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Testing models for transport systems dependent on periplasmic binding proteins

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A carrier model in which transport across the cytoplasmic membrane is mediated by a periplasmic binding protein (Krupka, R.M. (1992) Biochim. Biophys. Acta 1110, 1–10) is shown to account for many of the properties of these systems: (i) Michaelis-Menten kinetics; (ii) seemingly irreversible uptake; (iii) the absence of exchange transport and counter-transport; (iv) substrate half-saturation constants that in different systems may be lower or higher than the dissociation constant of the binding protein; (v) the high concentration of the binding protein in the periplasm and its weak association with the membrane component. The binding protein appears to function as a valve or rectifier that permits the substrate to enter the cell, but blocks exit in both the energized and de-energized states. The asymmetry depends on both the abruptness and the extent of the conformational change in the binding protein. Characteristically, these systems build up steep gradients across the membrane, circumstances in which such a valve might be important. In agreement with the mechanism, (a) the binding protein is missing in members of the same family of transporters that function in export of the substrate rather than import; and (b) in Gram-positive organisms, which have no periplasmic space, binding proteins function while anchored to the cytoplasmic membrane.

Introduction

In the preceding paper [1], kinetic equations were derived for transport models involving periplasmic binding proteins. Here, the experimental observations are compared with the predictions [2–9].

The transport scheme to be tested is shown in Fig. 1. To add to or dissociate from the outer carrier site, external substrate requires the binding protein, but internal substrate adds directly to the inner carrier site. The binding protein is an equilibrium mixture of two conformations, P′ and P; the former predominates in the absence of the substrate, the latter in its presence ($K_4 \gg 1$, and $K_6 \ll 1$ where $K_4 = [P']/[P]$ and $K_6 = [P'S]/[PS]$). Only conformation P adds to the membrane component of the system, and when it does so the substrate can move between the two proteins. The binding protein adds to the outward-facing but not to the inward-facing carrier. The point of coupling to ATP hydrolysis is left open, since in principle energy may be released anywhere along the cycle of carrier reactions [10]; the kinetic equations make it possible to determine the effects on the transport parameters of coupling at any particular step.

The transport constants referred to in the following discussion are as follows. K_s is the substrate dissociation constant of the binding protein: $K_s \approx K_4 K_5$, where $K_5 = [P][S_o]/[PS]$. K_{S_o} is the intrinsic substrate dissociation constant of the membrane carrier:

$$K_{S_o} = [C_o][S_o]/[C_oS] = K_5 k_{-1}/k_1 = K_5 k_{-1}/(k_1 K_4)$$

k_1 and k_{-1} are rate constants for binding protein-mediated transfer of the substrate to and from the external carrier site, respectively: $k_1 = k_8/K_7$ and $k_{-1} = k_{-8}/K_9$ (see Fig. 1). α is the ratio of internal to external substrate concentrations in the final steady-state; $\alpha = 1$ in a passive system, and $\alpha \gg 1$ in an active system. $[C_t]$ is the total concentration of carrier in the membrane, and $[P_t]$ is the total concentration of the binding protein.

The rate equations based on the reaction scheme in Fig. 1 involve the following experimental constants: V is a maximum flux, K a half-saturation constant, and small v the measured rate under a particular set of conditions. Attached to these symbols are subscripts and superscripts – subscripts indicating the substrate followed and its location either outside the cell, 'o', or inside, 'i', and superscripts indicating the type of exper-

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iment in which the constant is measured. A zero-trans experiment is indicated by a bar, '–', the minus sign denoting the complete absence of substrate in the trans (i.e., opposite) compartment; an equilibrium exchange experiment is indicated by a double bar, '=', the equal sign denoting an equal concentration of substrate in the trans compartment (or in active transport, denoting the concentration achieved in the final steady-state). Thus, $\bar{K}_{S_o}$ is the half-saturation constant, and $\bar{V}_{S_o}$ the maximum rate, measured in zero-trans uptake experiments. $\bar{K}_{S_i}$ and $\bar{V}_{S_i}$ are the corresponding constants in zero-trans exit experiments. $\bar{\bar{V}}_S$ is the maximum rate of equilibrium exchange.

Testing the mechanism

(i) The rate of substrate exit. In all systems examined, uptake appears to be an irreversible process, and neither net exit, nor exchange, nor counter-transport is detected [11–13]. The analysis of the model shows the reaction to be inherently asymmetrical. In exit, the required conformation of the free binding protein, P, is scarce, the equilibrium favouring P' ($K_4 \ll 1$). In entry, the required conformation of the complex, PS, is favoured by the equilibrium ($K_6 \ll 1$). As a result, exit is slower than uptake by a factor proportional to K_4. The asymmetry is apparent in the ratio of the maximum zero-trans exit and entry rates, $\bar{V}_{S_o}$ and $\bar{V}_{S_i}$:

$$\frac{\bar{V}_{S_i}}{\bar{V}_{S_o}} \approx \frac{[P_t]/K_7 + (1 + f_1/f_{-1})}{K_4(1 + f_2/f_{-2})k_1/k_{-1}} \qquad (1)$$

K_4 does not enter into the expression for α, the ratio of internal to external substrate concentrations in the final steady-state, which is large in the energized system and equal to unity in the de-energized system:

$$\alpha = f_{-1}f_2k_8k_{-2}K_9/(f_1f_{-2}k_{-8}k_2K_7K_8) \qquad (2)$$

Asymmetry therefore persists in the de-energized state. The substrate enters the cell easily but leaves with great difficulty, making the reaction seem irreversible.

(ii) Pathways for entry and exit. From the principle of microscopic reversibility the preferred reaction paths in the forward and reverse directions are necessarily the same if the alternative paths are of equivalent kinetic order (i.e., if the rate laws are of the same form with respect to reactant concentrations), but not if they are of differing kinetic order [14]. In the case of transport systems dependent on binding proteins, the alternative paths are not kinetically equivalent. The main pathway for entry, which involves the binding protein, is a function of the concentrations of the substrate and the binding protein, $[S_o]$ and $[P_t]$ in entry, and $[S_i]$ and $[P_t]$ in exit. Another pathway can be envisaged in which the substrate adds directly to the outer carrier site; here the rate is a function of $[S_o]$ in entry and $[S_i]$ in exit, independent of $[P_t]$. Experiment rules out the direct pathway in entry; the following argument rules it out in exit.

In the direct pathway,

$$C_o + S_o \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} C_oS$$

the dissociation constant for the carrier-substrate complex, k_{-3}/k_3, is equal to $[C_o][S_o]/[C_oS]$, which is given by

$$k_{-3}/k_3 = K_{S_o} = k_{-1}K_S/(k_1K_4) \qquad (3)$$

In exit (in the absence of the external substrate), the ratio of the rates of direct dissociation, and of binding protein-mediated dissociation, of the substrate from the outward-facing carrier-substrate complex, is equal to $k_{-3}/k_{-1}[P]$, where $[P] = [P_t]/K_4$ (in the absence of external substrate the total concentration of binding protein is given by $[P_t] = [P] + [P'] = [P](1 + K_4) \approx K_4[P]$). From Eqn. 3, this ratio of rates is given by

$$k_{-3}/(k_{-1}[P]) = k_3K_S/(k_1[P_t]) \qquad (4)$$

In entry, the rate of direct addition of the substrate to the outward-facing carrier is equal to $k_3[S_o][C_o]$; and with the external substrate concentration, $[S_o]$, set equal to K_S, the rate is $k_3K_S[C_o]$. This rate is to be compared with $k_1[P_t][C_o]$, the rate of addition mediated by the binding protein. (The first rate is half-maximal and the second maximal, but the 2-fold difference does not alter the conclusion). Experiment shows the ratio of these rates to be very small; i.e., $k_3K_S/(k_1[P_t]) \ll 1$. It follows from Eqn. 4 that $k_{-3}/k_{-1}[P]$, the ratio of exit via the binding protein-independent and binding protein-dependent pathways, is also very small.

(iii) Exchange transport. As noted, exchange transport cannot be detected. According to the kinetic analysis of the model, exchange should be extremely slow for two reasons. First, unidirectional exit suffers from the same scarcity as zero-trans exit of the required conformation of the free binding protein ($K_4 \gg 1$). Second, exchange is inhibited by the external substrate. The maximum exchange rate (with $K_4 \gg 1$ and $K_{11} \gg [P_t]$) is given by

$$\bar{\bar{V}}_S \approx \frac{k_{-1}[P_t][C_t]/(1 + f_2/f_{-2})}{K_4(1 + [S_o]/K_S)(1 + f_1/k_1[P_t])} \qquad (5)$$

The rate is seen to be inversely related to K_4 and to the substrate concentration. By forming the substrate complex, PS, the substrate removes the free binding protein required for dissociation of the substrate from

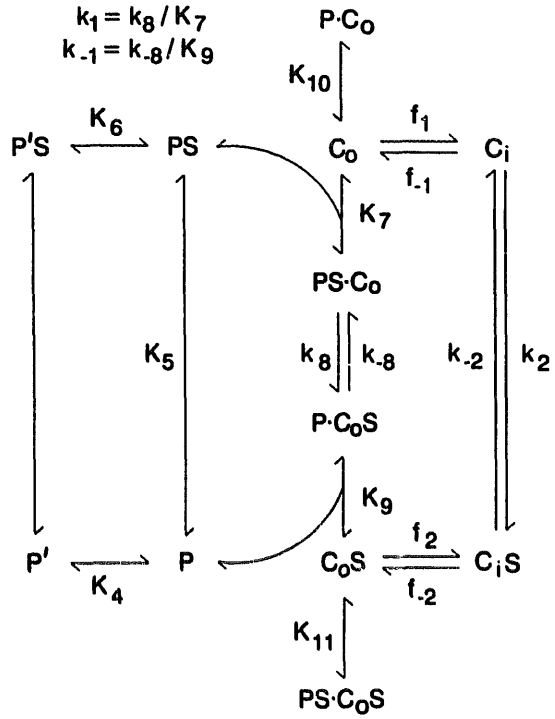

Fig. 1. Transport scheme involving a periplasmic binding protein. The binding protein P' transfers the external substrate S_o to the outward-facing carrier C_o. The external substrate cannot add to the carrier directly, but the internal substrate, S_i, adds directly to the inward-facing carrier. The carrier, imbedded in the cytoplasmic membrane, alternates between outward-facing and inward-facing forms, C_o and C_i. The binding protein is an equilibrium mixture of two conformations, P' and P, and only the latter combines with the carrier; free of substrate, the protein is mainly in conformation P' ($K_4 \gg 1$), and as the substrate complex in conformation PS ($K_6 \ll 1$). Though the binding protein combines with the outward-facing carrier, it does not interact with the inward-facing carrier. The carrier and binding protein form two types of complex: one productive, PS with C_o and P with C_oS, and one non-productive, P with C_o and PS with C_oS. The non-productive complex is at a dead-end in the reaction sequence and is therefore inhibitory. Steps considered to be in rapid equilibrium in the kinetic treatment of the model are indicated by single arrows, and steps that may be rate-limiting by double arrows.

the outer carrier site and therefore blocks the exit component of exchange. Consequently substrate entry, which depends on the release of the free outward-facing carrier, is also blocked. The inhibition constant is K_S, the dissociation constant for the binding protein, which in many cases is equal to the Michaelis constant for entry, $\bar{K}_{S_o}$; for this reason the inhibition may be strong at comparatively low substrate concentrations. As K_S is independent of α, the inhibition is equally strong in the de-energized state.

In theory, the substrate could inhibit in another way. PS could form a dead-end complex with the carrier, $PS \cdot C_oS$; the inhibition would then depend on K_{11} (Fig. 1) and on the steady-state concentration of C_oS. But where $\bar{K}_{So}$ is much larger than K_S the dead-end complex should be unimportant. The relative values of $\bar{K}_{S_o}$ and K_S are considered in section (vi) below.

(iv) Counter-transport. When de-energized cells previously loaded with unlabelled substrate are placed in a medium containing labelled substrate, the label would normally flow into the cell, rising above the equilibrium level and remaining high until the gradient of unlabelled substrate has ebbed away. This is called counter-transport. The labelled substrate accumulates because once inside the cell it has to compete with unlabelled substrate to move out again. In effect, the flow of unlabelled substrate down its concentration gradient, out of the cell, drives the labelled substrate in the opposite direction, because both move on the same carrier. In systems dependent on binding proteins, counter-transport is not observed; nor should it be, since exchange and unidirectional exit are so extremely slow. The absence of counter-transport, it may be noted, serves to demonstrate that exit is blocked in the de-energized system.

(v) Dependence on the substrate concentration. Substrate uptake invariably obeys Michaelis-Menten kinetics, with linear reciprocal plots over a wide concentration range [15–21]. Though the full rate expression for uptake includes terms in substrate concentration that give rise to non-Michaelis-Menten behaviour, the equation reduces to the Michaelis-Menten form under certain conditions: (i) K_4 is large (meaning that the free protein is largely in conformation P', which does not add to the carrier) and the dead-end complex $PS \cdot C_oS$ is not formed ($K_4 \gg 1$, $[P_t] \ll K_{11}$); (ii) the concentration of the binding protein is too low to saturate the carrier ($[P_t] \ll K_7$, $[P_t] \ll K_{11}$); (iii) $K_4 \gg 1$ and addition of the external substrate to the carrier site, governed by $k_1 = k_8/K_7$, is rate-limiting; (iv) $f_2 \gg (k_{-2} + f_{-2})$. The concentration of the binding protein is much less than saturating in some but not all cases. It is low in systems for arginine [22] and histidine [3] in *Escherichia coli*, but in the maltose system the concentration was reported to be saturating in one study and half-saturating in another [20,23]. In the histidine system of *Salmonella typhimurium* the concentration is saturating with respect to the maximum rate though not with respect to the substrate half-saturation constant [15].

(vi) Substrate half-saturation constants for the binding protein and for transport. In a list of experimental values put together by Wilson and Smith [24], the substrate half-saturation constant measured in entry experiments, $\bar{K}_{S_o}$, and the dissociation constant for the binding protein, K_S, agree within a factor of 2 in 12 cases, but $\bar{K}_{S_o}$ is the smaller in three cases (by factors of 3 to 6) and the larger in 1 case (by a factor of 6). In a list made later by Furlong [4], the values agree within a factor of 2 in 8 cases, but $\bar{K}_{S_o}$ is the smaller in one case (by a factor of 4) and the larger in seven cases (by

factors of 3 to 100). The kinetic analysis does show that the constants are not necessarily equal:

$$\bar{K}_{S_0} = \frac{K_S(1+f_1/f_{-1})\{k_{-2}+k_{-1}[P_t](f_{-2}+k_{-2})/(f_2K_4)\}/k_1[P_t]}{1+k_{-2}/f_{-1}+(k_{-2}+f_{-2})/f_2+\dfrac{(1+f_1/f_{-1})k_{-2}}{k_1[P_t]}+\dfrac{k_{-2}}{k_1K_7}}$$

$$= \frac{K_{S_0}(1+f_1/f_{-1})\{k_{-2}K_4/(k_{-1}[P_t])+(f_{-2}+k_{-2})/f_2\}}{1+k_{-2}/f_{-1}+(k_{-2}+f_{-2})/f_2+\dfrac{(1+f_1/f_{-1})k_{-2}}{k_1[P_t]}+\dfrac{k_{-2}}{k_1K_7}} \qquad (6)$$

A value of $\bar{K}_{S_0}$ equal to K_S is explained by any of the conditions listed above for Michaelis-Menten kinetics. A value less than K_S is explained if either $K_4 \gg 1$ and $K_{11} \gg [P_t]$, $K_4 \gg 1$ and k_1 is rate-limiting, or $f_7 \gg (k_{-2}+f_{-2})$. A value greater than K_S suggests that K_4 is large and the dead-end complex $PS \cdot C_0S$ is not formed ($K_4 \gg 1$ and $K_{11} \gg [P_t]$). From Eqn. 6, it appears that $\bar{K}_{S_0}$ might be large if the intrinsic substrate affinity of the membrane carrier is much lower than that of the binding protein (i.e., if $K_{S_0} \gg K_S$, where K_{S_0} is the dissociation constant for the carrier in the overall reaction $C_0 + S_0 \rightleftharpoons C_0S$). However, $K_{S_0} = K_S \cdot k_{-1}/(k_1K_4)$; therefore, if $K_{S_0} \gg K_S$, then $k_1K_4 \gg k_{-1}$, and this condition gives rise to non-Michaelis-Menten kinetics (as was shown in the preceding paper). It seems unlikely, therefore, that the model accounts for cases in which the kinetics are Michaelis-Menten and $\bar{K}_{S_0}$ is much larger than K_S.

The experimental ratios of the constants might be questioned, though, since affinities could differ in the periplasm and in dilute solution. The microenvironment can certainly distort dissociation constants, as in the case of immobilized enzymes [25] and 'crowding' effects in protein solutions [26]. The largest discrepancy between the constants is reported in the sulfate transport system of *Salmonella typhimurium*, where $\bar{K}_{S_0}$ is 100-times larger than K_S [4], and where the reciprocal plot in transport is linear over a wide concentration range [27]. K_S was measured at low ionic strength; at high ionic strength it was 30-times greater, where the factor would only be 3 [28]. It is possible that the real difference between the constants is never large.

(vii) The dependence of transport on the concentration of the binding protein, $[P_t]$. According to the analysis of the model, the maximum rate of uptake should rise to a limit as $[P_t]$ increases: $\bar{V}_{S_0} = a/(1 + K_P/[P_t])$. The substrate half-saturation constant may either rise or fall: $\bar{K}_{S_0} = b(1 + c/[P_t])/(1 + K_P/[P_t])$. a and b are constants, and

$$K_P = \frac{K_7(1+f_1/f_{-1})}{1+k_1K_7\{(1/f_{-1})+(1/k_{-2})+(1+f_{-2}/k_{-2})/f_2\}} \qquad (7)$$

$$c = f_2K_4/\{k_{-1}(1+f_{-2}/k_{-2})\} \qquad (8)$$

In the case of the maltose system, differing values for K_b have been reported. In a study in which the maltose binding protein was added back to mutant cells lacking binding protein, K_b was about 1 mM [20], which is the concentration in wild-type cells. In another study [23], the concentration of the binding protein was varied in mutants, and the half-maximal concentration was estimated to be 0.1 mM. The relationship between $\bar{V}_{S_0}$ and the binding protein concentration was sigmoid, however, not hyperbolic. This observation is understandable, in view of the known reversible absorption of the binding protein to the lambda receptor in the outer membrane [29], which would cause the free protein concentration, and therefore the maximum rate, to at first increase out of proportion to any increase in the total binding protein. Another complication is that the free binding protein is mainly a dimer and the substrate complex a monomer [30]. In this study, lowering the binding protein concentration by a factor of 25 reduced $\bar{V}_{S_0}$ 10-fold and doubled $\bar{K}_{S_0}$, trends suggesting that in the wild type $K_P < [P_t]$ and $c > [P_t]$. In the case of a mutant histidine transport system in *S. typhimurium* with 5-times the normal binding protein content, $\bar{V}_{S_0}$ was unchanged but $\bar{K}_{S_0}$ fell from 26 nM in the wild type to 6.6 nM in the mutant [15]; the implication is that in the wild type $K_P \ll [P_t]$ and $c \gg [P_t]$.

(viii) The concentration of the binding protein. The cell produces 20–50 copies of the binding protein for each copy of the membrane carrier, but much less would seem to be required if the interaction with the carrier were stronger. Where it has been measured (see above), the actual affinity for the carrier is low – K_P (Eqn. 5) may be in the range of 0.1 to 1 mM. Correspondingly, the concentration of the binding protein in the periplasm is high – about 1 mM. Why has selection pressure not evolved a more economical arrangement? Probably because stronger interaction would retard transport. The periplasm is a gel [31,32] in which diffusion of the binding protein is some 5000-times slower than in water [33]; and since a protein of this size diffuses about 10-times slower than a small molecule, the rate constant for formation of the carrier-protein complex, k(on), would be low, possibly $5 \cdot 10^4$ times lower than the diffusion-controlled rate constant for a small molecule. If the latter is taken to be $1 \cdot 10^9$ M^{-1} s^{-1}, the constant for the protein would be about $2 \cdot 10^4$ M^{-1} s^{-1}. Assuming that $K_7 = k(\text{off})/k(\text{on})$ is 0.1 mM, then $k(\text{off}) = 10^{-4}$ M $\times 2 \cdot 10^4$ M^{-1} $s^{-1} = 2$ s^{-1}. This rate is to be compared with the substrate dissociation constants for the binding protein, which vary between 2 and 90 s^{-1} (Miller et al. [34]), and with turnover numbers for transport, estimated to be no higher than 2 s^{-1} [29,34]. Hence, stronger attrac-

tion of the binding protein for the membrane carrier could make dissociation rate-limiting, reducing the transport rate. Considering that the concentration of the binding protein, which is only partly free [29], should not be less than K_7, the actual concentrations and affinities may be optimal.

(ix) The binding protein-carrier complex. It is understandable that the complex of the binding protein with the membrane carrier is hard to detect. As explained above, forming too tight a complex retards transport, and therefore binding ought to be, and is, weak. Further, the free protein is mainly in conformation P′, which does not interact with the carrier. In the presence of the substrate the conformation is correct, but on addition of the complex to the membrane component the substrate passes to the carrier site and the protein reverts to conformation P′. It is apparent that the complex will reach a steady state controlled by rate constants for the carrier. The half-saturating concentration of the binding protein, K_p, measured at a saturating concentration of external substrate, is given by Eqn. 7. That the binding protein does combine with the carrier, at least transiently, has been demonstrated in cross-linking studies [35].

(x) The non-productive complex $PS \cdot C_oS$. This complex, if formed, is a barrier to exit, but whether the inhibition would be an advantage depends on the effect on entry, also inhibited when PS adds to C_oS. Entry will be little inhibited at low steady-state concentrations of C_oS, i.e., where addition of external substrate to the carrier, governed by $k_1[PS]$, is slow relative to the rate of movement of the carrier-substrate complex, governed by f_2; and, understandably, this is one of the conditions for Michaelis-Menten kinetics. Under the same conditions the rate equation for exit retains the inhibitory $[S_o]$ terms.

At present, there is no way of deciding whether a non-productive complex is formed, though values of $\bar{K}_{S_o}$ larger than K_S were seen to argue against such a complex.

Testing transport systems for dependence on binding proteins

To fulfill its role in the mechanism in Fig. 1, the binding protein need not be free in the periplasmic space. The only requirement is that it should be able to interact with the membrane carrier, and it might do so while attached to the external surface of the cytoplasmic membrane adjacent to the carrier. In this light, the general test for the mechanism should focus, not on inactivation of the system and release of the binding protein through osmotic shock or disruption of the cell wall, but on the behaviour in transport, in particular the absence of equilibrium exchange at high substrate concentrations, and the absence of counter-transport in de-energized cells.

Possible implications of the mechanism

Two questions arise. What special property of the system justifies the added cost of the periplasmic binding protein, when a simple carrier could transport the substrate across the membrane? And why should the substrate induce a conformational change in the binding protein, when the added binding force involved impedes transfer of the substrate to the membrane component [1]? The answer may lie in the asymmetry imposed by the binding protein. The system facilitates entry but blocks exit in both the energized and de-energized states (Eqn. 1), suggesting that the binding protein functions as an inwardly directed valve, or rectifier, preventing substrate loss under all conditions.

The effective measure of asymmetry is the ratio of the maximum entry and exit rates, $\bar{V}_{S_o}$ and $\bar{V}_{S_i}$, not the half-saturation constants, $\bar{K}_{S_o}$ and $\bar{K}_{S_i}$. The constants are actually related to one another through α, the accumulation ratio [36]:

$$\alpha = (\bar{V}_{S_o}/\bar{V}_{S_i})/(\bar{K}_{S_o}/\bar{K}_{S_i}) \tag{9}$$

Under conditions of passive transport where $\alpha = 1$, $\bar{V}_{S_o}/\bar{V}_{S_i} = \bar{K}_{S_o}/\bar{K}_{S_i}$. In systems dependent on binding proteins, $\bar{V}_{S_o}/\bar{V}_{S_i} \gg 1$ (Eqn. 1) and therefore $\bar{K}_{S_o}/\bar{K}_{S_i} \gg 1$. With the substrate affinity far higher inside than outside, the exit rate may be maximal, but even in the de-energized system this maximum is very low.

An ordinary transport system involving only a membrane carrier can also e asymmetrical, but the asymmetry involves the rate constants determining the uptake ratio α and therefore depends on the energy state of the system. In terms of the ordinary carrier scheme in Fig 2, for example,

$$\alpha = f_{-1}f_2k_1k_{-2}/(f_1f_{-2}k_{-1}k_2) \tag{10}$$

and the maximum exit and entry rates, $\bar{V}_{S_i}$ and $\bar{V}_{S_o}$, are [37]

$$\bar{V}_{S_i} = k_{-1}f_1f_{-2}[C_i]/\{k_{-1}(f_1 + f_{-2}) + f_1(f_2 + f_{-2})\} \tag{11}$$

$$\bar{V}_{S_o} = k_{-2}f_{-1}f_2[C_i]/\{k_{-2}(f_{-1} + f_2) + f_{-1}(f_2 + f_{-2})\} \tag{12}$$

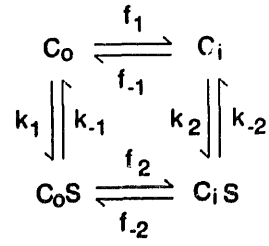

Fig. 2. The ordinary carrier model. The carrier alternates between outward-facing and inward-facing forms, C_o and C_i, respectively. External substrate adds to C_o to form the external complex, C_oS, and internal substrate adds to C_i to form the internal complex, C_iS.

Because α is high in active transport and equal to unity in a passive system, de-energization can result in an increased exit rate, with loss of accumulated substrate. In a system involving a periplasmic protein, asymmetry depends on the conformational change in the protein (governed by K_4 and K_6) and is therefore independent of α (Eqn. 2).

Observations in accord with a valve function

(i) Bacterial binding protein-dependent systems are members of a larger family of transporters whose membrane components are structurally related [8]. Included are the import systems we have been considering, and also export systems. Significantly, the binding protein component is always missing in the latter, as would be expected if the purpose of the binding protein is to block exit.

(ii) Transport systems involving binding proteins that are attached to the cytoplasmic membrane have been discovered in Gram-positive organisms, which lack the periplasm [38,39]. In the absence of a periplasm such an arrangement might have been predicted. Even if anchored adjacent to the membrane carrier, the binding protein could participate in the mechanism in Fig. 1.

(iii) A transport system that acts as a valve is likely to be of advantage in organisms other than bacteria. In agreement, systems dependent on binding proteins have been found in fungi and various metazoans [40]. The evidence for a similar mechanism is best in fungi, where water-soluble binding proteins involved in transport have been extracted from the cell wall [41–44]. In mammals, cobalamin transport in the intestine depends on soluble binding proteins and could involve such a mechanism [45].

(iv) Bacteria have two ways of pumping substrates into the cell chemically unchanged: ion-linked co-transport, and transport involving periplasmic proteins. Having two systems would be understandable if the functions were different. Periplasmic systems, with substrate constants of μM or less, scavenge substrates at low concentration in the medium and build up steep gradients across the membrane – greater than 10^5. With the pressure to escape strong, an inwardly directed value, which the binding protein supplies, might be vital. Co-transport systems [2] have lower substrate affinity, with half-saturation constants in the mM range, and build up flatter gradients – not above $2 \cdot 10^3$ – and here a valve might be dispensed with.

Additional tests of the mechanism

(i) As noted above, mutants have been isolated that carry out transport in the absence of a functional binding protein, though with low substrate affinity. Here, the analysis says, both exchange transport and counter-transport should be observed. This could be a decisive test, not only of the reaction scheme in Fig. 1 and the idea that the binding protein functions as a valve, but of all models, including those of Hengge and Boos [2] and of Quiocho [9], in which only one conformation of the binding protein – that of the substrate complex – interacts with the carrier.

(ii) A crucial feature of the model is the inability of the free binding protein, in its dominant conformation, to add to the membrane carrier. The rates of cross-linking between the two components [35] should be very low in experiments with protein freed of all substrate under denaturing conditions, and much higher with protein saturated with the substrate.

Energy input in active transport

The asymmetry of the mechanism is seen to be independent of which steps are coupled to ATP hydrolysis (since ATP is not involved in the carrier scheme). The kinetic treatment does provide a guide as to how energy could be used most efficiently. The important point is this: with too high a substrate affinity inside, exit soon balances entry and net uptake becomes extremely slow. Energy should therefore be applied to reduce the affinity of the inner carrier site. $\bar{K}_{S_o}$ is written above (Eqn. 6), and $\bar{K}_{S_i}$, the half-saturation constant in exit, is

$$\bar{K}_{S_i} = \frac{(1 + f_{-1}/f_1)\{K_{S_i} + (f_{-2}/k_2) + f_2 K_{S_i} K_4/(k_{-1}[P_i])\}}{1 + (f_{-2}/f_1) + (f_2 + f_{-2})K_4/(k_{-1}[P_i])} \quad (13)$$

In systems obeying Michaelis-Menten kinetics, the constants for zero-trans entry and exit are not independent but are related to the accumulation ratio α (Eqn. 9). The coupled step, which increases α (Eqn. 2), should be one that increases $\bar{K}_{S_i}/\bar{K}_{S_o}$ and not $\bar{V}_{S_i}/\bar{V}_{S_o}$. ATP could act directly on the internal site, converting the inward-facing carrier from a high-affinity to a low-affinity form, and leaving the $\bar{V}_{S_i}/\bar{V}_{S_o}$ ratio unchanged (Eqn. 1). Or it could act indirectly. In the mechanism of Hengge and Boos [2], ATP converts the free carrier from the inward-facing to the outward-facing form, which could make f_{-1}/f_1 large in the energized system. As a result, the internal affinity would be weakened (because $\bar{K}_{S_i}$ is proportional to $(1 + f_{-1}/f_1)$) and the external affinity strengthened somewhat (Eqn. 6), with little change in $\bar{V}_{S_i}/\bar{V}_{S_o}$.

Other interpretations

Other roles for the periplasmic binding proteins have already been suggested. These will now be considered.

(i) The membrane component would be most efficiently utilized if it were able to interact with binding

proteins for several different substrates. There would then be a saving on integral membrane proteins but little saving overall because the binding proteins are many times more abundant than the membrane component [2]. The mechanism is not feasible with widely dissimilar substrates, since substrate transfer from the binding protein to the carrier depends on the affinity of the membrane site [1]. As expected, most systems transport a single substrate. Joint systems are rare, but have been reported in the case of branched-chain amino-acids [3,46,47], and of histidine and arginine [48].

(ii) The function of the periplasmic protein, it has been suggested, could be to increase the substrate affinity of a membrane carrier that binds the substrate weakly [9]. One objection to this idea is that the membrane component could itself have been selected, in an evolutionary process, for high affinity. High affinity does not impair translocation, and a simpler system, without the periplasmic protein, would be more economical. But given a system requiring a binding protein, there is a difficulty. Lowering the intrinsic affinity of the carrier relative to that of the binding protein (i.e., raising K_{S_0} relative to K_S) impairs transport in any mechanism in which the substrate is transferred from the binding protein to the membrane component (Eqns. 39–41 in the preceding paper). At sufficiently high K_{S_0}, uptake is completely blocked.

It must not be concluded, either from the requirement for a binding protein, or from the low affinity of mutants that work without a functional binding protein, that the intrinsic affinity of the membrane carrier is low. The substrate half-saturation constant depends on both the affinity and the accessibility of the substrate site. Direct access to the site is blocked in normal cells. In the mutant, which functions without the binding protein, the obstruction may be partly but not wholly removed. The mutant system can be represented by the carrier model in Fig. 2, the half-saturation constant for which depends on whether substrate dissociation is a fast or a slow (rate-limiting) step, that is, whether access to the site is free or hindered [37]. If the step is fast, with k_{-1} and k_{-2} large relative to the rate constants for carrier reorientation, f_2, f_{-2} etc., the substrate constant for uptake is

$$\bar{K}_{S_0} = \frac{(1 + f_1/f_{-1})k_{-1}/k_1}{1 + f_2/f_{-1}} \qquad (14)$$

If the step is slow, with $k_{-1} \ll f_2$ (as when access to the outer site is made difficult in the mutant),

$$\bar{K}_{S_0} = \frac{(1 + f_1/f_{-1})f_2/k_1}{1 + f_2/f_{-1}} \qquad (15)$$

In the first case, the measured half-saturation constant is proportional to k_{-1}/k_1, the substrate dissociation constant for the outer carrier site. In the second, the half-saturation constant is proportional to f_2/k_1, which is much larger than k_{-1}/k_1 (since $f_2 \gg k_{-1}$); the measured affinity therefore underestimates the true affinity. In the wild type, where k_1 and k_{-1} are close to zero, the substrate constant will be too high to measure in the absence of the binding protein, and the maximum transport rate, being limited by k_1, will be extremely slow.

(iii) A related idea is that the concentration of the protein-substrate complex may be higher than expected from the kinetics of transport, and consequently that the binding protein, having trapped the substrate, presents it in more concentrated form to the carrier [3]. In fact, the binding protein is generally present at too low a concentration to fully saturate the carrier, and may therefore have the opposite effect. Aside from this, the conformational change in the binding protein, which impedes substrate transfer [1], would be a disadvantage.

(iv) It has been suggested that the purpose of the binding protein is to accelerate substrate diffusion through the periplasm, an idea rejected by Hengge and Boos [2]. The effect depends on a low solubility and therefore low mass flow of the substrate, where the amount diffusing can be increased by absorption to a binding protein. As diffusion is slower with larger molecules, passage of a freely soluble substrate would be retarded by adsorption to the protein. Brass and co-workers [33] suggested, as an alternative, that the substrate might be rapidly passed from one molecule of the binding protein to another, all the way from the outer to the inner membrane. At the same time they raised an objection to this hypothesis, which is that a concentrated solution of the galactose binding protein fails to increase the rate of substrate diffusion [49]. It might be pointed out too that if the periplasm were clogged by solid matter, as the hypothesis assumes, channels should open up in the absence of binding proteins, which make up one third of the protein released during osmotic shock [2]. Further, if facilitating substrate diffusion were the only role of binding proteins in transport, then spheroplasts and membrane vesicles should show normal transport, and shocked cells, with the protein missing, should be capable of transport at higher than normal substrate concentrations (because the substrates of transport systems that do not involve binding proteins diffuse, unassisted, through the periplasm at such concentrations). Another objection is that binding proteins are attached to the cytoplasmic membrane in Gram-positive organisms, which lack the periplasm, and in this circumstance it is hard to see how the protein would assist the approach of the substrate.

(v) The role of the binding protein could be to capture substrate molecules leaking out of the cell and

to deliver them to the carrier for re-importation, reducing the rate of loss [3,50,51]. But the rate of loss depends only on the concentration of the free substrate, which, as Hengge and Boos [2] have pointed out, is not affected by the binding protein. The situation is as follows. Let the rate of leakage across the cytoplasmic membrane be a constant, L; let the rate constant for diffusion across the outer membrane be k; and let the rate of uptake into the cytoplasm by the carrier system be $V/(1+K_m/[S])$, where [S] is the concentration of free substrate in the periplasm. Now [S] will rise until the rate of escape across the outer membrane and away from the cell, together with the rate of re-entry into the cytoplasm, equals the rate of leakage across the plasma membrane. In this steady-state, the free substrate concentration is determined by the equality

$$L = k[S] + V/(1 + K_m/[S]) \quad (16)$$

and is therefore independent of the concentration of the binding protein:

$$[S] = \frac{L - V - kK_m \pm ((V + kK_m - L)^2 + 4kLK_m)^{1/2}}{2k} \quad (17)$$

The total concentration of free and bound substrate is of course greater than [S]. In the comparison of two transport systems having the same V and K_m, one involving a periplasmic protein and the other not, the rates of substrate recovery by the cell, being the same function of the concentration of free substrate in either case, $v = V/(1 + K_m/[S])$, will be identical. The rate of leakage across the outer membrane, meanwhile, is $k[S]$, which is independent of the binding protein because [S] is independent of the binding protein.

Conclusion

The first of these alternative mechanisms applies only to closely related substrates. The others do not appear to be workable. None requires a binding protein capable of changing shape on addition of the substrate, especially as the conformational change, by hindering transfer of the substrate from the binding protein to the carrier, would be a disadvantage.

A valve mechanism, on the other hand, demands a conformational change that is both extensive, to enable the carrier to distinguish the loaded from the unloaded form of the binding protein, and abrupt, so that the form required in exit is present at a very low concentration. The conformational change is the price paid for the useful asymmetry of the system. With the conformation fixed (as P, with $K_4 = K_6 = 0$), the ratio of entry and exit rates depends solely on the rate constants that determine the uptake ratio, α; that is, the asymmetry introduced by the binding protein disappears [1]. With the interactions reversed, and the free binding protein but not the substrate complex able to add to the carrier, entry rather than exit is blocked by the binding protein [1].

Unexpectedly then, the model not only accounts for the kinetics of transport but provides a reason for the change in shape of the binding protein. The conformational change appears to be universal [3], and a mutant protein unable to undergo the normal conformational change was found to be inactive [52–54]. The observed transformation is so radical that one conformation, but not both, might be expected to form a complex with the membrane component [9].

In sum, the scheme in Fig. 1 is in accord with much experimental evidence on the structure of the system [3,5] and in its essential features resembles models suggested before [2,9]. It predicts kinetic behaviour not seen in other types of transport system and not predicted by other models, namely uptake that appears to be irreversible, and the absence of exchange and counter-transport. It accounts for the substrate-induced conformational change, for the high concentration of the binding protein and its weak association with the membrane component, and for the dependence of the substrate constants on the binding protein concentration. It can account for Michaelis-Menten kinetics. In addition, the mechanism explains why binding proteins are not present in members of the family of structurally related transporters that function in export rather than import, and it shows how binding proteins could function while attached to the cytoplasmic membrane in bacteria lacking the periplasmic space.

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References

1 Krupka, R.M. (1991) Biochim. Biophys. Acta 1110, 1–10.
2 Hengge, R. and Boos, W. (1983) Biochim. Biophys. Acta 737, 443–478.
3 Ames, G.F.-L. (1986) Annu. Rev. Biochem. 55, 397–425.
4 Furlong, C.E. (1987) *Escherichia coli* and *Salmonella typhimurium*. Cellular and Molecular Biology, Vol. 1 (Neidhardt, F.C., ed.), pp. 768–796, American Society for Microbiology, Washington, D.C.
5 Shuman, H.A. (1987) Annu. Rev. Genet. 21, 155–177.
6 Ames, G.F.-L. and Joshi, A.K. (1990) J. Bacteriol. 172, 4133–4137.
7 Higgins, C.F., Gallagher, M.P., Hyde, S.C., Mimmack, M.L. and Pearce, S.R. (1990) Phil. Trans. R. Soc. Lond. B 326, 353–365.
8 Higgins, C.F., Hyde, S.C., Mimmack, M.M., Gileadi, U., Gill, D.R. and Gallagher, M.P. (1990) J. Bioenerg. Biomembr. 22, 571–592.
9 Quiocho, F.A. (1990) Phil. Trans. R. Soc. Lond. B 326, 341–351.

10 Hill, T.L. and Eisenberg, E. (1981) Q. Rev. Biophys. 14, 463–511.
11 Parnes, J.R. and Boos, W. (1973) J. Biol. Chem. 248, 4429–4436.
12 Rosenberg, H., Gerdes, R.G. and Chegwidden, K. (1977) J. Bacteriol. 131, 505–511.
13 Silhavy, T.J., Ferenci, T. and Boos, W. (1978) in Bacterial Transport (Rosen, B.P., ed.), pp. 127–169, Marcel Dekker, New York.
14 Krupka, R.M., Kaplan, H. and Laidler, K.J. (1966) Trans. Faraday Soc. 62, 2754–2759.
15 Ames, G.F.-L. and Lever, J. (1970) Proc. Natl. Acad. Sci. USA 66, 1096–1103.
16 Robbins, A.R., Guzman, R. and Rotman, B. (1976) J. Biol. Chem. 251, 3112–3116.
17 Szmelcman, S., Schwartz, M., Silhavy, T.J. and Boos, W. (1976) Eur. J. Biochem. 65, 13–19.
18 Ferenci, T. (1980) Eur. J. Biochem. 108, 631–636.
19 Schweizer, H., Argast, M. and Boos, W. (1982) J. Bacteriol. 150, 1154–1163.
20 Brass, J.M., Ehmann, U. and Bukau, B. (1983) J. Bacteriol. 155, 97–106.
21 Poole, K. and Hancock, R.E.W. (1984) Eur. J. Biochem. 144, 607–612.
22 Celis, R.T.F. (1982) J. Bacteriol. 151, 1314–1319.
23 Manson, M.D., Boos, W., Bassford, P.J., Jr. and Rasmussen, B.A. (1985) J. Biol. Chem. 260, 9727–9733.
24 Wilson, D.B. and Smith, J.B. (1978) in Bacterial Transport (Rosen, B.P., ed.), pp. 495–557, Marcel Dekker, Inc., New York.
25 Goldstein, L. (1976) Methods Enzymol. 44, 397–443.
26 Ralston, G.B. (1990) J. Chem. Ed. 67, 857–860.
27 Dreyfass, J. (1964) J. Biol. Chem. 239, 2292–2297.
28 Pardee, A.B. (1966) J. Biol. Chem. 241, 5886–5892.
29 Boos, W. (1984) Biochem. Soc. Trans. 12, 141–146.
30 Richarme, G., Meury, J.M. and Bouvier, J. (1982) Ann. Microbiol. (Paris) 133A, 199–204.
31 Hobot, J.A., Carlemalm, E., Villiger, W. and Kellenberg, E. (1984) J. Bacteriol. 160, 143–152.
32 Van Wielink, J.E. and Duine, J.A. (1990) Trends Biochem. Sci. 15, 136–137.
33 Brass, J.M., Higgins, C.F., Foley, M., Rugman, P.A., Birmingham, J. and Garland, P.B. (1986) J. Bacteriol. 165, 787–794.
34 Miller, D.M., Olson, J.S., Pflugrath, J.W. and Quiocho, F.A. (1983) J. Biol. Chem. 258, 13665–13672.
35 Prossnitz, E., Nikaido, K., Ulbrich, S.J. and Ames, G.F.-L. (1988) J. Biol. Chem. 263, 17917–17920.
36 Krupka, R.M. (1989) Biochem. J. 260, 885–891.
37 Devés, R. and Krupka, R.M. (1979) Biochim. Biophys. Acta 556, 533–547.
38 Gilson, E., Alloing, G., Schmidt, T., Claverys, J.-P., Dudler, R. and Hofnung, M. (1988) EMBO J. 7, 3971–3974.
39 Alloing, G., Trombe, M.-C. and Claverys, J.-P. (1990) Mol. Microbiol. 4, 633–644.
40 Sennet, C., Rosenberg, L.E. and Mellman, I.S. (1981) Ann. Rev. Biochem. 50, 1053–1086.
41 Wiley, J. (1970) J. Bacteriol. 103, 656–662.
42 Stuart, W.D. and DeBusk, A.G. (1971) Arch. Biochim. Biophys. 144, 512–518.
43 Horak, J. and Kotyk, A. (1973) Eur. J. Biochem. 32, 36–41.
44 Opekarova, M., Kotyk, A., Horak, J. and Kholodenko, V.P. (1975) Eur. J. Biochem. 59, 373–376.
45 Grasbeck, R. and Kouvonen, I. (1983) Trends Biochem. Sci. 8, 203–205.
46 Landick, R., Oxender, D.L. and Ames, G.F.-L. (1985) in The Enzymes of Biological Membranes, Vol. 3 (Martonosi, A.N., ed.), pp. 577–615, Plenum Press, New York.
47 Landick, R. and Oxender, D.L. (1985) J. Biol. Chem. 260, 8257–8261.
48 Kustu, S.G. and Ames, G.F.-L. (1973) J. Bacteriol. 116, 107–113.
49 Silhavy, H.A., Boos, W. and Kalkar, H.M. (1974) in Biochemistry of Sensory Functions (Jaenicke, L., ed.), pp. 165–205, Springer-Verlag, Berlin.
50 Landick, L. and Oxender, D.L. (1982) in Membranes and Transport, Vol. 2 (Martonosi, A.N., ed.), pp. 81–88, Plenum Press, New York.
51 Stirling, D.A., Hulton, C.S.J., Waddell, L., Park, S.F., Stewart, G.S.A.B., Booth, I.R. and Higgins, C.F. (1989) Mol. Microbiol. 3, 1025–1038.
52 Kustu, S.G. and Ames, G.F.-L. (1974) J. Biol. Chem. 249, 6976–6983.
53 Manuck, B.A. and Ho, C. (1979) Biochemistry 18, 566–573.
54 Zukin, R.S., Clos, M.F. and Hirsch, R.E. (1986) Biophys. J. 49, 1229–1235.