, J.K., Baker, M.E., Rouch, D.A., Page, M.G.P., Skurray, R.A., Paulsen, I., Chater, K.F., Baldwin, S.A. and Henderson, P.J. (1992) *Curr. Opin. Cell. Biol.* 4, 684–695.
- 19 Marger, M.D. and Saier, M.H. (1993) *Trends Biochem. Sci.* 18, 13–20.
- 20 Poolman, B. (1990) *Mol. Microbiol.* 4, 1629–1636.
- 21 King, S.C. and Wilson, T.H. (1990) *Mol. Microbiol.* 4, 1433–1438.
- 22 Mitchell, P. (1963) *Biochem. Soc. Symp.* 22, 142–169.
- 23 Mitchell, P. (1990) *Res. Microbiol.* 141, 286–289.
- 24 Michels, P.A.M., Michels, J.P.J., Boonstra, J. and Konings, W.N. (1979) *FEMS Microbiol. Lett.* 5, 357–364.
- 25 Maloy, S.R. (1990) *The Bacteria*, Vol. XII, 203–224.
- 26 Heyne, R., De Vrij, W., Crielaard, W. and Konings, W.N. (1991) *J. Bacteriol.* 173, 791–800.
- 27 Golby, P., Cotton, N.P.J., Carver, M. and Jackson, J.B. (1992) *Arch. Microbiol.* 157, 125–130.
- 28 Ambudkar, S.V. and Rosen, B.P. (1990) *The Bacteria*, Vol. XII, 247–271.
- 29 Sweet, G., Gandor, C., Voegelé, R., Wittekindt, N., Beuerle, J., Truniger, V., Lin, E.E.C. and Boos, W. (1990) *J. Bacteriol.* 172, 424–430.
- 30 Postma, P.W., Epstein, W., Schuitema, A.R.J. and Nelson, S.O. (1984) *J. Bacteriol.* 158, 351–353.
- 31 Heller, K.B., Lin, E.E.C. and Wilson, T.H. (1980) *J. Bacteriol.* 144, 274–278.
- 32 Voegelé, R.T., Sweet, G.D. and Boos, W. (1993) *J. Bacteriol.* 175, 1087–1094.
- 33 DiMarco, A.A. and Romano, A.H. (1985) *Appl. Env. Microbiol.* 49, 151–157.
- 34 Barnell, W.O., Yi, K.C. and Conway, T. (1990) *J. Bacteriol.* 172, 7227–7240.

- 35 Baldwin, S.A. (1992) in *Molecular aspects of transport proteins*, New Comprehensive Biochem. Vol. 21 (De Pont, J.J.H.H.M., ed.), pp. 169–217, Elsevier, Amsterdam.
- 36 Konings, W.N. and Michels, P.A.M. (1980) in *Diversity of Bacterial Respiratory Systems* (Knowles, C.J., ed.), CRC Press, Boca Raton.
- 37 Uratani, Y. (1985) *J. Biol. Chem.* 260, 10023–10026.
- 38 Russell, J.B., Strobel, H.J., Driessen, A.J.M. and Konings, W.N. (1988) *J. Bacteriol.* 170, 3531–3536.
- 39 Konings, W.N., Poolman, B. and Driessen, A.J.M. (1989) *CRC Crit. Rev. Microbiol.* 16, 419–476.
- 40 Driessen, A.J.M., Ubbink-Kok, T. and Konings, W.N. (1988) *J. Bacteriol.* 170, 817–820.
- 41 Driessen, A.J.M., Van Leeuwen, C. and Konings, W.N. (1989) *J. Bacteriol.* 171, 1453–1458.
- 42 Speelmans, G., Poolman, B. and Konings, W.N. (1993) *J. Bacteriol.*, 175, 2060–2066.
- 43 Poolman, B., Molenaar, D., Smid, E.J., Ubbink, T., Abee, T., Renault, P.P. and Konings, W.N. (1991) *J. Bacteriol.* 173, 6030–6037.
- 44 Van der Rest, M., Abee, T., Molenaar, D. and Konings, W.N. (1991) *Eur. J. Biochem.* 195, 71–77.
- 45 Konings, W.N. and Rosenberg, H. (1978) *Biochim. Biophys. Acta* 211, 158–168.
- 46 Seol, W. and Shatkin, A.J. (1992) *J. Biol. Chem.* 267, 6409–6413.
- 47 Cairney, J., Higgins, C. and Booth, I.R. (1984) *J. Bacteriol.* 160, 22–27.
- 48 Hama, H., Shimamoto, T., Tsuda, M. and Tsuchiya, T. (1987) *Biochim. Biophys. Acta* 905, 231–239.
- 49 Kalman, M., Gentry, D.R. and Cashel, M. (1991) *Mol. Gen. Genet.* 225, 379–386.
- 50 Tolner, B., Poolman, B. and Konings, W.N. (1992) *J. Bacteriol.* 174, 2391–2393.
- 51 Reizer, J., Reizer, A. and Saier, M.H. Jr. (1990) *Res. Microbiol.* 141, 1069–1072.
- 52 Wilson, D.M. and Wilson, T.H. (1987) *Biochim. Biophys. Acta* 904, 191–200.
- 53 Hirata, H., Kambe, T. and Kagawa, Y. (1984) *J. Biol. Chem.* 259, 10653–10656.
- 54 Hama, H. and Wilson, T.H. (1992) *J. Biol. Chem.* 267, 18371–18376.
- 55 De Vrij, W., Bulthuis, R.A., Van Iwaarden, P.R. and Konings, W.N. (1989) *J. Bacteriol.* 171, 1118–1125.
- 56 Dimroth, P. (1987) *Microbiol. Rev.* 51, 320–340.
- 57 De Vrij, W., Speelman, G., Heyne, R.I.R. and Konings, W.N. (1990) *FEMS Microbiol. Rev.* 75, 183–200.
- 58 Dibrov, P.A. (1991) *Biochim. Biophys. Acta* 1056, 209–224.
- 59 Speelmans, G., De Vrij, W. and Konings, W.N. (1989) *J. Bacteriol.* 171, 3788–3795.
- 60 Chen, Ch-Ch., Tsuchiya, T., Yamane, Y., Wood, J.M. and Wilson, T.H. (1985) *J. Membr. Biol.*, 84, 157–164.
- 61 Lombardi, F.J. and Kaback, H.R. (1972) *J. Biol. Chem.* 247, 7844–7857.
- 62 Van der Rest, M., R. Sieve, Abee, T., Schwartz, E., Oosterhelt, D. and Konings, W.N. (1992) *J. Biol. Chem.*, 267, 8971–8976.
- 63 Fujimura, T., Yamato, I. and Anraku, Y. (1983) *Biochemistry* 22, 1954–1959.
- 64 Fujimura, T., Yamato, I. and Anraku, Y. (1983) *Biochemistry* 22, 1959–1965.
- 65 Deguchi, Y., Yamato, I. and Anraku, Y. (1990) *J. Biol. Chem.* 265, 21704–21708.
- 66 Milner, J.L., Grothe, S. and Wood, J.M. (1988) *J. Biol. Chem.* 263, 14900–14905.
- 67 Wood, J. (1988) *J. Membr. Biol.* 106, 183–202.
- 68 Harold, F.M. and Levin, E. (1974) *J. Bacteriol.* 117, 1141–1148.
- 69 Locher, H., Poolman, B. and Konings, W.N. (1993) *J. Bacteriol.* 175, 1075–1080.
- 70 Konings, W.N., Poolman, B. and Driessen, A.J.M. (1992) *FEMS Microbiol. Rev.* 88, 93–108.
- 71 Belliveau, J.W. and Lanyi, J.K. (1978) *Arch. Biochem. Biophys.* 186, 98–105.
- 72 Ando, A., Yabuki, M. and Kusaka, I. (1981) *Biochim. Biophys. Acta* 640, 179–184.
- 73 Bröer, S. and Krämer, R. (1991) *Eur. J. Biochem.* 202, 137–143.
- 74 Brey, R.N., Beck, J.C. and Rosen, B.P. (1978) *Biochem. Biophys. Res. Commun.* 83, 1588–1594.
- 75 Brey, R.N., Rosen, B.P. and Sorensen, E.N. (1980) *J. Biol. Chem.* 255, 39–44.
- 76 Brey, R.N. and Rosen, B.P. (1979) *J. Biol. Chem.* 254, 1957–1963.
- 77 Nakamura, T., Tokuda, H. and Unemoto, T. (1984) *Biochim. Biophys. Acta* 776, 330–336.
- 78 Jayakumar, A., Epstein, W. and Barnes, E.M. Jr. (1985) *J. Biol. Chem.* 260, 7528–7532.
- 79 Yamaguchi, A., Udagawa, T. and Sawai, T. (1990) *J. Biol. Chem.* 265, 4809–4813.
- 80 Yamaguchi, A., Iwasaki-Ohba, Y., Ono, N., Kaneko-Ohdera, M. and Sawai, T. (1991) *FEBS Lett.* 282, 415–418.
- 81 Maloney, P.C. (1990) *J. Bioenerg. Biomem.* 22, 509–523.
- 82 Anantharam, V., Allison, M.J. and Maloney, P.C. (1989) *J. Biol. Chem.* 265, 7244–7250.
- 83 Winkler, H.H. (1976) *J. Biol. Chem.* 251, 389–396.
- 84 Driessen, A.J.M., Poolman, B., Kiewiet, R. and Konings, W.N. (1987) *Proc. Natl. Acad. Sci. USA* 84, 6093–6097.
- 85 Driessen, A.J.M., Smid, E.J. and Konings, W.N. (1988) *J. Bacteriol.* 170, 4522–4527.
- 86 Kashiwagi, K., Miyamoto, S., Suzuki, F., Kobayashi, H. and Igarashi, K. (1992) *Proc. Natl. Acad. Sci. USA* 89, 4529–4533.
- 87 Ambudkar, S.V., Sonna, L.A. and Maloney, P.C. (1986) *Proc. Natl. Acad. Sci. USA*, 83, 280–284.
- 88 Bröer, S. and Krämer, R. (1990) *J. Bacteriol.* 172, 7241–7248.
- 89 Foucaud, C. and Poolman, B. (1992) *J. Biol. Chem.*, 267, 22087–22094.
- 90 Verhoogt, H., Smit, H., Abee, T., Gamper, M., Driessen, A.J.M., Haas, D. and Konings, W.N. (1992) *J. Bacteriol.* 174, 1568–1573.
- 91 Huber, R.E., Lytton, J. and Fung, E.B. (1980) *J. Bacteriol.* 141, 528–533.
- 92 Engel, P., Krämer, R. and Unden, G. (1992) *J. Bacteriol.*, 174, 5533–5539.
- 93 Driessen, A.J.M. and Konings, W.N. (1990) *The Bacteria*, vol. XII, 449–478.
- 94 Poolman, B., Driessen, A.J.M. and Konings, W.N. (1987) *J. Bacteriol.* 169, 5597–5604.
- 95 Molenaar, D., Bosscher, J.S., Ten Brink, B., Driessen, A.J.M. and Konings, W.N. (1993) *J. Bacteriol.*, in press.
- 96 Lühti, E., Bauer, H., Gamper, M., Brunner, F., Villeval, D., Mercenier, A. and Haas, D. (1990) *Gene* 87, 37–43.
- 97 Seep-Feldhaus, A.H., Kalinowski, J. and Pühler, A. (1991) *Mol. Microbiol.* 5, 2995–3005.
- 98 Meng, S.-Y. and Bennett, G.N. (1992) *J. Bacteriol.* 174, 2659–2669.
- 99 Laubinger, W. and Dimroth, P. (1989) *Biochemistry* 27, 7194–7198.
- 100 Boyer, P.D. (1988) *Trends Biochem. Sci.* 13, 5–7.
- 101 Tolner, B., Poolman, B. and Konings, W.N. (1992) *Mol. Microbiol.*, 6, 2845–2856.
- 102 Poolman, B., Royer, T.J., Mainzer, S.E. and Schmidt, B.F. (1989) *J. Bacteriol.* 171, 244–253.
- 103 Schmidt, B.F., Royer, T., Yoast, S. and Poolman, B. (1990) *FEMS Microbiol. Rev.* 87, p18.
- 104 Leong-Morgenthaler, P., Zwahlen, M.C. and Hottinger, H. (1991) *J. Bacteriol.* 173, 1951–1957.
- 105 Mizushima, K., Awakihara, S., Kuroda, M., Ishikawa, T., Tsuda, M. and Tsuchiya, T. (1992) *Mol. Gen. Genet.*, 234, 74–80.

- 106 Baldwin, S.A. and Henderson, P.J.F. (1989) *Ann. Rev. Physiol.* 51, 459–471.
- 107 Maloney, P.C. (1990) *FEMS Microbiol. Rev.* 87, 91–102.
- 108 Foster, D.L., Boublik, M. and Kaback, H.R. (1983) *J. Biol. Chem.* 258, 31–34.
- 109 Vogel, H., Wright, J.K. and Jahnig, F. (1985) *EMBO J.* 4, 3625–3631.
- 110 Radding, W. (1991) *J. Theor. Biol.* 150, 239–249.
- 111 Calamia, J. and Manoil, C. (1990) *Proc. Natl. Acad. Sci. USA* 87, 4937–4941.
- 112 Botfield, M.C., Naguchi, K., Tsuchiya, T. and Wilson, T.H. (1992) *J. Biol. Chem.* 267, 1818–1822.
- 113 Allard, J.D. and Bertrand, K.P. (1992) *J. Biol. Chem.* 267, 17809–17819.
- 114 Lloyd, A.D. and Kadner, R.J. (1990) *J. Bacteriol.* 172, 1688–1693.
- 115 Manoil, C. and Beckwith, J. (1985) *Proc. Natl. Acad. Sci. USA* 82, 8129–8133.
- 116 Von Heyne, G. (1986) *EMBO J.* 5, 3021–3027.
- 117 Anderson, H., Bakker, E. and von Heyne, G. (1992) *J. Biol. Chem.* 267, 1491–1495.
- 118 Midgley, M. (1987) *Microbiol. Sci.* 4, 125–127.
- 119 Gheysen, G., Herman, L., Breynne, P., Gielen, J., Van Montague, M. and Depicker, A. (1990) *Gene* 94, 155–163.
- 120 Morimyo, M. (1991) *GenEMBL database acc. no. M62732*.
- 121 Grinius, L., Dreguniene, G., Goldberg, E.B., Liao, C.-H. and Projan, S.J. (1992) *Plasmid* 27, 119–129.
- 122 Tate, C.G., Muir, J.A.R. and Henderson, P.J.F. (1992) *J. Biol. Chem.* 267, 6923–6932.
- 123 Muramatsu, S. and Mizuno, T. (1989) *Nucleic Acids. Res.* 17, 4378.
- 124 Pao, G.M., Wu, L.-F., Johnson, K.D., Höfte, H., Chrispeels, M.J., Sweet, G., Sandal, N.N. and Saier, M.H. (1991) *Mol. Microbiol.* 5, 33–37.
- 125 Poolman, B., Modderman, R. and Reizer, J. (1992) *J. Biol. Chem.*, 267, 9150–9157.
- 126 Maiden, M.C.J., Davis, E.O., Baldwin, S.A., Moore, D.C.M. and Henderson, P.J.F. (1987) *Nature* 325, 641–643.
- 127 Yamaguchi, A., Someya, Y. and Sawai, T. (1992) *J. Biol. Chem.* 267, 19155–19162.
- 128 Yamaguchi, A., Ono, N., Akasaki, T., Noumi, T. and Sawai, T. (1990) *J. Biol. Chem.* 265, 15525–15530.
- 129 Yamaguchi, A., Nakatani, M. and Sawai, T. (1992) *Biochemistry* 31, 8344–8348.
- 130 Yamaguchi, A., Kimura, T., Someya, Y. and Sawai, T. (1993) *J. Biol. Chem.*, 130, 6496–6504.
- 131 McKenna, E., Hardy, D. and Kaback, H.R. (1992) *Proc. Natl. Acad. Sci. USA* 89, 11954–11958.
- 132 MacArthur, M.W. and Thornton, J.M. (1991) *J. Mol. Biol.* 218, 397–412.
- 133 Brandl, C.J. and Deber, C.M. (1986) *Proc. Natl. Acad. Sci. USA* 83, 917–921.
- 134 Von Heyne, G. (1991) *J. Mol. Biol.* 218, 499–503.
- 135 Lolkema, J.S., Püttner, I.B. and Kaback, H.R. (1988) *Biochemistry* 27, 8307–8310.
- 136 Consler, T.G., Tsolas, O. and Kaback, H.R. (1991) *Biochemistry* 30, 1291–1298.
- 137 Barsukov, I.L., Abdulaeva, G.V., Arseniev, A.S. and Bystrov, V.F. (1990) *Eur. J. Biochem.* 192, 321–327.
- 138 Menezes, M.E., Roepe, P.D. and Kaback, H.R. (1990) *Proc. Natl. Acad. Sci. USA* 87, 1638–1642.
- 139 Altenbach, C., Marti, H., Khorana, H.G. and Hubbell, W.L. (1990) *Science* 248, 1088–1092.
- 140 Todd, A.P., Cong, J., Levinthal, C., Levinthal, W. and Hubbell, W.L. (1989) *Proteins: Struct., Funct., Genet.*, 6, 294–302.
- 141 Dornmair, K., Corin, A.S., Wright, J.K. and Jähnig, F. (1985) *EMBO J.* 4, 3633–3638.
- 142 Lakey, J.H., Baty, D. and Pattus, F. (1991) *J. Mol. Biol.* 218, 639–653.
- 143 King, S.C., Hansen, C.L. and Wilson, T.H. (1991) *Biochim. Biophys. Acta* 1062, 177–186.
- 144 Lee, J.-I., Hwang, P.P., Hansen, C. and Wilson, T.H. (1992) *J. Biol. Chem.* 267, 20758–20764.
- 145 Sahin-Tóth, M., Dunten, R.L., Gonzalez, A. and Kaback, H.R., (1992) *Proc. Natl. Acad. Sci. USA* 89, 10547–10551.
- 146 Dunten, R.L., Sahin-Tóth, M. and Kaback, H.R. (1993) *Biochem.*, 32, 3139–3145.
- 147 Costello, M.J., Escaig, J., Matsushita, K., Viitanen, P.V., Menick, D.R. and Kaback, H.R. (1987) *J. Biol. Chem.* 262, 17072–17082.
- 148 Goldkorn, T., Rimon, G. and Kaback, H.R. (1983) *Proc. Natl. Acad. Sci. USA* 80, 3322–2326.
- 149 Ambudkar, S.V., Anantharam, V. and Maloney, P.C. (1990) *J. Biol. Chem.* 265, 12287–12291.
- 150 Bibi, E., Verner, G., Chang, C.-Y., Kaback, H.R. (1991) *Proc. Natl. Acad. Sci. USA* 88, 7271–7275.
- 151 Calamia, J. and Manoil, C. (1992) *J. Mol. Biol.* 224, 539–543.
- 152 Wrubel, W., Stochaj, U., Sonnewald, U., Theres, C. and Ehring, R. (1990) *J. Bacteriol.* 172, 5374–5381.
- 153 Bibi, E. and Kaback, H.R. (1992) *Proc. Natl. Acad. Sci. USA* 89, 1524–1528.
- 154 Bibi, E., Stearns, S.M. and Kaback, H.R. (1992) *Proc. Natl. Acad. Sci. USA* 89, 3180–3184.
- 155 Roepe, P.D., Zbar, R.I.S., Sarkar, H.K. and Kaback, H.R. (1989) *Proc. Natl. Acad. Sci. USA* 86, 3992–3996.
- 156 McKenna, E., Hardy, D., Pastore, J.C. and Kaback, H.R. (1991) *Proc. Natl. Acad. Sci. USA* 88, 2969–2973.
- 157 McKenna, E., Hardy, D. and Kaback, H.R. (1992) *J. Biol. Chem.* 267, 6471–6474.
- 158 Botfield, M.C. and Wilson, T.H. (1989a) *J. Biol. Chem.* 264, 11643–11648.
- 159 Ahrem, B., Hoffschulte, H.K. and Müller, M. (1989) *J. Cell Biol.* 108, 1637–1646.
- 160 Von Heyne, G., Wickner, W. and Dalbey, R. (1988) *Proc. Natl. Acad. Sci. USA* 85, 3363–3366.
- 161 Saier, M.H., Werner, P.K. and Müller, M. (1989) *Microbiol. Rev.* 53, 333–366.
- 162 Dalbey, R.E. (1990) *Trends Biochem. Sci.* 15, 253–257.
- 163 Ehrman, M. and Beckwith, J. (1991) *J. Biol. Chem.* 266, 16530–16533.
- 164 Wickner, W., Driessen, A.J.M. and Hartl, F.-U. (1991) *Annu. Rev. Biochem.* 60, 101–124.
- 165 Ehring, R., Beyreuther, K., Wright, J.K. and Overath, P. (1980) *Nature* 283, 537–540.
- 166 Werner, P.K., Saier, M.H. and Müller, M. (1992) *J. Biol. Chem.* 267, 24523–24532.
- 167 Ito, K. and Akiyama, Y. (1991) *Mol. Microbiol.* 5, 2243–2253.
- 168 Yamato, I. (1992) *J. Biochem.* 111, 444–450.
- 169 McGovern, K. and Beckwith, J. (1991) *J. Biol. Chem.* 266, 20870–20876.
- 170 Von Heyne, G. (1989) *Nature* 341, 456–458.
- 170a Fersht, A.R. (1985) *Enzyme structure and mechanism*, pp. 317–331, Wiley, New York.
- 171 Olsen, S.G. and Brooker, R.J. (1989) *J. Biol. Chem.* 264, 15982–15987.
- 172 Lolkema, J.S. and Walz, D. (1990) *Biochemistry* 29, 10120–10126.
- 173 Lolkema, J.S., Carrasco, N. and Kaback, H.R. (1991) *Biochemistry* 30, 1284–1290.
- 174 Kaczorowski, G.J., Robertson, D.E. and Kaback, H.R. (1979) *Biochemistry* 18, 3697–3704.
- 175 Kaczorowski, G.J. and Kaback, H.R. (1979) *Biochemistry* 18, 3691–3697.

- 176 Garcia, M.-L., Viitanen, P., Foster, D.L. and Kaback, H.R. (1983) *Biochemistry* 22, 2524–2531.
- 177 Viitanen, P., Garcia, M.L., Foster, D.L., Kaczorowski, G.J. and Kaback, H.R. (1983) *Biochemistry* 22, 2531–2536.
- 178 Page, M.G.P. (1987) *Biochim. Biophys. Acta* 897, 112–126.
- 179 Page, M.G.P., Rosenbusch, J.P. and Yamato, I. (1988) *J. Biol. Chem.* 263, 15897–15905.
- 180 Cohn, D.E. and Kaback, H.R. (1980) *Biochemistry* 19, 4237–4243.
- 181 Damiano-Forano, E., Bassilana, M. and Leblanc, G. (1986) *J. Biol. Chem.* 261, 6893–6899.
- 182 Bassilana, M., Damiano, E. and Leblanc, G. (1985) *Biochem. Biophys. Res. Commun.* 129, 626–631.
- 183 Bassilana, M., Pourcher, T. and Leblanc, G. (1987) *J. Biol. Chem.* 262, 16865–16870.
- 184 Bassilana, M., Pourcher, T. and Leblanc, G. (1988) *J. Biol. Chem.* 263, 9663–9667.
- 185 Hirata, H., Altendorf, K. and Harold, F.M. (1973) *Proc. Natl. Acad. Sci. USA* 70, 1804–1808.
- 186 Schuldiner, S. and Kaback, H.R. (1977) *Biochemistry* 14, 5451–5461.
- 187 Mogi, T. and Anraku, Y. (1984) *J. Biol. Chem.* 259, 7791–7806.
- 188 Chen, C.-C. and Wilson, T.H. (1986) *J. Biol. Chem.* 261, 2599–2604.
- 189 Yamato, I., Ohsawa, M. and Anraku, Y. (1990) *J. Biol. Chem.* 265, 2450–2455.
- 190 Cairney, J., Booth, I.R. and Higgins, C.F. (1985) *J. Bacteriol.* 164, 1218–1223.
- 191 Grothe, S., Krogsrud, R.L., McClellan, D.J., Milner, J.L. and Wood, J.M. (1986) *J. Bacteriol.* 166, 253–259.
- 191a Culham, D.E., Lasby, B., Marangoni, A.G., Milner, J.L., Steer, B.A., Van Nues, R.W. and Wood, J.M. (1993) *J. Mol. Biol.* 229, 268–276.
- 192 Cairney, J., Booth, I.R. and Higgins, C.F. (1985) *J. Bacteriol.* 164, 1224–1232.
- 193 Levy, S.B. (1988) *ASM News* 54, 418–421.
- 194 Kaneko, M., Yamaguchi, A. and Sawai, T. (1985) *FEBS Lett.* 193, 194–198.
- 194a Neyfakh, A., Bidnenko, V.E. and Chen, L.B. (1991) *Proc. Natl. Acad. Sci. USA* 88, 4781–4785.
- 194b Bissonnette, L., Champetier, S., Buisson, J.-P. and Roy, P.H. (1991) *J. Bacteriol.* 173, 4493–4502.
- 195 Taglicht, D., Padan, E. and Schuldiner, S. (1993) *J. Biol. Chem.*, 268, 5382–5387.
- 196 Bassilana, M., Damiano, E. and Leblanc, G. (1984) *Biochemistry* 23, 1015–1022.
- 197 Bassilana, M., Damiano, E. and Leblanc, G. (1984) *Biochemistry* 23, 5288–5294.
- 198 Macnab, R.M. and Castle, A.M. (1987) *Biophys. J.* 52, 637–647.
- 199 Taglicht, D., Padan, E. and Schuldiner, S. (1991) *J. Biol. Chem.* 266, 11289–11294.
- 200 Padan, E., Maisler, N., Taglicht, D., Karpel, R. and Schuldiner, S. (1989) *J. Biol. Chem.* 264, 20297–20302.
- 201 Pinner, E., Kotler, Y., Padan, E. and Schuldiner, S. (1993) *J. Biol. Chem.* 268, 1729–1736.
- 202 Pinner, E., Padan, E. and Schuldiner, S. (1992) *J. Biol. Chem.* 267, 11064–11068.
- 203 Schuldiner, S. and Fishkes, H. (1978) *Biochemistry* 17, 706–710.
- 204 Pan, J.W. and Macnab, R.M. (1990) *J. Biol. Chem.* 265, 9247–9250.
- 205 Maloney, P.C., Ambudkar, S.V., Thomas, J. and Schiller, L. (1984) *J. Bacteriol.* 158, 238–245.
- 206 Sonna, L.A. and Maloney, P.C. (1988) *J. Membr. Biol.* 101, 267–274.
- 207 Ramos, S. and Kaback, H.R. (1977) *Biochemistry* 16, 4271–4275.
- 208 Leblanc, G., Rimón, G. and Kaback, H.R. (1980) *Biochemistry* 19, 2522–2528.
- 209 Goldrick, D., Yu, G.-Q., Jiang, S.-Q., Hong, J.-S. (1988) *J. Bacteriol.* 170, 3421–3426.
- 210 Ambudkar, S.V., Larson, T.J. and Maloney, P.C. (1986) *J. Biol. Chem.* 261, 9083–9086.
- 211 Sonna, L.A., Ambudkar, S.V. and Maloney, P.C. (1988) *J. Biol. Chem.* 263, 6625–6630.
- 212 Baron, C. and Thompson, T.E. (1975) *Biochim. Biophys. Acta* 382, 276–285.
- 213 Racker, E., Violand, B., O'Neal, S., Alfonzo, M. and Telford, J. (1979) *Arch. Biochem. Biophys.* 198, 470–477.
- 214 Newman, M.J. and Wilson, T.H. (1980) *J. Biol. Chem.* 255, 10583–10586.
- 215 Maloney, P.C. and Ambudkar, S.V. (1989) *Arch. Biochem. Biophys.* 269, 1–10.
- 216 In't Veld, G., de Vrije, T., Driessen, A.J.M. and Konings, W.N. (1992) *Biochim. Biophys. Acta* 1104, 250–256.
- 217 Jain, M.K. and Zakim, D. (1987) *Biochim. Biophys. Acta* 906, 33–68.
- 218 Rigaud, J.-L., Levy, D. and Seigneuret, M. (1991) *Trends Biomembr. Bioenerg.* 1, 11–27.
- 219 Wilson, D.M., Ottina, K., Newman, M.J., Tsuchiya, T., Ito, S. and Wilson, T.H. (1985) *Membr. Biochem.* 5, 269–290.
- 220 Henderson, P.J.F., Kagawa, Y. and Hirata, H. (1983) *Biochim. Biophys. Acta* 732, 204–209.
- 221 Ambudkar, S.V. and Maloney, P.C. (1986) *J. Biol. Chem.* 261, 10079–10086.
- 222 Hirata, H. (1986) *Meth. Enzymol.* 125, 485–491.
- 223 Frijlink, M.J., Abee, T., Laanbroek, H.J., De Boer, W. and Konings, W.N. (1992) *Arch. Microbiol.* 157, 389–393.
- 224 Maloney, P.C., Anantharam, V. and Allison, M.J. (1992) *J. Biol. Chem.* 267, 10531–10536.
- 225 Driessen, A.J.M., Zheng, T., In't Veld, G., Op den Kamp, J.A.F. and Konings, W.N. (1988) *Biochemistry* 27, 865–872.
- 226 Seto-Young, D., Chen, C.-C. and Wilson, T.H. (1985) *J. Membr. Biol.* 84, 259–267.
- 227 Chen, C.-C. and Wilson, T.H. (1984) *J. Biol. Chem.* 259, 10150–10158.
- 228 Uratani, Y. and Aiyama, A. (1986) *J. Biol. Chem.* 261, 5450–5454.
- 229 In't Veld, G., Driessen, A.J.M., Op de Kamp, J.A.F. and Konings, W.N. (1991) *Biochim. Biophys. Acta* 1065, 203–212.
- 230 Uratani, Y., Wakayama, N. and Hoshino, T. (1987) *J. Biol. Chem.* 262, 16914–16919.
- 231 In't Veld, G., Driessen, A.J.M. and Konings, W.N. (1992) *Biochim. Biophys. Acta* 1108, 31–39.
- 232 Maiden, M.C.J., Jones-Mortimer, M.C. and Henderson, P.J.F. (1988) *J. Biol. Chem.* 263, 8003–8010.
- 233 Newman, M.J., Foster, D.L., Wilson, T.H. and Kaback, H.R. (1981) *J. Biol. Chem.* 256, 11804–11808.
- 234 Foster, D.L., Garcia, M.L., Newman, M.J., Patel, L. and Kaback, H.R. (1982) *Biochemistry* 21, 5634–5638.
- 235 Wright, J.K. and Overath, P. (1984) *Eur. J. Biochem.* 138, 497–508.
- 236 Li, J. and Tooth, P. (1987) *Biochemistry* 26, 4816–4823.
- 237 Dornmair, K. (1988) *FEBS Lett.* 235, 35–39.
- 238 Matsushita, K., Patel, L., Gennis, R.B. and Kaback, H.R. (1983) *Proc. Natl. Acad. Sci. USA* 80, 4889–4893.
- 239 Viitanen, P., Garcia, M.L. and Kaback, H.R. (1984) *Proc. Natl. Acad. Sci. USA* 81, 1629–1633.
- 240 Dornmair, K., Overath, P. and Jähnig, F. (1989) *J. Biol. Chem.* 264, 342–346.
- 241 Hanada, K., Yamato, I. and Anraku, Y. (1987) *J. Biol. Chem.* 262, 14100–14104.

- 242 Hanada, K., Yamato, I. and Anraku, Y. (1988) *J. Biol. Chem.* 263, 7181–7185.
- 243 Ruan, Z.-S., Anantharam, V., Crawford, I.T., Ambudkar, S.V., Rhee, S.Y., Allison, M.J. and Maloney, P.C. (1992) *J. Biol. Chem.* 267, 10537–10543.
- 244 Tsuchiya, T., Lopilato, J. and Wilson, T.H. (1978) *J. Membr. Biol.*, 42, 45–59.
- 245 Püttner, I.B., Sarkar, H.K., Poonian, M.S. and Kaback, H.R. (1986) *Biochemistry* 25, 4483–4485.
- 246 Püttner, I.B., Sarkar, H.K., Padan, E., Lolkema, J.S. and Kaback, H.R. (1989) *Biochemistry* 28, 2525–2533.
- 247 Carrasco, N., Antes, L.M., Poonian, M.S. and Kaback, H.R. (1986) *Biochemistry* 25, 4486–4488.
- 248 Menick, D.R., Carrasco, N., Antes, L., Patel, L. and Kaback, H.R. (1987) *Biochemistry* 26, 6638–6644.
- 249 Lee, J.A., Püttner, I.B. and Kaback, H.R. (1989) *Biochemistry* 28, 2540–2544.
- 250 Kraut, J. (1977) *Annu. Rev. Biochem.* 46, 331–358.
- 251 King, S.C. and Wilson, T.H. (1989) *Biochim. Biophys. Acta* 982, 253–264.
- 252 King, S.C. and Wilson, T.H. (1989) *J. Biol. Chem.* 264, 7390–7394.
- 253 King, S.C. and Wilson, T.H. (1990) *J. Biol. Chem.* 265, 3153–3160.
- 254 Franco, P.J. and Brooker, R.J. (1991) *J. Biol. Chem.*, 266, 6693–6699.
- 255 Matzke, E.A., Stephenson, L.J. and Brooker, R.J. (1992) *J. Biol. Chem.* 267, 19095–19100.
- 256 Pourcher, T., Sarkar, H.K., Bassilana, M., Kaback, H.R. and Leblanc, G. (1990) *Proc. Natl. Acad. Sci. USA* 87, 468–472.
- 257 Pourcher, T., Bassilana, M., Sarkar, H.K., Kaback, H.R. and Leblanc, G. (1992) *Biochem.*, 31, 5225–5231.
- 258 Yamaguchi, A., Adachi, K., Akasaka, T., Ono, N. and Sawai, T. (1991) *J. Biol. Chem.* 266, 6045–6051.
- 259 Gerchman, Y., Olami, Y., Rimon, A., Taglicht, D., Schuldiner, S. and Padan, E. (1993) *Proc. Natl. Acad. Sci. USA*, in press.
- 260 Carrasco, N., Püttner, I.B., Antes, L.M., Lee, J.A., Larigan, D., Lolkema, J.S., Roepe, P.D. and Kaback, H.R. (1989) *Biochemistry* 28, 2533–2539.
- 261 Poolman, B. (1993) Manuscript in preparation.
- 262 Pourcher, T., Zani, M.-L. and G. Leblanc, G. (1993) *J. Biol. Chem.*, 268, 3209–3215.
- 263 Eisenberg, D., Weiss, R.M. and Terwilliger, T.C. (1984) *Proc. Natl. Acad. Sci. USA* 81, 140–144.
- 264 Wilson, D.M. and Wilson, T.H. (1992) *J. Bacteriol.* 174, 3083–3086.
- 265 Pourcher, T., Deckert, M., Bassilana, M. and Leblanc, G. (1991) *Biochem. Biophys. Res. Commun.* 178, 1176–1181.
- 266 Zani, M.-L., Pourcher, T. and Leblanc, G. (1993) *J. Biol. Chem.*, 268, 3216–3221.
- 267 Behr, J.-P., Dumas, P. and Moras, D. (1982) *J. Am. Chem. Soc.* 104, 4540–4543.
- 268 Hinkle, P.C., Hinkle, P.V. and Kaback, H.R. (1990) *Biochemistry* 29, 10989–10994.
- 269 Yamaguchi, A., Akasaka, T., Ono, N., Someya, Y., Nakatani, M. and Sawai, T. (1992) *J. Biol. Chem.* 267, 7490–7498.
- 270 McMurtry, L.M., Stephan, M. and Levy, S.B. (1992) *J. Bacteriol.* 174, 6294–6297.
- 271 Fox, C.F. and Kennedy, E.P. (1965) *Proc. Natl. Acad. Sci. USA* 51, 891–899.
- 272 Kaback, H.R. and Barnes, E.M. (1971) *J. Biol. Chem.* 246, 5523–5531.
- 273 Robillard, G.T. and Konings, W.N. (1982) *Eur. J. Biochem.* 127, 597–604.
- 274 Konings, W.N. and Robillard, G.T. (1982) *Proc. Natl. Acad. Sci. USA* 79, 5480–5484.
- 275 Van Iwaarden, P., Driessen, A.J.M., Menick, D.R., Kaback, H.R. and Konings, W.N. (1991) *J. Biol. Chem.* 266, 15688–15692.
- 276 Van Iwaarden, P., Pastore, J.C., Konings, W.N. and Kaback, H.R. (1991) *Biochemistry* 30, 9595–9600.
- 277 Mitaku, S., Wright, J.K., Best, L. and Jähnig, F. (1984) *Biochim. Biophys. Acta* 776, 247–258.
- 278 Neuhaus, J.M., Soppa, J., Wright, J.K., Riede, I., Blöcker, H., Frank, R. and Overath, P. (1985) *FEBS Lett.* 185, 83–88.
- 279 Van Iwaarden, P., Driessen, A.J.M., Lolkema, J.S., Kaback, H.R. and Konings, W.N. (1993) *Biochemistry*, in press.
- 280 Yamato, I. and Anraku, Y. (1988) *J. Biol. Chem.* 263, 16055–16057.
- 281 Poolman, B., Konings, W.N., Robillard, G.T. (1983) *Eur. J. Biochem.* 135, 41–46.
- 282 Huang, A.-M., Lee, J.-I., King, S.C. and Wilson, T.H. (1992) *J. Bacteriol.* 174, 5436–5441.
- 283 Roepe, P.D. and Kaback, H.R. (1989) *Biochemistry* 28, 6127–6132.
- 284 Overath, P., Weigel, U., Neuhaus, J.-M., Soppa, J., Seckler, R., Riede, I., Bocklage, H., Müller-Hill, B., Aichele, G. and Wright, J.K. (1987) *Proc. Natl. Acad. Sci. USA* 84, 5535–5539.
- 285 Collins, J.C., Permeth, S.F. and Brooker, R.J. (1989) *J. Biol. Chem.* 264, 14698–14703.
- 286 Franco, P.J., Eelkema, J.A. and Brooker, R.J. (1989) *J. Biol. Chem.* 264, 15988–15992.
- 287 King, S.C. and Wilson, T.H. (1990) *J. Biol. Chem.* 265, 9638–9644.
- 288 Brooker, R.J. and Wilson, T.H. (1985) *Proc. Natl. Acad. Sci. USA* 82, 3959–3963.
- 289 Brooker, R.J., Fiebig, K. and Wilson, T.H. (1985) *J. Biol. Chem.* 260, 16181–16186.
- 290 Mieschendahl, M., Büchel, D., Bocklage, H. and Müller-Hill, B. (1981) *Proc. Natl. Acad. Sci. USA* 78, 7652–7656.
- 291 Markgraf, M., Bocklage, H. and Müller-Hill, B. (1985) *Mol. Gen. Genet.* 198, 473–475.
- 292 Eelkema, J.A., O'Donnell, M.A. and Brooker, R.J. (1991) *J. Biol. Chem.* 266, 4139–4144.
- 293 Püttner, I.B. and Kaback, H.R. (1988) *Proc. Natl. Acad. Sci. USA* 85, 1467–1471.
- 294 Persson, B., Roepe, P.D., Patel, L., Lee, J. and Kaback, H.R. (1992) *Biochemistry* 31, 8892–8897.
- 295 Page, M.G.P. and West, I.C. (1982) *Biochem. J.* 204, 681–688.
- 296 Brooker, R.J. (1991) *J. Biol. Chem.* 266, 4131–4138.
- 297 Gram, C.D. and Brooker, R.J. (1992) *J. Biol. Chem.* 267, 3841–3846.
- 298 Brooker, R.J. (1990) *J. Biol. Chem.* 265, 4155–4160.
- 299 King, S.C. and Wilson, T.H. (1990) *J. Biol. Chem.* 265, 9645–9651.
- 300 Seto-Young, D., Bedu, S. and Wilson, T.H. (1984) *J. Membr. Biol.* 79, 185–193.
- 301 Brooker, R.J., Myster, S.H. and Wilson, T.H. (1989) *J. Biol. Chem.* 264, 8135–8140.
- 302 Tsuchiya, T. and Wilson, T.H. (1978) *Membr. Biochem.* 2, 63–79.
- 303 Kawakami, T., Akizawa, Y., Ishikawa, T., Shimamoto, T., Tsuda, M. and Tsuchiya, T. (1988) *J. Biol. Chem.* 263, 14276–14280.
- 304 Niiya, S., Yamasaki, K., Wilson, T.H. and Tsuchiya, T. (1982) *J. Biol. Chem.* 257, 8902–8906.
- 305 Tsuchiya, T., Oho, M. and Shiota-Niiya, S. (1983) *J. Biol. Chem.* 258, 12765–12767.
- 306 Shiota, S., Yazyu, H. and Tsuchiya, T. (1984) *J. Bacteriol.* 160, 445–447.
- 307 Yazyu, H., Shiota, S., Futai, M. and Tsuchiya, T. (1985) *J. Bacteriol.* 162, 933–937.
- 308 Botfield, M.C. and Wilson, T.H. (1988) *J. Biol. Chem.* 263, 12909–12915.

- 308 Ohsawa, M., Mogi, T., Yamamoto, H., Yamato, I. and Anraku, Y. (1988) *J. Bacteriol.* 170, 5185–5191.
- 309 Myers, R.S. and Maloy, S.R. (1988) *Mol. Microbiol.* 2, 749–755.
- 310 Yazyu, H., Shiota-Niiya, T., Shimamoto, T., Kanazawa, H., Futai, M. and Tsuchiya, T. (1984) *J. Biol. Chem.* 259, 4320–4326.
- 311 Kamata, H., Akiyama, S., Morosawa, H., Ohta, T., Hamamoto, T., Kambe, T., Kagawa, Y. and Hirata, H. (1992) *J. Biol. Chem.* 267, 21650–21655.
- 312 Quijcho, F.A. (1990) *Phil. Trans. R. Soc. Lond. B* 326, 341–351.
- 313 Mowbray, S.L. (1992) *J. Mol. Biol.* 227, 418–440.
- 314 Sixma, T.K., Pronk, S.E., Kalk, K.H., Van Zanten, B.A.M., Berghuis, A.M. and Hol, W.G.J. (1992) *Nature* 355, 561–564.
- 315 Lolkema, J.S., Dijkstra, D.J. and Robillard, G.T. (1992) *Biochemistry* 31, 5514–5521.
- 316 Saier, M.H. (1989) *Microbiol. Rev.* 53, 109–120.
- 317 Postma, P.W., Ruijter, G.J.G., Van der Vlag, J. and Van Dam, K. (1992) in *Molecular mechanisms of Transport* (Palmieri, F. and Quagliariello, E., eds.), pp. 97–105, Elsevier, Amsterdam.
- 318 Postma, P.W. and Lengeler, J.W. (1985) *Microbiol. Rev.* 49, 232–269.
- 319 Peterkofsky, A., Svenson, I. and Amin, N. (1989) *FEMS Microbiol. Rev.* 63, 103–108.
- 320 Osumi, T. and Saier, M.H. (1982) *Proc. Natl. Acad. Sci. USA* 79, 1457–1461.
- 321 Nelson, S.O., Wright, J.K. and Postma, P.W. (1983) *EMBO J.* 2, 715–720.
- 322 Kuroda, M., De Waard, S., Mizushima, K., Tsuda, M., Postma, P. and Tsuchiya, T. (1992) *J. Biol. Chem.* 267, 18336–18341.
- 323 Dean, D.A., Reizer, J., Nikaido, H. and Saier, M.H. (1990) *J. Biol. Chem.* 265, 21005–21010.
- 324 De Boer, M., Broekhuizen, C. and Postma, P.W. (1986) *J. Bacteriol.* 167, 393–395.
- 325 Nelson, S.O., Schuitema, A.R.J. and Postma, P.W. (1986) *Eur. J. Biochem.* 154, 337–341.
- 326 Postma, P.W., Broekhuizen, C.P., Schuitema, A.R.J., Vogler, A.P. and Lengeler, J.W. (1988) in *Molecular basis of Biomembrane Transport* (Palmieri, F. and Quagliariello, E., eds.), pp. 43–52, Elsevier, Amsterdam.
- 327 Wilson, T.H., Yunker, P.L. and Hansen, C.L. (1990) *Biochim. Biophys. Acta* 1029, 113–116.
- 328 Kühnau, S., Reyes, M., Sieverstein, A., Shuman, H.A. and Boos, W. (1991) *J. Bacteriol.* 173, 2180–2186.
- 329 Romano, A.H., Brino, G., Peterkofsky, A. and Reizer, J. (1987) *J. Bacteriol.* 169, 5589–5596.
- 330 Niiya, S., Moriyama, Y., Futai, M. and Tsuchiya, T. (1980) *J. Bacteriol.* 144, 192–199.