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Interpreting the effects of site-directed mutagenesis on active transport systems

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Abstract

Single amino acid substitutions in the lactose permease of *Escherichia coli* are known to elicit behaviour, such as the transformation of an active into a passive system, not explained by current co-transport models. The behaviour, it is shown, can be explained by an expanded reaction scheme that takes account of the required alternation of the carrier, in the course of the coupled reaction, between mobile and immobile conformations or between conformations that bind either only one substrate or both substrates. The extended model links such behaviour to altered conformational equilibria or binding regions. Thus, mutations that affect the equilibrium between a mobile one-site conformation of the free carrier and an immobile conformation having sites for both substrates allow passive transport of the second substrate in an ordered mechanism, and mutations in a secondary substrate binding region that affects this conformational change allow passive transport of the first substrate. Mutations in regions interacting with a substrate in the transition state in carrier movement, as well as in the initial binding sites, can also be distinguished. The analysis applies to both primary and secondary active transport.

Key words: Uncoupled transport; Cotransport; Mutation; Lactose permease; Calcium pump; Transport kinetics

1. Introduction

Single amino acid substitutions in the lactose permease of *Escherichia coli* (a lactose-H^+ co-transporter) can have effects on transport not predicted by any of the usual carrier models. Consequently the link between mutation and the process of translocation or coupling may remain obscure, and studies of site-directed mutagenesis may fail to yield the promised insight into mechanism. Consider the kind of behaviour that has been found [1]. Substitution at Glu-235 abolishes active transport; it is the coupling of the movement of the two substrates, not the capacity for transport, that is impaired, for the altered carrier does facilitate the downhill entry of lactose. Surprisingly, downhill exit is not detected, even though the altered system, being passive, must allow substrates to equilibrate; in fact, the system does facilitate the exchange of internal and external lactose, a process in which the substrate is carried out as well as in. Another substitution, at His-322, also abolishes active transport; the altered carrier facilitates the uncoupled transport of lactose, both entry and exit, while all steps dependent on protonation or deprotonation appear to be defective. Other substitutions, at Ala-177 or Tyr-236, lead to an uncoupled transport of H^+. The transport mechanism in the wild-type involves an ordered addition of substrates, H^+ first and lactose second, as in the general co-transport scheme in Fig. 1, with S representing H^+ and T representing lactose [2,3] – in such a mechanism only the free carrier and the ternary complex can be mobile. One wonders how lactose can be transported at all, once the addition of H^+ to the carrier has been blocked, as in His-322 substitution; or why active transport and facilitated transport are alternative modes – one might have expected active transport to be an exclusive function.

Our models for active transport are evidently incomplete, and it is not hard to see what they leave out: they do not explain why some forms of the carrier are mobile and others are immobile, why some forms bind only one substrate and others bind both – they simply postulate a series of predetermined changes in the

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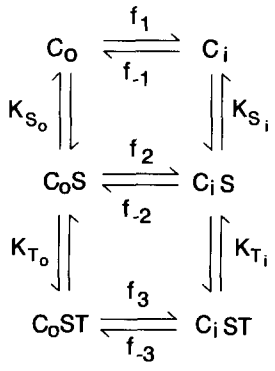


Fig. 1. Symmetrical ordered co-transport mechanism. The order of addition of substrates to the carrier is the same on the two sides of the membrane – S first and T second. The carrier alternates between two conformations, C_o and C_i, in which substrate sites face either outward or inward, respectively. Substrates in the outer or inner compartment add to these, forming an outward-facing or inward-facing complex. For transport to be coupled, C_oS and C_iS must be immobile (i.e., $f_2, f_{-2} \ll f_1, f_{-1}, f_3, f_{-3}$).

properties of the carrier over the course of the transport reaction. When the models are expanded to account for the known sequence of steps, the behaviour becomes predictable.

In an active transport system the mobility of the carrier, and sometimes its substrate specificity and enzyme activity as well, must be strictly controlled. In the familiar co-transport scheme in Fig. 1 the substrates add to the carrier in fixed order – S first, T second: only S adds to the free carrier but both S and T are bound in the ternary complex; and while the free carrier and the ternary complex are mobile, the binary complex with a single substrate is immobile (otherwise S could be transported alone, passively, abolishing active transport). The transport cycle is governed by what may be called a set of 'rules' [4]; the rules describe the reaction sequence but leave the underlying mechanisms unspecified.

These underlying mechanisms will first be examined: one, a simple mechanism for an ordered addition of substrates, in which a substrate stabilizes an immobile carrier conformation containing a site for the companion substrate; the other, a simple mechanism in which a substrate catalyzes the movement of an immobile carrier form, a process that may be called vectorial catalysis. The two mechanisms are then introduced into standard co-transport models; the result in each case is an expanded scheme containing no intermediate not implicit in the original model, but exhibiting new properties. The expanded schemes make definite predictions regarding the effects of shifts in individual conformational equilibria and also regarding the effects of modifications of specific binding regions – not only the regions to which the substrates are initially bound but also ancillary regions that come into play in the course of the translocation cycle. Hence, single amino acid residues in the transport protein may be linked to particular steps in the transport reaction. It is shown that the expanded scheme for a symmetrical ordered co-transport model can account for the puzzling behaviour of mutant forms of the lactose permease.

At the outset it may be helpful to define terms. Carrier 'movement', or carrier 'reorientation', is the process in which the binding site for a transported substrate, initially exposed on one side of the membrane, becomes exposed on the other side; a 'mobile' carrier form is capable of movement, an 'immobile' form is not. 'Coupling' refers to the processes in which the movements of two substrates are obligatorily linked, or, in primary active transport, in which the movement of one substrate is contingent on chemical reaction of the other substrate. In 'uncoupled transport' one substrate moves (or reacts) independently of the other; 'slippage' is another name for uncoupled transport. The 'coupling ratio' is the rate of the coupled relative to the uncoupled reaction (in principle, the latter can never be reduced to zero in any coupled system). 'Passive transport', which is facilitated transport by a carrier system, allows substrates to equilibrate across the membrane but cannot produce a concentration gradient. The subscripts 'o' and 'i' refer to the two sides of the membrane, 'out' and 'in', respectively.

2. Mechanisms involved in coupling

2.1. Ordered addition of substrates

In the ordered co-transport scheme in Fig. 1, the binary complex is immobile, the free carrier and the ternary complex, mobile: S, adding first, immobilizes the carrier and exposes a binding site for the second substrate, T. To account for the behaviour, two different carrier conformations are postulated, as in Fig. 2

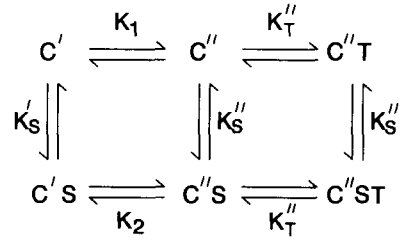


Fig. 2. Reaction scheme accounting for an ordered addition of substrates – S first and T second. The carrier exists in two conformations: C', with a site for S but none for T, and C'', with sites for both S and T. The free carrier is predominantly in conformation C' ($K_1 = [C']/[C''] \gg 1$), while the complex with S may be predominantly in conformation C'' ($K_2 = [C'S]/[C''S] \ll 1$). Substrate T can add to the two-site conformation of the free carrier, forming C''T, which is expected to be fully mobile (see text). The simplifying assumption is made that S and T bind to the two-site conformation independently.

[5]. The reasoning is as follows. Since only one of the substrates can add to the free carrier, a conformation exists containing a site for this substrate alone (C′); and since at a later stage of the reaction both substrates are bound, another conformation exists in which both sites are exposed (C″). The one-site conformation C′ and the two-site conformation C″ will be in equilibrium, though one may be favoured; a bound substrate molecule induces a conformational change by virtue of its preference for one or the other. To ensure that S binds first, the free carrier is in the one-site conformation C′ ($K_1 = [C']/[C''] \ll 1$); this conformation, therefore, has to be mobile. The complex with S, which contains a site for T, is not mobile; accordingly it must be in the other conformation C″S, and this conformation has to be immobile($K_2 = [C'S]/[C''S] \ll 1$). Note that K_1 and K_2 are related to K'_S and K''_S,the substrate dissociation constants of the two carrier forms:

$$K''_S/K'_S = K_2/K_1 \ll 1 \quad (1)$$

The dissociation constants for T, either in the absence of S (K_T), or in the presence of a saturating concentration of S (K_{ST}), are

$$\begin{aligned} K_T &= ([C'] + [C''])[T]/[C''T] \\ &= [C''](1 + K_1)[T]/[C''T] \\ &= K''_T(1 + K_1) \approx K_1\, K''_T \end{aligned} \quad (2)$$

$$\begin{aligned} K_{ST} &= ([C'S] + [C''S])[T]/[C''ST] \\ &= [C''S](1 + K_2)[T]/[C''ST] \\ &= K''_T(1 + K_2) \approx K''_T \end{aligned} \quad (3)$$

The affinity of T is seen to be higher in the presence than in the absence of S by the factor K_1, where $K_1 \gg 1$. As a result, the free carrier forms a complex almost exclusively with S. It is apparent that the strictness of ordering depends on the abruptness of the change in conformation from C′ to C″, which is governed by K_1. In a random mechanism, substrate T adds as readily to the free carrier as to the complex of S: $K_T = K_{ST}$; in a perfectly ordered mechanism, T does not add to the free carrier at all: $K_T = \infty$; it follows that the ratio of random to ordered addition is a function of K_{ST}/K_T, equal to K_1 (Eqs. (2) and (3)).

When the substrate adds to the free carrier, the binding forces are utilized to shift the conformational equilibrium. The abruptness of the shift, which depends on the ratio of dissociation constants for the tight and loose complex, K''_S/K'_S, limits the ratio of coupled to uncoupled transport (as will now be shown).In consequence, a limiting value can be found for K''_S/K'_S, whose size may indicate what additional interactions are required in the tight complex.

Uncoupled transport cannot be completely eliminated because T should catalyze carrier movement whether it adds to C″S or to C″. The uncoupled flow of T will be proportional to the concentration of C″T, the coupled flow to the concentration of C″ST. In the absence of S,

$$[C''T] = [C_t]/(1 + K_T/[T]) = [C_t]/(1 + K_1K''_T/[T]) \quad (4)$$

and in the presence of a saturating concentration of S,

$$[C''ST] = [C_t]/(1 + K_{ST}/[T]) = [C_t]/(1 + K''_T/[T]) \quad (5)$$

(where $[C_t]$ is the total carrier concentration). Let the two rates, coupled ($\bar{v}_{ST}$) and uncoupled ($\bar{v}_T$), be measured at a concentration ($[T] < K_{ST}$) that in coupled transport is less than saturating (and in uncoupled transport, very much less than saturating). The ratio of coupled to uncoupled flux, equal to the ratio of the concentrations of C″ST and C″T is then (from Eqs. (1), (4), and (5))

$$\bar{v}_{ST}/\bar{v}_T \approx K_i = K_2\, K'_S/K''_S \ll K'_S/K''_S \quad (6)$$

The important result here is that K'_S/K''_S is very much larger than the coupling ratio, $\bar{v}_{ST}/\bar{v}_T$, for the second substrate, T. Binding in C″S, therefore, has to be very strong.

The dissociation constant for the tight complex, K''_S, is roughly equal to $\bar{K}_{So}$, the experimental half-saturation constantfor external S extrapolated to $[T] = 0$, divided by the coupling ratio.The relationship is found as follows. From rate equations derived forthe co-transport scheme in Fig. 1 [6], the experimental constant $\bar{K}_{So}$ is a function of the dissociation constant of the free carrier, K_S:

$$\bar{K}_{So} = K_S(f_1 + f_{-1})/(f_3 + f_{-1}) \approx K_S \quad (7)$$

where f_1, f_{-1} and f_3 are rate constants for carrier movement, and where

$$\begin{aligned} K_S &= ([C'] + [C''])[S]/([C'S] + [C''S]) \\ &= [C''](1 + K_1)[S]/\{[C''S](1 + K_2)\} \approx K_1K''_S \end{aligned} \quad (8)$$

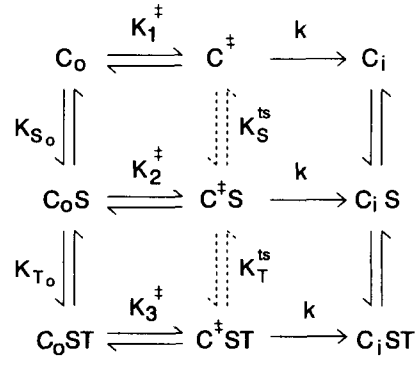


Fig. 3. Symmetrical ordered co-transport mechanism (as in Fig. 1), showing the transition states in carrier movement, $C^{\ddagger}$, $C^{\ddagger}S$ and $C^{\ddagger}ST$.

From Eqs. (1), (6), (7), and (8)

$$K''_S \approx K_S/K_1 \approx \bar{K}_{So}(\bar{v}_T/\bar{v}_{ST}) \quad (9)$$

2.2. Vectorial catalysis

In the co-transport scheme in Fig. 1, where the binary complex is immobile and the ternary complex is mobile, the role of the second substrate, T, is to convert the carrier from an immobile to a mobile form; in effect, to catalyze carrier movement. Binding forces are found to play a similar role, whether a substrate is required to shift a conformational equilibrium, as in the case treated above, or to increase the rate of a conformational change, as in the present case. By introducing the transition state in carrier reorientation into the reaction scheme, as in Fig. 3, it may be shown [7] that the virtual substrate dissociation constant in the transition state, K_T^{ts}, can be estimated from experimentally measured constants:

$$K_{To}/K_T^{ts} > \bar{V}_{STo}/\bar{V}_{So} \quad (10)$$

$$K_T^{ts} = \bar{K}_{STo}\bar{V}_{So}/\{\bar{V}_{STo}(1 + f_1/f_{-1})\}$$

$$< \bar{K}_{STo}\bar{V}_{So}/\bar{V}_{STo} \quad (11)$$

$\bar{V}_{STo}$ is the maximum rate of coupled transport, i.e., uptake in the presence of saturating concentrations of both substrates, S and T; $\bar{V}_{So}$ is the maximum rate of uncoupled transport of S, i.e., uptake in the absence of external T; and $\bar{K}_{STo}$ is the half-saturating concentration of the external T in the presence of saturating S [6]. According to Eq. (11), the affinity of T in the transition state is higher than the apparent affinity by a factor greater than the coupling ratio $\bar{V}_{STo}/\bar{V}_{So}$ (i.e., the ratio of coupled to uncoupled rates for substrate S). Why such a relationship exists may be seen in the diagram in Fig. 4. The increment in the binding energy of T accompanying the conversion of the initial complex to the transition-state complex is seen to be equal to the difference in the activation energies for movement of the binary and ternary substrate complexes. Since uncoupled transport depends on the mobility of the former, while coupled transport depends on the mobility of the latter, the ratio of coupled to uncoupled transport rates is directly related to the increased binding force in the transition state. ($\bar{V}_{STo}/\bar{V}_{So}$ is less than, rather than equal to, K_{To}/K_T^{ts} [7] because the coupled rate is limited not only by movement of the ternary complex (f_3) but of the free carrier (f_{-1}, where $f_{-1} \approx f_3$), while the uncoupled rate is limited solely by movement of the binary complex (f_2, where $f_2 \ll f_{-1}$).)

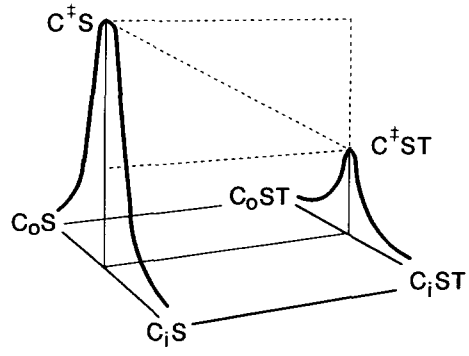


Fig. 4. Energy profile for catalysis of carrier movement by the substrate. C_oS and C_iS are the outward-facing and inward-facing conformations of the binary complex, respectively, and C_oST and C_iST the corresponding forms of the ternary complex; $C^‡S$ and $C^‡ST$ are the transition states in transfer of the substrate site from one side of the membrane to the other. Coupled transport depends on the low mobility of the binary complex, in that the ratio of coupled to uncoupled flux is proportional to the ratio of the mobilities of the binary and ternary complexes. The transition state $C^‡S$ must therefore be at a higher energy level (vertical axis) than $C^‡ST$, and by exactly the same amount the free energy for formation of the complex with T is more favourable in the transition state than in the ground state. Consequently the tightness of coupling is directly related to the tightness of substrate binding in the transition state.

3. Binding forces

To avoid wasting metabolic energy the ratio of coupled transport to slippage should of course be large. The ratio is determined by the strength of substrate binding forces (Eqs. (6), (9), (10), (11)), which implies that the carrier must be capable of binding the substrate very tightly at certain stages of the reaction. Forces of the required magnitude could be attained in a chelate complex, in which the carrier encloses the substrate and interacts with it from all sides. The formation of such a complex can account for rate-limiting dissociation of transported substrates, for the occluded state of transported cations in ATP-driven pumps, for the highly favourable reaction of inorganic phosphate with the calcium pump, and for various characteristic properties of the anion exchanger of red cells [8,9]. In the case of the anion exchanger, the K_m for chloride ion is about 65 mM, while in the transition state the virtual dissociation constant, from the coupling ratio of 4×10^4 [10,11], would be 1–2 μM (Eq. (10)). By hypothesis, the substrate (the chloride ion) is loosely bound at a surface site (which allows for a large increase in the binding force), and in the transition state the site closes over the ion, forming an inclusion complex – the rearrangement of residues at the binding site sets in train a wider conformational change, allowing the substrate site to shift from one side of the membrane to the other. Among other things the hypothesis accounts for the way substrate specificity is expressed: anions of widely different structure have almost the same affinity but are transported at rates that vary by factors of up to 10^4. Because the initial

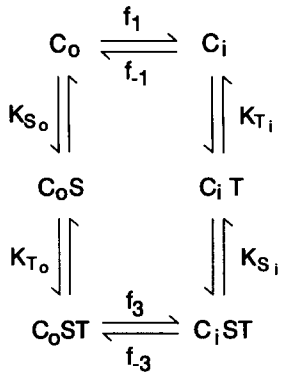


Fig. 5. Asymmetrical ordered co-transport mechanism. The order of substrate addition is different on the two sides of the membrane: S first on the outside, T first on the inside. The binary complex of each substrate fails to move across the membrane because on the opposite side the corresponding complex is missing.

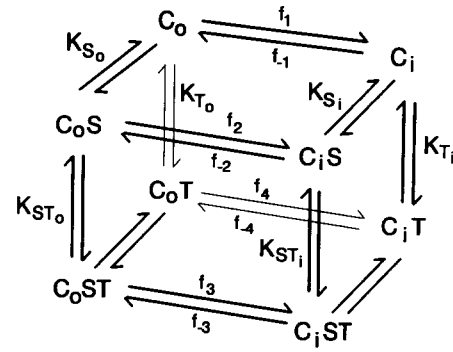
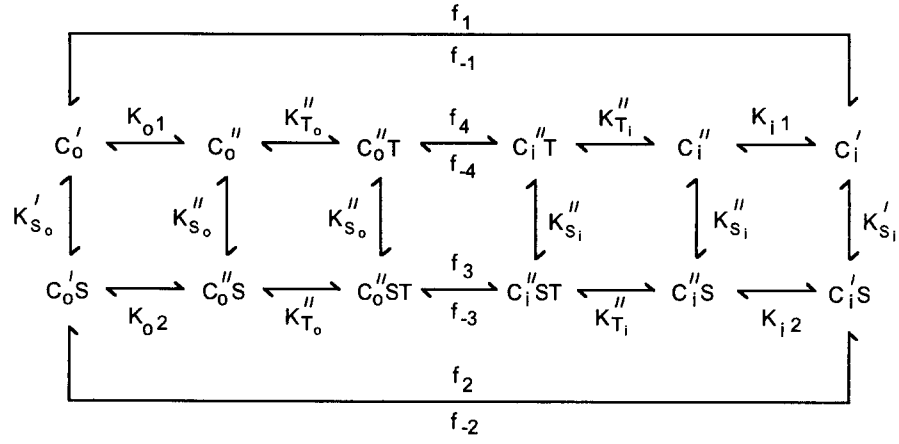


Fig. 6. Random-order co-transport mechanism. Either substrate, S or T, can add to the free carrier. For coupling, the binary complex with each substrate must be immobile, the complex with both substrates mobile: the implication is that each substrate, alone, prevents the carrier from moving, while the two substrates together (in the ternary complex) catalyze carrier movement.



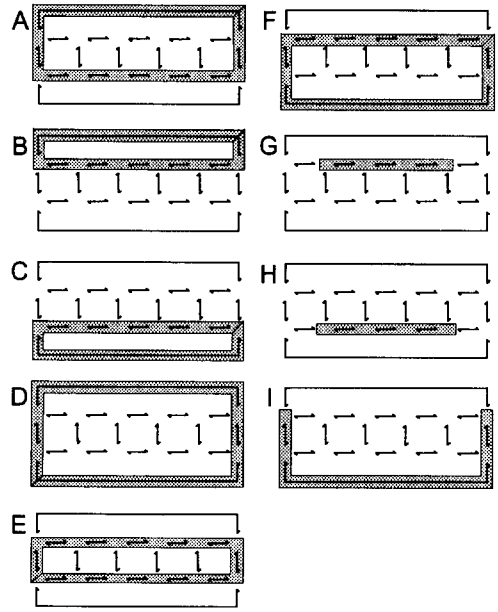


Fig. 7. Expanded scheme for a symmetrical ordered co-transport mechanism (as in Fig. 1, S followed by T on both sides of the membrane; subscript o refers to the outer surface of the membrane, subscript i to the inner surface). The scheme accounts for the immobility and altered specificity of the binary carrier-substrate complex. Lower diagram: potential translocation paths opened up through displacement of conformational equilibria in the reaction scheme above: A, coupled path; B and C, passive flow of T (in C the flow depends on the presence of S); D and E, passive flow of S (in E the flow depends on the presence of T); F, exchange of S for T; G, H and I, exchange paths.

complex is loose there is little specificity in binding; but because the transition-state complex is tight, the maximum exchange rate is highly sensitive to the structure of the substrate.

4. Co-transport mechanisms

The driving and driven substrates could add to the carrier in fixed or random order, depending on the mechanism, and an ordered mechanism may be either symmetrical or asymmetrical [12]. In a symmetrical mechanism the order is the same on the two sides of the membrane: in Fig. 1, S followed by T. In an asymmetrical mechanism the order is different on the two sides – S followed by T outside, T followed by S inside, as in Fig. 5. A random mechanism is shown in Fig. 6. (The gradients of the two substrates, it may be noted, will be coupled whatever the order of addition of the driving and driven substrates.) Expanded carrier schemes may be drawn for each mechanism by incorporating the scheme in Fig. 2 for a substrate-induced conformational change: the symmetrical mechanism, Fig. 7; the asymmetrical mechanism, Fig. 8; the random mechanism, Fig. 9. In these schemes, K_1 and K_2 in Fig. 2 enter on both sides of the membrane, becoming K_{o1} and K_{o2} outside and K_{i1} and K_{i2} inside. The effects on transport of changes in these constants, and the nature of associated target sites, are now considered.

5. Transport in uncoupled systems

The potential for passive flow is implicit in the reaction schemes for co-transport, Figs. 7–9. The reason is that one form of binary complex of each substrate is mobile (namely C′S and C″T in the model for the control of the carrier conformation, Fig. 2). Possible translocation paths are traced out in the diagrams in the lower part of each figure, one path for active transport (cycle A), and more than one for exchange and for the uncoupled translocation of S or T. Normally, the uncoupled flow is negligible because the

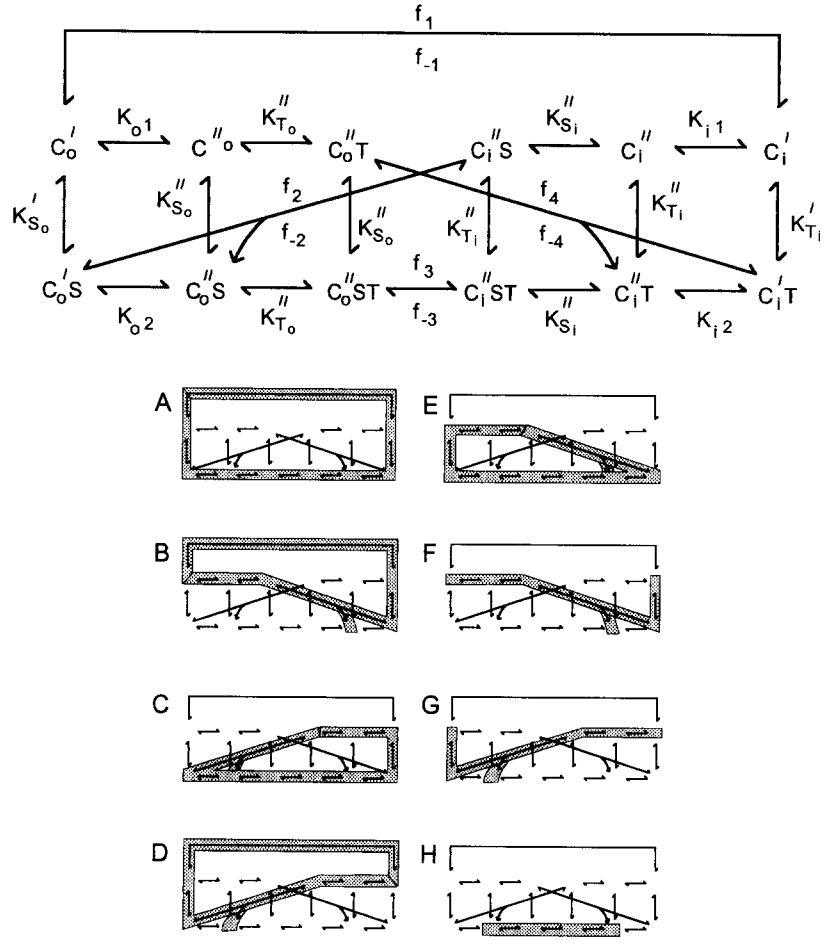


Fig. 8. Expanded scheme for an asymmetrical ordered co-transport mechanism (as in Fig. 5, S followed by T outside, and T followed by S inside). Lower diagram: potential translocation paths: A, coupled path; B and C, passive flow of T (in C the flow depends on the presence of S); D and E, passive flow of S (in E the flow depends on the presence of T); F, G and H, exchange paths.

concentrations of the mobile forms of the binary complex are very low, but mutations that alter K_1 and K_2 could increase their concentrations, allowing the system to work passively. Rate expressions for passive transport in mutant systems are given in the Appendix.

5.1. Net transport

In the symmetrical ordered mechanism (Fig. 7) uncoupled transport of the second substrate, T, results from a decrease in K_{o1} or K_{i1}, which shifts the free carrier equilibrium towards C''_o or C''_i and allows the mobile binary complex, C''_oT or C''_iT, to be formed (cycle B). Depending on how far the constants fall, transport may be partially or wholly uncoupled; an increase in the constants has little effect. Uncoupled transport of the first substrate, S, results from an increase in K_{o2} and K_{i2}, which favours the mobile forms C'_oS and C'_iS (cycle D). Transport of S could be slowed by a decrease in K_{i2} and a shift in equilibrium towards C''_iS, a form in which the substrate is very tightly bound, and whose dissociation rate may be limited by the rate of conversion of the tight to the loose complex, C''_iS to C'_iS (Eq. (1): $K_2 \ll 1$ and $K''_S \ll K'_S$). Rate-limiting dissociation of the first substrate in an ordered mechanism has actually been observed in

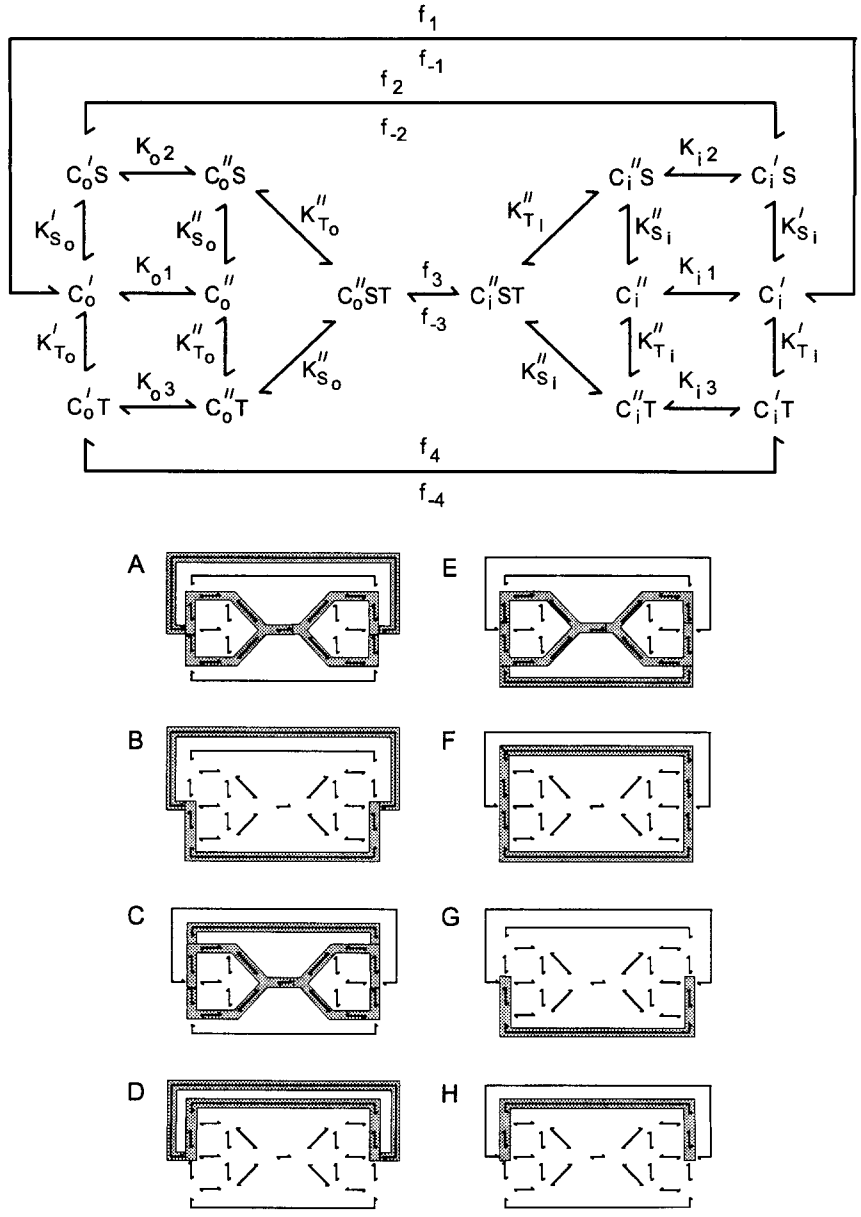


Fig. 9. Expanded scheme for a random-order co-transport mechanism (as in Fig. 6). Lower diagram: potential translocation paths: A, coupled path; B and C, passive flow of T (in C the flow depends on the presence of S); D and E, passive flow of S (in E the flow depends on the presence of T); F, G and H, exchange paths.

one coupled system [13], and it is reasonable to suppose that a decline in K_{i2} in a mutant might reduce the entry rate. A further point of interest is that because the reverse step, conversion of the loose to the tight complex, is not impeded by this shift in equilibrium, entry but not exit may be blocked if the shift occurs on only one side of the membrane (K_{i2} but not K_{o2} in Fig. 7).

The asymmetrical ordered mechanism (Fig. 8) works in a different way. A decrease in K_{o1} allows the mobile complex of T to be formed on the outside (C''_oT), leading to uncoupled transport of T (cycle B); a decrease in K_{i1}, on the other hand, allows the mobile complex with the other substrate to be formed on the inside (C''_iS), leading to uncoupled transport of S (cycle D).

In the random mechanism (Fig. 9) the situation is different again: a shift in the equilibrium of either binary complex to favour the mobile form (C'_oT or C'_oS) allows uncoupled transport to proceed (substrate T via cycle B, set in motion by an increase in K_{o3} or K_{i3}, and substrate S via cycle D, set in motion by an increase in K_{o2} or K_{i2}).

5.2. Exchange transport

Exchange proceeds through segments of cycles. In the normal system, exchange involves the central ternary complex C''_oST and C''_iST, but in mutants other routes can become functional, as Figs. 7–9 show. Thus, in the symmetrical ordered mechanism (Fig. 7) exchange may proceed not only through the ternary complex, route H, but through the binary complex: route G in the case of T (dependent on a decrease in K_{o1} and K_{i1}), and route I in the case of S (dependent on an increase in K_{o2} and K_{i2}). In cycle F, dependent on a simultaneous decrease in K_{o1} and K_{i1} and increase in K_{o2} and K_{i2}, S exchanges for T.

5.3. One-way passive transport

A mutant form of the lactose permease (at Glu-325) that is defective in active transport is reported to transport lactose downhill into but not out of the cell [1]. This seems odd: the mutant is a passive transport system, and a passive system can only allow substrates to equilibrate. Could a passive carrier act as a one-way valve, permitting flow in only one direction? The answer is that it might *appear* to do so – even though at equilibrium the unidirectional fluxes in opposite directions must be equal. In a passive system the initial entry and exit rates under zero-trans conditions (with no substrate in the opposite compartment) are necessarily identical at sufficiently low (and equal) substrate concentrations. If the system obeys the Michaelis–Menten law these rates (v) are governed by V/K_m ratios ($v = V[S]/K_m$), and though V/K_m for entry must equal V/K_m for exit [14], the maximum rates (V) may differ provided the half-saturation constants (K_m) also differ. The implication is that in the mutant system V and K_m for lactose are far higher in entry than in exit. The substrate concentration in the experiments, though in the normal range, would then have been saturating inside; the exit rate would have been maximal but, still, too low to detect (see Appendix).

6. Target sites

A mutation could shift an equilibrium in the mechanism in Fig. 2, shunting the flow from the coupled to

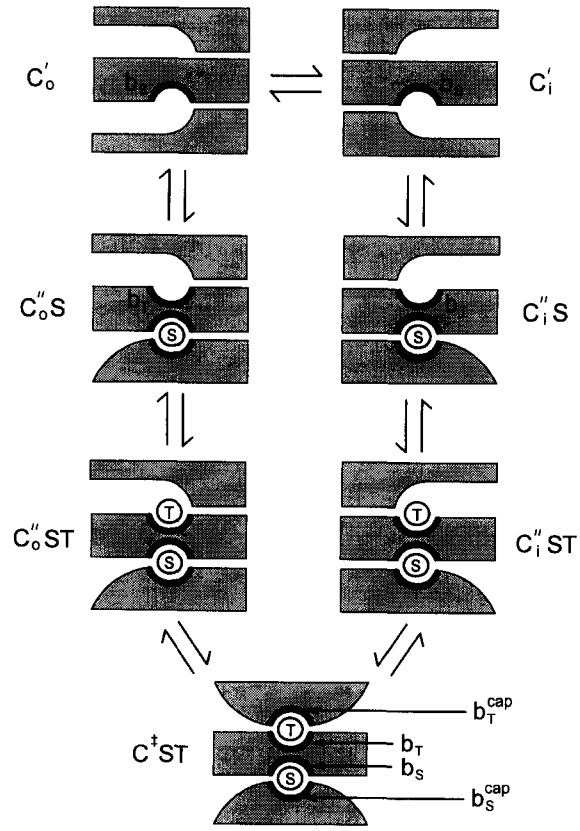


Fig. 10. A mobile-barrier model corresponding to the symmetrical ordered co-transport scheme in Fig. 7. Substrate binding regions are represented by indentations in the outline, substrates (S or T) by circles. The substrate bound first (S) induces a conformational change (C′ to C″) in which two new binding regions appear, one enclosing the already-bound substrate, the other specific for the substrate adding second. This second substrate, once bound, catalyzes carrier movement: it increases the *rate* of interconversion of the inward-facing and outward-facing conformations by becoming more strongly bound in the transition state, $C^{\ddagger}ST$. Whether the substrate induces a conformational change or increases the rate of a conformational change, binding is greatly strengthened in the inclusion complex formed from the initial complex, and the increased binding force serves to alter the carrier conformation.

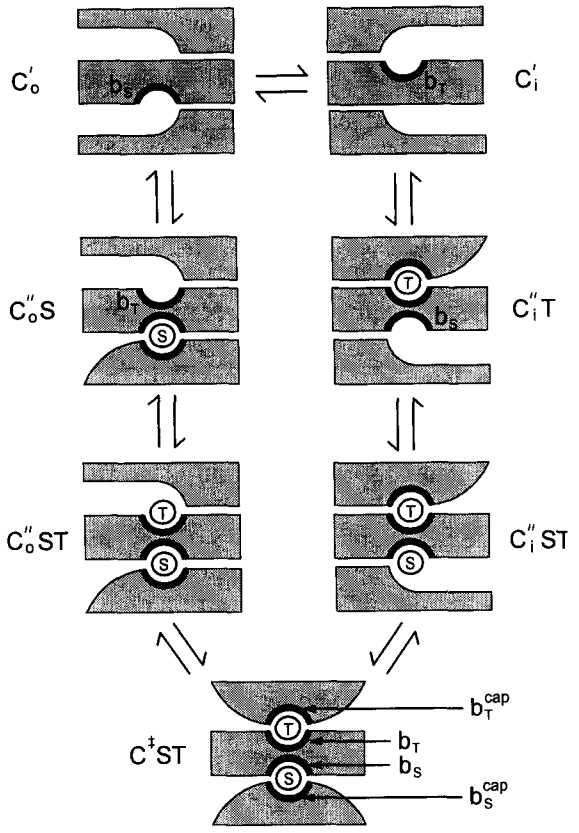


Fig. 11. A mobile-barrier model, as in Fig. 10, representing the asymmetrical ordered reaction scheme in Fig. 8.

an uncoupled path; and depending on the physical relationship between binding sites in the inward-facing and outward-facing carrier, a single mutation might or might not disturb the corresponding equilibria on either side of the membrane (K_{o1} and K_{i1}, or K_{o2} and K_{i2}). To see how this could happen, possible carrier structures may be visualized: the diagram in Fig. 10 represents a symmetrical co-transport model corresponding to the scheme in Fig. 7, and the diagram in Fig. 11, an asymmetrical model corresponding to the scheme in Fig. 8. The mechanism shown is of the 'gated-barrier' variety [15–17] – the channels between the stationary substrate sites and the compartments inside and outside the cell alternately open and close, lifting the barrier on only one side at a time. The binding site for each substrate is made up of two parts – one, preformed, holds the substrate loosely; the other, not preformed, caps the bound substrate, forming an inclusion complex. The two sites for substrate S are labelled b_S and b_S^{cap}, respectively, and those for substrate T, b_T and b_T^{cap}. An inclusion complex of S is represented by C''_oS in Fig. 10 or 11, an inclusion complex of T by C''_iT in Fig. 11. In the transition state $C^{\ddagger}ST$, both substrates are shown as being enclosed. It is the favourable energy of interaction in the inclusion complex that drives the conformational change or stabilizes the transition state in carrier reorientation. Depending on the structure of the carrier a binding site in the outward-facing and inward-facing forms could comprise the same or different amino acid residues; if the same, the effects of mutation on the inner and outer constants, and therefore on entry and exit, should be the same; otherwise they may be different.

6.1. The substrate-induced conformational change

The equilibrium governed by K_2 in Fig. 2 ($K_2 = [C'S]/[C''S]$) depends on the relative affinities of substrate S (K'_S/K''_S) in the surface complex, conformation C', and the tight complex involving both sites, conformation C''. Modification of the capping site, by lowering the affinity in C''S, should increase K_2 without affecting the equilibrium of the free carrier, governed by K_1 ($K_1 = [C']/[C'']$). A shift in K_1, on the other hand, might induce a comparable shift in K_2, with no change in the K'_S/K''_S ratio.

6.2. Vectorial catalysis

Catalysis of carrier reorientation by substrate T (f_3 and f_{-3} in Fig. 7) depends on a sharp increase in the binding force in the transition state (Fig. 4, Eq. (10)). The increase is assumed to result from interaction at an ancillary binding region, b_T^{cap} in Fig. 10, and modification of this region should reduce the rate of coupled transport. Catalysis of carrier movement, it may be noted, is required in the symmetrical ordered mechanism, Fig. 7, and the random mechanism, Fig. 9, but may be absent from the asymmetrical ordered mechanism, Fig. 8.

6.3. The gating mechanism

In a gated channel mechanism, gates on the outer and inner sides of the membrane open and close coordinately – that is, when one is open the other is closed. Mutation in these regions could disturb the equilibrium between inward-facing and outward-facing forms (f_1/f_{-1}, f_2/f_{-2}, f_3/f_{-3}, f_4/f_{-4} in Fig. 7), as well as the rates of interconversion. The effects on individual transport experiments may be judged by referring to the general rate equations for co-transport [6].

6.4. Carrier reorientation

Mutation in a hinge region, around which the carrier swings between outward-facing and inward-facing conformations, could impede all reorientation rates

(f_1, f_{-1}, f_2, f_{-2}, f_3, f_{-3}, f_4, f_{-4} in Fig. 7) and therefore block both coupled and uncoupled transport.

7. Lactose permease mutants

There is on hand a wealth of information on mutants of the lactose permease of *E. coli* [1], and this information may be drawn upon to illustrate the application of the coupling theory. The transport mechanism is reported to be ordered – in loading the carrier, H^+ adds first, followed by lactose [2,3]. The order in unloading has not been determined; certain mutants, however, exhibit behaviour explainable by a symmetrical but not by an asymmetrical mechanism, as explained in the Appendix – namely passive entry but not exit of lactose, the second substrate in loading the carrier. The mechanism, therefore, is probably of the symmetrical ordered type, as in Fig. 1, S being hydrogen ion and T lactose.

Substitution of His-322 by Asn, Gln, or Lys abolishes active transport; in its place is an uncoupled entry and exit of lactose, without an accompanying movement of H^+. The system appears to be defective in all steps involving protonation or deprotonation: normally the affinity for external lactose rises with $\Delta\mu H^+$, but not in the mutant. (Substitution of Arg-302 by Leu, His, or Lys has similar effects.) Two of the cycles in Fig. 7, B and C, give rise to passive transport of substrate T (lactose). Cycle B is set in motion by shifts in the free carrier equilibria to favour C''_o and C''_i ($K_{o1} \leq 1$ and $K_{i1} \leq 1$, whereas normally $K_{o1} \gg 1$ and $K_{i1} \gg 1$). Probably the simplest hypothesis is that K_{o2} and K_{i2} also decline, with the result that dissociation of S (H^+) is slow enough to block movement through the active cycle, A (as explained above). On this interpretation, His-322 and Arg-302 play a role in the equilibrium between the C′ and C″ conformations on both sides of the membrane, stabilizing the one-site, mobile form, C′; when these amino acids are replaced, therefore, the equilibrium shifts to favour the two-site form C″.

Substitution at Glu-325 (by Ala, Gln, Val, His, Cys, or Trp) has a more complicated effect: there is no active transport; there is uncoupled transport of lactose (without an accompanying movement of H^+) – but only in entry, not in exit; nevertheless, equilibrium exchange proceeds. (Replacement of Lys-319 by Leu has similar effects.) A possible explanation is that K_{o1} decreases, initiating one-way entry of T via cycle B, while K_{o2} increases, initiating uncoupled entry of S via cycle D, which dissipates the gradient of the driving substrate S and eliminates active uptake. Exchange still proceeds via a segment of the coupled path, route H. In this interpretation, Glu-325 and Lys-319 could be constituents of b_S^{cap}, the ancillary site for S (H^+), since weakened binding in the inclusion complex can be expected to favour C'_oS as against C''_oS; these residues are apparently present in the site in the outward-facing but not the inward-facing carrier. In the absence of S the mutation at this site is assumed to stabilize C''_o.

Other mutations result in a partially uncoupled transport of lactose [19], behaviour that may be explained by less decisive shifts in the equilibrium constants K_{o1} and K_{i1}, K_{o2} and K_{i2}.

There are also mutants that catalyze the uncoupled transport of H^+ (Ala-177 replaced by Val, or Tyr-236 replaced by His, Asn, Phe, or Ser) [20]. The behaviour may be explained by cycle D, which becomes functional if the equilibria governed by K_{o2} and K_{i2} shift to favour C'_oS and C'_iS over C''_oS and C''_iS. The explanation may be that Ala-177 and Tyr-236 are constituents of b_S^{cap}, the ancillary site for S (H^+), in both the inward-facing and outward-facing conformations.

Though the details of the interpretation are uncertain, it seems clear that all the mutated residues play some role in the one-site to two-site conformational change that accounts for the ordered addition of substrates and for immobilization of the binary complex with H^+. His-322 and Arg-302 may directly influence this conformational change, independent of the substrate (H^+). Other residues could be constituents of b_S^{cap}, the ancillary binding site for H^+ – Ala-177 and Tyr-236 in both the inward-facing and outward-facing carrier, Glu-325 and Lys-319 in only the outward-facing form. Depending on the structure of the carrier, overlapping but not entirely identical sequences could be present in substrate sites in the inner and outer carrier forms (see Fig. 10); Ala-177 and Tyr-236 appear to be constituents of both sites, Glu-325 and Lys-319 of only one. The existence of an elaborate binding site for the hydrogen ion is not unexpected: the observation of active transport systems in which the pH gradient can be replaced by a Na^+ gradient has suggested that it is not a protonated acid group in the carrier that is translocated, but H_3O^+ [18], which like Na^+ could form an inclusion complex at the transport site.

8. ATP-driven pumps

A co-transport system draws on the free energy of the concentration gradient of the driving substrate, an ATP-driven pump on the energy released in ATP hydrolysis; nevertheless, the mechanisms responsible for coupling appear to be fundamentally similar [5,8]. To understand why, notice that the kinetic reaction scheme for active transport has been shown to be the same, whether the driving substrate is transported across the membrane, like Na^+ or H^+, or is not transported, like ATP in an ATP-driven pump [21,22]. A reaction scheme, Fig. 1 for example, specifies the car-

rier forms, inward-facing or outward-facing, to which substrates are bound but not the location of the substrates in either an inner or outer compartment. It follows that relationships demonstrated between rate and equilibrium constants apply to either system, including the fundamental relationships between coupling ratios and binding forces in successive carrier states (Eqs. (6) and (10)). These relationships hold not only for ATP but even for the phosphorylated derivative of a transport protein produced in the course of reaction with ATP; the evidence on the calcium pump indicates that the phosphate substituent, while covalently bonded at the carrier site, interacts non-covalently, drawing the carrier into an altered conformation [8]. In every case, the conformational changes responsible for coupling are driven by non-covalent binding forces between a substrate and the transport protein.

The driving force, even in primary active transport, is, in effect, a concentration gradient. The equivalent of the electrochemical gradient of a transported ion is the concentration of ATP relative to its hydrolysis products; in either case free energy is derived from the tendency of the system to move toward the final equilibrium concentrations. Because the driving substrate, ATP, is a chemically different molecule when it approaches the carrier than when it leaves, it can enter and exit on the same side of the membrane – whereas the driving ion in co-transport necessarily exits on the opposite side. It follows that with such a mechanism the reaction of any metabolite could power active transport, provided the equilibrium sufficiently favours the products of the reaction. In this light the existence of other primary pumps is understandable: not only H^+-transporting pyrophosphate-driven carriers [23], but Na^+-transporting systems [24] driven by the decarboxylation of either oxaloacetate [25], methylmalonyl-CoA [26], or glutaconyl-CoA [27], where the carrier protein is a specific decarboxylase, or by the oxidation of NADH, where the carrier protein is an NADH–ubiquinone oxidoreductase [28] In investigating the mechanism of pumping, attention should be focused less on the bond energy of the driving substrate, or on the enzymic properties of the carrier, than on the linkage between the process of translocation and the conversion of reactants to products.

The similarity in the mechanisms of primary and secondary active transport suggests that it should be possible for mutations to uncouple ATP hydrolysis from translocation. How this can happen may be illustrated in the case of the calcium pump. The pump mechanism, originally proposed by Makinose [29], appears to be asymmetrical-ordered, as in the scheme in Fig. 5: calcium ion (S) adds to the outward-facing free carrier, forming an immobile complex which on reaction with ATP (T_o) is converted to a phosphoryl-carrier derivative that is mobile; inorganic phosphate (T_i) reacts with the inward-facing free carrier to form an immobile phosphoryl derivative, which on addition of Ca^{2+} becomes mobile [30] (the phosphoryl derivative is immobile in the sense that the calcium binding site is locked on one side of the membrane; on addition of Ca^{2+} the site rotates, releasing Ca^{2+} on the opposite side). Expansion of the asymmetrical ordered model yields the general scheme in Fig. 7, as we have seen, or, with ATP and its reaction products drawn in, the equivalent scheme in Fig. 12 (where $S = 2Ca^{2+}$). This mechanism is shown to account for experimental observations on the pump that cannot be explained by the usual models (see the accompanying paper, [31]). In this mechanism, displacement of the equilibrium gov-

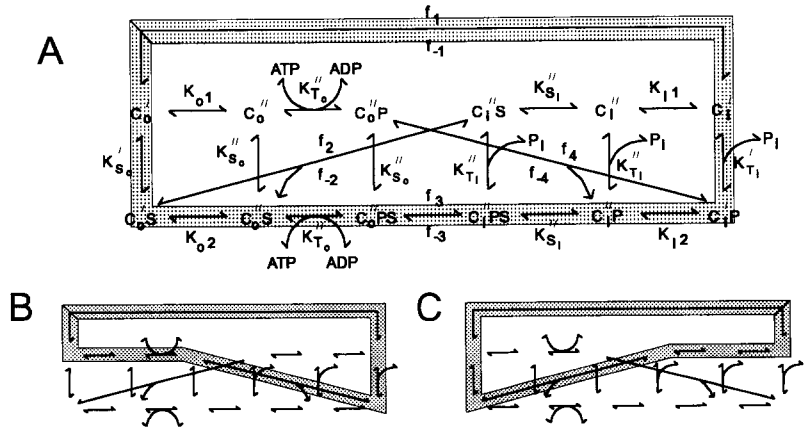


Fig. 12. An asymmetrical ordered mechanism for the calcium pump. The coupled path is traced out in A, above; below are paths for the uncoupled hydrolysis of ATP (B) and for the uncoupled transport of Ca^{2+} (C). S represents 2 Ca^{2+}. Phosphorylation of the carrier by either ATP or inorganic phosphate, P_i, plays the same structural role as addition of a mobile substrate in the asymmetrical ordered co-transport mechanism (Figs. 5 and 8).

erned by K_{o1} allows the second substrate in the ordered mechanism, ATP, to form a mobile complex with the free carrier, here a phosphoryl-carrier derivative; the resulting uncoupled cycling of the system would allow ATP to be hydrolyzed in the absence of Ca^{2+} (Fig. 12B). Uncoupled ATPase activity does not appear to have been noticed in studies of site-directed mutagenesis [32], but mutants of this kind, if discovered, would be directly relevant to the problem of vectorial coupling. The coupling theory, it may be noted, should apply not only to membrane pumps, including the F_1F_0-ATPase, but to ATP-driven molecular motors such as actomyosin, kinesin, and RNA polymerase [8].

9. The design and interpretation of experiments

The recognition of the coupling mechanisms implicit in models for active transport may make it possible to dissect out various functional regions in carrier proteins, by means of site-directed mutagenesis. Two different regions in the transport proteins interact with each substrate, one the binding site proper, which most directly affects the apparent affinity; the other an ancillary region that only comes into contact with the bound substrate as the carrier conformation changes (Figs. 10, 11). Alteration of the ancillary site for the first substrate bound to the carrier has predictable effects on uncoupled transport, and the degree of uncoupling, partial or total, indicates how strong a role a mutated amino acid plays. In the inward-facing and outward-facing carrier conformations each of these regions could be composed of identical, or overlapping, or entirely different, amino acid residues; whether or not a mutated residue plays the same role in the two carrier states may be judged by the relative effects on entry and exit. The translocation paths in the various co-transport mechanisms are many (Figs. 7–9), and single mutations can give rise to complex patterns of behaviour; in designing and interpreting experiments this complexity might be turned to advantage, since a particular pattern may be characteristic of one transport mechanism or of a lesion in some defined region of the carrier.

A mutation that transforms an active into a passive system – in the case of an ATP-driven pump, that uncouples transport from ATP hydrolysis and ATP hydrolysis from transport – targets the steps responsible for vectorial coupling, the links between free energy and movement. In these steps, the transport protein is switched from one state to another – mobile or immobile, having or not having a given substrate site, with or without a specific enzyme activity. As such switches are key elements in any coupled vectorial process, uncoupled mutants are likely to be invaluable tools in the study of active transport mechanisms.

Appendix

(A) Coupled and uncoupled rates

In treating the symmetrical ordered mechanism in Fig. 7, the case of rate-limiting carrier movement will be considered first. Here, $\bar{V}_{STo}$, the maximum rate of coupled entry under zero-trans conditions (cycle A, with saturating concentrations of external S and T), depends on conversion of the external to the internal substrate complex (C''_oST to C''_iST) and of the internal to the external free carrier (C'_i to C'_o). The former is governed by the rate constant f_3. The latter depends on f_{-1} and on the proportion of the free carrier in the mobile form (C'_i rather than the immobile form C''_i); hence the rate-constant is equal to $f_{-1}[C'_i]/([C'_i]+[C''_i]) = f_{-1}/(1+1/K_{i1})$. In the fully coupled system, K_{o1} and $K_{i1} \gg 1$, while K_{o2} and $K_{i2} \ll 1$, as seen above. Expressions for maximum rates can be written by inspection: Stein and Lieb [33] have pointed out that a reciprocal rate constant is equivalent to a resistance, and the total resistance in a cycle is the sum of individual resistances; the overall rate, then, is inversely proportional to this total resistance, which is a sum of reciprocal rate constants. Hence the maximum rate of coupled entry is given by

$$\bar{V}_{STo} = [C_t]\{1/f_{-1} + 1/f_3\}^{-1} \qquad \text{(A-1)}$$

where $[C_t]$ is the total carrier concentration. Similarly, an expression may be written for the maximum rate of uncoupled entry of S in the absence of T (cycle D in Fig. 7), with K_{o2} and $K_{i2} \geq 1$; K_{o1} and $K_{i1} \gg 1$:

$$\bar{V}_{So} = [C_t]\{1/f_{-1} + (1 + 1/K_{o2})/f_2\}^{-1} \qquad \text{(A-2)}$$

and for the maximum rate of uncoupled entry of T in the absence of S (cycle B in Fig. 7), with K_{o1} and $K_{i1} \leq 1$:

$$\bar{V}_{To} = [C_t]\{1/f_4 + (1 + 1/K_{i1})/f_{-1}\}^{-1} \qquad \text{(A-3)}$$

If the rates of reorientation of the various carrier forms are similar, it is apparent from Eqs. (A-1)–(A-3) that the rates of uncoupled transport in an uncoupled mutant can be comparable to the rate of coupled transport in the wild-type. In mutants the uncoupled flow of S or T makes it impossible to build up a concentration gradient, even if the coupled cycle A is functional; hence active transport will not be seen.

Steps other than carrier movement could of course be rate-limiting, this being a matter to be decided by experiment. In coupled entry (cycle A) the conforma-

tional changes governed by K_{o2} and K_{i2} could be slow steps. In uncoupled entry of T (cycle B) the conformational changes governed by K_{o1} or K_{i1} could be slow, while in uncoupled entry of S (cycle D) no conformational change other than carrier reorientation is involved. In all cases coupled and uncoupled transport could be equally fast.

(B) One-way passive transport

(i) The symmetrical ordered mechanism (Fig. 7)

Passive transport of T. To account for one-way flow through cycle B, let the equilibrium of the outward-facing free carrier between one-site and two-site conformations, governed by K_{o1}, be altered in the mutant, while the equilibrium on the inside, governed by K_{i1}, is unchanged. In the normal system, before mutation, the two-site conformations (C''_o and C''_i) as well as the mobile binary complex with the second substrate, T, are present at very low concentrations, ($K_{o1} = [C'_o]/[C''_o] \gg 1$ and $K_{i1} = [C'_i]/[C''_i] \gg 1$); consequently, uncoupled transport of T is negligible. In a mutant with $K_{o1} \leq 1$, the two-site conformation C''_o is abundant and the mobile complex with T is readily formed, allowing T to be transported inward. On the inside, with K_{i1} unchanged ($[C'_i]/[C''_i] \gg 1$), the two-site conformation C''_i is scarce and little of the mobile complex with T is formed. As a result, transport could be one-way – inward. But in predicting the direction of flow it has to be remembered that the equilibria governed by K_{o1} and K_{i1} are in a cycle of reactions and that the product of the constants in a cycle is itself a constant (unity); a change in one constant is therefore compensated by changes in others. In cycle B, a decrease in K_{o1} without a corresponding decrease in K_{i1} will be attended by an increase in f_1/f_{-1} or a decrease in f_4/f_{-4} (assuming that the substrate dissociation constants are unchanged), and this may affect the flux. If the carrier reorientation steps are rate-limiting (and other steps fast by comparison), the maximum rate of zero-trans entry of T, $\bar{V}_{To}$, is a function of f_4 and $f_{-1}/(1 + 1/K_{i1})$, and the maximum rate of exit, $\bar{V}_{Ti}$, a function of f_{-4} and $f_1/(1 + 1/K_{o1})$:

$$\bar{V}_{To} = [C_t]\{1/f_4 + (1 + 1/K_{i1})/f_{-1}\}^{-1} \quad \text{(A-4)}$$

$$\bar{V}_{Ti} = [C_t]\{1/f_{-4} + (1 + 1/K_{o1})/f_1\}^{-1} \quad \text{(A-5)}$$

where $[C_t]$ is the total carrier concentration. These rates, with either f_1/f_{-1} increasing or f_4/f_{-4} decreasing, do not explain one-way passive entry, because a fall in f_{-1} or f_4 retards uncoupled entry of T through cycle B, and a rise in f_1 or f_{-4} accelerates uncoupled exit; hence $\bar{V}_{To} < \bar{V}_{Ti}$. That is, entry is slower than exit, not faster. However, even exit may be too slow to detect – internal T will be very weakly bound because the free carrier inside is overwhelmingly in the one-site form C'_i, which does not bind T.

One-way entry via cycle B, Fig. 7, can be explained, however, if the conformational changes C''_i to C'_i and C''_o to C'_o, governed by K_{o1} and K_{i1}, are rate-limiting. With the help of the computer program provided by Runyan and Gunn [34] expressions for the maximum rates of entry and exit via cycle B, Fig. 7 – $\bar{V}_{To}$ and $\bar{V}_{Ti}$ respectively – and the corresponding half-saturation constants – $\bar{K}_{So}$ and $\bar{K}_{Si}$ – have been derived, on the assumption that carrier reorientation steps (governed by f_1, f_{-1}, f_4, and f_{-4}) are rapid equilibria and that substrate dissociation is a rapid step:

$$\bar{V}_{To} = k_{-o1}k_{i1}/\{F_1(k_{i1} + k_{-i1}/F_{-1} + k_{-o1}/F_1)\} \quad \text{(A-6)}$$

$$\bar{V}_{Ti} = k_{o1}k_{-i1}/\{F_{-1}(k_{o1} + k_{-i1}/F_{-1} + k_{-o1}/F_1)\} \quad \text{(A-7)}$$

$$\begin{aligned}\bar{K}_{To} = {} & [(k''_{-Ti}/F_4 + k''_{-To}/F_4) \\ & \times \{k_{o1}k_{i1} + k_{o1}k_{-i1}/F_{-1} + k_{-o1}k_{i1}/F_1\}] \\ & \times [(k''_{-Ti}k''_{To}/F_4) \\ & \times \{k_{i1} + k_{-i1}/F_{-1} + k_{-o1}/F_1\}]^{-1}\end{aligned} \quad \text{(A-8)}$$

$$\begin{aligned}\bar{K}_{Ti} = {} & [(k''_{-Ti}/F_{-4} + k''_{-To}/F_4) \\ & \times \{k_{o1}k_{i1} + k_{o1}k_{-i1}/F_{-1} + k_{-o1}k_{i1}/F_1\}] \\ & \times [(k''_{Ti}k''_{-To}/F_4) \\ & \times \{k_{o1} + k_{-i1}/F_{-1} + k_{-o1}/F_1\}]^{-1}\end{aligned} \quad \text{(A-9)}$$

where $k_{o1}/k_{-o1} = K_{o1}$ and $k_{i1}/k_{-i1} = K_{i1}$; also $F_1 = 1 + f_1/f_{-1}$, $F_{-1} = 1 + f_{-1}/f_1$, $F_4 = 1 + f_4/f_{-4}$, $F_{-4} = 1 + f_{-4}/f_4$. Given that $K_{o1} \leq 1$ and $K_{i1} \gg 1$, and that in compensation $f_4/f_{-4} \ll 1$, $\bar{V}_{To}$ can be much larger than $\bar{V}_{Ti}$. This may be confirmed by assigning arbitrary values to the constants in Eqs. (A-6)–(A-9): let $K_{o1} = 1$, $K_{i1} = 10^4$, $f_4/f_{-4} = 10^{-4}$, $f_1/f_{-1} = 1$; $k_{o1} = k_{-o1} = k_{i1} = 10^2$; $k_{-i1} = 10^{-2}$; $k_{-To} = k_{-Ti} = k_{To}[T_o] = k_{Ti}[T_i] = 10^5$; $[T_o] = [T_i]$; then $\bar{V}_{To} = 10^2/3$, $\bar{V}_{Ti} = 10^{-2}/3$, $\bar{K}_{To}/[T_o] = 10$, $\bar{K}_{Ti}/[T_i] = 10^{-3}$. With substitution of these values of the maximum rates and half-saturation constants into the Michaelis–Menten equation, the rate of entry is $v = \bar{V}_{To}/(1 + \bar{K}_{To}/[T_o]) \approx 3$, and the rate of exit $v = \bar{V}_{Ti}/(1 + \bar{K}_{Ti}/[T_i]) \approx 0.0033$. The model, then, does give a satisfactory account of the behaviour of lactose permease mutants.

In cycle C, Fig. 7, transport of T is dependent on S: the carrier site moves across the membrane bearing both S and T, and returns bearing only S. In effect, S catalyzes the transport of T; but as there is no net movement of S, transport can only be downhill. An

increase in K_{i2} with no shift in K_{o2} can give rise to uncoupled flow in one direction, depending on which steps are rate-limiting, as above.

Transport of S. One-way flow via cycles D and E is possible, as above.

Transport of one substrate but not the other. A fall in K_{o1} and K_{i1} is seen to initiate cycles B and E: in cycle B, T is transported alone, and in cycle E the transport of S depends on T. Similarly, a rise in K_{o2} and K_{i2} initiates cycles C and D: in D, S is transported alone, and in cycle C, the transport of T depends on S. In either case the active system is converted to a passive system for both substrates, though passive flow may depend on the presence of the companion substrate. Still, one-way entry of only one of the substrates is possible: an increase in K_{o2} with no change in K_{i2} allows S to be transported inward (cycle D), but cycle C, carrying T, only runs in the direction of exit. Similarly, a decrease in K_{o1} with no change in K_{i1} allows T to be transported inward (cycle B), but cycle E, carrying S, only runs in exit.

(ii) The asymmetrical ordered mechanism (Fig. 8)

In the symmetrical mechanism some carrier forms are mobile, others are not, whereas in the asymmetrical mechanism, at least in the simplest version, all carrier forms are inherently mobile: in the transport scheme in Fig. 5, certain forms fail to move across, not because the protein is inherently rigid, but because the corresponding complex on the opposite side of the membrane does not exist. (In reality, as the ordering model in Fig. 2 makes clear, and as shown in the complete co-transport scheme in Fig. 8, the missing complex is present, but at a very low concentration.) Because all carrier forms can be mobile, the shift in the equilibrium of C′ and C″ conformations that allows the complex of the second substrate to form on one side of the membrane gives rise to two-way, not one-way, traffic, as is apparent from cycles B and D, Fig. 8. There can, however, be a one-way passive transport if substrate dissociation is blocked on one side of the membrane as a result of a fall in K_{o2} or K_{i2}. The asymmetry of the system has an interesting consequence: dissociation of T can be blocked inside but not outside, so that one-way exit of T is possible but not one-way entry (cycle A or C, initiated by a decrease in K_{i1} and K_{i2}); the reverse is true for substrate S (cycle A or E).

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