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# BACTERIAL ANION EXCHANGE: REDUCTIONIST AND INTEGRATIVE APPROACHES TO MEMBRANE BIOLOGY

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**Summary**

Studies of two different bacterial anion exchange proteins (antiporters) led us to conclude that both reductionist and integrative approaches contribute to progress in understanding membrane biology. We have used a reductionist perspective in applying cysteine scanning mutagenesis to probe individual amino acid positions of UhpT (uptake of hexose phosphate transporter), the carrier responsible for transport of glucose 6-phosphate by *Escherichia coli*. This work has established experimental criteria that should allow one to identify and localize the translocation pathway in such membrane proteins. An integrative view is exemplified by work with OxlT (oxalate transporter), the carrier used by an anaerobe *Oxalobacter formigenes* to catalyze the antiport of divalent oxalate and monovalent formate. The activity of OxlT is functionally coordinated with that of a cytosolic oxalyl decarboxylase; together, these vectorial and scalar activities constitute a metabolic proton pump, allowing *O. formigenes* to display decarboxylative phosphorylation. The role played by OxlT argues that membrane carriers can assume unanticipated emergent properties when their biochemical functions are properly articulated in relation to other aspects of cell function.

## Studies with UhpT, a phosphate-linked anion exchange protein

### *Background*

Carriers related to the protein known as UhpT (for the uptake of hexose phosphates) catalyze an obligatory anion exchange. Such porters are termed '$P_i$-linked', because in each case $P_i$ (inorganic phosphate) is accepted with relatively low affinity while some selected organic phosphate substrate is accepted with high affinity (Maloney *et al.* 1990). Genetic and biochemical criteria identify three such $P_i$-linked porters in *Escherichia coli* and *Salmonella typhimurium*: UhpT is involved in the transport of glucose 6-phosphate (G6P); GlpT accepts glycerol 3-phosphate as its high-affinity substrate; and PgtP favors phosphoenolpyruvate and triose phosphates. Phenotypic tests point to other important examples in organisms such as *Streptococcus lactis* and *Staphylococcus aureus* (Maloney *et al.* 1990).

The study of UhpT and similar sugar phosphate antiporters has been under way for some time. Indeed, in 1953 a description of the $P_i$–self exchange reaction in *S. aureus*

Key words: anion exchange, cysteine scanning mutagenesis, site-directed mutagenesis, sulfhydryl reagents, substrate translocation pathway, membrane carrier, indirect proton pumps, UhpT, OxlT.

established the field of membrane biology in microorganisms (Mitchell, 1953). Somewhat later, work with *E. coli* focused on the unusual regulation of UhpT, whose expression is controlled by extracellular, but not intracellular, G6P (Winkler, 1966; Island and Kadner, 1993). It is now clear that this regulatory asymmetry reflects a signal transduction. Information transfer is initiated by occupancy of an external site on a membrane receptor (the UhpC protein); the signal then moves *via* phosphorylations catalyzed by the UhpB protein to a transcriptional regulator, UhpA, whose activity recruits synthesis of UhpT itself (Island and Kadner, 1993; Kadner *et al.* 1994).

### *A biochemical model*

That UhpT emerges once again as a model for transport (rather than regulation) reflects the considerable progress made in the past decade. In particular, biochemical studies have proved that UhpT functions as an anion exchange mechanism (Sonna *et al.* 1988). These efforts have also provided the tools needed for analysis of UhpT in solubilized and reconstituted systems (Ambudkar and Maloney, 1986; Sonna *et al.* 1988); that work has established that the monomer is the minimal functional unit (Ambudkar *et al.* 1990).

A biochemical model of UhpT function has derived from considerations of substrate selectivity and reaction stoichiometry, as noted in *S. lactis* (Ambudkar *et al.* 1986), but the model is fully consistent with what is known of other examples of UhpT (discussed in Maloney *et al.* 1990). Those experiments suggest that UhpT favors monovalent over divalent $P_i$, yet makes no substantial discrimination between monovalent and divalent forms of G6P. These and other conclusions, along with the observation that antiport of phosphate against sugar phosphate is an electroneutral event, can be easily understood if UhpT possesses a bifunctional active site that accepts two negative charges. More importantly, these charges would be taken *either* as a pair of monovalent anions *or* as a single divalent species (Ambudkar *et al.* 1986; Maloney *et al.* 1990). This reaction mechanism would therefore resemble that of the ionophore A23187, whose paired carboxyl groups accept either two protons or a single divalent cation. Note that for UhpT this mechanism preserves an electroneutral reaction at the expense of a pH-dependent variable stoichiometry, for at a molecular level substrates will exchange at either a 2:1 or a 2:2 ratio, depending on the nature of the substrate(s) and the pH at either membrane surface (Ambudkar *et al.* 1986). Perhaps most striking, in the presence of a pH gradient (alkaline inside) one may even imagine that *E. coli* takes up two sugar phosphate monoanions ($2HG6P^-$) from the relatively acidic medium in exchange for a single divalent sugar phosphate ($G6P^{2-}$) from the relatively alkaline interior (Fig. 1). Despite its unusual character, this reaction, a masquerade of a proton-linked symport, fits well with the known cell biology of UhpT in *E. coli* (see Maloney *et al.* 1990).

### *Molecular studies of UhpT*

The biochemical basis of exchange *via* UhpT is reasonably well-established, and further insight requires the application of molecular biology. Early progress in this area came from the laboratories of Kadner, Boos and Hong, who provided the amino acid sequences of UhpT, GlpT and PgtP (Friedrich and Kadner, 1987; Eiglmeier *et al.* 1987; Goldrick *et al.* 1988), and this information has now been supplemented with the results of

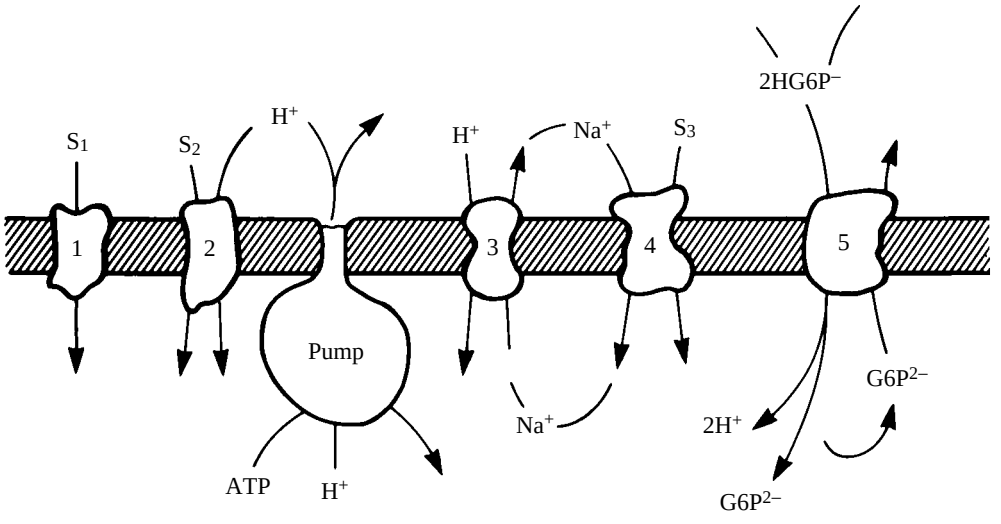


Fig. 1. Chemiosmotic circuits at the *Escherichia coli* inner membrane. In this simplified diagram of events at the *E. coli* inner membrane, a proton-motive force is established using ATP hydrolysis by the $F_1F_o$-ATPase. This outward $H^+$ movement is used to drive a number of subsidiary reactions, represented here by a population of chemiosmotic porters (1–5). The porters shown include examples of uniport (1), symport (2, 4) and antiport (3, 5). As described in the text, one mode of UhpT activity is represented by porter 5. $S_1$, $S_2$ and $S_3$ are substrates; G6P, glucose 6-phosphate. From Maloney (1987).

gene fusions in UhpT, GlpT and UhpC (Gott and Boos, 1988; Island *et al.* 1992). The combined data show that these proteins resemble many other secondary carriers in having a hydrophobic core consisting of 12 transmembrane segments (Fig. 2), with cytosolic N and C termini. A more recent analysis also shows that UhpT and its relatives (GlpT, PgtP, UhpC) constitute one of the five clusters of the major facilitator superfamily (MFS), a collection of 67 related carriers that includes examples from both prokaryotes (e.g. LacY, AraE) and eukaryotes (e.g. the GLUT proteins) (Marger and Saier, 1993). As is typical of carriers within this superfamily (Griffith *et al.* 1992; Marger and Saier, 1993), the 12 transmembrane segments of UhpT are organized as two groups of six segments each, connected by a central cytoplasmic loop, suggesting that the overall structure may have pseudo-twofold symmetry. Although there is no internal evidence of this asymmetry in UhpT, other carriers in the MFS show clear homology between the N-terminal and C-terminal groups of transmembrane segments (Maiden *et al.* 1987; Griffith *et al.* 1992), supporting both the idea of symmetry and the suggestion that such carriers may operate as covalent heterodimers (Maloney, 1990, 1994).

These early experiments have answered questions concerning the two-dimensional organization of UhpT (Fig. 2), but to address more complex issues – for example, the mechanisms underlying substrate selectivity and reaction stoichiometry – new approaches are needed. For this reason, we have undertaken a program of cysteine scanning mutagenesis, a strategy in which cysteine is systematically targeted to strategic

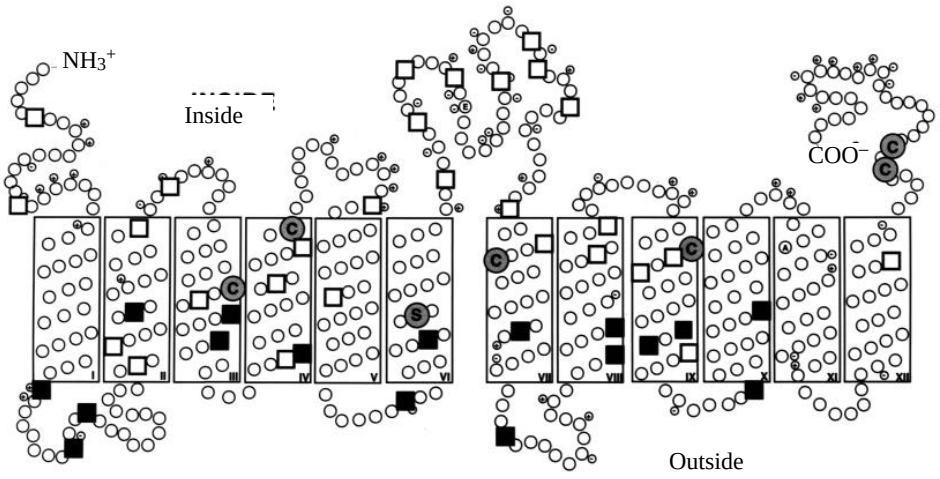


Fig. 2. The topology of UhpT. The UhpT amino acid sequence (small circles) is arranged in two dimensions to show its overall organization in the membrane (Yan and Maloney, 1993). This topology, which differs in only minor details from that reported by Island *et al.* (1992), is based upon gene fusion experiments with both UhpT (Island *et al.* 1992) and its relative GlpT (Gott and Boos, 1988); fusion junctions in GlpT were placed at their homologous positions within the UhpT sequence to derive the pattern shown here. Open squares show fusions indicating an intracellular location of the reporter gene (LacZ+ or PhoA− phenotype); filled squares are consistent with an external position of the fusion joint (LacZ− or PhoA+). The enlarged circles show the six UhpT cysteines (residues 108, 143, 265, 331, 436 and 438) as well as serine-199.

locations within the protein. This general methodology was used in early studies of bacteriorhodopsin (Altenbach *et al.* 1990) and is currently being used to probe functional domains in the acetylcholine receptor ion channel (Akabas *et al.* 1992) and a possible $H^+$-coupling region in LacY (Sahin-Toth and Kaback, 1993). In work with UhpT, our immediate objective is to examine new cysteine variants with SH-reactive probes that resemble the natural substrate in size and charge. In this way, we should be able to identify the translocation pathway and locate the residues that G6P encounters while passing through UhpT. With this information in hand, it is almost certain that we will be better able to understand the origins of substrate selectivity and stoichiometry.

### *The role of cysteines in UhpT*

As the first step in applying cysteine scanning mutagenesis to UhpT, we asked whether the cysteines normally present in UhpT are essential to function (see Fig. 2). Site-directed mutagenesis was used to replace, one at a time, each of the six UhpT cysteines with an isosteric serine residue (Yan and Maloney, 1993). These variants were then used, along with *in vitro* restriction enzymology, to obtain a complementary set of derivatives, each containing a single cysteine at one of the normal positions. Assays of transport by both sets of mutants showed that UhpT function was retained at normal or near normal levels in all variants, even in the cysteine-less derivative (Yan and Maloney, 1993).

This analysis of UhpT cysteines shows that this amino acid plays no essential role in antiport, and such findings have considerably simplified the approach to our long-term goal, since manipulation of old cysteines or the addition of new ones can now take place against a neutral background. It may be that bacterial systems are especially favorable for such work, since the same conclusion has been reached for LacY (van Iwaarden *et al.* 1991) and GalP (cited in Henderson, 1993). By contrast, cysteines may not be so readily eliminated in other settings. There is a disulfide linkage in the membrane sector of the plasma membrane proton pump of *Neurospora crassa* (Rao and Scarborough, 1990), and the nature of several mitochondrial carriers is profoundly altered by cysteine-directed agents (Dierks *et al.* 1990*a*,*b*).

### *A residue in the translocation pathway of UhpT*

UhpT-type proteins are inhibited by low concentrations of mercury and mercurials (Mitchell, 1953; Maloney *et al.* 1990). Using the UhpT variants containing single cysteines, we sought to identify the residues responsible for this phenotype. Such an effort seemed worthwhile, if only because this information, along with use of permeant and impermeant mercurials, might be relevant to confirming the suspected locations of cysteines in UhpT (see Fig. 2).

Studies of inhibition by a lipid soluble agent, *p*-chloromercuribenzoic acid (PCMB), showed unambiguously that mercurial sensitivity was attributable to reactions at both C143 and C265 (Fig. 2). Note that PCMB is membrane-permeant in its protonated form and, therefore, that its attack on UhpT might occur from either the extracellular or intracellular surface. To distinguish these alternatives, we next used *p*-chloromercuribenzosulfonate (PCMBS), a hydrophilic membrane-impermeant derivative of PCMB. We anticipated that PCMBS would not affect G6P transport by intact cells. We also expected that studies of everted membrane vesicles would be informative, since a latent sensitivity to PCMBS might become evident.

These arguments were justified by the behavior of C143. PCMBS failed to inhibit transport by intact cells expressing the single-cysteine UhpT variant with cysteine at position 143; nevertheless, G6P accumulation by everted vesicles was substantially blocked (Table 1). This result shows that C143 is exposed to the cytoplasm, and only to the cytoplasm, and verifies the topological model of UhpT in this region (Fig. 2). By contrast, when we tested the UhpT variant containing only C265, PCMBS blocked transport by *both* intact cells and everted vesicles. This important finding shows that water-soluble, membrane-impermeant PCMBS has access to C265 from *both* internal and external phases (Yan and Maloney, 1993) (Table 1).

Clearly, UhpT contains a translocation pathway through which G6P moves. Thus, our finding that PCMBS can approach C265 from either membrane surface can be understood readily if this anionic probe moves through UhpT using a pre-existing substrate translocation pathway. To test this possibility in the simplest way, we asked whether G6P could protect C265 against an attack by PCMBS – one might expect this protection to occur if the two anions used the same pathway. Indeed, substrate protection could be readily documented, whether the attack by PCMBS was from the extracellular medium or from the cytoplasmic surface (not shown, see Yan and Maloney, 1993). Such findings

Table 1. *PCMBS-sensitivity of three single-cysteine derivatives of UhpT*

| | Relative activity after PCMBS treatment | |
|---|---|---|
| UhpT variant | Intact cells | Everted vesicles |
| C143 | 0.95 | 0.35 |
| C265 | 0.25 | 0.15 |
| S199C | 0.22 | 0.92 |

Glucose 6-phosphate transport activity was measured for intact cells or for $P_i$-loaded everted membrane vesicles, using strains expressing the indicated single-cysteine variant of UhpT. Cells or vesicles were exposed for 10 min to 0.2 mmol $l^{-1}$ PCMBS and, after removal of the inhibitor, initial rates of glucose 6-phosphate transport were measured as described by Yan and Maloney (1993). From Yan and Maloney (1993, 1994) and from their unpublished experiments.

PCMBS, *p*-chloromercuribenzosulfonate.

strongly suggest that C265 is one of the residues that lines the translocation pathway of UhpT.

### *Engineering a PCMBS-sensitive site into UhpT*

The experiments summarized in the preceding section suggest that C265 lies on the UhpT substrate translocation pathway. Equally important, this work shows how one might use targeted cysteine mutagenesis to identify residues that constitute a functional domain. To validate the approach further, we have now begun to implant cysteines into new locations, and one of these new derivatives merits special comment. Starting with the cysteine-less variant of UhpT, we engineered a Ser→Cys mutation at position 199. Analysis of S199C suggests that this variant provides an essential control that justifies extended use of this overall strategy. The S199C derivative is inhibited by PCMBS from the external surface (intact cells), but *not* from the cytoplasmic surface (everted vesicles) (Table 1). This finding has great practical value, for it suggests that our tacit assumption regarding the polarity of everted membrane vesicles is verifiable by controls using UhpT itself.

Together, these UhpT variants (Table 1) define the experimental limits that should allow identification of residues in the translocation pathway of this membrane carrier. In one case (S199C), the hydrophilic and impermeant PCMBS inhibits only from the extracellular phase; in another instance (C143), PCMBS attacks only from the cytoplasmic phase; whereas in the remaining example (C265), inhibition is found from either surface. It is this last attribute that marks residues lining the translocation pathway.

Fig. 3 describes the population of UhpT cysteine substitution mutants to be examined while holding these experimental criteria in mind. We envision this collection as supporting two kinds of investigations. Because at least one residue on transmembrane segment VII lies on the transport pathway, we plan an initial ‘vertical’ experiment to examine the full length of segment VII. Among other things, this survey should tell us whether the transport pathway involves small or large numbers of residues; moreover, if several such residues are found, it may be possible to use the patterns of reactivity

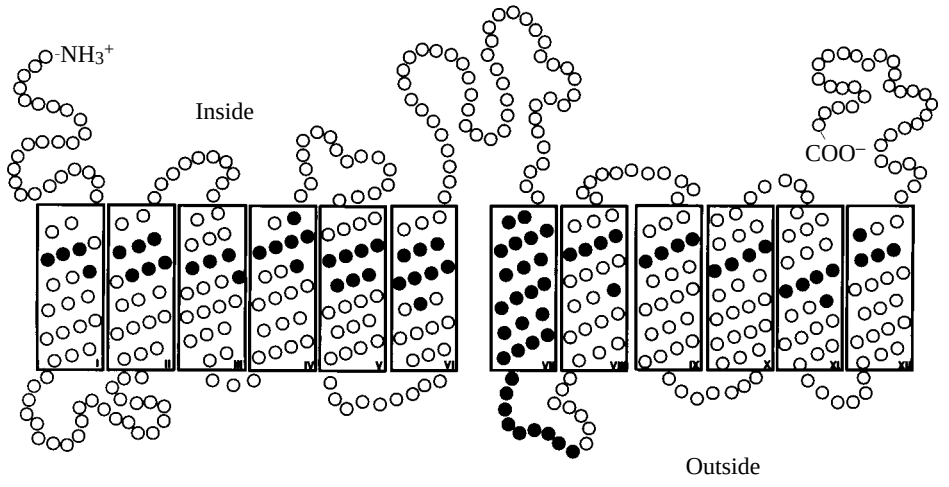


Fig. 3. Cysteine substitution mutants in UhpT. The UhpT amino acid sequence is shown as in Fig. 2. Each filled circle represents a UhpT variant in which at the indicated position, and only at that position, there is a cysteine residue (R.-T. Yan and P. C. Maloney, unpublished results).

towards PCMBS as an indirect probe of local structure, as has been argued for positional-reactivity in the acetylcholine receptor (Akabas *et al.* 1992). Early results from this study are encouraging and suggest that the pathway has considerable size; it is also likely that transmembrane segment seven is structured as an alpha-helix, at least between positions 261 and 273.

We anticipate that a 'horizontal' experiment will sample a relatively small number of residues on all transmembrane segments. In this case, we should be able to make rapid preliminary decisions about whether a segment does or does not contribute to the transport pathway. At the moment, we have only generated the necessary reagents for this series of trials (Fig. 3); their analysis has not yet begun. On completion of the vertical and horizontal experiments, we may even be able to generate informed models of the tertiary structure of this membrane carrier (Maloney, 1994) and, since UhpT belongs to the major facilitator superfamily (Marger and Saier, 1993), these inferences may be more broadly applicable.

### Studies with OxlT – carriers that partake in proton-motive metabolic cycles

The topics emphasized above are typical of those in the current literature – that is, much work in this field is directed to an understanding of membrane protein structure. Nevertheless, we should not forget that these transporters operate in the world of biology and that they might assume significant new roles in new settings; these 'emergent' functions are as equally worthy of study as are issues dealing with protein structure. In the microbial world, this integrative view is exemplified by OxlT and similar carriers that participate in proton-motive metabolic cycles. In effect, these carriers work as proton pumps.

*Discovery of OxlT*

OxlT is an anion exchange carrier (antiporter) found in the Gram-negative anaerobe *O. formigenes*, a cell that derives all of its metabolic energy from the decarboxylation of oxalate ($^{-}OOC–COO^{-}$), a process that yields the simple end-products, formate ($HCOO^{-}$) and $CO_2$ (Allison *et al.* 1985). A study of oxalate transport by this cell (Anantharam *et al.* 1989) identified an anion exchange reaction involving the one-for-one electrogenic antiport of *divalent* oxalate and *monovalent* formate. The properties of OxlT, the protein responsible for this exchange (Ruan *et al.* 1992), suggest that it is a typical membrane carrier. The stoichiometry of exchange and the rate with which it occurs further suggest that OxlT is the major pathway by which oxalate and formate move into and out of *O. formigenes.*

*An indirect proton pump*

Although the biochemical characteristics of OxlT are of intrinsic interest (e.g. OxlT has a turnover number higher than that of any other organic substrate carrier), OxlT merits special attention for the manner in which its function is integrated within overall cell

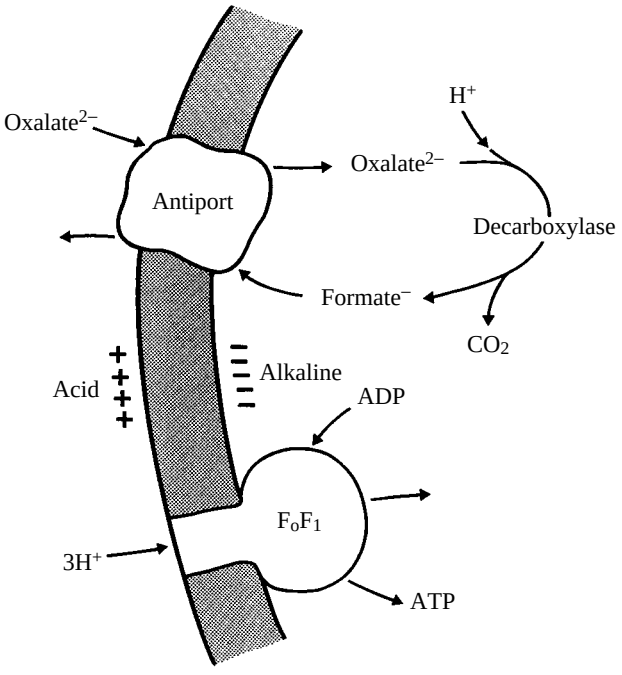


Fig. 4. OxlT participates in the indirect proton pump of *Oxalobacter formigenes.* The diagram describes decarboxylative phosphorylation in *O. formigenes.* As outlined in the text, a proton-motive force is established by the combined activity of the vectorial OxlT antiport reaction and the scalar internal oxalyl decarboxylation. Entry of divalent oxalate in exchange for monovalent formate generates an electrical potential, negative inside; consumption of a proton during decarboxylation generates an internal alkalinity. Three antiport cycles provide sufficient energy for the synthesis of one ATP. From Anantharam *et al.* (1989).

physiology – OxlT, in combination with the cytosolic decarboxylase, functions as an entirely new kind of proton pump (Anantharam *et al.* 1989; Ruan *et al.* 1992). This 'indirect' or 'metabolic' proton pump is illustrated in Fig. 4. Note that OxlT transports both the precursor ($oxalate^{2-}$) and product ($formate^{-}$) of decarboxylation and that decarboxylation itself consumes a single internal proton. Consequently, each completed cycle of this pathway is accompanied by net inward movement of a single negative charge and the disappearance of a single internal proton. This cycle is the thermodynamic equivalent of an *outwardly* directed proton pump, one whose stoichiometry is $1H^+$/cycle. Three cycles would extrude $3H^+$ and establish a proton-motive gradient sufficient to drive ATP synthesis by 'decarboxylative phosphorylation.'

### *Other proton-motive metabolic cycles*

With this prototype in mind, it has been possible to search for similar examples, and in recent years a number of proton-motive decarboxylation cycles have been identified (Table 2) (see also Maloney, 1994; and the summary by Poolman and Konings, 1993). In several cases, there is direct evidence in support of the hypothesis, whereas in other instances the required players are present but suitable experiments are not yet available. I believe it is notable that these systems often show acidic pH optima (K. Abe, unpublished results). For this reason, it will be of interest to consider such cycles in connection with the acid-inducible amino acid decarboxylases found in *E. coli* (Meng and Bennet, 1992). Decarboxylation-linked proton-motive cycles may be especially well adapted to highly acidic conditions, since these new proton pumps are not limited by the traditional thermodynamic restraints associated with ion transport. That is, if $CO_2$ is freely disseminated, the cycle becomes functionally irreversible and the sustainable proton-motive gradient will be limited by the transmembrane concentration gradient of the precursor. With sufficiently high internal levels of a scalar partner, cytosolic levels of precursor might become extraordinarily small. Accordingly, these metabolic cycles may function as 'tunable' proton pumps.

The proton-motive metabolic cycles described in Table 2 resemble the prototype based on OxlT (Fig. 4). This resemblance is not essential, however, since on theoretical grounds these cycles require only a one-for-one stoichiometry between entry of negative charge (exit of positive charge) and consumption of internal protons (or external hydroxyls). There are many ways this final result might be achieved. In fact, consideration of this principle suggests a role for such cycles in the evolution of primitive systems, perhaps as a way of generating a proton-motive force without requiring complex metabolic or physical structures (Maloney and Wilson, 1993). Note also that the presently known cycles are rather simple, involving as they do only a pyridoxal- or CoA-linked decarboxylation system (Table 2). Again, simplicity is not essential, and more complex examples have been suggested. For example, acetate, formate or bicarbonate can serve as substrates for synthesis of methane. If these precursors enter as anions, the collection of internal reactions leading to methane production can be viewed as the scalar portion of a metabolic proton pump – for each anion that moves inward, the path to methanogenesis ensures that a single internal proton will be consumed (Ruan *et al.* 1992).

Table 2. *Likely proton-motive metabolic cycles*

| Precursor/Product[1] | Organism |
|---|---|
| Aspartate/Alanine | *Lactobacillus*[2] |
| Glutamate/GABA | *Lactobacillus*[3] |
| Histidine/Histamine | *Lactobacillus* (Molenaar *et al.* 1993) |
| Lysine/Cadaverine | *Escherichia* (Meng and Bennet, 1992) |
| Malate/Lactate | *Lactobacillus* (Poolman *et al.* 1991; Olson *et al.* 1991) |
| Oxalate/Formate | *Oxalobacter* (Anantharam *et al.* 1989) |
| Phenylalanine/Phenethylamine | *Lactobacillus*[3] |
| Tyrosine/Tyramine | *Pediococcus*[3] |

[1]Conversion of precursor into product requires carrier-mediated entry of precursor into the cell, followed by an internal decarboxylation and export of product by way of the same or a different carrier. The net exchange of precursor and product brings in a single negative charge; a single cytosolic proton is consumed during decarboxylation as carbon dioxide (not shown) is generated. The organic product need not be excreted, but may be used biosynthetically; in which case, requirements of a proton pump may be satisfied by other restraints, e.g. in the case of divalent anion entry ($oxalate^{2-}$), a proton pump is preserved if precursor entry is accompanied by hydroxyl export (or proton entry) *via* by the same or different carrier (see Anantharam *et al.* 1989; Ruan *et al.* 1992).

[2]K. Abe and P. C. Maloney (unpublished results).

[3]K. Abe, H. Hayashi and T. Higuchi (unpublished results).

Indirect proton pumps appear to be restricted to bacterial systems, but because the possibility of a wider distribution has not been carefully examined, it is probably too soon for strong generalizations. Most importantly, the role played by OxlT argues that membrane carriers can assume unanticipated emergent properties if their biochemical functions are properly articulated in relation to other aspects of cell function. This principle will surely be found in all cell types, wherever membrane carriers are found.

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