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Binding proteins enhance specific uptake rate by increasing the substrate–transporter encounter rate

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Microorganisms rely on binding-protein assisted, active transport systems to scavenge for scarce nutrients. Several advantages of using binding proteins in such uptake systems have been proposed. However, a systematic, rigorous and quantitative analysis of the function of binding proteins is lacking. By combining knowledge of selection pressure and physiochemical constraints, we derive kinetic, thermodynamic, and stoichiometric properties of binding-protein dependent transport systems that enable a maximal import activity per amount of transporter. Under the hypothesis that this maximal specific activity of the transport complex is the selection objective, binding protein concentrations should exceed the concentration of both the scarce nutrient and the transporter. This increases the encounter rate of transporter with loaded binding protein at low substrate concentrations, thereby enhancing the affinity and specific uptake rate. These predictions are experimentally testable, and a number of observations confirm them.

Introduction

Microorganisms use a variety of uptake systems to take up nutrients from their environment [1]. Some of these systems rely on extra-cytoplasmic binding proteins (BP), where the substrate molecule first binds to a BP before it is transported into the cell. The BP-substrate complex interacts with a trans-membrane transporter where the substrate is released from the BP, and transported over the membrane. These BP-dependent transporters are ubiquitous in prokaryotic cells and are also found in eukaryotes and Archaea. In Gram-negative bacteria, the binding proteins float freely in the periplasmic space, whereas, in other cell types, they are anchored to the membrane or the transport protein [2–4].

BP-dependent transport systems have a wide range of substrates, including sugars, amino acids, and metals [5]. Generally, BPs bind substrates with very high affinities, exhibiting dissociation constants in the nano- to micromolar range. They can transport against very large substrate gradients, up to a 10 000 fold difference [4–6], and hence require the input of free energy [6]. Most of these systems have an ATP-binding cassette

Abbreviations
BP, binding protein; BPD, model, binding protein-dependent model; MPB, maltose-binding protein.

and use free energy derived from ATP-hydrolysis [3,7], where as some are of the tripartite ATP-independent periplasmic transporter type or tripartite tricarboxylate transporters, both of which use the sodium electrochemical gradient as a driving force [8–10].

Although the structure, mechanics and kinetics of these systems have been well studied [3,5,6,11,12], the functions of BPs in BP-dependent transport are still uncertain, especially because many nutrients imported by these systems can be taken up by other, non-BP-dependent mechanisms too [3]. Examples are phosphate transport systems in *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter johnsonii* [13–15], or glucose uptake in *Agrobacterium radiobacter* [16] and *E. coli*. The latter is mediated by a glucose-PTS or by the BP-dependent system Mgl [17,18], (although Mgl is typically considered to be a maltose BP, it also binds glucose strongly, with a dissociation-constant of only 0.2 μM) [19]. In all these cases, BP-dependent uptake systems are used at low substrate concentrations, whereas the alternative transporters are used at high substrate concentrations. The obvious conclusion would be that the binding protein with its high affinity for substrate plays a role in substrate scavenging. However, there is no reason to assume that non-BP-dependent systems could not have an equal high affinity for their substrates.

Several ideas have been put forward regarding the beneficial functions of the binding proteins in transport. Common suggestions are that the BPs enhance the affinity of the transport system or the diffusivity of the substrate, or that they increase the free or total solute concentration in the periplasm [3,6,20]. These postulated benefits each depend on specific conditions and are subject to criticism.

The diffusion of substrate to transporter was suggested to be limiting at low substrate concentrations, as a result of the viscosity of the periplasm and the small size of the substrate [21]. This hypothesis shifts the problem from diffusion of the substrate to diffusion of the binding protein. However, the lateral diffusion constants measured for the maltose binding protein in the periplasm are very low, up to 50–100 times lower than the diffusion constants of similar sized proteins in the cytosol [21]. Brass *et al.* [21] postulated that the density of binding proteins in the periplasm could result in a semi-ordered chain where the proteins only move within a small space and rotate, passing the substrate molecule along the chain from the outer membrane to the inner. However, this would require a stable structuring of the periplasm that has not yet been observed. It is also incompatible with the fact that most BPs encapsulate the substrate molecule and bind it tightly. It is hard to see how a tightly bound substrate molecule could be rapidly passed on to the next BP without the input of external energy.

Despite the appeal of postulated benefits of BPs, they remain debatable as long as they are not quantitatively derived from basic kinetic mechanisms and physical and thermodynamic constraints. Without such an account one cannot rule out the occurrence of trade-offs, in that two acclaimed benefits cannot be simultaneously attained as a result of physical-chemical incompatibility, or that the conditions under which they are said to occur violate basic kinetic or thermodynamic requirements, such as the thermodynamic constraint of reactions only proceeding in the direction of free energy reduction. Furthermore, to substantiate claims of benefit, the putative fitness effects of binding-protein mediated transporters should be quantified in comparison to alternative transporters.

Here, we offer an unbiased, systematic analysis of BP functionality. We analyze the kinetic mechanism of BP-dependent transporters constrained by thermodynamic relations and diffusion limits. We characterize the state of maximal transport activity per unit of transporter and report conditions under which BP-dependent transporters outperform non-BP dependent transporters. We show that current experimental data on BP-dependent transporters meet these conditions. We also show that BPs are primarily advantageous at low nutrient concentrations. At high nutrient concentrations, the costs of BP synthesis exceed its benefits and BP-independent systems are more favorable.

Results

Global hypothesis

We now give a chain of reasoning that provides a hypothesis from which we can start filling in the details.

Natural selection

Transport systems, in particular BP-dependent systems and alternative non-BP-dependent systems, are subject to natural selection, (i.e. fitness has increased by adoption of particular catalytic schemes, components and expression patterns for these transporters).

Fitness costs and benefits

The operational fitness benefits (during the transport catalytic cycle) result from the transport reaction itself. The operational costs result from the investment of

biological free energy in the transport reaction. The initial costs (during synthesis of the transporter) result from the burden put on protein synthesis capacity or on occupancy of the membrane (proportional to the membrane-located subunits of a transporter).

Maximization of benefit-to-cost ratio

It follows that, at a given operational free energy investment, the benefit-to-cost ratio, (i.e. the specific transport rate), defined as the transport rate per amount of transport protein or per membrane area occupied by the transporter, will be subject to natural selection. Natural selection will maximize the specific transport rate bounded by physico-chemical constraints, in particular those imposed by thermodynamics and molecular movement.

Interpreting function

The ultimate function of a biological component (as in 'ultimate cause') is its contribution to fitness of the organism. It follows that the ultimate biological function of binding proteins as well as the molecular details of the transporter and of its catalytic cycle (rate constants, free energy differences), must be stated in terms of their effect on the specific transport rate.

Discussion of fitness costs and benefits

In the second point, we assume that the synthesis or membrane occupation of an inactive transporter would have a negative effect on fitness, (i.e. present a fitness cost), because it would displace other proteins that do have catalytic activity from resources like protein synthesis capacity or membrane space. An underlying assumption is, therefore, that these internal resources are limited or, more accurately, that they will be exploited by natural selection up to a limit [22]. An active transporter may be able to overcome the negative effect of resource occupancy on fitness by providing substrate for metabolism. The negative effect of protein burden on fitness, specifically on growth rate, has been proposed and experimentally demonstrated previously [23–27]. The precise details of a putative membrane burden, whether through a limit on the membrane surface or on membrane protein insertion machinery, do not matter much for the argument. The fact that even a slight over-expression of membrane proteins causes growth arrest indicates that cells operate close to a capacity limit for such proteins [28]. We show below that maximization of specific activity either constrained by the protein synthesis capacity or membrane capacity leads to qualitatively similar conclusions.

Detailed hypothesis

We propose that binding proteins are beneficial at low substrate concentrations because they increase the specific transport rate as a result of a simultaneous increase of the number of binding sites and the effective substrate concentration (Fig. 1A,B).

The effective substrate for BP-dependent transport systems is the BP-substrate complex, and the effective substrate concentration is thus the concentration of the BP-substrate complex. (For a BP independent system, the effective substrate concentration is just the free substrate concentration). Although the free substrate concentration outside and in the periplasm will be equal, the total substrate concentration (bound plus free) in the periplasm increases by its binding to BPs [6,20]. When the affinity of BP for substrate is sufficiently high, and when the BP concentration in the periplasm is higher than the free substrate concentra-

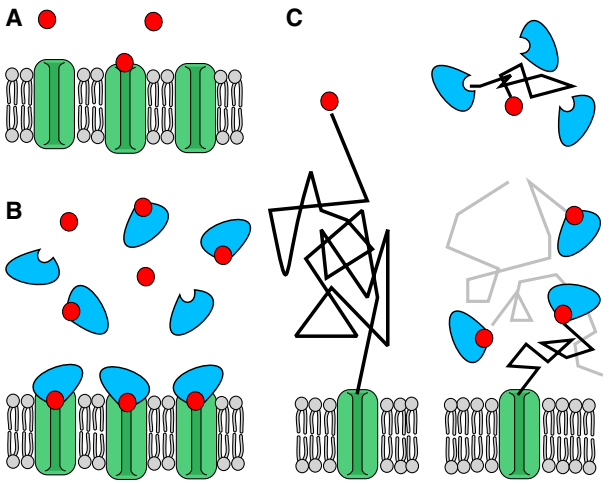

Fig. 1. Illustration of the hypothesis that binding proteins enhance the specific uptake rate. (A) When substrate is scarce, transmembrane transporter proteins are used inefficiently because they are mostly idle. (B) In binding protein (BP) dependent uptake systems, the BP-substrate complex (BS) is the effective substrate for the transporter. High levels of BPs increases the number of binding sites for substrate. If the affinity of the BPs is high, also the effective substrate concentration will also be strongly increased. This decreases the average time between substrate–transporter binding events, and thus allows for more efficient usage of the transporters. This is particularly beneficial to a cell when membrane space is limited. (C) The use of BPs makes the binding of substrate to the transporter a two-step process: (1) the binding of substrate to the BP, and (2) binding of the BS to the transporter. To enhance the specific uptake rate, both steps individually need to be 'faster' than direct substrate–transporter binding.

tion, the effective substrate concentration is raised in the periplasm. Binding proteins also increase the number of substrate binding sites per transporter [29]. Both effects may increase the specific transport rate without synthesis of additional transport protein. Thus, they have no burden on membrane occupancy, or only a small additional burden on protein synthesis capacity, because of the relatively small size of BPs. Below, we quantitatively specify the conditions under which an increase of binding sites and effective substrate concentration improve the specific transport rate.

Testing the hypothesis

There are a few ways to put a hypothesis about the ultimate function of a biological component to the test: (a) directly, by showing theoretically or experimentally that the extant option leads to the highest fitness among alternative options, and (b) indirectly, by showing that parallel evolution has led to similar solutions in many organisms. Clearly, BP-dependent transport should be compared with non-BP-dependent transport (Fig. 1A,B). Such a comparison is obvious because organisms often express such alternatives transporters for the same substrate. The preferred use of BP-dependent transporters at low substrate concentrations suggests that the BP-independent system has the highest specific uptake rate at low substrate concentrations, whereas, at high substrate concentrations, when the free substrate concentration in the periplasm is in the range of the concentration of the BP-substrate complex, alternative non-BP-dependent transporters have a higher specific uptake rate.

A comparison between BP dependent and non-BP-dependent transporters is complicated by the fact that they typically differ in the free energy input. An ATP-driven, BP-dependent transporter may, for example, outperform a proton motive force-driven transporter merely as a result of the difference in free energy input. This thus raises two different questions: what is the optimal free energy input in transport given the prevailing extracellular substrate concentration? In addition why are binding proteins beneficial (regardless of the free energy input)? In the present study, we only focus on the latter question. The former question is very hard to answer, because it is not clear how the cost of extra free energy input relates to the potential benefit of increased transport. However, in the Discussion, we speculate on why both low free energy input-BP-dependent and high free energy input-BP-independent system are not (or hardly ever) found in nature.

As a result of this difference in free energy input, it is not feasible to test the hypothesis directly in an experiment. Apart from the question of whether it is technically possible to construct non-BP-dependent transporters with equal energy input from BP-dependent one, the experimental manipulation of existing transporters itself would likely lead to suboptimal performance. Therefore, we test our hypotheses using mathematical models of transport systems, in the following set-up:

(1) We construct kinetic schemes of the reaction cycles of a BP-dependent and an alternative transporter. Both schemes will have the same input of free energy.
(2) For each scheme, we calculate optimal kinetic constants that maximize the specific transport rate at a particular substrate concentration. This step simulates the evolutionary optimization of the transporter at that substrate concentration.
(3) Additionally, we calculate specific transport rates with randomly sampled kinetic constants to be able to evaluate the generality of the conclusions and assess suboptimal situations.
(4) We compare the rates of both transporter models under different substrate concentrations to evaluate if, and under what conditions, either transport mechanism outperforms the other.

In addition to these numerical simulations, it is possible to derive, under quite general assumptions, an analytical expression describing strict requirements for outperformance of BP-dependent transporters over alternative transporters.

The predictions following from these theoretical investigations can be compared with experimentally measured properties of BP-dependent transporters. Indirect experimental evidence for the hypothesis is accumulated from investigations on many of these transporters. Given the generality and simplicity of the predictions to follow, we conclude that this should be feasible with the experimental approaches currently available.

Minimal models of active transport systems

We compare two models of active substrate transport: a BP-dependent model (BPD-model) and a model without BPs (null-model). To make a fair comparison, the models need to be similar in every respect except for a few relevant characteristics. This is best achieved with a minimal model that only considers the general features of the uptake system. Compared to detailed models with complex kinetic schemes, such simple models are more tractable, more intuitive to understand, and they reduce the risk of introducing model artefacts.

The reaction schemes we use are depicted in Fig. 2. We model BP-dependent uptake as a five step process, consisting of:

(1) Perisplasmic substrate (S_o) to BP binding to form a BP-substrate complex (BS): $S_o + B \overset{1}{\rightleftharpoons} BS$
(2) Binding of the BS complex to the transporter forming a transporter-BS complex TBS: $BS + T \overset{2}{\rightleftharpoons} TBS$
(3) Transportation of the substrate over the membrane: $TBS \overset{3}{\rightleftharpoons} TB + S_i$
(3*) ATP hydrolysis: $ATP \overset{3*}{\rightleftharpoons} ADP + P_i$
(4) Release of the BP from the transporter: $TB \overset{4}{\rightleftharpoons} T + B$

ATP binding and hydrolysis are usually considered to be coupled to the import process [30–32]. However, coupling of ATP hydrolysis or any other free energy dissipating reaction to this step in particular is not required from first principles (thermodynamics), which is why we considered the ATP hydrolysis and the transport reaction independently and labeled them as 3 and 3*, respectively. We therefore do not model the coupling of ATP hydrolysis to a transport step explicitly. Rather, it is incorporated as an overall equilibrium constant of the transport cycle

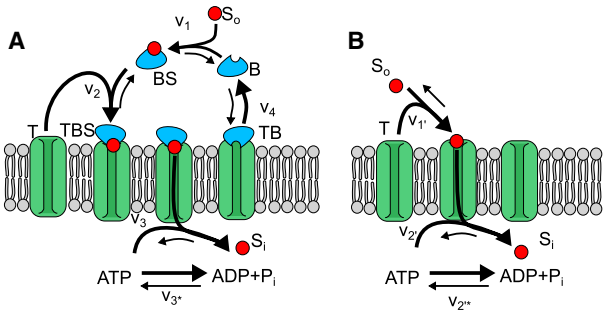

Fig. 2. Minimal models of binding protein dependent and independent transport systems. (A) Binding protein depend (BP-dependent) transport is modeled as a four step process: (1) binding of substrate to BP to form the BP-substrate complex BS, (2) BS to transporter binding. (3) substrate transport over the membrane and (4) release of the BP from the transporter. (B) The 'null-model' to test our hypothesis against is a two-step process: (1) substrate to transporter binding and (2) transport of the substrate over the membrane. All individual steps have simple mass action kinetics and are reversible. BPs are always involved in active transport. We do not explicitly model the energy coupling to the transport process, but incorporate it as an overall equilibrium constant $K_{eq} > 1$. The free substrate concentration outside the cell is kept fixed. For Gram-negative bacteria, we implicitly assume that transport over the outer membrane is not limiting. However, including outer membrane transport does not qualitatively affect the results (Fig. S3). Model details can be found in Doc. S3. The results shown in the study do not dependent on the details of these models, nor on the details of the parameters used (Fig. S3).

$$K_{eq} \equiv \exp(-\Delta_r\mu^0/RT) > 1$$

where $\Delta_r\mu^0$ represents the molar Gibbs free energy of the driving reaction under physiological conditions. This overall equilibrium constant applies to the net reaction of the BP-dependent system: $S_o + ATP \rightleftharpoons S_i + ADP + P_i$ (as the binding protein occurs on both sides of the reaction it drops out).

In the null-model, nutrient uptake is a two step process consisting of:

(1') Substrate to transporter binding to form transporter-substrate complex (TS): $T + S_o \overset{1'}{\rightleftharpoons} TS$
(2') Transport of the substrate over the membrane: $TS \overset{2'}{\rightleftharpoons} T + S_i$
(2'*) ATP hydrolysis: $ATP \overset{2'*}{\rightleftharpoons} ADP + P_i$

Again, ATP hydrolysis is incorporated implicitly.

For both models, each individual step is reversible and is modeled by mass-action kinetics. In what follows, rate constants k refer to the BPD-model and k' to the null-model. The overall equilibrium constant, K_{eq}, is related to the equilibrium constants of the four reactions mentioned above according to Wegscheider's condition for this model as:

$$K_{eq} \equiv \exp(-\Delta_r\mu^0/RT) = \frac{k_{1f}}{k_{1r}} \cdot \frac{k_{2f}}{k_{2r}} \cdot \frac{k_{3f}}{k_{3r}} \cdot \frac{k_{4f}}{k_{4r}} \quad (1)$$

where $k_{1f} \ldots k_{4f}$ and $k_{1r} \ldots k_{4r}$ are the forward and reverse rate constants, respectively. For each step i (i = 1…4) their ratio equals an equilibrium constant that is equivalent to a molar standard free Gibbs energy for that step, $\Delta_i\mu^0$. Wegscheider's condition can, therefore, also be written as:

$$\Delta_r\mu^0 = \Delta_1\mu^0 + \Delta_2\mu^0 + \Delta_3\mu^0 + \Delta_4\mu^0, \quad with\ \Delta_i\mu^0 \equiv -RT\ln\left(\frac{k_{if}}{k_{ir}}\right) \quad (2)$$

The interpretation of this constraint is that although ATP hydrolysis happens at the transporter, the derived driving force can be distributed unequally over different steps of the entire transport cycle. For example, tight binding of the substrate to the BP decreases the chemical potential of the BS complex, μ^0_{BS}; it makes it more negative. This effectively increases the driving force of BS formation, $\Delta_1\mu^0$ at the expense of the driving force of the substrate transport over the membrane, $\Delta_3\mu^0$. In other words, free energy can be 'invested' indirectly in substrate binding. A detailed description of this reasoning can be found in Doc. S2.

The concentration terms in the models need some discussion. For Gram-negative bacteria, the substrate, transporter and BP concentrations are defined in terms

of mol·L^{-1} of periplasm. For Gram-positive bacteria, which lack a periplasm, defining the concentration of transporters and binding proteins is less straightforward. The most sensible definition, and the one used here, is to use moles of transporter and BP divided by the volume around the cell that the BPs can reach (which is given by the length of their anchor to the membrane). We use these somewhat unconventional units for concentrations to avoid having to introduce scaling factors between the different molecular species (i.e. the binding of one unit BP to one unit transporter results in one unit BP-transporter complex). However, the precise definition of volume that is used for the concentrations is not critical because the most important quantity, BPs per transporter, is unambiguously defined. Model details are provided in Doc S3.

Enhanced uptake rate requires increasing the number of substrate binding sites and the effective substrate concentration

Before we proceed to study the models introduced above, we will discuss a strict requirement for increased uptake through the use of BPs. The derivation of this requirement is general, in the sense that it only considers substrate- and protein association rates and is independent of other mechanistic details, including differences in driving force. The derivation is based on the assumptions that, (a) at low substrate concentration, the nutrient uptake rate per transporter is dictated by rate at which the transporter encounters its substrate and (b) that the processes of substrate binding to either a binding site on a BP or on a transporter do not differ physically and therefore have the same rate constant. The requirement, formulated in Eqn (3) below, is derived in Doc. S1. There, we estimate both the BS-transporter encounter rate and the free substrate–transporter encounter rate for a fixed periplasmic substrate concentration $[S_o]$. The premise is that BPs only confer a benefit when the BS-transporter encounter rate exceeds the free substrate–transporter encounter rate. The BS-transporter encounter rate depends on the total binding protein concentration, $[B_{tot}]$, the transporter concentration $[T_{tot}]$, the free substrate concentration $[S_o]$ and the the dissociation constant of BP-substrate binding reaction, K_D. The result of this analysis states that BPs enhance the substrate–transporter encounter rate only when the following condition for $[B_{tot}]$ is met:

$$[B_{tot}] > [T_{tot}] + \alpha_{on}(K_D + [S_o]) \qquad (3)$$

Equation (3) has an intuitive interpretation. To enhance the substrate–transporter encounter rate, two sub-conditions need to be true: both the substrate-BP encounter rate and the BS-transporter encounter rate must be higher than the substrate–transporter encounter rate without BPs (Fig. 1C). Equation (3) implies that enhanced uptake requires at least $[B_{tot}] > [T_{tot}]$, (i.e. the binding proteins should be more abundant than the transporters). This ensures an increase in the number of substrate binding sites per transporter, and hence the BP-substrate encounter rate is enhanced compared to the transporter-substrate encounter rate in the null model.

Furthermore, in order to increase the BS-transporter encounter rate compared to the the substrate–transporter encounter rate, the effective substrate concentration $[BS]$ needs to be higher than the free substrate concentration $[S_o]$. At equilibrium of binding, the BS to free substrate ratio $[BS]/[S_o]$ equals $[B_{tot}]/(K_D + [S_o])$ (Eqn S7). Thus, the effective substrate concentration $[BS]$ is raised only when $[B_{tot}] > (K_D + [S_o])$. The factor α_{on} accounts for the fact that BPs and substrate molecules have different diffusion coefficients, and a slower diffusion of BPs reduces the encounter rate. The second term in Eqn (3) thus implies that the BS-transporter encounter rate is enhanced. Because BPs are larger than substrate molecules, they have a lower diffusion constant and hence $\alpha_{on} > 1$. An upper bound on α_{on} is deduced when the association rates are fully diffusion limited, which implies $\alpha_{on} < D_S/D_{BP}$, where D_S and D_{BP} are the diffusion coefficients of substrate and BP, respectively (for details see Doc. S1). For convenience, in this derivation, we have assumed a fixed periplasmic substrate concentration. However, Eqn (3) is also valid if we include transport over the outer membrane, and interpret $[S_o]$ as the extracellular substrate concentration (Doc. S1).

In summary, our hypothesis states that, when BPs are present at high concentrations and have a high affinity for their substrate, they increase the substrate–transporter encounter rate and thereby the uptake rate per transporter. This requires a simultaneous increase of the number of substrate binding sites per transporter and of the effective substrate concentration, as indicated by Eqn (3). These arguments are summarized in Fig. 1.

Binding proteins enhance transporter uptake rate at low substrate concentrations

We now turn to kinetic models of the binding-protein dependent and independent transport mechanisms. This allows us to simultaneously assess effective substrate concentration enhancement and the partitioning of Gibbs free energy of ATP hydrolysis in the catalytic

cycle. Below, the concentrations of components and enzyme forms in the model are expressed relative to the transporter concentration, and are denoted by lower case symbols. Thus, substrate, binding protein and transporter reaction intermediate concentrations are denoted as $x \equiv [X]/[T_{tot}]$. The specific uptake rates (per transporter) in the BPD and null-models are denoted by j and j', respectively, and the binding constant of substrate to BP is expressed as $\kappa_D = K_D/[T_{tot}]$. Using these definitions a dimensionless version of Eqn (3) was formulated that allows an easier comparison of transporters [see Eqn (S8) in Doc. S1]

To compare the steady state uptake rate of the BPD-model relative to the null-model, we consider the models discussed above for a particular set of parameter values, which we discuss below. We choose these parameters such that Eqn (3) is satisfied, and to loosely reflect some assumptions on typical properties of condition BP-dependent uptake systems function under. However, the following results do not qualitatively depend on these particular choices, which we illustrate later by random parameter sampling. We use a BP to transporter ratio of 10 (i.e. $b_{tot} = 10$), $s_o = 1/100$ and $s_i = 1$. We set $K_{eq} = 1000$, which corresponds to $\Delta\mu^0 = -17$ kJ $\cdot$ mol^{-1} (or approximately 60% of the total Standard Gibbs free energy of ATP hydrolysis to account for non-perfect efficiency). We assume that the binding of substrate to a binding site on a binding protein or on the transporter are biophysically similar processes and hence set the rates of formation of BS and TS complexes equal ($k'_{1f} = k_{1f}$). We also assume that the translocation of substrate across the membrane is a similar process in both transporters and hence set $k'_{2f} = k_{3f} = 1$. By choosing the parameters in this way, both models have the same maximal turnover (at infinite substrate and BP concentrations), which allows for a fair comparison between these models. The forward rate constants $k_{1f} = 20$ and $k_{2f} = 5$, which reflects the assumption that that binding of substrate to BP faster than that of BS to transporters, which in turn is faster than transport over the membrane. Finally, we set $k_{4f} = 5$. These parameters and forward rate constants are kept fixed. Subsequently, the backward rate constants (the four k_{ir} and two k'_{ir}) that maximize the steady state uptake rates j and j' of the BPD and null-models under the constraint (1) are calculated. This optimization simulates an evolutionary process whereby kinetic constants are adjusted to maximize the rate per transporter.

Under these conditions and with the rate constants given above, the BP-dependent transporter has a 3.7-fold higher steady state uptake rate (i.e. $j = 0.44$ and $j' = 0.12$).

Changes in the rate constants occur upon evolutionary time scales, whereas a transport system should be able to function for a range of substrate concentrations. We therefore assessed the influence of s_o on the relative performance of the models. We did this by calculating the uptake rates as functions of s_o but otherwise keeping all the parameters the same (i.e. without re-optimizing the backwards rate constants; Fig. 3). Figure 3 shows that, at low substrate concentrations, the uptake rate attained by the BP-dependent system is higher than that of the null-model, whereas with increasing s_o, at some point, the null-model becomes more efficient, which is also what we expected based on equation (3). This holds when the uptake rate per transporter (Fig. 3B, solid green line) as well as the uptake rate per total protein (dashed, yellow line), is considered. In the calculation of the specific rate per total protein we assume that the amount of protein in the BP is 20-fold smaller than in the transporter complex, i.e. the protein-specific rate equals $j/([b_{tot}/20] + 1)$. Thus, the BP-dependent system has a higher affinity for substrate but a slightly lower maximal uptake at saturating substrate concentrations. The same pattern is observed when an outer membrane transport step is included in the model, or when alternative reaction schemes are used, indicating that our results are not dependent on details of the model. (c.f. Doc. S3 and Fig. S3). Similar results are obtained when a lower free energy input is used (Fig. S1) and when the k_{ir}'s are optimized for each value of s_o (Fig. S2).

The risk of drawing conclusions that depend on particular parameter choices can be eliminated by comparing the steady state uptake rate of the BPD-model and the null-model in a large set of models with random parameters. We also calculated the relative substrate–transporter encounter rate [Eqn (S5)] for each of these models. This allows us to asses the validity of Eqn (3). The k_{if}'s for the BPD-model as well as s_o, s_i and b_{tot} are randomly drawn from a broad, log-normal distribution.

For every random parameter combination, the k_{ir}'s are again calculated such that they maximize the steady state uptake rate j under the constraint (1). Because by definition $\alpha_{on} = k_{1f}/k_{2f}$ and $\kappa_D = k_{1r}/k_{1f}$, we can calculate both the relative uptake rate, j/j', and relative substrate–transporter encounter rate. Figure 4 shows a scatterplot of the relative uptake rate against the estimated relative substrate–transporter encounter rate. It is clear that increased encounter rates lead to higher relative uptake rates, and that indeed $j > j'$ only when condition (3) is satisfied. The random sampling results are in good agreement with our hypothesis; Irrespective of model details, BPs can enhance

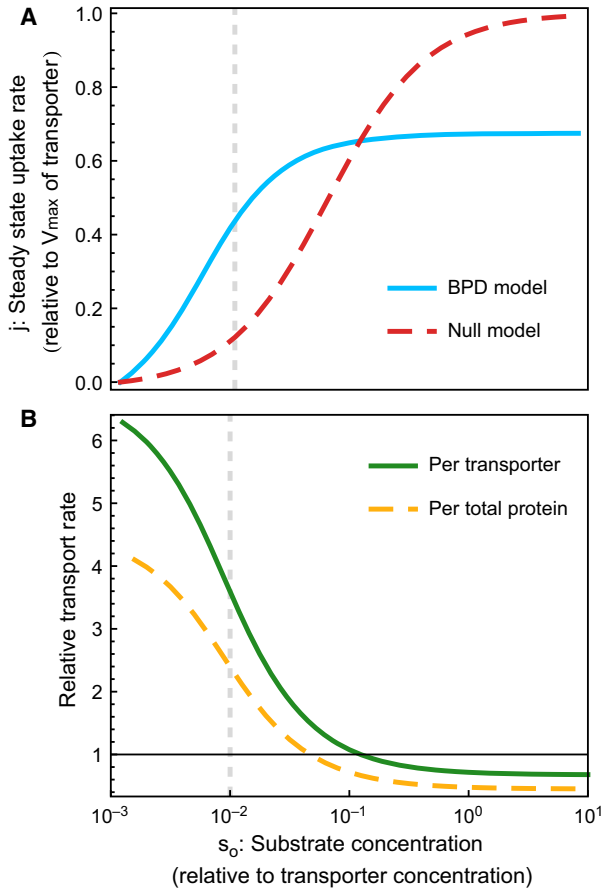

Fig. 3. Binding proteins enhance uptake at low substrate concentrations. (A) The steady state specific uptake rate of the BP-dependent system (j, blue, solid line) compared to the null-model (j', red, dashed line), and (B) the ratio between them (green, solid line). At low substrate concentrations, j is significantly higher j'. Also, the uptake rate per total invested protein (i.e. transporter and BP combined, where it is assumed that a BP protein is 1/20-th the size of the transporter proteins) is higher (dashed, yellow line), albeit less so. As expected, at high substrate concentrations BP-dependent uptake is less efficient than in the null-model. The vertical dashed gray line indicates the b_{tot} for which the model was optimized. The following parameters were used: $b_{tot} = 10$, $s_i = 1$ and $\Delta_r\mu^0 = -5.8$ kJ·mol^{-1}·K^{-1} (corresponding to $K_{eq} = 1000$). For the BPD-model, the forward rate constants are $k_{1f} = 20$, $k_{2f} = 5$, $k_{4f} = 5$. The reverse rate constants were obtained by optimizing j at $s_o = 1/100$ under constraint (3). This gives $k_{1r} = 0.23$, $k_{2r} = 2.1$, $k_{3r} = 1$ and $k_{4r} = 1$. For the null-model $k'_{1f} = 20$ and optimizing j' yields $k_{1r} = k_{2r} = 0.14$.

uptake rate but only when their concentration is sufficiently high. This conclusion even holds if the reverse rate constants are not optimized but randomly sampled (Fig. S4). The derivation of the relative substrate–transporter encounter rate are provided found in Doc. S1, and details of the sampling procedure are provided in Doc. S4.

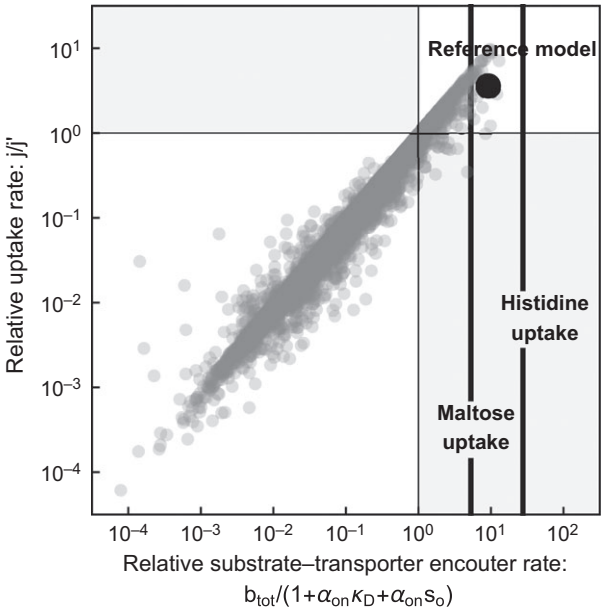

Fig. 4. Parameter sampling confirms estimated minimally required BP concentration to enhance uptake. For randomly sampled parameters, the steady state uptake rate of the BPD-model, j, relative to that of the null-model, j', is plotted against the substrate–transporter encounter rate. Note that the parameter sampling spans many orders of magnitude. The vertical axis indicates $b_{tot} = 1 + \alpha_{on}\cdot(\kappa_D + s_o)$ and the horizontal axis $j = j'$. The relative uptake rate correlates very well with relative substrate transporter encounter rate. Because the latter depends on the concentration and other general characteristics of the BPs, this can be used to find the requirements for enhanced uptake by BPs, which is: $j > j'$ or $b_{tot} > 1 + \alpha_{on}\cdot(\kappa_D + s_o)$ (c.f. Eqn (S8) in Doc. S1). Regions when this requirement is not true (i.e. $j > j'$ when $b_{tot} < 1 + \alpha_{on}\cdot(\kappa_D + s_o)$ and *vice versa*) are gray. Less than 4% of the 10 000 samples are within the gray regions. Moreover, there is no instance were $b_{tot} < 1 + \alpha_{on}\cdot(\kappa_D + s_o)$ when $j > j'$, indicating that it is a very strict condition. Interestingly, the BP dependent maltose uptake system in *E. coli* and histidine uptake system in *S. typhimurium* are well within the region of enhanced uptake. (Estimation of the relative encounter rates for these systems is indicated by vertical lines. For details, see Discussion). The model used in the rest of the study, which represents biologically plausible parameters, is indicated by a black dot. The sampling procedure is described in the main text and in Doc. S4.

Binding proteins enhance affinity but this is not the result of strong BP-substrate binding

It is often stated that BPs have a high affinity for their periplasmic substrate and that they therefore bind 'strongly' to the substrate. However, this cannot be the complete story, because there is no *a priori* reason why binding of substrate to the transporter could not be equally strong. To see this, it is instructive to consider the standard Gibbs free energy profile of the whole transport cycle of the optimized models. This is shown

in Fig. 5A,B. Indeed, for the BPD-model a large fraction of the total $\Delta_r\mu^0$ is invested in substrate to BP binding. Because $\kappa_D \equiv k_{1r}/k_{1f} = \exp(-\Delta_1\mu^0)$ this can be interpreted as an investment in strong substrate binding. However, a comparison with optimized *TS* dissociation constant $\kappa'_D \equiv k'_{1r}/k'_{1f} = \exp(-\Delta_1\mu^0)$ shows that, in the null-model, substrate binding is stronger; $\kappa_D = 0.1$ versus $\kappa'_D = 0.07$. This means that the increased effective substrate concentration of the BP-dependent system should not simply be attributed to the strong substrate-BP binding, but rather to the high BP concentration.

A further illustration of this view is that the K_M (i.e. the substrate concentration for which the half-maximal uptake rate is attained) of BP-dependent uptake strongly decreases with b_{tot}, as shown in Fig. 6A. High BP concentrations are required to reduce the K_M. Because this reduction is achieved by increasing the effective substrate concentration, it is the BP concentration that is at least equally important as its affinity. At very high BP concentrations, the transporter is saturated with BP-complex and no further increase in affinity and uptake can be attained by further increasing b_{tot} (Fig. 6). At this point, the uptake rate per total protein will start decreasing as the extra protein burden of adding BPs outweighs the increase in uptake rate.

The importance of the high BP concentration is further underscored by contrasting the driving force, or (molar) Gibbs free energy differences, $\Delta_i\mu$, at each transport cycle step with the standard Gibbs free energy, the $\Delta_i\mu^0$'s. [The $\Delta_i\mu$ values take into account the concentrations of the reaction partners, e.g. $\Delta_1\mu_r \equiv \Delta_1\mu_r^0 + RT\ln(bs/b \cdot s_o)$]. Figure 5C,D shows the Gibbs free energy profile at steady state of the BP-dependent and the null-model, respectively. Specifically,

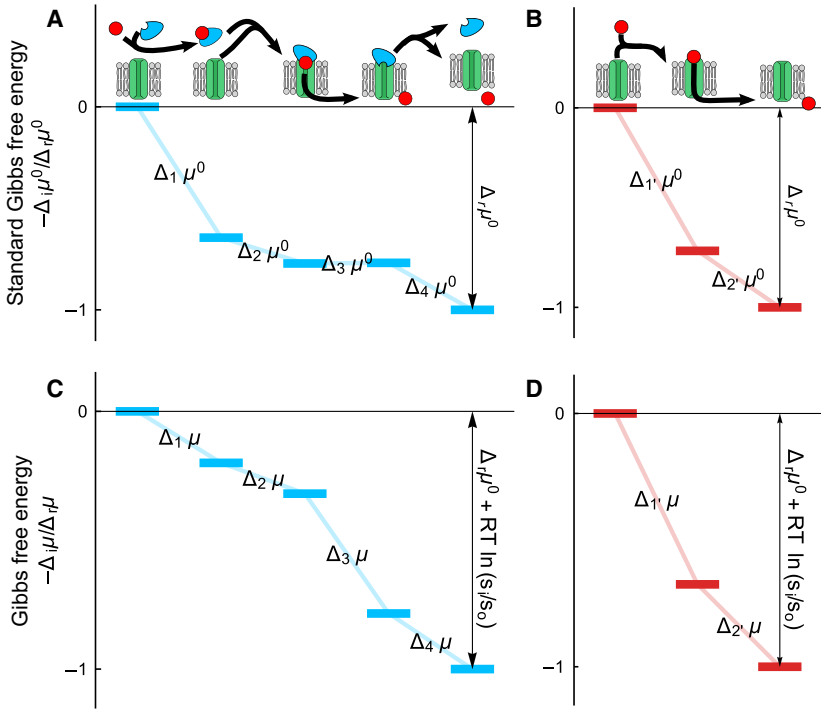

Fig. 5. Gibbs free energy profiles of optimized systems show optimal energy 'investment'. (A, B) The standard Gibbs free energy profile of the BPD (A) and null (B) models along the transportation cycle. Models were optimized for $s_o = 1/100$ by optimizing reverse rate constants under the constraint (1). In both cases, a large 'investment' is made in the strong binding of substrate to the BP or transporter, as can be seen from the large $\Delta_1\mu^0$ and $\Delta'_1\mu^0$. Note that the standard Gibbs free energy is an intrinsic system property and is independent of the extracellular substrate concentration. The $\Delta_i\mu^0$ values are defined according to Eqn (2). (C, D) The Gibbs free energy profile of the same transporters (taking into account the reaction partners), when they are at steady state (with $s_o = 1/100$). Despite the approximately equally strong binding of the substrate to BP and transporter, the driving force remaining for transport is much higher for the BP-dependent system (compare $\Delta_3\mu+\Delta_4\mu$ with $\Delta'_2\mu$). Because the high BP concentration makes the first two steps in BP-dependent transport relatively fast, these steps are relatively close to equilibrium, leaving a larger part of the driving force for the transport step. Indeed, this effect is much more pronounced when b_{tot} is increased and less pronounced when b_{tot} is decreased, as shown in Fig. S7, illustrating the relevance of the BP concentration. $\Delta_1\mu_r \equiv \Delta_1\mu_r^0 + RT\ln(bs/b \cdot s_o)$, $\Delta_2\mu_r \equiv \Delta_2\mu_r^0 + RT\ln(tbs/t \cdot bs)$, $\Delta_3\mu_r \equiv \Delta_3\mu_r^0 + RT\ln(s_i \cdot tb/tbs)$ and $\Delta_4\mu_r \equiv \Delta_4\mu_r^0 + RT\ln(b \cdot tf/tb)$.

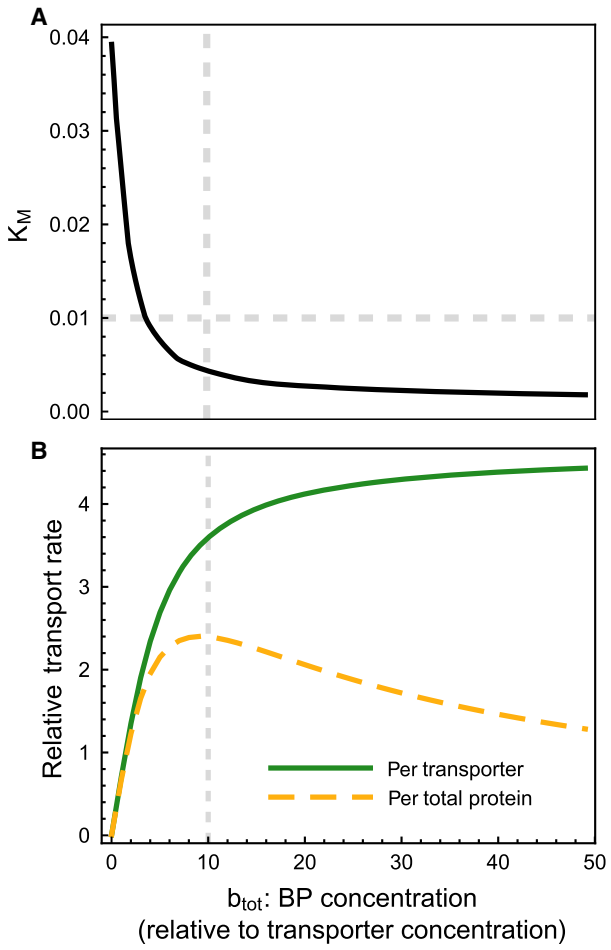

Fig. 6. High binding protein concentrations increase affinity. (A) The K_M as a function of b_{tot} of the BPD-model. K_M is defined as the s_o for which the half maximal uptake rate is attained, in the absence of intracellular substrate ($s_i = 0$). The affinity increases strongly with increasing b_{tot}. This emphasizes the interpretation that BPs enhance affinity not by binding the substrate strongly, but , instead, as a result of facilitating substrate to transporter binding by increasing binding site and effective substrate concentration. At some point the transporters are saturated with BP, and the decrease in K_M levels off. (B) Green line: specific uptake rate relative to the null-model, j/j', as function b_{tot}. This increases hyperbolically, leveling off at the approximately the same b_{tot} as the K_M. Yellow line: steady state uptake rate per total amount of protein, $\frac{j/(1+b_{tot}/20)}{j'}$, where it is assumed that a BP protein is 1/20-th the size of a transporter protein. As the increase in j/j' starts leveling off this starts decreasing, because the gain in uptake rate of an extra BP is less then the cost. Vertical and horizontal dashed gray lines indicate the b_{tot} and s_o for which the model was optimized, respectively. The same parameters as those shown in Fig. 3 were used.

the driving force of the actual transport (and BP release) step in the BPD-model $\Delta_3\mu+\Delta_4\mu$ is much larger than for the null-model, $\Delta'_2\mu$. Our result showing that, in the null model, the majority of the free energy is invested in substrate binding appears to be at odds with previous analysis on the basic energetics of transport catalysis, in which it has been argued that it is unfavorable to invest the majority of the free energy in substrate binding [33,34]. The argument is that when most free energy is invested in substrate binding, there is no driving force remaining for translocation over the membrane and this step therefore becomes slow and inefficient. However, this argument is heuristic and qualitative, whereas our results are quantitatively derived from basic kinetics mechanisms, taking into account thermodynamic constraints. Specifically, the argument does not take into account that, at low substrate concentrations, substrate to transporter binding becomes limiting, and it is more favorable to invest in this step at the expense of translocation of the substrate over the membrane. Indeed, for both models, the fraction of free energy invested in substrate binding decreases with the substrate concentration, when substrate binding becomes less limiting (Fig. S5).

The reason is that a high concentration of BPs facilitates TBS formation, making the first two steps relatively fast and thus close to equilibrium. So, despite the relatively large standard Gibbs free energy differences in the binding process, at steady state, the Gibbs free energy difference invested in binding is relatively low. As a consequence, a higher fraction of the total driving force remains for the actual translocation step. This is also reflected in the higher saturation of the transporter with BPs (Fig. S6). The size of this effect crucially depends on the BP concentration, it is much stronger for very large b_{tot} and much weaker for relatively low b_{tot} (Fig. S7).

Discussion

Here, we have argued that binding proteins function to enhance the cellular nutrient uptake rate when nutrients are scarce. This is achieved by increasing the effective substrate concentration, and hence the rate at which substrate molecules bind to transporter proteins, allowing these to function more efficiently. This result is independent of the mechanistic details of the transport system. It also hints towards an explanation for the absence of low free energy input-BP-dependent transport systems; BPs are only beneficial when the extracellular nutrient concentration is very low, and therefore high free energy input is required to overcome the the large concentration gradients. Conversely, under conditions where a high free energy input is required (because the extracellular nutrient concentration is very low), BPs are also required to

attain a reasonable uptake rate, which might explain the absence of ATP-driven, BP-independent transport systems. Because it is not possible to make a sensible experimental comparison between binding protein dependent and other active transport systems, this hypothesis is difficult to directly test experimentally, which is why we resorted to models of kinetic mechanisms of BP-dependent and independent transport. Our analysis obeys basic kinetic and thermodynamic principles. We have discovered several quantitative requirements on the binding characteristics and concentrations of the binding proteins [e.g. those stated in Eqn (3)] that specify important characteristics of BP-dependent systems. These we consider as experimentally testable predictions.

It is interesting to note that the requirements are found to be in line with what is generally known about binding proteins, such as the high affinity for their substrate (typically in the nM to μM range [5]) and high concentration compared to transporter proteins [6,35] that appear to saturate the transporters [1]. A recent study found that in *E. coli* all binding proteins are synthesized at much higher rates than the membrane bound transporters [36]. In the case of Gram-positive bacteria, where BPs are anchored by lipids to the cell membrane, very high BP to transporter ratios cannot be attained. Still, there are typically at least two BPs per transporter complex, which is in line with our hypothesis [29]. Our hypothesis can be falsified by compiling concentration measurements of many different BPs and transporters, as well as K_D measurements of these BPs, and testing whether they meet the condition for increased substrate encounter rate [Eqn (3) or (S8)]. Unfortunately, quantitative data on BP to transporter ratios, absolute transporter concentrations, and the rates of BP to transporter binding, which are required to calculate b_{tot} and α_{on}, are currently lacking.

For the maltose uptake system in *E. coli* and the histidine transport system in *Salmonella typhimurium* sufficient data are available to make sensible estimates of all relevant quantities. In *E. coli*, there are 500–1000 copies of the maltose transporter per cell and 3–4×10^4 copies of the maltose binding protein (MBP) per cell [6]. With a periplasmic volume of 0.13 μm^3 (BioNumber ID:100012) [37] this gives a periplasmic BP concentration of 380 μM and a transporter concentration of 12.5 μM and hence $b_{tot} = 30$ (using the most conservative estimate of 1000 transporters and 3×10^4 MBPs per cell). Because the K_D of maltose-MBP binding is 1 μM [6], we have $\kappa_D = 0.08$. The value of α_{on} is more difficult to estimate because data on the rate of BP to transporter binding are lacking. We can, however, use estimates of the diffusion constants based on the molecular weight of maltose and MBP to establish an upper bound of α_{on}. Empirically, the cytoplasmic diffusion constant D and molecular weight W are related through $D \propto M^{-0.7}$ [38]. Because $M_{MBP} =$ 42.5 kDa and $M_{maltose} = 0.35$kDa, we find that $\alpha_{on} = 29$. For substrate concentrations in the order of the K_D, this gives $j/j' \approx 5.5$, (i.e. a more then five fold increase in substrate–transporter encounter rate). Interestingly, the MBP-independent system becomes favorable when $j/j' < 1$, which happens maltose at concentrations higher than 140 μM. We emphasize that we consistently used the most conservative estimations. Especially the assumption that the binding processes are diffusion limited might lead to a severe underestimation of the true benefit of BPs. Substrate binding actually appears to be almost two orders of magnitude slower than the diffusion limit [39], making a value of $\alpha_{on} \approx 1$ more realistic. This would imply $j/j' \approx 25$ for maltose concentrations in the micromolar range, and BP-dependent transport would outperform the null-model as long as the maltose concentration would be below 4.5 mM. Hence, the stoichiometric and kinetic properties of the maltose uptake system in *E. coli* are clearly in line with our hypothesis. Similarly, the concentration of the histidine binding protein HisJ in *S. typhimurium* is between 1–10 mM and exceeds the transporter by more than 30-fold, (i.e. $b_{tot} = 30$) [35]. Assuming that the K_D is the same as for HisJ-histidine binding in *E. coli* (40 nM, [5]) we get $\kappa_D = 1.2 \cdot 10^{-3}$. Since $M_{HisJ} = 28$ kDa and $M_{histidine} = 0.16$ kDa, the most conservative estimate gives $\alpha_{on} = 37$. For a histidine concentration in the order of the K_D this gives $j/j' = 27$ and $j > j'$ for histidine concentrations below 866 μM. The estimation of j/j' for these transport system are indicated in Figs 4 and S4 as vertical lines.

Our hypothesis requires strong substrate to BP binding, implying the need of indirect investment of energy from (e.g. ATP-hydrolysis) in tight substrate binding to make this process thermodynamically favorable. Although this is thermodynamically definitely possible (Doc. S2) we have not discussed how this might by implemented mechanistically. One scenario is that ATP-hydrolysis is partially used to energize the BP. Interestingly, in ATP-binding cassette transport systems, BPs can have an open conformation, where the binding site is exposed and substrate can easily bind, and a closed conformation where the substrate binding site is much less accessible. There is a substantial body of evidence that BPs are typically open in the absence of substrate and that closing of the protein is induced by substrate binding, thereby making escape of the substrate unlikely [11]. How-

ever, thermodynamics dictates that in order for the closed conformation to remain closed when bound to substrate, it must have a lower energy (i.e. lower μ^0) than the open-conformation. One could speculate that when BP is bound to the transporter during the transport step, some free energy from ATP hydrolysis is invested in opening the BP conformation. There is an inherent risk to this strategy. In the absence of substrate, thermal fluctuations can cause BP in an open conformation to spontaneously switch to the closed state. Because there is a considerable free energy barrier between the open and closed conformations [40], the closing of BPs in the absence of substrate will be prevented. This scenario would also explain why the closed liganded and unliganded BPs bind to the transporter with equal affinity [35], which initially appears to be puzzling from a functional point of view. We speculate that to return the closed, unliganded BP to the open state, and thereby allow it to bind substrate again, ATP-hydrolysis at the transporter is required in a void transport cycle. Indeed, ligand free BPs stimulate ATP-hydrolysis by transporters *in vivo* [41].

Materials and methods

All calculations were preformed using MATHEMATICA, version 10 (Wolfram Research, Champaign, IL, USA). Model details are provided in Doc. S3. In summary, the rates depicted in Fig. 2 were modeled by mass action kinetics. Steady states were calculated using the NSolve or Solve function. Optimizations were performed using the FindMaximum function. A notebook containing the models and functions used to analyze them is available in Doc.S5.

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Author contributions

E.B., F.B, B.T. and D.M. conceived the ideas. S.M. and E.B. carried out the theoretical and simulation work. All authors wrote the paper.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Doc. S1. Derivation minimally required BP concentration.

Doc. S2. Investment of free energy in substrate-BP binding.

Doc. S3. Model definitions.

Doc. S4. Parameter sampling procedure.
Fig. S1. Model comparison at low energy input.
Fig. S2. Maximal uptake rate as function of substrate concentration.
Fig. S3. Comparison of alternative model topologies.
Fig. S4. Results of parameter sampling without model optimization.
Fig. S5. Free energy investment in substrate binding.
Fig. S6. Effect of $[B_{tot}]$ on effective substrate concentration and transporter occupancy.
Fig. S7. Free energy profiles for different $[B_{tot}]$.
Doc. S5. Model file.