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Uncoupled Active Transport Mechanisms Accounting for Low Selectivity in Multidrug Carriers: P-Glycoprotein and SMR Antiporters

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Abstract. The extraordinarily low substrate specificity of P-glycoprotein conflicts with the notion that specific substrate interactions are required in the control of the reaction path in an active transport system. The difficulty is shown to be overcome by a half-coupled mechanism in which the ATP reaction is linked to carrier transformations, as in a fully coupled system, but in which the transported substrate plays a passive role. The mechanism, which requires no specific interaction with the substrate, brings about uphill transport. A half-coupled mechanism is directly supported by two observations: (i) almost completely uncoupled ATPase activity in purified P-glycoprotein, and (ii) a pattern of substrate specificity like that of passive systems, where maximum rates for different substrates vary little (unlike active systems, where maximum rates vary greatly). The mechanism accommodates other findings: partial inhibition of ATPase activity by an actively transported substrate; simultaneous binding and translocation of more than one substrate molecule; and stimulation or inhibition of the transport of one substrate molecule by another. A half-coupled system associated with an internal competitive inhibitor should behave as if tightly coupled, in agreement with the effects of the synthetic peptide, polytryptophan. The degree of coupling in the intact system is yet to be determined, however. A half-coupled ATPase mechanism could originally have evolved in a flippase, where immersion of the carrier in its substrate, the membrane lipid, precludes uncoupled ATP hydrolysis. These concepts may have wider application. An uncoupled antiport mechanism, driven by a proton gradient rather than ATP, can explain low selectivity in the SMR multidrug carriers of bacteria, and a half-coupled mechanism for the ion-driven cotransport of water (the substrate in which the carrier site is immersed) can explain a recently proposed uphill flow of water.

Key words: Coupling mechanisms — Binding energy — Half-coupling — Flippase — Ion/water cotransport — Polytryptophan

Introduction

Resistance of cancer cells to cytotoxins can be brought about by P-glycoprotein, an ATP-driven pump that expels chemical agents used in chemotherapy [3, 14, 38, 47]. The system is unselective, most substrates never having been encountered by the organism before. Substrates differ widely in chemical structure, in molecular weight, and in shape; typically they are hydrophobic, amphipathic, and contain a planar ring system, but there are exceptions; most are positively charged but some are neutral. Oddly, two different substrate molecules can be bound at once [50], and the pair may be transported faster than either alone [38]. In the case of drug molecules and certain oligopeptides, the binding sites are separate but adjoining [42], which shows that interaction with a particular part of the translocation site is not required for coupling. Even chemosensitizers, which counteract the resistance mechanism by interfering with drug export, are probably transported substrates; their effective competition with other substrates may be due to their rapid diffusion, and recycling, within the membrane bilayer [12, 38].

The low specificity essential in a carrier of such wide-ranging capability is retained by the isolated pump and is therefore intrinsic. Clearly, the translocation step, in which the substrate is carried across the membrane, is insensitive to the structure of the substrate. Yet in active transport this step is expected to be specific. The reason it should be so is that control of the reaction sequence in a coupled process depends on systematic changes in the properties of the carrier, which have to be brought about

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through interactions with the substrates; a shift in the state of a carrier protein will depend on multiple interactions of the substrate at the binding site and should therefore involve complimentary structures and a high degree of specificity.

All things considered, the low selectivity of the multidrug carrier is puzzling. In contrast to a coupled system, a mechanism is called for in which carrier movement is little affected by the structure of the substrate, or even the number of substrates, occupying the translocation site. Two things, which may be connected, come to mind. First, in ordinary facilitated (i.e., passive) transport, the free carrier rotates in the membrane about as readily as the substrate complex does; it follows that the translocation step must be largely independent of the substrate and could, therefore, be unselective. Second, purified preparations of P-glycoprotein have almost completely uncoupled ATPase activity [42], from which it may be inferred that the processes of substrate movement and ATP hydrolysis are partly disengaged.

Could translocaton of the substrate be a passive step in a controlled ATP reaction sequence? This question will be explored here. From the principles underlying coupled and uncoupled transport, together with the implications for substrate specificity, we find that the pattern of specificity of P-glycoprotein is that of a passive, not a coupled, system. And we are able to show, from an examination of the kinetics of ATPase models, that a mechanism in which the transported substrate plays a passive role, accounting for low selectivity, can still pump the substrate uphill. With the translocation step passive and the substrate binding region large, there is no reason why two substrate molecules should not be handled at once, with either positive or negative interactions between them. The analysis reveals that with substrates of very low affinity the half-coupled system has a double advantage over a coupled system, eliminating the requirement for specific interaction and, in taking up the substrate, making full rather than partial use of available binding interactions. The analysis also shows that a half-coupled mechanism behaves as if tightly coupled if the driving reaction is blocked by an endogenous inhibitor that competes with the driven substrate.

Not all drug export systems are driven by ATP. There are comparably unselective carriers in bacteria, including the SMR family of multidrug efflux proteins, which are driven by a proton gradient. The electrogenic mechanism is antiport, with substrates exchanging for H^+ [15, 25, 29]. Here, too, a mechanism involving passive substrate movement is found to account for low specificity and uphill transport.

Active and Passive Transport Mechanisms

The passive glucose carrier and the tightly coupled chloride-bicarbonate exchange carrier (the anion exchanger), both of red cells, exhibit fundamentally different patterns of substrate specificity. With the first, the maximum rates for different monosaccharides are much the same, but the affinities are up to 9,000 times lower than with glucose; with the second, the affinities of various anions are little different from those of the true substrates, but the maximum rates are up to 10,000 times lower [20, 21].

In explaining the contrasting behavior, we should first be clear about the basis of coupling. An asymmetric mechanism, one with much lower substrate affinity on the unloading than the loading side of the membrane, avoids tying up the carrier as the product complex, but does not, by itself, produce a concentration gradient; as proof, a passive system, the carrier model for example, can be asymmetric. Nor is an intermediate in the reaction sequence that incorporates the energy of the phosphate bond of ATP necessary. On the contrary, high energy intermediates, which by definition are relatively unstable and therefore form slowly, as well as low energy intermediates, which are relatively stable and therefore react slowly, only depress the rate of turnover by forming bottlenecks in the reaction. The general conclusion is that the rate of transport or of enzyme catalysis is optimal if all intermediates are about equally stable, achieved by using substrate binding forces to adjust energy levels [1, 18].

Quite simply, active transport depends on a linkage between a driving process, poised at disequilibrium but seeking equilibrium, and a driven process, entangled in the first and dragged toward disequilibrium. For coupling, the two processes are combined in a single reaction sequence, whose free energy change is that for both. The transport protein is required to guide the reaction along the coupled path, cutting off uncoupled side reactions, and this depends on abrupt changes in specificity, mobility, and catalytic properties in the course of the reaction. Partial changes result in slippage, which is the uncoupled reaction, one process without the other. The coupled reaction scheme in Fig. 1 illustrates the point. For the substrates to add to the carrier in order—T first, S second—the specificity of the carrier must change: the free carrier binds only the first substrate, T, while the binary complex binds S and T. The mobility of the carrier is also required to change: the free carrier is mobile (it has the ability to turn about-face in the membrane); the complex with one substrate is immobile; and the complex with both substrates is mobile again. But for these shifts, the system would fail: if the free carrier or the ternary complex were immobile there could be no net transport, and if the binary complex were mobile, the system would be uncoupled—T would be transported in the absence of S.

Shifts in the state of the carrier, it can be shown, would have to be driven by changes in substrate binding energy. Once the substrate has added to the carrier in an

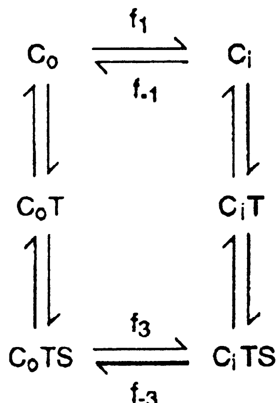

Fig. 1. A cotransport model for substrates T and S. The carrier C is a mixture of outward-facing and inward-facing forms, C_o and C_i, respectively. The substrates add in order, T followed by S. Coupling depends on the immobility of the binary complex, that is, on its inability to undergo an about-face in the membrane, C_oT to C_iT.

initial complex, secondary binding interactions can be used either to stabilize an altered carrier state or to catalyze the transformation of one state to another, such as the about-face of the carrier in the membrane. The larger the increment in binding energy, the more complete, or the faster, the change, and the higher the ratio of coupled to uncoupled rates. The relationship may be expressed as follows: [19, 21, 22, 23]:

$$Rate_{(coupled)}/Rate_{(uncoupled)} \leq K_{(initial\ state)}/K_{(final\ state)} \quad (1)$$

where $K_{(initial\ state)}$ and $K_{(final\ state)}$ are substrate dissociation constants before and after the transformation. The equation applies to primary active transport, secondary active transport, and exchange transport. Tight coupling is seen to depend on large increments in binding energy and, therefore, on an array of interactions, some in the initial complex, some in the second. Hence, bonding should be specific.

In a passive system, carrier movement can be independent of substrate binding forces. The reason is apparent in the carrier model in Fig. 2, where net transport depends on both the free carrier and the substrate complex being mobile. If the free carrier were immobile, the system would only catalyze exchange, the exit of one substrate molecule coupled to the entry of another, the rate of which will depend on the substrate's ability to catalyze the about-face of the carrier. Catalysis can be shown to depend on increased substrate binding energy in the transition state, and in this case $K_{(final\ state)}$ in Eq. 1 represents the virtual dissociation constant for the transition-state complex.

The contrasting patterns of specificity in the coupled anion exchanger and the passive glucose carrier can be understood in the light of these prinicples [20, 21]. In

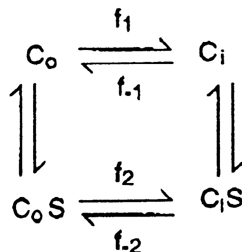

Fig. 2. The carrier model. For net transport, both the free carrier and the substrate complex must be mobile ($f_1 \approx f_2$ and $f_{-1} \approx f_{-2}$). If only the complex is mobile ($f_1 = f_{-1} = 0$) there is no net transport; inward and outward substrate movements are then coupled, and the system is an obligatory exchanger.

the passive system the rate of carrier movement is largely independent of the substrate, and therefore any needed specificity must be achieved in the binding step; hence good and bad substrates differ in affinity, not rate. In the coupled exchanger, movement of the carrier is catalyzed by the substrate, and the greater the increase in bonding energy in the transition state complex, the faster the rate (Eq. 1). Potential binding interactions should not be squandered in the first complex but should be used in the transition state, and as binding here does not affect the affinity, good and bad substrates should differ in rate, not affinity.

It would be wrong to conclude, however, that in passive transport the rate of carrier movement is absolutely fixed. On the one hand, large substrate analogues that protrude from the translocation site can sterically interfere with carrier movement. In the case of the choline system of red cells, an analogue slightly larger than the substrate may be transported slowly and have low affinity. A still larger analogue, containing a nonpolar substituent that is strongly adsorbed outside the substrate site proper, may have much higher affinity than the parent molecule and yet completely block the translocation step; it is then an inhibitor—a nontransported substrate analogue [8]. On the other hand, the true substrate, offering no interference, may increase the rate of carrier movement, making exchange faster than net transport. The acceleration factor is at least 2 for the glucose carrier [51] and at least 5 for the choline carrier [8], both in red cells; nevertheless, net transport rates for good substrates, being limited by movement of the free carrier, are similar. Obligatory exchange and facilitated transport systems are seen to differ only with respect to the relative mobility of the free carrier and the substrate complex. The implication is that active and passive systems are not fundamentally different, an idea in accord with the finding that active and passive carriers for the same substrate may belong to a single gene family and have similar amino acid sequences.

In all forms of coupled transport, not just exchange, rates depend on substrate binding forces. Consider, for

example, three possible models for active transport. Bear in mind that reaction schemes for the contransport of two substrates and for ATP-driven transport are kinetically equivalent [49], so that the models apply to both primary and secondary active transport. If the substrates add in order, as in the fully coupled scheme in Fig. 1, the first to add (T) causes the carrier to become immobile, and the second, the transported substrate S, has to catalyze the about-face of the carrier, just as it does in an exchange reaction; the specificity patterns should therefore be the same. If the order of addition is random, both substrates are required to catalyze the translocation step, with the same result. If the transported substrate S adds first, it must convert the carrier to an immobile form with an exposed site for T. The extent of the transformation depends on the strength of binding in the altered state, and the less complete the conversion, the smaller the proportion of the carrier able to bind T and the slower, therefore, the coupled reaction. Note that Eq. 1 for the ratio of coupled and uncoupled rates applies to a variety of transport models. It applies to both the driving and driven substrates, and to mechanisms in which the substrate stabilizes an altered carrier state or catalyzes the conversion of one state to another. It applies to models with substrate sites alternating between inner and outer positions, as in Fig. 1, and to those with sites stationed on both sided of the membrane; to those in which the driving and driven substrates add to the carrier in fixed order and those in which the addition is random; and to those in which the driving substrate is ATP and those in which it is a transported ion. In every case, rate is a function of an increment in binding energy and should therefore be sensitive to the structure of the substrate.

The Pattern of Substrate Specificity of P-Glycoprotein

In view of the contrasting patterns of substrate specificity in active and passive transport, the pattern exhibited by P-glycoprotein could be diagnostic. The affinities of various substrates have been reported, but not maximum transport rates. However, maximum ATPase rates have been measured in the presence of substrates [39, 42, 50], and these will be proportional to the transport rates, whatever the transport mechanism, coupled, partly coupled, or uncoupled. At saturating concentrations of both the transported substrate and ATP, when there can be no complex with either alone and therefore no uncoupled reaction, the ATPase reaction must cycle through inward-facing and outward-facing carrier states, involving movement of a carrier-substrate complex, which is the translocation step. The rates of the translocation step and of ATP hydrolysis, being parts of the same cycle, are equal, and it follows that the ATPase reaction should give a true measure of the maximum transport rate. Indeed, it should give a better measure than could be obtained in transport studies because net transport is slowed by any recycling of the lipid-soluble substrate in the lipid bilayer; some undetermined fraction of the substrate, once pumped outward, may diffuse back, to be pumped out again.

Table 1. ATPase activity of P-glycoprotein in the presence of various drugs [39]

Drug	K_D (μM)	$V_{(ATP,S)}$
N-acetyl-leucyl-leucyl-norleucinal	40	2.4
N-acetyl-leucyl-leucyl-methioninal	65	2.0
Leupeptin	100	2.0
Pepstatin A	68	2.3
Valinomycin	0.63	2.4
Cyclosporin A	0.01	1.4
Nonactin	25	2.5
Vinblastine	0.7	1.4
Verapamil	0.9	2.2

The protein (from Cinese Hamster ovary cells) is embedded in plasma membrane vesicles. The maximum rate of ATP hydrolysis in the presence of a saturating concentration of the drug, relative to the rate in its absence, is listed ($V_{(ATP,S)}$), as well as the half-saturating concentration of the drug in the stimulation of ATP hydrolysis (K_D).

Maximum ATP hydrolysis rates in the presence of a saturating drug concentration, relative to the rate in the absence of a drug, $V_{(ATP,S)}$, are listed in Table 1; the half-saturating drug concentration in the same assay, K_D, is also listed. The measurements were made with membrane vesicles from Chinese hamster ovary cells. The affinities vary by a factor of 10,000, the rates by a factor of only 1.8. Similar observations on the purified (and isolated) pump are listed in Table 2. Here maximum ATP hydrolysis rates, $V_{(ATP)}$, were determined in the presence of a high (but not quite saturating) concentration of a drug, relative to the rate in the absence of drugs. Half-saturating concentrations, K_D, determined by fluorescence quenching, are also listed. Rates are corrected according to the formula $V_{(ATP,S)} = V_{(ATP)} + (V_{(ATP)} - 1) K_D/[S]$, where $V_{(ATP,S)}$ is the relative ATP hydrolysis rate at saturating concentrations of both ATP and the substrate S; the assumption is that ATP hydrolysis proceeds along paths involving either the unloaded carrier or the substrate complex (in Fig. 3, the steps governed by f_{-2} and f_{-3}). The affinities vary by a factor of over 200, the rates by a factor of less than 4. Similar results on the purified protein were obtained in another laboratory, where affinities for a different group of compounds varied by a factor of 100 and maximum rates by a factor of less than 2 [50].

The pattern is unmistakably that of a passive rather than an active system, supporting the idea that the translocation step is passive. But will a half-coupled system move substrates against a concentration gradient? Can the driving process, even though free to proceed on its

Table 2. ATPase rates and substrate affinities determined with solubilized, purified P-glycoprotein from Chinese hamster ovary cells

Drug (S)	[S] (μM)	K_D (μM)	$V_{(ATP)}$	$V_{(ATP,S)}$
None			1.00	
Trifluoperazine	10	7.7	1.58	2.03
Colchicine	100	158	1.71	2.83
Daunorubicin	50	10.5	1.26	1.31
Vinblastine	20	0.77	0.80	0.79
Verapamil	10	2.4	1.37	1.46

Maximum rates of ATP hydrolysis ($V_{(ATP)}$) in the presence of transported substrates at defined concentrations [42] together with half-saturation constants (K_D) determined by fluorescence quenching [38] are listed. The last column shows the calculated maximum rate $V_{(ATP,S)}$ for a saturating concentration of both ATP and the substrate, S, according to the formula $V_{(ATP,S)} = V_{(ATP)} + (V_{(ATP)} - 1)\, K_D/[S]$.

own, sufficiently entangle the driven process to pump against a gradient? The question can be answered by kinetic analysis, which gives the final concentration gradient as a function of the free energy supplied in the driving reaction.

Uphill Pumping by Uncoupled Transport Systems

Half-Coupled Models for ATP-driven Transport

Obviously, no concentration gradient would be built up if the substrate could be translocated without in some way involving ATP, for the substrate, having been moved across the membrane in one direction, would freely move back again. The half-coupled model in Fig. 3 is feasible, since no complex is formed with the transported substrate S by itself. Here, the substrates add in order—first the driving substrate ATP (T), then S. All carrier forms are mobile, as in passive transport: the inward-facing and outward-facing states of the free carrier are interconvertible, as are the two states of the complex with ATP or of the ternary complex with ATP and the transported substrate. The addition of ATP does not convert the carrier to an immobile state, as it would in a fully coupled system, but it does cause the binding site for the transported substrate, unavailable in the free carrier, to become exposed. And the hydrolysis of ATP (T) to give ADP + phosphate (D) is coupled to a carrier transformation, an about-face of the carrier, inward-facing to outward-facing ($C_iT \rightarrow C_oD$ and $C_iTS \rightarrow C_oDS$). Transport can be unselective because S, not required to catalyze the translocation step, only has to occupy the carrier site. The scheme includes an inhibitor A inside the cell that adds in competition with S but not T. But for the inhibitor, the carrier would be an uncoupled ATPase in the absence of S. The inhibitor acts as a nontransported substrate analogue, and in blocking carrier movement, blocks the associated ATP reaction.

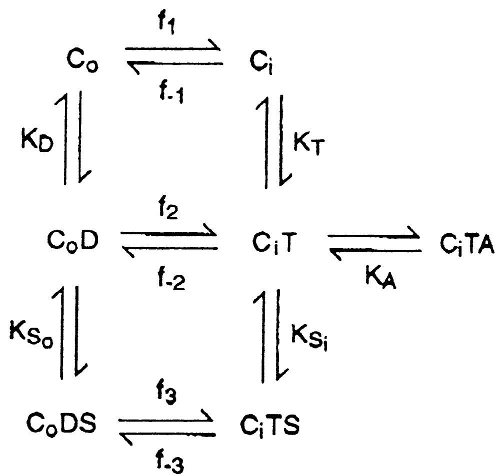

Fig. 3. A half-coupled model for ATP-driven exit of substrate S. Unlike a coupled system, all carrier forms are mobile. The mechanism is ordered: ATP, represented by T, binds to the inward-facing free carrier, C_i, while the hydrolysis products ADP + Pi, represented by D, bind to the outward-facing free carrier, C_o; only when these have added can the transported substrate, S, bind. Associated with the system is an internal inhibitor A, which is competitive with S and uncompetitive with T (A has the properties of a nontransported substrate analogue). The model is uncoupled in the sense that S does not alter the carrier state and is not required for carrier movement, but is coupled in the sense that the binding of ATP (or ADP + Pi) exposes the site for S, and that reaction of ATP at the ATPase active centre is linked to movement of the site for the transported substrate (C_i to C_o).

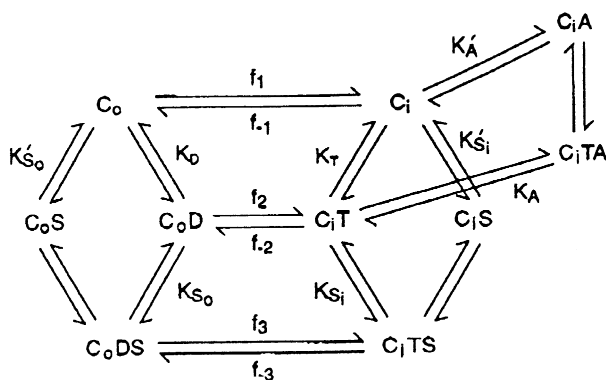

Fig. 4. A half-coupled ATPase model in which ATP (T) and the transported substrate (S) add in random order. The free carrier is assumed to be a mixture of two forms, one mobile and with no exposed substrate sites, the other immobile and with exposed substrate sites. The transported substrate plays a passive role, while ATP, in the same step in which it is hydrolyzed, catalyzes movement of the immobile form of the carrier. Associated with the system is an internal inhibitor A, competitive with S and noncompetitive with T.

An objection to the scheme in Fig. 3 should be recognized at once. ATP and the transported substrate have been observed to add independently to P-glycoprotein [27, 40, 41], which means that the order of addition is random. The related model in Fig. 4 meets the objection:

both S and T add to the free carrier, which is mobile, but the complex with S by itself is immobile. As the substrate is to play a passive role, the free carrier is now assumed to be an equilibrium mixture of two forms, one mobile but lacking exposed sites for ATP or S, the other immobile and having exposed sites. S and ATP bind to the immobile form, and ATP (or ADP + Pi) catalyzes the carrier reorientation step. As in the first model, the hydrolysis of ATP is coupled to an about-face of the carrier.

The analysis in the Appendix demonstrates that both models can pump the substrate S uphill and conserve ATP in the absence of S. Were coupling perfect, the final concentration gradient would depend only on the [ATP]/[ADP] ratio, which is the measure of the free energy available to do work (Eq. A8). The half-coupled models produce this same gradient if the affinity for ATP is very low, but in this case transport is very slow. At the other extreme, with very high affinity, transport becomes passive regardless of the ATP to ADP ratio (Eq. A9). Active transport fails because, with none of the carrier free, the reaction cycles through C_iT, C_iTS, C_oDS, C_oD (Figs. 3 and 4)—substrate exit involves ATP hydrolysis as usual (C_iTS to C_oDS) but the return of the carrier is accompanied by the resynthesis of ATP (C_oD to C_iT). With no net hydrolysis of ATP, no free energy is released and no osmotic work is done. An intermediate affinity would be best—something in the range of the cellular ATP concentration, where the rate and the concentration gradient can both be high (*see* Appendix).

An Uncoupled Model for Electrogenic Drug-Proton Antiport

Figure 5 shows an uncoupled carrier model for the passive transport, and exchange, of two substrates, S and H. All carrier forms are mobile—the free carrier and the complex with either substrate. For transport, nothing more than occupation of the carrier site is demanded (binding can be specific, but this is not a requirement of the mechanism). Associated with the carrier is an internal competitive inhibitor, A.

In a coupled exchange mechanism the free carrier would be immobile ($f_1 = f_{-1} = 0$) and substrates would have to catalyze carrier movement, with the consequences for specificity we have noted.

The analysis in the Appendix, with S representing the driven and H the driving substrate, shows that the uncoupled model can pump the substrate uphill. If the concentration of H is saturating, the gradient in S is the same as with a coupled system, (Eq. A21), but if the concentration is low, the proton gradient is poorly tapped and S tends to equilibrate (Eq. A22).

Slippage

The coupling ratio—the ratio of the maximum rate of coupled transport to the maximum rate of slippage—is a measure of the efficiency of energy conversion. Efficiency, it is found, always depends on intrinsic relative to apparent binding energies. In a half-coupled mechanism involving an endogenous inhibitor, the coupling ratio, Eq. A17, is a function of the actual substrate affinity relative to the apparent affinity measured in the presence of the inhibitor. A similar relationship is found for the uncoupled antiporter, Eq. A29. In either case, only part of the total binding energy shows up as affinity, the rest being used to displace the inhibitor. The inhibitor is responsible for coupling; the greater its concentration or affinity, the higher the coupling ratio. With a fully coupled mechanism the relationship between the coupling ratio and binding energy, Eq. 1, is similar in form, but its basis is different. Binding is a two-step process, part of the potential bonding energy being used in an initial complex, the rest in an altered complex. The increment, which drives a carrier transformation responsible for coupling, does not contribute to the apparent substrate affinity.

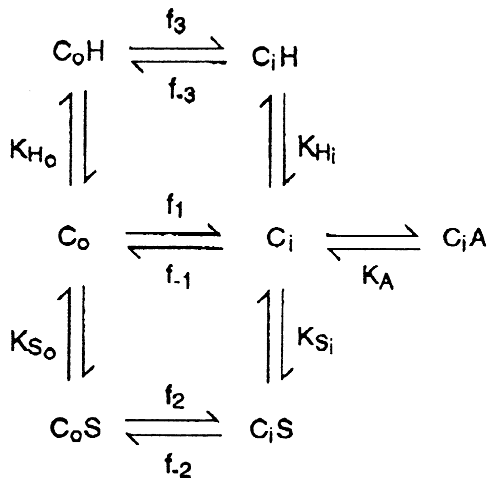

Fig. 5. An uncoupled antiport model for two transported substrates, H and S (H being a hydrogen ion). Associated with the system is an internal competitive inhibitor, A.

In spite of the similarities, the implications are different. In a half-coupled or uncoupled system, any bonding, including unspecific hydrophobic bonding, is sufficient, since mere occupation of the carrier site is needed; in a coupled system the substrate enters into a coordinated two-step process of bonding, dependent on complimentary structures of the substrate and binding site.

Usable Affinity: The Advantage of Loose Coupling

Tight coupling, while it conserves the energy of the driving reaction, has one disadvantage. The tighter the coupling, the lower the substrate's apparent affinity in relation to its intrinsic affinity, as we have just seen. Cou-

pling is paid for in the currency of binding energy, whatever the mechanism. It follows that if a system is required to pick up a wide variety of foreign chemicals, coupling efficiency might be sacrificed in the interest of usable affinity. In this light, a half-coupled mechanism with a very low coupling ratio has two advantages; first, it eliminates the requirement for specific substrate interactions, and second, it makes full use of available bonding interactions.

The Behavior of P-Glycoprotein

ATP and Transported Substrates Alter the Conformation of P-Glycoprotein

Conformational changes have been demonstrated in the intact system through changes in immunoreactivity in the presence or absence of substrates and ATP [17, 28]. In experiments with the purified protein, ATP, but not verapamil, caused a population of slowly exchanging amide hydrogens to become more accessible, while ATP and verapamil together caused a population of rapidly exchanging hydrogens to become less accessible [46]. And in experiments in which conformational changes were signaled by quenching of flurescence of the purified protein covalently labeled with a fluorescent probe close to the ATP site, ATP and substrates were found to have independent and additive effects [27].

These observations are not inconsistent with a passive role for the transported substrate, for in any system a substrate can induce a conformational change by shifting the partition between outward-facing and inward-facing carrier forms. It will do so if the dissociation constants of the outer and inner binding sites differ, and in an active transport system, binding should be stronger on the loading than on the unloading side of the membrane. In the case of P-glycoprotein, the affinity should be higher inside. In Fig. 2, which is a passive carrier model, the relationship between the constants in the cycle of reactions is $K_{si}/K_{so} = (f_1/f_{-1})/(f_2/f_{-2})$; if $K_{si} < K_{so}$, then $f_1/f_{-1} < f_2/f_{-2}$, where the partition of the free carrier is governed by f_1/f_{-1}, the partition of the complex by f_2/f_{-2}.

Another way for a passively transported substrate to shift the carrier conformation is implicit in the reaction scheme in Fig. 4. The free carrier here is a mixture of two forms, one mobile, with no exposed substrate sites, the other immobile, and with exposed substrate sites. Either substrate, therefore, converts all the carrier to the latter form.

The Substrates Add to the Carrier in Random Order

ATP is hydrolyzed by P-glycoprotein in the absence of substrates, and many studies of substrate binding have been carried out in the absence of ATP. Fluorescence assays have confirmed that the binding of ATP and drugs is independent, and have also shown that the affinity of substrates is not significantly altered by prior addition of ATP [27, 40]. The random mechanism in Fig. 4 agrees with the observations.

Substrates Alter ATP Hydrolysis Rates

While most substrates slightly accelerate ATP hydrolysis, vinblastine inhibits the activity of the purified protein by 20% (Table 2), and gramicidin D, an unusually large substrate, inhibits in proteoliposomes by 50% [6]. Again the model in Fig. 4 can explain the findings. In the absence of S the rate is governed by f_{-2}, and in the presence of S by f_{-3}; and depending on which is larger, the rate may rise or fall. (It will be remembered that even in passive systems a substrate can raise or lower the rate of the translocation step). An inhibitory allosteric site is probably not involved, for while vinblastine inhibits the carrier from Chinese hamster, it accelerates that from humans [4].

The Carrier Binds and Transports More than one Substrate Molecule at a Time

P-glycoprotein carries drug molecules that differ widely in size and structure, and also leucyl oligopeptides varying in length from L2 to L5 [43]. And one substrate may accelerate the reaction of another. N-acetyl-leucyl-leucyl-tyrosine amide (L2) stimulates the transport of colchicine, and colchicine stimulates transport of the peptide. On the other hand, peptides do not stimulate the reaction of vinblastine, and some inhibit (35% inhibition by L4). Colchicine, verapamil, and cyclosporin stimulate L2 transport at lower concentrations but inhibit at higher concentrations; vinblastine inhibits at all concentrations while methotrexate is without effect. From studies of the labeling of the transporter with azidopine and tamoxifen aziridine it is concluded that the binding region occupied by verapamil, azidopine, or colchicine does not overlap the peptide site, while the region occupied by cyclic peptides, cyclosporin A and valinomycin, does. The nonuniformity of the binding regions is emphasized by other studies showing that Hoechst 33342 and rhodamine occupy separate regions, which vinblastine and actinomycin D overlap [36]. It appears that the mobile region in P-glycoprotein comprises two main areas, one large, nonpolar, and unspecific, where drugs are absorbed; the other smaller and more specific, where oligopeptides are absorbed. The two areas must be linked, in view of the mutual transport stimulation of a peptide and colchicine, and they are probably adjacent, judging by the overlap of the peptide site by cyclosporin A and valinomycin.

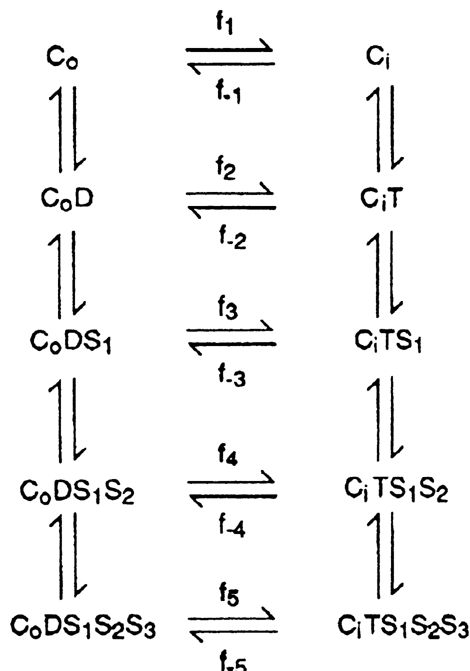

Fig. 6. A half-coupled ATPase model, based on that in Fig. 3, which allows for the binding and transport of more than one substrate molecule at a time, S_1, S_2, and S_3.

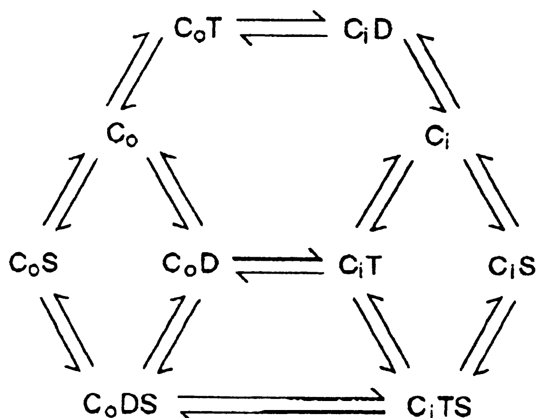

Fig. 7. A half-coupled ATPase model involving reaction of two ATP per carrier cycle. The substrates add to the carrier in random order, and the transported substrate, *S*, plays a passive role. Both the free carrier and its complex with *S* are immobile; ATP (*T*) catalyzes the about-face of the carrier as it undergoes reaction, both outward movement (C_iTS to C_oDS and C_iT to C_oD) and, completing the cycle, inward movement (C_oT to C_iD).

In a half-coupled mechanism, where no specific substrate bonding interactions are required, a variety of nonpolar molecules could be taken up and moved if the binding site is large and nonpolar. Hydrophobic bonding depends on the avoidance of nonpolar surfaces by water (since water prefers to bond with water), rather than attraction between nonpolar surfaces, and is therefore the least specific force for complex formation. The unusual structure of P-glycoprotein [14], composed of two nearly identical halves, may explain the ability to absorb more than one large substrate molecule at a time. Indeed, the N- and C-terminal halves of the protein have been shown to bind a photoactive substrate derivative independently [9].

An expanded half-coupled model is shown in Fig. 6 (for the sake of simplicity, the scheme is based on the ordered mechanism in Fig. 3). The model allows three substrate molecules to bind, S_1, S_2, and S_3. The exit reaction is governed by f_{-3}, f_{-4}, and f_{-5}. Where $f_{-3} < f_{-4}$, each substrate stimulates reaction of the other. Where $f_{-3} > f_{-4}$ each substrate inhibits the other. In the case of a drug that at rising concentrations first stimulates and then inhibits peptide transport the relationship is $f_{-3} < f_{-4} > f_{-5}$. The basis of inhibition or stimulation could be, respectively, crowding (steric hindrance), or facilitation through positive interactions, in the course of the translocation step.

This model, which lacks allosteric sites but allows more than one substrate molecule to add in the same binding region, can account for complex interactions among substrates, including negative cooperativity (e.g., vinblastine with certain dihydropyridines [13]) and positive cooperativity (e.g., enhancement of azidopine binding by prenylamine [30], and enhancement of tamoxifen aziridine binding by colchicine [31]). It also accounts for noncompetitive inhibition by vanadate of the verapamil stimulation of ATPase activity [26].

P-glycoprotein has Two ATPase Sites, Both Functioning in Transport [38]

The kinetics of the ATPase are Michaelis-Menten, as if a single ATP reacts [10, 43], and accordingly, if 2 ATP are consumed per cycle, the reactions would be consecutive. The role of the two ATPase sites is uncertain, however. In the scheme in Fig. 7, two ATP molecules react at different stages of the transport cycle. The behavior will be similar to that in Fig. 4, since both schemes involve random addition of ATP and the transported substrate, but the underlying mechanisms differ: in Fig. 7 the free carrier is immobile and all carrier movement is catalyzed by ATP or ADP.

Km for ATP is Unusually High, in the Range of the Cellular Concentration, 0.4 – 0.8 mm

Low ATP affinity in a loosely coupled P-glycoprotein could have the function of limiting the waste of ATP at low ATP concentrations, when systems with higher affinity would be favored. Or the half-coupled mechanism itself may dictate this condition: in the case of the model

in Fig. 4, transport was seen to be passive if the affinity is very high, and to be active but very slow if the affinity is very low; in between, the rate and concentration gradient can both be sizable. But it must be remembered that the model in Fig. 4 is incomplete, for two ATPase sites, not one, are involved in transport. The model in Fig. 7, with two ATP reacting, transports actively when the affinity is high, though other 2-ATP models may be imagined that behave in the same way as the model in Fig. 4.

Stoichiometry

For several reasons the true stoichiometry—the number of ATP molecules hydrolyzed for each substrate molecule transported—is not easily determined: substrates can recycle in the membrane bilayer; more than one drug molecule may be bound, depending on the concentration; and—a point that must now be emphasized—the system is at least partly uncoupled. Widely varying values, as high as 50, have been reported [34, 38]. An ingenious solution to the problem was provided by Eytan et al. [11] who measured the entry, into proteoliposomes, of rubidium ions bound to the transported substrate valinomycin. Valinomycin can recycle, but not the ion. The difficulty of accounting for uncoupled ATPase activity, in the absence of the transported substrate, was overcome by means of polytryptophan, whose inhibition of the P-glycoprotein ATPase is reversed, competitively, by valinomycin. In these experiments the stoichiometry was found to be between 1.2 and 2. The same result was obtained in an experiment that measured the parallel reductions in Rb^+ transport and ATP hydrolysis brought about by an ATPase inhibitor, vanadate or oligomycin, which does not compete with valinomycin. The reaction scheme in Fig. 4 accounts for a value of 1, that in Fig. 7 for a value of 2. The full complexity of the mechanism, however, would require us to consider the possibility that, on average, more than one substrate molecule may be bound and transported.

A half-coupled mechanism, by itself, complicates matters. In another no less careful study, the stoichiometry was calculated from rates of vinblastine transport in intact cells relative to rates of vinblastine-stimulated ATP hydrolysis in crude membranes [2]. The pump was assumed to be tightly enough coupled to contribute nothing to the basal ATP hydrolysis rate; hence, the ATPase activity of the pump could be taken as that stimulated by vinblastine, contrary to the finding of Eytan et al. [11] with the more highly purified preparation. By calculation, 2.8 molecules of ATP were hydrolyzed for each molecule of vinblastine transported, which is in reasonable agreement with a stoichiometry of 1.2 to 2. But compensating errors are a strong possibility: too low an estimate of ATP hydrolysis, due to partial uncoupling in the membrane preparation, balanced by too low an estimate of transport, due to vinblastine recycling in the membrane.

Coupling

The coupling ratio—the ratio of ATP hydrolysis rates in the presence and absence of a transported substrate, which is a measure of the tightness of coupling—has never been found to be high, as it should be in a tightly coupled system. In an ideal uncoupled system, with the substrate having no effect whatever on the rate of ATP hydrolysis or carrier movement, the ratio of rates in the presence and absence of the substrate would be unity, but as noted earlier, substrates can significantly raise or lower rates even in facilitated transport. A reasonably unambiguous value for the coupling ratio in proteoliposomes is provided by the study of Eytan et al. [11], described in the last section; from the data in their Fig. 2, the ratio of the polytryptophan-inhibitable ATPase activity in the presence and absence of valinomycin is about 3.6 (the inhibitor is essential in revealing that part of the basal activity due to P-glycoprotein). In various studies the ratio of rates in the presence and absence of substrates was similar to this, between 2 and 5 with most substrates, and the value was not significantly different whether measured with a crude membrane preparation, with membrane systems of different degrees of purity, or with the purified protein (as may be seem in data summarized by Shapiro and Ling [33]). With pure P-glycoprotein the ratios for widely dissimilar drug molecules are between 1.4 and 2 in one study [50], and between 0.8 and 2.8 in another (Table 2). Allowing for steric effects, the conclusion is that the ATPase is intrinsically uncoupled. But what of the intact system, in whole cells?

As the analysis in the Appendix demonstrates, a half-coupled mechanism behaves as if coupled in the presence of an inhibitor with the right properties. However, coupling ratios are most conveniently measured with purified P-glycoprotein or with isolated membranes, in the course of whose preparation any inhibitor in the cell could well have been lost. The degree of coupling in whole cells is not easily determined, but this knowledge will be essential for a full understanding of the multidrug carrier.

The Inhibitor Hypothesis and Polytryptophan

From the kinetic treatment of the half coupled model in Fig. 4 (Appendix), the inhibitor that makes a half-coupled system behave as if coupled would act in the same way as a nontransported substrate analogue: it would bind in competition with transported substrates but would inhibit ATP hydrolysis noncompetitively; and

it would be internal, binding at the inner transport site. The endogenous inhibitor could resemble substrates in being partly nonpolar, and so bind at the carrier site, but might be much larger than even the largest substrates, and so interfere sterically with carrier movement. The reversible inhibitor polytryptophan, which was introduced by Eytan et al. [11], and which played an important part in the experimental determination of stoichiometry, fits the description. This synthetic compound contains hydrophobic residues and is large—5.4 kDa; it inhibits ATP hydrolysis, and the inhibition is reversed through competition with the transported substrate. Hence, polytryptophan probably binds at the transport site that in the cell faces inward (the loading side). It remains to be seen whether such an inhibitor occurs naturally. A protein or large peptide is a possibility, which could either be in the cytoplasm, or, considering that P-glycoprotein [32, 35, 37, 48] and the related *Lactococcus* multidrug carrier [5] take up substrates directly from the membrane, loosely associated with the inner surface of the membrane.

If there is no endogenous inhibitor, and if the intact system is in fact very weakly coupled, another role for P-glycoprotein comes to mind. When not extruding foreign chemicals, it might, instead of wasting ATP, be gainfully employed as a flippase, transferring lipid molecules from the inner to the other leaflet of the membrane. The idea that the multidrug carrier is a flippase has been debated in the past [7, 16, 30, 45], but the evidence is inconclusive. Suggestively, P-glycoprotein, like a flippase, takes up substrates from the membrane rather than the internal medium. And another member of the same gene family (class III Pgp) specifically moves lipid—phosphatidyl choline—into the external medium [45].

Polytryptophan and the Reaction Mechanism

Polytryptophan has the same effects as our hypothetical inhibitor: it increases the coupling ratio, i.e., the tightness of coupling, by inhibiting uncoupled ATP hydrolysis in the absence of a transported substrate, but ceasing to inhibit in the presence of a substrate. As the expected effect of an inhibitor on the half-coupled model has actually been observed, the proposed coupling mechanism must be feasible, and conversely, the fact that P-glycoprotein responds to polytryptophan as it does supports a half-coupled mechanism.

Polytryptophan should be useful as an experimental tool, whatever the status of the proposed endogenous inhibitor. The character of the inhibition is to be related to the pump mechanism. With the random model in Fig. 4, the inhibition of ATP hydrolysis by a nontransported substrate analogue is noncompetitive, but with the ordered model in Fig. 3 the inhibition is uncompetitive. In the case of a random model, an inhibitor that competes with both ATP and the transported substrate can also give rise to coupling (here C_iTA in Fig. 4 is not formed. Depending on the openness of the transport site on the two sides of the membrane (referring to the orientation in the cell), polytryptophan may bind to the carrier on both the inner and outer sides, or only inside. And if the inhibitor can bind on both sides, it might add on only one side at a time or on both simultaneously, depending on the transport mechanism, whether of the alternating-site or fixed-site type; experimentally, the inhibition would be, respectively, a function of the first or second power of the inhibitor concentration. There are also questions as to whether bound polytryptophan will hold the isolated protein in a particular conformation, and at a particular stage in the reaction of ATP.

Origin of a Half-Coupled Mechanism

A transport ATPase immersed in its substrate should be under no selection pressure to be fully coupled, for with the substrate saturating there is no substrate-free complex to react and therefore no uncoupled reaction of ATP. For this reason the mechanism can just as well be half-coupled. Now, a flippase, whose function is to move lipids from one leaflet of the membrane bilayer to the other, is such a carrier; it is embedded in the cell membrane and its substrates are membrane lipid molecules. A flippase, if it had such a mechanism, could rather directly have given rise to a multidrug carrier. In fact, the structures are closely related.

Recent findings of Sharom and coworkers [40] may have a bearing on the problem. In experiments on P-glycoprotein embedded in artificial membranes, the substrate binding constants were proportionate to their solubility in the lipid phase. The observed log-log relationship would indicate that the free energy of solution adds to the free energy of binding. A simple concentration effect, in which the substrate in the lipid bilayer is presented to the carrier in concentrated form and therefore has a proportionately higher apparent affinity, seems unlikely. The difficulty is that by however much the partition between water and the membrane is favored, the partition between the membrane and the carrier site is disfavored. The reasoning is as follows. Since the substrate equilibrates between water and lipid, and between lipid and the carrier site, its free energy (that is, its activity or escape tendency) in water, in the lipid phase, and at the binding site, will be the same. Then, unless the free energy of the binding site is a function of the substrate's lipid solubility, which seems unlikely, the partition between the aqueous solution and the carrier will be independent of the lipid. But there is another, simpler, interpretation. Suppose that lipid is strongly ad-

sorbed at the carrier site, remaining in place throughout the transport cycle, and that the substrate adds to this lipid-coated site. Affinity and lipid solubility are then directly related. In support of the hypothesis, lipid remains tightly associated with P-glycoprotein after purification (50–60 lipid molecules per mole [42]), and the adsorbed lipid is required for ATPase activity [10, 44].

With this hypothesis in mind, speculation as to the provenance of multidrug transporters may be carried a step further. Selection pressure would favor the evolution of a mutant flippase with altered specificity, one that is able to indiscriminately bind harmful foreign chemicals. Paradoxically, a sharp increase in lipid affinity could have the desired effect. The lipid, if very tightly bound, would be inefficiently transported—because of slow dissociation—but drug molecules, adsorbed in the lipid-coated site, could be taken up indiscriminately, and moved outward. The idea is testable at two points. First, flippase mechanisms should be only weakly coupled. Second, any flippase activity of P-glycoprotein should be highest in membranes composed of the most weakly bound lipids (those whose dissociation is fastest).

A multidrug carrier created in this way should be highly satisfactory. The half-coupled mechanism allows active transport to be unspecific; the carrier, taking up the substrate directly from the membrane, ejects intruding chemicals before they have damaged cellular constituents; and the ability of foreign molecules to enter the cell, and the ability of the cell to pump them out again, depend on the same factor—solubility in the lipid membrane.

The Applicability of Uncoupled Mechanisms

Looking back over this discussion, we can see that two general ideas have come out. The first is half-coupling. The analysis has shown that the concept of uncoupled, but energy driven, transport against a concentration gradient is not limited to one type of model: the driving energy can be supplied in the form of ATP or an ion gradient, and the reaction sequence can be that of either cotransport or antiport. Such mechanisms are characterized by contrasting roles of the driving and driven substrates. The driving substrate enters into bonding interactions linked to shifts in the state, presumably the conformation, of the pump protein. The driven substrate is exempt from this requirement, and to be pumped uphill it only has to be present at the transport site.

The second idea concerns a pump immersed in its substrate; such a pump, a flippase for example, should have no need to be fully coupled, as explained above. Most pumps are immersed in water: what if water is to be actively transported? Zeuthen and Stein cite evidence to show that many salt carriers—those channelling Na^+, K^+, and Cl^-—function as water pumps [52]. The observations suggest that water is cotransported with the ions, and that the flow, driven by an ion gradient, can run counter to the transmembrane chemical potential of water. The authors envisage a model in which the bound ions induce a conformational change in the carrier protein, opening up a cavity that fills with a good many water molecules. Translocation ensues, with release of the ions and the water on the far side of the membrane. The conformation then changes again, eliminating the cavity, and the carrier returns empty, to begin another cycle. This is essentially an ordered cotransport model. Were the mechanism fully coupled, the first substrate (the ions) would induce a conformational change that both exposes the site for the second substrate and immobilizes the carrier (otherwise the first substrate would be transported independently, and the system would be completely uncoupled). The second substrate (water) would then bind and catalyze the translocation step, a process dependent on greatly increased affinity in the transition state. But in a water pump there is no need for full coupling. The surrounding water, which saturates the site, should convert the first complex to the complex of both substrates, ions plus water, bypassing the uncoupled reaction. The mechanism, then, may be expected to be half-coupled, avoiding the catalytic step. Even though this water pump involves secondary active transport, and P-glycoprotein primary active transport, the two systems may, in this light, be similar; both mechanisms being half-coupled, and both carriers containing a large transport cavity, into which an assortment of substrate molecules can enter.

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Appendix

Half-Coupled ATPase Models

The reaction schemes in Figs. 3 and 4 are uncoupled in the sense that ATP is freely hydrolyzed in the absence of the transported substrate S, and that the role of S is passive. But ATP hydrolysis (ATP $\rightleftharpoons$ ADP + Pi, represented as $T \rightleftharpoons D$) is coupled to a carrier transformation, the about-face of the carrier in the membrane (C_iT to C_oD).

In the model in Fig. 3 the substrates add to the carrier C in order: first the driving substrate T (*ATP*) and then the driven substrate S. As in a passive system, all carrier forms—the free carrier, the complex with the driving substrate, and the complex with both substrates—are mobile; that is, the site for S freely moves from an inward-facing to an outward-facing stance (C_i to C_o, where the subscripts i and o mean *in* and *out*, respectively). ATP (T) binds only to the inward-facing carrier, and ADP + Pi (D) only to the outward-facing carrier. ATP is not translocated because the catalytic site is fixed on one side of the membrane. While the free carrier has no binding site for S, the addition of T to C_i or of D to C_o causes the site to become exposed. The system is completed by an endogenous inhibitor A inside the cell, competitive with the transported substrate S but not with ATP or ADP. The inhibitor behaves as a nontransported substrate analogue.

In the model in Fig. 4, the substrates add to the carrier in random order, but the substrate complex, C_iS or C_oS, is immobile. The translocation step, the about-face of the carrier, only occurs as ATP undergoes reaction, the role of S being passive throughout.

Rate equations will be derived for the random model in Fig. 4, which subsumes the ordered model in Fig. 3. A convenient method of derivation has been described by Stein [47]. On the assumption that all substrate additions are equilibrium steps, the unidirectional rates for the exit and entry of substrate S, v_{TSi} and v_{DSo}, respectively, are

$$v_{TSi} = f_{-3}\, t \cdot s_i\, (f_1 + f_2 d + f_3 d \cdot s_o)[C]/Denom \quad \text{(A1)}$$

$$v_{DSo} = f_3\, d \cdot s_o\, (f_{-1} + f_{-2} t + f_{-3} t \cdot s_i)[C]/\text{Denom} \quad \text{(A2)}$$

$Denom = \{1 + t + t \cdot a + t \cdot s_i + a' + s_i'\}\{f_1 + f_2 d + f_3 d \cdot s_o\} + \{1 + d + d \cdot s_o + s_o'\}\{f_{-1} + f_{-2} t + f_{-3} t \cdot s_i\}$ where $s_i = [S_i]/K_{Si}$, $s_o = [S_o]/K_{So}$, $t = [T]/K_T$, $d = [D]/K_D$, $s_i' = [S_i']/K_{Si}'$, $s_o' = [S_o]/K_{So}'$, $a = [A]/K_A$, $a' = [A]/K_A'$. $[C]$ is the total carrier concentration. Note that dropping the primed terms yields rate equations for the ordered model in Fig. 3.

Concentration Gradients

In the final-steady state, when there is no further net substrate movement, the ratio of external and internal concentrations of S is found by equating the unidirectional exit and entry rates, Eqs. A1 and A2:

$$(s_o/s_i)_{final} = \frac{f_{-3} t (f_1 + f_2 d)}{f_3 d (f_{-1} + f_{-2} t)} \quad \text{(A3)}$$

Eq. A3 may be written in a more useful form by introducing obligatory relationships among the constants, found in the following way. When both ATP and the transported substrate are at equilibrium, $f_1[C_o] = f_{-1}[C_i]$, $f_2[C_oD] = f_{-2}[C_iT]$, and $f_3[C_oDS] = f_{-3}[C_iTS]$. From the second of these three expressions,

$$f_2[C_o][D]/K_D = f_{-2}[C_i][T]/K_T \quad \text{(A4)}$$

and therefore,

$$([T]/[D])_{equil} = K_{eq} = \frac{f_2[C_o]K_T}{f_{-2}[C_i]K_D} = \frac{f_2 f_{-1} K_T}{f_{-2} f_1 K_D} \quad \text{(A5)}$$

Similarly, from the third expression,

$$([S_o]/[S_i])_{equil} = 1 = \frac{f_2 f_{-3} K_{So}}{f_{-2} f_3 K_{Si}} \quad \text{(A6)}$$

By substituting Eqs. A5 and A6 into Eq. A3, the gradient is found to be

$$([S_o]/[S_i])_{final} = \frac{K_{So} f_{-3}(f_2 + f_1 K_D/[D])}{K_{Si} f_3 (f_{-2} + f_{-1} K_T/[T])} \quad \text{(A7a)}$$

$$= \frac{1 + f_1 K_D/(f_2[D])}{1 + f_{-1} K_T/(f_{-2}[T])} = \frac{1 + f_{-1} K_T/(f_{-2} K_{eq}[D])}{1 + f_{-1} K_T/(f_{-2}[T])} \quad \text{(A7b)}$$

A Fully Coupled System ($f_2 = f_{-2} = 0$)

From Eq. A7a, with $f_2 = f_{-2} = 0$,

$$([S_o]/[S_i])_{final} = \frac{K_{So} f_{-3} f_1 K_D [T]/[D]}{K_{Si} f_3 f_{-1} K_T} = ([T]/[D])/K_{equil} \quad \text{(A8)}$$

As expected, the final concentration gradient in a perfectly coupled system depends on the ratio of the concentration of ATP to ADP and Pi, in relation to the equilibrium value.

A Half-Coupled System

(i) If the affinity of the ATPase is so high that $[T]/K_T >> 1$ and $[D]/K_D >> 1$, the system behaves as a passive transporter. There is no concentration build-up and no net hydrolysis of ATP: from Eq. A7b,

$$([S_o]/[S_i])_{final} = 1 \quad \text{(A9)}$$

(ii) If the affinity of the ATPase is extremely low, such that $[T]/K_T << 1$ and $[D]/K_D << 1$, the gradient in the final steady-state, Eq. A7a, will be identical to that in the fully coupled system, Eq. A8. The systems are equivalent because under these conditions f_2 and f_{-2} drop out of Eq. A7a; in the fully coupled system the constants are zero. The system continues to be effective in active transport at much higher affinities, however. From Eq. A7a, if $[T]/K_T = 1$ and $[D]/K_D = 1$, the final concentration gradient is $(1 + f_1/f_2)/(1 + f_{-1}/f_{-2})$; and from Eq. A5 for the ATP equilibrium constant, $K_{eq} << 1$, we find that $f_1/f_2 >> f_{-1}/f_{-2}$ (provided K_T and K_D are not too different, a realistic assumption). We may conclude that a substantial concentration gradient can be built up if the dissociation constants are comparable to the cellular concentrations of ATP and ADP, but that they should not be much lower.

Transport is then active, and the rate roughly half-maximal, perhaps the optimal arrangement.

Slippage

In the absence of the transported substrate *S*, C_oDS and C_iTS drop out of Figs. 3 and 4, and the scheme reduces to a simple one-substrate carrier model. The initial rate of ATP hydrolysis (*T* to *D*, with $[D] = 0$) is given by [24]

$$v_{Ti} = \frac{f_1 f_{-2}\, t\, [C]}{(f_1 + f_{-1} + f_1 a') + t(f_1 + f_{-2} + f_1 a)} \tag{A10}$$

The maximum rate of slippage (with t very large) is therefore

$$V_{Ti} = f_1 f_{-2}\, [C]/(f_1 + f_{-2} + f_1\, a) \tag{A11}$$

This rate may be compared with the coupled rate—the maximum rate of ATP hydrolysis in the presence of *S*, which is the same as the maximum rate of exit of *S* (Eq. A1, with *t* and s_i very large and with $d = s_o = 0$):

$$V_{TSi} = f_1 f_{-3}\, [C]/(f_1 + f_{-3}) \tag{A12}$$

The ratio of these is the coupling ratio—the ratio of ATP hydrolysis rates in the presence and absence of a transported substrate:

$$V_{TSi}/V_{Ti} = \frac{f_{-3}\,(f_1 + f_{-2} + f_1 a)}{f_{-2}\,(f_1 + f_{-3})} \tag{A13}$$

The inhibitor *A* is seen to reduce slippage, V_{Ti}, relative to the coupled rate, V_{TSi}.

The relationship between tight coupling and binding energy is similar in coupled and half-coupled systems. In a coupled system an increment in binding energy drives a conformational change; in a half-coupled system, substrate binding energy is used to displace a competitive inhibitor. The apparent substrate affinity, in the presence of the inhibitor, may be compared with the intrinsic substrate affinity, in its absence. From Eq. A1, the zero trans rate of coupled exit (at saturating *T* and with $d = s_o = 0$) is

$$v_{TSi} = \frac{f_1 f_{-3}\, s_i\, [C]}{(f_1 + f_{-2} + f_1\, a) + s_i\,(f_1 + f_{-3})} \tag{A14}$$

from which the K_m for internal *S* is

$$Km_{(Si)} = K_{Si}\,(f_1 + f_{-2} + f_1\, a)/(f_1 + f_{-3}) \tag{A15}$$

The ratio of *Km* values in the presence and absence of the inhibitor is therefore

$$Km'_{(Si)}/Km_{(Si)} = (f_1 + f_{-2} + f_1\, a)/(f_1 + f_{-2}) \tag{A16}$$

This ratio of intrinsic to apparent affinity, Eq. A16, is seen to be directly related to the coupling ratio in Eq. A13:

$$(V_{TSi}/V_{Ti}) = \frac{(1 + f_1/f_{-2})}{(1 + f_1/f_{-3})}\,(Km'_{(Si)}/Km_{(Si)}) \approx (Km'_{(Si)}/Km_{(Si)}) \tag{A17}$$

Eq. A17 may be compared with Eq. 1 for a coupled system.

An Uncoupled Antiport Mechanism

An exchange model capable of accounting for low substrate specificity in binding and translocation is shown in Fig. 5. This uncoupled system, in which both the free carrier and the substrate complex are mobile, is like an ordinary passive carrier, except in having transport sites for two different substrates, *H* and *S*. The driving ion, *H*, could be bound with a high degree of specificity but is not required to alter the carrier, neither its mobility nor the accessibility of the other substrate site. The molecule to be cleared from the cell, *S*, which only has to occupy the carrier site, can be bound and transported indiscriminately. A competitive inhibitor *A* is included, which in the absence of *S* blocks waste of the gradient in *H*.

Rate equations for the uncoupled system may be written directly from a general treatment of the kinetics of transport [24]. To simplify the problem, the mobility constants for the various carrier forms are assumed to be identical: $f_1 = f_2 = f_3$ and $f_{-1} = f_{-2} = f_{-3}$. The exit rate for *S* is

$$v_S = \left\{\frac{V_{si}}{K_{Si}}([S_i] - \alpha[S_o]) + \frac{V_{si}}{K_{Si}K_{Ho}}([S_i][H_o] - [S_o][H_i]\alpha/\beta)\right\}/Denom \tag{A18}$$

$Denom = s_i + h_i + s_o s_i + h_o h_i + s_o h_i + s_i h_o + (1 + a)\{1 + s_o + h_o\}$ where $s_i = [S_i]/K_{Si}$; $h_i = [H_i]/K_{Hi}$; $s_o = [S_o]/K_{So}$; $h_o = [H_o]/K_{Ho}$; $a = [A]/K_A$;

$$\alpha = ([S_i]/[S_o])_{equilibrium}$$

$$\beta = ([H_i]/[H_o])_{equilibrium}$$

α and β will be functions of the membrane potential if *H* and *S* are charged species (*H* may be a proton, H^+).

For comparison, the rate of exchange transport by a perfectly coupled system, in which the free carrier is immobile ($f_1 = f_{-1} = 0$) and the postulated inhibitor *A* is absent, is

$$v_S = \frac{V_{Si}}{K_{Si}\,K_{Ho}}\,\frac{([S_i][H_o] - [S_o][H_i]\alpha/\beta)}{Denom} \tag{A19}$$

Denom is as in Eq. A18 with $a = 0$.

The Final Concentration Gradient in S

In the passive system, the ratio of external and internal substrate concentrations in the final steady-state, where there is no further net transport, is found by setting Eq. A18 equal to zero:

$$([S_o]/[S_i])_{final} = \frac{\{1 + [H_o]/K_{Ho}\}}{\alpha\,\{1 + [H_i]/\beta K_{Ho}\}} \tag{A20}$$

$[H_o]/\beta[H_i]$ is related to the proton-motive force for the cell. If $[H_o] >> K_{Ho}$ and $[H_i] >> \beta K_{Ho}$, then

$$([S_o]/[S_i])_{final} = \beta[H_o]/\alpha H_i \tag{A21}$$

But if $[H_o] << K_{Ho}$ and $[H_i] << \beta K_{Ho}$,

$$([S_o]/[S_i])_{final} = 1/\alpha \tag{A22}$$

In the coupled system, the final gradient in *S* (found by setting Eq. A19 equal to zero) is the same as in Eq. A21.

Slippage

The tightness of coupling is given by the ratio of the maximum coupled and uncoupled rates, which here is the ratio of the maximum rates of entry of H in the presence and absence of S. The rate equation for the entry of H is [24]

$$v_{Ho} = \frac{V_o}{K_{Ho}}([H_o] - [H_i]/\beta) + \frac{V_{Ho}}{K_{Ho}K_{Si}}([H_o][S_i] - [H_i][S_o]\alpha/\beta)/Denom \tag{A23}$$

where Denom is the same as in A18. The uncoupled rate is that in the absence of S: with $[S_o] = [S_i] = 0$, $[H_o] >> [H_i]/\beta$, $h_o >> 1$, and a $>> h_i$, the maximum rate is

$$V_{Ho} = \frac{V_{Ho}([H_o] - [H_i]/\beta)}{K_{Ho}\, Denom} = V_{Ho}/(1 + a) \tag{A24}$$

The maximum coupled entry rate is that with the same concentrations of H and A but with saturating S inside ($[S_i] >> [H_i]$ and $s_i >> a$):

$$V_{HoSi} \approx V_{Ho} \tag{A25}$$

The coupling ratio is therefore

$$V_{HoSi}/V_{Ho} \approx 1 + a \tag{A26}$$

Again, tight coupling is related to binding energy. The ratio of the apparent to the intrinsic substrate dissociation constant is given by the ratio of Km values in the presence and absence of the inhibitor (as in Eq. A17). In exit (Eq. A18), the half-saturating concentration for S_i, with the concentration of H_o high and the concentrations of S_o and H_i low, is

$$[S_i]_{1/2} = K_{Si}(1 + a) \tag{A27}$$

Hence the ratio of Km values in the presence and absence of A is

$$Km'_{Si}/Km_{Si} = (1 + a) \tag{A28}$$

Therefore, from Eq. A26,

$$V_{HS}/V_H \approx Km'_{Si}/Km_{Si} \tag{A29}$$