ELSEVIER

Biochimica et Biophysica Acta 1236 (1995) 1–9

BBA Biochimica et Biophysica Acta

Interpreting the effects of specific protein modification on antiport coupling mechanisms: the case of the aspartate/glutamate exchanger

Richard M. Krupka *

London Research Centre, Agriculture Canada, 1391 Sandford St., London, Ont., N5V 4T3 Canada

Received 16 June 1994; accepted 27 October 1994

Abstract

Reaction of two cysteine residues in the aspartate/glutamate carrier of mitochondria is reported to abolish exchange but to actuate a passive one-way exit of extremely low substrate affinity and specificity, but with the same activation energy as antiport (Dierks, T., Salentin, A. and Krämer, R. (1990) Biochim. Biophys. Acta 1028, 281–288). This behaviour, reminiscent of a channel, becomes understandable when the required control over carrier mobility by the substrate is allowed for. Whether the transport mechanism involves a substrate site alternately exposed on opposite sides of the membrane or sites simultaneously exposed on both sides, and whether the substrate acts by converting an immobile carrier conformation to an inherently mobile intermediate or by stabilizing the transition state in carrier movement, the same fundamental relationship emerges: the ratio of coupled to uncoupled rates (antiport relative to net flux) is limited by the ratio of substrate dissociation constants in successive carrier conformations, one immobile, the other mobile; the increment in the binding energy in the two forms must therefore be large. Shifts in the equilibrium between these conformations and shifts in their relative affinities for the substrate can account for the properties of the modified transport system, which, it is concluded, functions as a carrier, not a channel.

Keywords: Uncoupled transport; Mutagenesis; Protein modification; Antiport model; Vectorial coupling; Channel

1. Introduction

Small changes in the structure of a transport protein can give rise to behaviour not easily explained by conventional carrier models. Reaction of the glutamate/aspartate exchanger of mitochondria with thiol reagents blocks antiport, the coupled reaction, while opening up a path for passive exit – but not passive entry; the exit route is channel-like rather than carrier-like in being unspecific and of low affinity, yet has the same activation energy as exchange transport in the unmodified system [1–3]. And single amino acid substitutions uncouple the lactose permease of *Escherichia coli*, a co-transport system, permitting independent movement of the sugar or the driving hydrogen ion; with some mutants passive flow of the sugar appears to be in only one direction, into the cell but not out of it [4]. The effects of mutation on co-transport were accounted for earlier [5] by vectorial coupling theory, which relates the tightness of coupling to substrate-induced conformational changes. The similar problem of exchange transport is dealt with here. The relationship between coupling and substrate binding forces in successive carrier conformations is given for two antiport models, one in which a substrate binding site is exposed alternately on opposite sides of the membrane, and one in which substrate sites are simultaneously exposed on both sides. The implications for transport are illustrated with reference to experimental observations on the aspartate/glutamate exchanger.

2. The basis of coupling

For substrate translocation into the cell to be coupled to translocation out, which is the condition for antiport, the properties of the carrier, most crucially its mobility, have to be controlled at every stage of the reaction. ("Mobility" refers to the carrier transformation involved in substrate movement across the membrane.) To the extent that control is weak, inward and outward flux will be independent, as in facilitated, or passive, transport. Control depends on

* Corresponding author. Fax: +1 (519) 6455476

SSDI 0005-2736(94)00259-2

specific interactions between the carrier and substrate – the substrate, adding to an immobile form of the carrier, promotes a conformational change required in the translocation step; and the abruptness of the change, which depends directly on the increase in the substrate binding force in the altered carrier form, determines how tightly the reaction is coupled. Hence the increment in the binding force is related to the ratio of coupled to uncoupled rates (called the *coupling ratio)*:

$$\text{rate}_{(\text{coupled})}/\text{rate}_{(\text{uncoupled})} < K_{(\text{initial state})}/K_{(\text{induced state})} \quad (1)$$

where K is a substrate dissociation constant. This relationship appears to be fundamental, applying to all coupled vectorial processes [6].

To control the mobility of the carrier, a substrate may either increase the *rate* at which one conformation is converted to another, by binding more strongly in the transition state, or it may shift the *equilibrium* between immobile and mobile conformations, by binding more strongly to the latter. The two cases will be dealt with separately, even though the first is general: that is, acceleration of the translocation step involves a stabilization of the transition state whether or not a substrate-induced conformational change precedes this step. The importance of mobile and immobile intermediates in the reaction is to be decided by experiment.

As the ratio of coupled to uncoupled rates has to be large (so as not to waste metabolic energy), substrate binding should be comparatively weak in the initial complex and strong in the complex derived from it. The large increment in binding energy has suggested that a surface complex could be converted to an inclusion, or chelate, complex, where the increased binding energy is largely entropic in origin [7].

3. Antiport models

Models for exchange-only transport are of two kinds. In one, which is based on the ordinary carrier model, a substrate site, in effect, rotates from one side of the membrane to the other; the system catalyzes exchange and not net transport if the substrate complex is able to rotate while the free carrier cannot (Fig. 1). In the other model, which may be called a bilateral model, substrate sites are

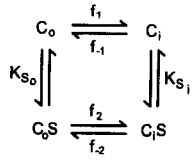

Fig. 1. The carrier model. The carrier rotates between two conformations, C_o, in which the substrate site faces outward, and C_i, in which the site faces inward; the former binds the substrate, S, in the outer compartment; the latter, the substrate in the inner compartment. The condition for antiport is that only the substrate complex is mobile ($f_2, f_{-2} \gg f_1, f_{-1}$).

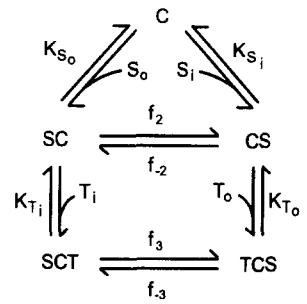

Fig. 2. The bilateral model. Binding sites in the carrier, C, for the substrates S and T are simultaneously exposed on both sides of the membrane, allowing the carrier to form a ternary complex in which a substrate molecule from each compartment is bound: S_o and T_o in the outer compartment, S_i and T_i in the inner. S and T can be different substrates or the same substrate (one labelled), and the order of addition need not be fixed. SC is the complex with external substrate, CS the complex with the internal substrate. The condition for antiport is that substrate translocation occurs in the ternary but not in the binary complex ($f_3, f_{-3} \gg f_2, f_{-2}$).

simultaneously exposed on both sides of the membrane; exchange is allowed and net transport is excluded if substrates move across the membrane only when both sites are occupied (Fig. 2). The properties of the models are different, as are the requirements for coupling.

3.1. The carrier model

3.1.1. Transition-state model

The simplest kinetic scheme for exchange-only transport includes only the transition state between outward-facing and inward-facing carrier forms, as in Fig. 3. Net transport is prohibited if the free carrier is immobile, as noted above. The relationship between the coupling ratio and binding forces depends on the fact that the maximum exchange rate is determined by the mobility of the substrate complex (governed by f_2 and f_{-2} in Fig. 1), while the maximum rate of net transport is determined by the mobility of the free carrier (governed by f_1 and f_{-1}, which are much smaller than f_2 and f_{-2}). The ratio of exchange to net transport therefore depends on the ability of the substrate, when bound at the carrier site, to increase the rate of carrier reorientation. The following relationship was demonstrated earlier (Ref. [7], Eq. (11)):

$$\tilde{K}^{s}_{S_o}/K^{ts}_{S} \approx \overline{\overline{V}}_S/\overline{V}_{S_i} < K_{S_o}/K^{ts}_{S} \quad (2)$$

$\overline{\overline{V}}_S$, the coupled rate, is the maximum rate of exchange transport, and $\overline{V}_{S_i}$, the uncoupled rate, is the maximum rate of exit in the absence of external substrate; $\overline{\overline{V}}_S/\overline{V}_{S_i}$, is the coupling ratio. K_{S_o} is the external substrate dissociation constant, K^{ts}_{S} the virtual substrate dissociation constant in the transition state, and $\tilde{K}^{s}_{S_o}$ the experimental constant, the half-saturating concentration of external substrate measured with a saturating concentration of substrate inside:

$$\tilde{K}^{s}_{S_o} \approx K_{S_o}/(1 + f_2/f_{-2}) \quad (3)$$

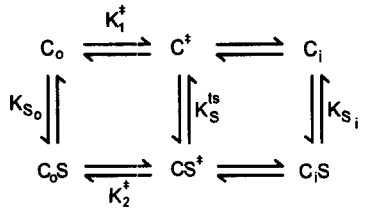

Fig. 3. The carrier model (see Fig. 1), showing the transition state in movement of the substrate site from exposure outside (o) to exposure inside (i).

In an exchange-only system $\overline{\overline{V}}_S \gg \overline{V}_{S_i}$ and therefore both K_{S_o} and $\tilde{K}^s_{S_o}$ are much larger than K^{ts}_S. It is apparent that catalysis of the carrier conformational change (which may be called vectorial catalysis) depends on there being very much stronger substrate binding in the transition state than in the initial complex.

3.1.2. Model with mobile and immobile intermediates

In the expanded scheme in Fig. 4, involving intermediates in translocation, the mobility of the outward-facing or inward-facing carrier is determined by an equilibrium between inherently immobile and mobile conformations (denoted by a single prime and a double prime, respectively). The requirement for exchange-only transport is that the free carrier be predominantly in the immobile conformation:

$$K_{o1} = [C'_o]/[C''_o] \gg 1 \quad (4)$$

To account for its mobility, the substrate complex must be partly in the mobile conformation, C''_oS:

$$K_{o2} = [C'_oS]/[C''_oS] \ll K_{o1} \quad (5)$$

And to induce this conformational change the substrate must be bound far more strongly in the mobile than in the immobile conformation:

$$K'_{S_o} = [C'_o][S_o]/[C'_oS] \gg K''_{S_o} = [C''_o][S_o]/[C''_oS] \quad (6)$$

The relationship between the coupling ratio and the binding force is found as in the first case: exchange depends on the mobility of the substrate complex, net flux on the mobility of the free carrier; these rates depend on

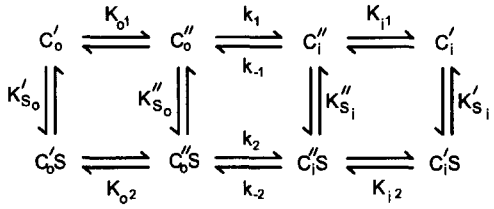

Fig. 4. The carrier model (see Fig. 1) involving immobile and mobile carrier conformations, indicated by a single and double prime, respectively. For antiport, the mobile conformation of the free carrier, C''_o, must be assumed to be unstable ($[C'_o]/[C''_o] = K_{o1} \gg 1$ and $[C'_i]/[C''_i] = K_{i1} \gg 1$), and the mobile conformation of the substrate complex, C''_oS, stable by comparison ($[C'_oS]/[C''_oS] = K_{o2} \ll K_{o1}$ and $[C'_iS]/[C''_iS] = K_{i2} \ll K_{i1}$).

the equilibrium between mobile and immobile conformations (for the sake of simplicity the mobile conformations of the free carrier and of the substrate complex are assumed to move at the same rate: $k_1 = k_2$); and the equilibrium shifts in the presence of the substrate, owing to stronger binding in the mobile form. Hence the ratio of exchange to net transport depends on the equilibrium of the free carrier, governed by K_{o1}, and on the substrate dissociation constants in the mobile and immobile conformations. The following relationship is found (Ref. 7, Eq. (18)):

$$\tilde{K}^s_{S_o}/K''_{S_o} = \overline{\overline{V}}_S/\overline{V}_{S_i} < K'_{S_o}/K''_{S_o} \quad (7)$$

where $\tilde{K}^s_{S_o}$ is the experimental half-saturation constant (Eq. (3), with $K_{S_o} = K'_{S_o}/(1 + 1/K_{o2})$). Since

$$\overline{\overline{V}}_S \gg \overline{V}_{S_i}$$

it follows that $K'_{S_o} \gg K''_{S_o}$ and $\tilde{K}^s_{S_o} \gg K''_{S_o}$.

3.2. The bilateral model

3.2.1. Transition-state model

In a bilateral model (Fig. 2), where substrate sites are exposed on both sides of the membrane at the same time, the role of the substrate may simply be to catalyze the translocation step, as in Fig. 5. The assumption is that the two substrates together stabilize the transition state far more effectively than either substrate does alone; the mobility of the ternary complex, which determines the exchange, or coupled, rate, is therefore high, while that of the binary complex, which determines the rate of net, or uncoupled transport, is low. The relationship between binding energy and coupling was shown earlier to be the same as for the carrier model: Eq. (2), above (Ref. 7, Eq. (24)).

3.2.2. Model with mobile and immobile intermediates

When in addition to the transition state an inherently mobile intermediate is formed in the step in which a substrate is transferred across the membrane, as in Fig. 6, the relationship between coupling and binding energy is found as follows. The carrier is assumed to exist in two conformations, one immobile (C′) and one mobile (C″).

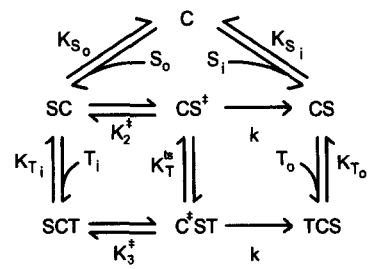

Fig. 5. Bilateral model (see Fig. 2) showing the transition state in the translocation step.

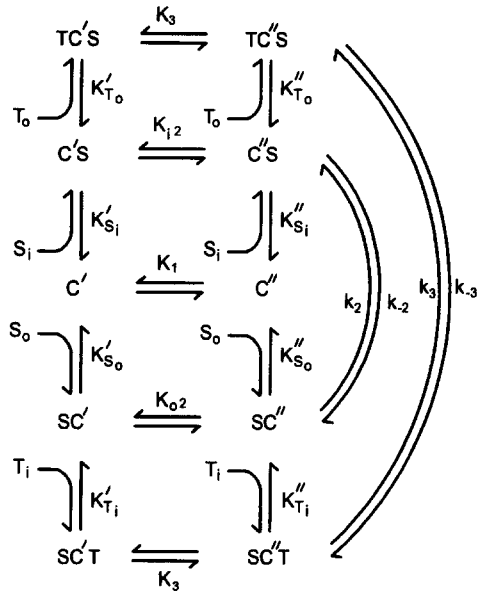

Fig. 6. Bilateral model (as in Fig. 2) involving intermediates in transport. Immobile and mobile carrier conformations are indicated by a single and double prime, respectively. The condition for antiport is that the mobile form of the binary substrate complex is unstable ($[SC']/[SC''] = K_{o2} \gg 1$ and $[C'S]/[C''S] = K_{i2} \gg 1$), while the mobile conformation of the ternary complex is stable by comparison ($[SC'T]/[SC''T] = [TC'S]/[TC''S] = K_3 \ll K_{o2}$); further, $K_1 = [C']/[C''] \gg K_{o1}$, $K_{i2} \gg K_3$, and consequently $K''_{S_o} \ll K'_{S_o}$, $K''_{S_i} \ll K'_{S_i}$, $K''_{T_o} \ll K'_{T_o}$, $K''_{T_i} \ll K'_{T_i}$. S and T can be the same substrate (one labelled) or different substrates. In a more general reaction scheme either substrate could add first, and substrate bound on one side could affect the affinity on the other side.

To exclude net transport, the complex with a single substrate molecule must be mainly in the immobile conformation:

$$K_{i2} = [C'S]/[C''S] \gg 1 \quad (8)$$

$$K_{o2} = [SC']/[SC''] \gg 1 \quad (9)$$

The free carrier, too, may be assumed to be in the immobile form:

$$K_1 = [C']/[C''] \gg 1 \quad (10)$$

The ternary complex, on the other hand, is partly in the mobile form. The immobile binary complex will be converted to the mobile ternary complex ($SC' \rightarrow SC''T$) if the substrate binding force is far stronger in the mobile than in the immobile conformation; the hypothesis is that the equilibrium begins to shift toward the mobile form when the first substrate molecule binds and shifts further with the second:

$$[C']/[C''] \gg [SC']/[SC''] \gg [SC'T]/[SC''T] \quad (11)$$

$$K_1 \gg K_{o2} \text{ or } K_{i2}; \text{ and } K_{o2} \text{ or } K_{i2} \gg K_3 \quad (12)$$

The ratio of coupled to uncoupled transport is found from the relative rates of reorientation of the ternary and binary complex (the transition $SC''T \rightleftarrows TC''S$, which is responsible for exchange transport, compared with $SC'' \rightleftarrows C''S$, responsible for net transport). The maximum rate of uncoupled entry of substrate S is proportional to the fraction of the carrier as the mobile binary complex (this rate is measured at a saturating concentration of the external substrate, in the absence of internal substrate, where all the carrier is in the form of the complex SC' or SC''):

$$\overline{V}_{S_o} = k_2[C_t][SC'']/([SC'] + [SC'']) = k_2[C_t]/(1 + K_{o2}) \quad (13)$$

where $[C_t]$ is the total carrier concentration. Similarly, the maximum rate of coupled entry, with all the carrier in the form of the ternary complex, is:

$$\overline{\overline{V}}_{ST} = k_3[C_t]/(1 + K_3) \quad (14)$$

Given that the constants in a cyclic reaction are related to one another:

$$K_3 K'_{T_i} = K_{o2} K''_{T_i} \quad (15)$$

it follows that if $K_{o2} \gg 1$ and if SC'' and SC''T have the same mobility ($k_2 = k_3$), then

$$\overline{\overline{V}}_{ST}/\overline{V}_{S_o} \approx K_{o2}/(1 + K_3) < K_{o2}/K_3 \quad (16)$$

Therefore,

$$\overline{\overline{V}}_{ST}/\overline{V}_{S_o} < K'_{T_i}/K''_{T_i} \quad (17)$$

4. Implications for an antiport mechanism

The coupling ratio is seen to depend on the increment in binding strength in the induced compared with the initial conformation – this is true of the carrier model in Fig. 1 and of the bilateral model in Fig. 2, whether there is catalysis of the carrier reorientation step through stabilization of the transition state (Eq. (2)), or induction of a conformational change to produce a reaction intermediate with altered properties (Eqs. (7) and (17)). Substrate binding needs to be comparatively weak in the initial complex in order to allow for a sufficiently large increase in binding energy in the complex derived from it, the affinity in which will be much higher than the experimental half-saturation constant suggests. Transition state stabilization accounts for various experimental observations on the anion exchanger of red cells, including a strikingly different pattern of substrate specificity in the exchanger than in facilitated transport systems [8,9]. And stabilization of intermediates in the transport reaction explains how replacement of a single amino acid can convert an active into a passive co-transport system, even a system that brings about one-way passive flow, as in the case of the lactose permease of *E. coli* [4].

If, as suggested above, a large increment in binding energy depends on the formation of a tight inclusion complex from a comparatively loose surface complex, then the substrate site may comprise two regions, one to which the substrate initially binds, the other closing over the

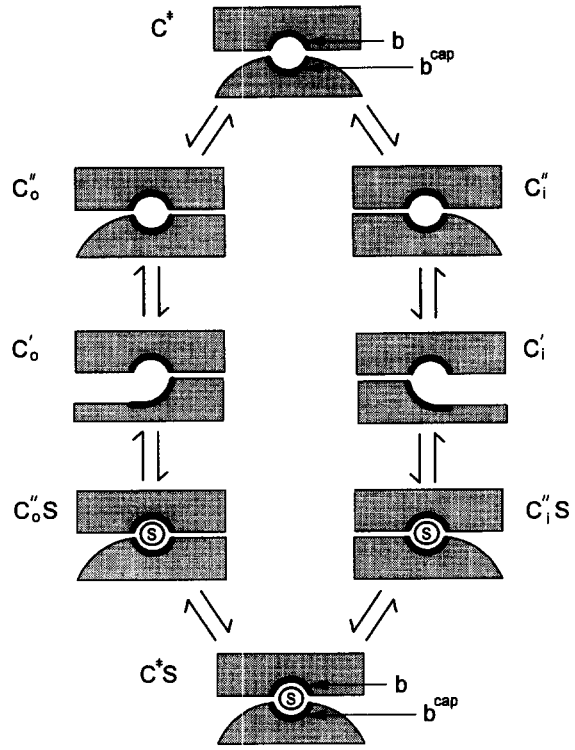

Fig. 7. Diagram of a hypothetical gated-channel version of the carrier model (in which the carrier alternates between inward facing and outward-facing forms, as in Fig. 1). The region of the carrier to which the substrate S initially attaches is labelled b, and the region that subsequently moves into place to form an inclusion complex with the substrate is labelled b^{cap}. Immobile and mobile carrier intermediates are included, as in Fig. 4. The immobile carrier forms, C'_o or C'_i, are in an open conformation, that is, accessible to the substrate. The mobile carrier forms C''_o and C''_i are in a closed conformation, with the two parts of the substrate site, b and b^{cap}, drawn together and enclosing a bound substrate molecule, or in the free carrier blocking substrate access.

substrate and forming additional bonds as the conformation is altered. The increased binding energy in an inclusion complex could serve to stabilize either the transition state in carrier reorientation or an intermediate in the transport reaction, as discussed earlier [7]. The diagrams in Figs. 7 and 8, corresponding to the carrier model in Fig. 4 and the bilateral model in Fig. 6, respectively, illustrate such processes; the mechanisms, involving gated-channels, are hypothetical, but serve here as a basis for discussion.

5. Target sites

Mutation or chemical substitution in a transport protein could conceivably affect any of the steps in the expanded reaction schemes in Figs. 4 and 6. Substrate binding and translocation could be disrupted, altering affinity, specificity, or flux; or the equilibrium of mobile and immobile conformations could be disturbed, uncoupling the inward and outward flow. The binding site for each substrate is, by hypothesis, divided; the separate regions are labelled b and b^{cap} in the diagrams in Figs. 7 and 8 – the first, the primary site, is preformed and holds the substrate loosely; the second, an ancillary site, and not necessarily preformed, caps and encloses the bound substrate molecule. Depending on the structure of the carrier the equivalent outward-facing and inward-facing binding regions could comprise the same or different amino acid residues: if the same, the inner and outer constants (K_{o1} versus K_{i1} and K_{o2} versus K_{i2} in the carrier model in Fig. 4, and K_{o2}

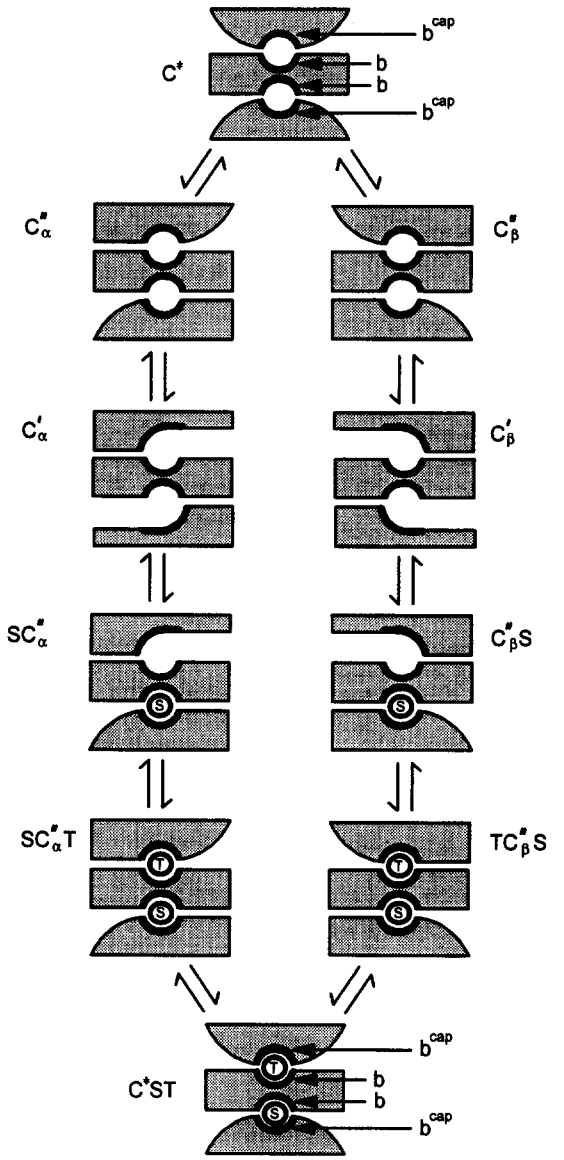

Fig. 8. Diagram of a hypothetical dual gated-channel version of the bilateral model (in which substrate sites are simultaneously available on both sides of the membrane). Immobile and mobile carrier intermediates, indicated by a single and double prime as in Fig. 6, are represented by open and closed conformations, respectively; b and b^{cap} are the two regions into which each substrate site is initially divided. Substrate sites in a pair of anti-parallel carriers undergo concerted rotation, one site becoming exposed inside as the other is exposed outside. Rotation produces isomers, labelled α and β, which could have different properties. The diagram does not include all possible conformational states – those with one site occluded and those with both.

versus K_{i2} in the bilateral model in Fig. 6) may be affected in the same way; otherwise, they may be affected differently.

5.1. The substrate-induced conformational change

The equilibria between mobile and immobile forms of the carrier-substrate complex depend partly on the relative affinities of the substrate in the surface complex (the open conformation, involving only the primary site and marked by a single prime) and the inclusion complex (the closed conformation, involving both sites and marked by a double prime). Modification of the capping site, by lowering the affinity in C″S, should increase K_{o2} or K_{i2}. In the case of the carrier model, Fig. 4, this need not affect the equilibrium of the free carrier, governed by K_{o1} or K_{i1}, whereas a shift in K_{o1} or K_{i1} might induce a comparable shift in K_{o2} or K_{i2} with no change in the relative substrate affinities in the two forms.

5.2. Vectorial catalysis

Catalysis of carrier reorientation by the substrate (f_2 and f_{-2} in the carrier model, Fig. 1, and f_3 and f_{-3} in the bilateral model, Fig. 2) depends on a sharp increase in the binding force in the transition state (Eq. (3)). The increase is assumed to result from interaction at an ancillary binding region, b^{cap} in Figs. 7 and 8, and modification of this region could reduce the transport rate.

5.3. The gating mechanism

In a mechanism based on gated channels, gates on the outer and inner sides of the membrane open and close coordinately – that is, when one is open the other is closed. (In consequence a substrate molecule can diffuse up to an exposed binding site but, until the carrier conformation changes, not beyond it.) Mutation in these regions could disturb the equilibrium between inward-facing and outward-facing forms (f_1/f_{-1}, f_2/f_{-2}, f_3/f_{-3} in Figs. 1 and 2), as well as the rates of interconversion of the two forms.

5.4. Carrier reorientation

Mutation in a hinge region, around which the carrier swings between outward-facing and inward-facing conformations, could impede all reorientation rates (f_1, f_{-1}, f_2, f_{-2}, f_3, f_{-3} in Figs. 1 and 2) and therefore block both coupled and uncoupled transport.

6. Aspartate / glutamate exchange

The effects of thiol reagents on the aspartate/glutamate exchanger of mitochondria have been described in a series of important papers by Krämer and co-workers [1–3,10]. Various mercury compounds, including some that do not penetrate the lipid membrane, react with two cysteine residues in the transport protein. After reaction of just one, cys(a), all translocation is blocked. After reaction of both, cys(a) and cys(b), a route is opened for uncoupled exit; uncoupled entry, nevertheless, is not detected, nor is exchange. Cys(a) but not cys(b) is protected by the substrate. In passive exit the specificity of the system is lost – sulfate, not transported by the intact system, is translocated at about the same rate as aspartate. The affinity for the substrate inside falls to a very low level – the K_m for internal aspartate is 2.8 mM in the intact system but at least 200 mM in the modified system. Surprisingly, the affinity for the substrate outside remains high, and external aspartate, while not transported, strongly inhibits uncoupled exit; the external specificity is also maintained, since sulfate does not block exit.

Uncoupled exit of 16 mM aspartate is as fast after treatment with thiol reagents as is exchange transport in the intact system, implying, from the difference in affinities, that the maximum rate of uncoupled flow is at least 10-times higher than the maximum rate of exchange. Yet, in spite of the difference in rates, the activation energy is the same in uncoupled exit by the modified system as in exchange transport by the intact system. Several types of experiment, including the incorporation of purified protein into liposomes, show that passive exit depends on the same transport protein as antiport.

From the kinetics of aspartate/glutamate exchange, the transport mechanism involves the formation of a ternary complex containing a substrate molecule from each compartment, as in the bilateral model in Fig. 2. Aspartate and glutamate in the same compartment compete for the carrier, suggesting that the same site binds either substrate.

6.1. Adjustment of constants in the bilateral model, Fig. 6

The experimental findings on the aspartate/glutamate exchanger may be explained by shifts in constants in the kinetic scheme in Fig. 6. To account for the inhibition of exchange transport, assume that one of the substrate binding sites is blocked after reaction of cys(a); let K'_{T_i} and K'_{T_o} become very large. A single substrate molecule, S in Fig. 6, can add to the carrier, though the complex is immobile (very little of the mobile complex, SC″ or C″S, is formed since K_{i2} and K_{o2}, from Eqs. (8) and (9), are necessarily large); the second substrate molecule, T, is unable to add. Then, with the binary complex immobile and the ternary complex not formed, both net transport and exchange transport are blocked. To account for uncoupled exit under circumstances where entry is blocked, assume that after reaction of cys(b) the equilibrium of the two forms of the binary complex with internal substrate, governed by K_{i2}, shifts to favour the mobile conformation, C″S, but with no corresponding shift in the external equi-

librium, governed by K_{o2}. On the inside, therefore, a significant proportion of the substrate complex is in the mobile conformation, C″S, whereas on the outside, with K_{o2} unchanged, the substrate complex is predominantly in the immobile conformation, SC′. As a result there is passive flow outward but not inward (the anomaly of one-way passive transport is considered below). To account for lowered affinity inside assume that reaction at cys(b) not only shifts the equilibrium from C′S to C″S but interferes with substrate binding at the inner site, greatly increasing K'_{S_i}; and with this lowering of affinity may be expected a lowering of specificity. As reaction at cys(b) has little effect on the outer site – on neither the external equilibrium governed by K_{o2} nor substrate affinity – external substrate adds to the carrier, forming the binary complex SC′; but as this complex is immobile the carrier is held in an inactive state and therefore all transport, including exit, is blocked.

The maximum rate of passive transport is higher than the maximum rate of exchange, yet the activation energies are the same: how so? Given that transport rates are determined partly by the proportion of carrier in the mobile conformation, dependent on the equilibrium constants K_{i2} and K_3, and partly by the translocation steps governed by k_{-2} and k_{-3}, passive exit and exchange will have the same activation energy if K_{i2} and K_3 are independent of temperature while k_{-2} and k_{-3} have the same temperature dependence (and possibly the same value); and passive exit will be faster than exchange (by a factor of at least ten) if in the modified system K_{i2} is significantly more favourable to the mobile conformation than is K_3 in the intact system; i.e., $K_2 < K_3$ and $K_3 \geq 10$.

6.2. Possible structural changes

A hypothetical model such as that in Fig. 8 may be helpful in interpreting or designing experiments, allowing us to visualize channels, gates, and sites that interact with the substrate. Here, sites in a pair of anti-parallel gated-channels are shown undergoing concerted rotation, one becoming exposed inside as the other is exposed outside. This arrangement is consistent with the finding that an external, non-penetrating thiol reagent attacks a site at which the internal substrate is bound, for both sites – inner and outer – become exposed outside in the course of the transport reaction.

Substrates are reported to protect cys(a) but not cys(b) against thiol reagents. The first reaction, of cys(a), blocks antiport, but apparently not by blocking the translocation step, since after reaction of cys(b) as well as cys(a) a translocation function – passive transport – is regained. As a working hypothesis cys(a) may be assumed to be within one of the substrate binding sites: consequently a bound substrate molecule blocks the reaction of the thiol reagent and after reaction the substrate is unable to bind; further, because the substituent is enclosed in the substrate cavity in place of the substrate, the substituted thiol group does not interfere with the translocation step. The cys(a) derivative of the carrier, therefore, binds one substrate molecule, not two, and as in the intact carrier the binary complex is immobile; consequently both antiport and net transport are blocked. On this hypothesis cys(a) could be located in region b in the diagram in Fig. 8, where the substrate initially interacts. Supposing this site to be in the upper carrier unit in the diagram, K'_{T_o} and K'_{T_i} become large. The substituent may neither favour nor disfavour the closed conformation, C″, and therefore have no effect on the rate-limiting step in carrier rotation (k_2, k_3 etc.).

Cys(b) is asymmetrically positioned in the transport protein. It appears to be associated with the second substrate site, not the first, where cys(a) is located – in Fig. 8, cys(b) would be in the lower rather than the upper section of the dual carrier structure. Unlike cys(a), cys(b) cannot lie within a substrate site (since the substrate gives no protection) but it could be in a region which in the inward-facing gated-channel structure comes to lie on the periphery of the site. In this position it might disturb both the binding of the internal substrate and the equilibrium of the internal substrate complex, favouring the mobile (closed) conformation.

7. One-way passive flow

The observation of passive flow in only one direction appears to be contrary to physical principles. The substrate must be able to move both ways, and at equilibrium the inward and outward flows must be identical. But the rates *can* be unequal under zero trans conditions (with no substrate in the opposite compartment). In a passive system obeying Michaelis-Menten kinetics the V/K_m ratios for zero-trans entry and zero-trans exit are necessarily equal, but not the constants themselves [11]. Consequently, at sufficiently low (and equal) substrate concentrations the rates, given by $V[S]/K_m$, will be identical; at saturating concentrations, however, the rates are determined by the values of V, which can differ by any amount provided the K_m values differ by the same factor. The implication is that in the modified aspartate/glutamate exchanger V and K_m are far higher in exit than in entry, V being so low in entry that even at a saturating substrate concentration the rate is below the experimentally detectable level. In agreement, in passive exit the rate is high but the affinity is very low, whereas in passive entry the rate is very low but the affinity is high.

8. Carriers and channels

In theory the concept of a carrier is continuous with the concept of a channel [12], but actual carriers and channels are probably different in both structure and function. In

carrier-mediated transport, even in facilitated, i.e. passive, transport, movement of the substrate is linked to a sequence of carrier conformational changes. The linkage is the basis of all vectorial coupling, in that conformational changes can be tied on one hand to movement of one component of a system, and on the other hand to movement of another component; in consequence free energy drained from one process can be used to do work on the other. (As discussed earlier [6], this view of vectorial coupling encompasses even ATP-driven pumps, where the substrate enters the pump in one form – ATP – and leaves in another form – ADP and P_i – and where this passage is obligatorily linked to a series of conformational changes that in turn are linked to substrate movement.) In contrast to a carrier mechanism the effect of substrate passage on channel structure would be too slight or too undirected to be harnessed for useful work. Channels and carriers differ in just this way – in the possibility of reliably linking induced conformational changes to other cellular processes. Nevertheless it can hardly be doubted that channel structures may be component parts of carrier proteins, as suggested in gated-channel models. Hydrophilic regions in trans-membrane segments of intrinsic membrane proteins, including carriers, appear to be evidence for channels.

The channel-like behaviour of the modified aspartate/glutamate exchanger seems at first to implicate an underlying channel structure, a point discussed by Dierks and co-workers [2]; for if treatment with thiol reagents were to inactivate – and open – the gating machinery involved in antiport, and to destroy the inner but not the outer substrate site, the carrier could become a simple channel with a substrate site at one end. But this hypothesis would not explain why the activation energies for coupled transport and passive exit are the same. Nor would it explain why aspartate and glutamate, strongly bound at the outer site, and sulfate, not bound at all, are indistinguishable in passive transport. The observations suggest instead that a carrier mechanism is involved, and that a conformational change – the same conformational change – is rate-limiting in antiport and passive exit.

9. Summing up

The mathematical relationship between the coupling ratio and the binding energy in successive substrate complexes is rigorous as well as general, applying to antiport, symport, and ATP-driven pumps [6], and to the two main transport models – the carrier model and a bilateral model. From the mathematical relationship it may be deduced that at one or more stages in any coupled vectorial process the carrier interconverts between loose and tight forms of the substrate complex, and that the difference in the binding energy is large. A reaction scheme incorporating such conformational changes, in Fig. 6, accounts for the complicated behaviour of the modified aspartate/glutamate exchanger, supporting the general theory. The hypothetical mechanism in Fig. 8, which corresponds to the scheme in Fig. 6 but is not otherwise based on definite evidence, could be helpful in interpreting experiments.

These transformations in the carrier structure, between the loose and tight complex, are central to an understanding of coupling. A sharply increased binding energy may entail displacements of sections of the transport protein outside the site of initial interaction, as in the diagrams Figs. 7 and 8; the increased binding force in the resulting chelate complex could drive a wider conformational change, altering the properties of the carrier. Certain observations do, indeed, suggest that the substrate site is initially divided. In the lactose permease of *E. coli*, amino acids important in substrate recognition have been identified in six helices thought to form a channel; the binding site, made up of residues on opposite sides of the channel, would presumably converge to interact with the substrate [13]. And in the case of the anion exchanger of red cells two substrate ions appear to be capable of binding on the same side of the membrane and of being transported together, as if the site is divided in two, each part holding a substrate ion [8].

The readiness with which structural modification gives rise to the uncoupled reaction points to a delicate balance between coupled and uncoupled paths. (i) Chemical substitution at cysteine residues abolishes antiport but opens up a route for facilitated transport in several mitochondrial transport systems: the aspartate/glutamate exchanger (as discussed above), the ATP/ADP exchanger [1], the carnitine carrier [14], and the inorganic phosphate carrier [15]. (ii) Single amino acid substitutions in the lactose permease of *E. coli* [4] abolish co-transport but permit facilitated transport. (iii) A single amino acid substitution in yeast plasma membrane ATPase weakens coupling, the rate of ATP hydrolysis rising 3.5-fold while the transport rate falls by 20% [16]. (iv) In aqueous dimethyl sulfoxide the calcium pump catalyzes uncoupled ATP hydrolysis [18,19]. (v) Mutants of the histidine permease of *E. coli*, which function in the absence of the periplasmic binding protein, have ATPase activity uncoupled from transport [17]. (vi) An isolated component, MalK, of the membrane complex of the maltose transport system of *Salmonella typhimurium* (a binding-protein dependent system) exhibits uncoupled ATPase activity [20]. (vii) The classic case of an uncoupled vectorial reaction is myosin ATPase; myosin, when separated from other components of the contractile apparatus, is an uncoupled ATPase [21,22]. In the last two cases the restraint on enzyme activity imposed by the coupling nexus is removed on dissociation of the components of the system.

References

[1] Dierks, T., Salentin, A., Heberger, C. and Krämer, R. (1990) Biochim. Biophys. Acta 1028, 268–280.

[2] Dierks, T., Salentin, A. and Krämer, R. (1990) Biochim. Biophys. Acta 1028, 281–288.
[3] Stappen, R., Dierks, T., Broer, A. and Krämer, R. (1992) Eur. J. Biochem. 210, 269–277.
[4] Kaback, H.R., Bibi, E. and Roepe, P.D. (1990) Trends Biochem. Sci. 15, 309–314.
[5] Krupka, R.M. (1994) Biochim. Biophys. Acta 1193, 165–178.
[6] Krupka, R.M. (1993) Biochim. Biophys. Acta 1183, 105–113.
[7] Krupka, R.M. (1989) J. Membr. Biol. 109, 151–158.
[8] Krupka, R.M. (1989) J. Membr. Biol. 109, 159–171.
[9] Krupka, R.M. (1990) J. Membr. Biol. 117, 69–78.
[10] Dierks, T., Riemer, E. and Krämer, R. (1988) Biochim. Biophys. Acta 943, 231–244.
[11] Krupka, R.M. (1989) J. Biochem. 260, 885–891.
[12] Lauger, P. (1980) J. Membr. Biol. 57, 163–178.
[13] Collins, J.C., Permuth, S.F. and Brooker, R.J. (1989) J. Biol. Chem. 264, 14698–14703.
[14] Indiveri, C., Tonazzi, A., Dierks, T., Krämer, R. and Palmieri, F. (1992) Biochim. Biophys. Acta 1140 53–58.
[15] Stappen, R. and Krämer, R. (1993) Biochim. Biophys. Acta 1149, 40–48.
[16] Serrano, R. and Portillo, F. (1990) Biochim. Biophys. Acta 1018, 195–199.
[17] Petronilli, V. and Ames, G.F.L (1991) J. Biol. Chem. 266, 16293–16296.
[18] De Meis, L. and Inesi, G. (1985) Biochemistry 24, 922–925.
[19] Inesi, G., Kurzmack, M., Nakamoto, R., De Meis, L. and Bernhard, S.A. (1980) J. Biol. Chem. 255, 6040–6043.
[20] Walter, C., Honer zu Benztrup, K. and Schneider, E. (1992) J. Biol. Chem. 267, 8863–8869.
[21] Engelhardt, W.A. and Lyubimova, W.V. (1939) Nature 144, 668–669.
[22] Kielley, W.W. (1961) The Enzymes, 2nd Edn. (Boyer, P.D., Lardy, H. and Myrback, K., eds.), Vol. 5, pp. 159–168, Academic Press, New York, NY.