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Review Letter

Ordered binding model as a general mechanistic mechanism for secondary active transport systems

Ichiro Yamato

Department of Biological Science and Technology, Science University of Tokyo, 2641 Yamazaki, Noda-shi, Chiba 278, Japan

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The mechanistic mechanism of secondary active transport processes has not been fully elucidated Based on substrate binding studies dependent on coupling cation concentrations of the glutamate, melibiose, lactose and proline transport carriers in *Escherichia coli*, the ordered binding mechanism was proposed as the energy coupling mechanism of the transport systems This ordered binding mechanism satisfactorily explained the properties of the secondary active transport systems Thus, this mechanism as the general energy coupling mechanism for the transport systems is discussed

Secondary active transport, Ordered binding mechanism; Energy coupling mechanism

1. INTRODUCTION

Since P. Mitchell proposed the chemiosmotic theory in 1961 [1], the mechanisms of energy transduction systems have been studied extensively. These systems, such as oxidative phosphorylation and active transport, are now explained thermodynamically by proton (or ion) circuits. Recent thermodynamical and kinetic observations have indicated that the coupling is tight (1:1) especially for lactose transport [2–4]. The thermodynamic relation for general systems for secondary active transport of a neutral substrate tightly coupled with the flow of a co-substrate having a positive charge (see Fig 1) is formulated as [4–6]:

$$\exp(\Delta\mu_s/RT)=[S]_i/[S]_o=([X]_o/[X]_i)\exp(-F\Delta\psi/RT)$$
$$=\alpha_i k_{-c}k_s K_{Xi}K_{Si}[X]_o/(\alpha_o k_c k_{-s}K_{Xo}K_{So}[X]_i),$$

where $\Delta\psi$, R, T, F, and $\Delta\mu_s$ represent the electrical potential difference across the membrane, the gas constant, the temperature, the Faraday constant and the electrochemical potential difference of the substrate inside and outside the membrane, respectively. In this case, the binding steps of the substrate and co-substrate to the carrier are thought to be in local equilibrium and the rate limiting steps are postulated to be the translocation steps of the carrier or carrier–substrate complex The existence of a closed membrane system is essential.

This formulation indicates that the electrochemical potential of the coupling cation or proton across the membrane drives the accumulation of substrate. The chemical potential and electrical potential may affect the rate constants of translocation (k_c, etc.) and, or the apparent affinity for substrate at the surface of the membrane. In other words, this formulation (Fig. 1) assumes that the flows of the substrate and the co-substrate are tight in the coupling process, but does not necessarily mean any unique mechanistic mechanism of the coupling process. Then, depending on the value of α, this general model of the mechanism can become (1) an ordered binding model [7–12] when $\alpha\to 0$, K_S or $K_X\to\infty$ and αK_S or αK_X = a finite number, (2) a random binding model [4,6,13] when $\alpha = 1$, or (3) a selective binding model [14,15] when α is between 0 and 1. Thus in order to distinguish any unique type of mechanistic mechanism of the secondary active transport process, detailed kinetic studies of substrate binding and transport reactions are necessary. In eukaryotic systems [4,16,17], the transport activity was also demonstrated to be dependent on the concentration of co-substrate, and especially in some cases, the apparent affinity for substrate has been shown to increase in the presence of the higher concentration of the coupling cation And on this basis, it was postulated in some cases that the transport mechanism is the ordered binding model But mere demonstration of the dependence of the affinity (K_t) for substrate in the transport reaction on the coupling cation concentration is not enough to distinguish any one of the mechanistic mechanisms of the secondary active transport process, because even the random binding model of the coupling mechanism can explain the apparent dependence of affinity on the cation concentration Only detailed binding studies of Na^+, K^+-ATPase and Ca^{2+}-ATPase have been performed [18,19], and in such cases, the transport reaction seemed to be ruled by the ordered binding mechanism.

Correspondence address Ichiro Yamato, Department of Biological Science and Technology, Science University of Tokyo, 2641 Yamazaki, Noda-shi, Chiba 278, Japan. Fax. (81) (471) 25 1841

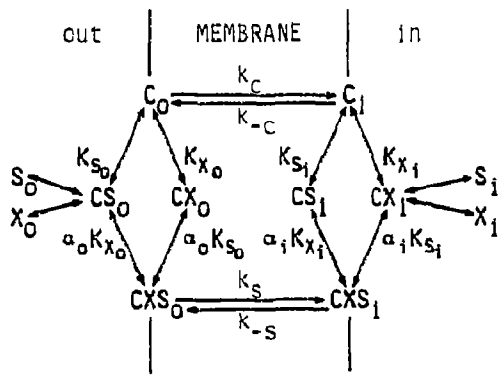

Fig. 1. General model for the transport cycle of a secondary active transport protein. C, S and X are the carrier, substrate and the symported coupling cation (co-substrate), respectively. k_c, k_{-c}, k_s, and k_{-s} are the rate constants of translocation as shown in the figure. K_s and K_x are the dissociation constants of substrate and co-substrate, respectively, to the carrier, and α is a constant. Subscripts i and o represent inside and outside the membrane.

However, in cases of F_1, F_0-ATPase, respiratory proton pump, and secondary active transport systems in general, the ordered binding mechanism has not been yet fully challenged from the point of view of the binding properties.

Bacterial secondary active transport systems are susceptible to the detailed studies of binding properties owing to the easiness of the cloning of carrier genes and of the amplification of carrier proteins. I have been studying the mechanisms of several secondary active transport systems in *Escherichia coli*, especially details of the binding reactions dependent on coupling cations [8,12,20], and in this review, I describe the studies of the kinetic properties of the carrier activities including transport and binding processes and the derivation of the ordered binding model on the basis of binding properties of carrier proteins. Then I propose the ordered binding model as an energy coupling mechanism for secondary active transport that should be generally applicable, and useful even in studies of other energy transducing systems. The biochemical characterization and molecular biological studies of carriers, including site-directed mutagenesis are not discussed in detail.

Na^+/glutamate (gltS), *Na^+/melibiose* (melB), *Na^+/proline* (putP) *and H^+/lactose* (lacY) *symport systems*

Glutamate, melibiose, proline, and lactose carriers in *E. coli* are products of the *gltS* [21,22], *melB* [23,24], *putP* [25,26], and *lacY* [27,28] genes, respectively.

These substrates have been demonstrated to be symported with the coupling cations (Na^+ or H^+) in *E.coli* cells and membrane vesicles, especially by the observations of concomitant movement of the coupling cations with their respective substrates [2,3,29–35].

Among these transport systems, H^+/lactose symport system has been the most extensively studied [4,6,8,11,13–15,36–38]. However, details of the molecular mechanism of H^+ symport are not fully understood; this is partly due to the fact that the coupling cation is H^+, and partly that the coupling mechanism has only been studied on the basis of the properties of the transport activity without considerations of the binding properties, especially of the dependence of the binding activity on pH. Therefore, several types of models and mechanisms have been proposed; namely, a random binding model [4,6,13], a selective binding model [14,15,36], an ordered binding model [8,11], or a proton or charge relay mechanism [37–39]. Thus, the study of binding properties of the substrate to the carrier depending on the coupling cation is inevitable.

Substrate binding properties of glutamate (gltS), *melibiose* (melB), *proline* (putP) *and lactose* (lacY) *transport carriers*

The binding of glutamate, melibiose, proline and lactose to their respective carriers have been studied in detail and shown to be dependent on the coupling cation concentration [8,9,12,20,40,41]. Detailed kinetic studies of substrate binding to the carriers showed that the cations bind first to the carriers, and that through formation of the cation/carrier binary complexes, the carriers acquire an affinity for their substrates (Fig. 2). This suggests that the cation binding induces a conformational change of the carrier to create a binding site for the substrate [8,12,20]. At present, there is no evidence that the cation affecting the binding is the coupling cation, but as the same kind of cation is concerned with both reactions (binding and transport reactions of a carrier), it is simplest to consider that this is so.

In the case of glutamate, a proton also seems to be concerned with the binding and transport cycle [7,20], but since this involvement of a proton in the transport has not been fully elucidated, I will not discuss it further here.

The ordered binding mechanism for glutamate (gltS), *melibiose* (melB), *proline* (putP) *and lactose* (lacY) *transport*

Admitting the assumption that the cation affecting the binding is the coupling cation, the transport cycle can be simplified as follows (for simplicity, I will describe the example for glutamate transport cycle; see ref. [7]; Fig 3A).

(1) The vacant carrier with its binding site for coupling ion oriented to the outside binds the ion, and this induces a conformational change of the carrier to create a binding site for glutamate.

(2) The Na^+/carrier binary complex binds glutamate with high affinity, and this again induces a conformational change by which the carrier becomes oriented inside and outside.

(3) The Na^+/glutamate/carrier ternary complex becomes

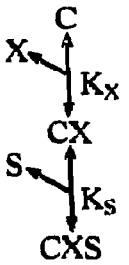

Fig. 2. Binding mechanism of substrate to a secondary active transport carrier. This mechanism has been shown for several secondary active transport carriers [8,9,11,12,20,41]. In this model, a cation, X, binds first to the carrier, enabling the CX complex to bind the substrate. K_s and K_x are the dissociation constants of substrate and co-substrate (cation), respectively, to the carrier. For details, see text.

oriented inside by a conformational change. Usually this step is thought to be the rate limiting step of the transport cycle. This step also seems to be enhanced by $\Delta\psi$.

(4) The Na^+/glutamate/carrier ternary complex oriented inside undergoes similar reactions in the reverse order resulting in the release of first glutamate and then Na^+.

(5) The ion binding site of the vacant carrier becomes reoriented to the outside, resuming the initial state. Thus similarly to the binding reaction, Na^+ binding is thought to cause increase in affinity for glutamate of the carrier in the transport reaction.

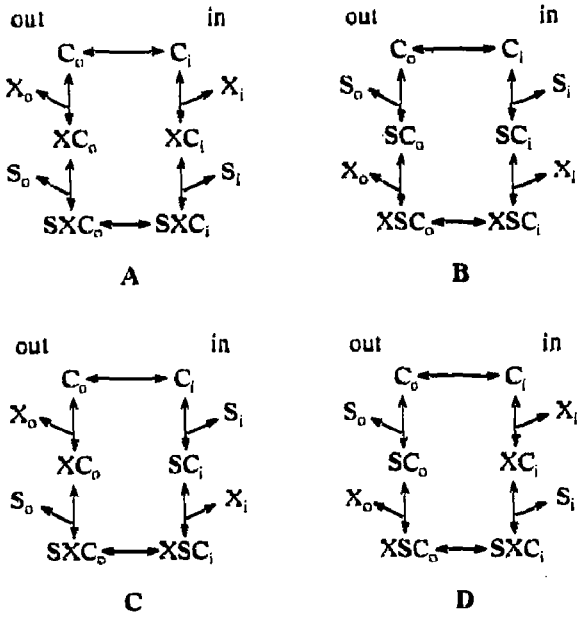

Fig. 3. Ordered binding mechanisms for secondary active transport systems. For explanation, see the legend to Fig. 1. (A) X binds first to C enabling the CX complex to bind S outside the membrane and S dissociates first, then X dissociates to resume the vacant carrier inside the membrane. (B) Outside the membrane, S binds first, then X binds and inside the membrane, X dissociates first, then S dissociates. (C) Outside the membrane, X binds first, then S binds and inside the membrane, X dissociates first, then S dissociates. (D) Outside the membrane, S binds first, then X binds and inside the membrane, S dissociates first, then X dissociates. For secondary active transport systems for GltS, PutP and LacY proteins, the models B to D are excluded as discussed in the text.

If the binding affinities for the substrate and the cation are extraordinarily asymmetrical, the other ordered binding models in Fig. 3 (Fig. 3B, C and D) can not be excluded. But if the binding affinities of the carrier are not extraordinary, these other ordered binding models are excluded from the substrate binding properties strictly dependent on the presence of the coupling cation. Furthermore, the random binding model (Fig. 1) corresponds to the model that allows the four possibilities in Fig. 3 in an equal degree. Thus by the same reason, the binding properties of substrate dependent on the cation exclude the random binding model for the secondary active transport mechanism.

The kinetic parameters of the glutamate transport based on this ordered binding model using the binding parameters were formulated as follows [7]:

$$K_t = \frac{1.5}{[H^+]} + \frac{220}{[Na^+]}$$

$$V_{max} = k_s C_t$$

,where K_t and V_{max} are the apparent affinity and maximum velocity in the transport reaction of glutamate, respectively, and k_s and C_t are the translocation rate constant in the forward reaction and the total concentration of the carrier in membrane, respectively. This formulation could explain quantitatively the transport properties of the glutamate carrier [7]. The apparent affinities for Na^+ and glutamate in the transport cycle were in the same order of magnitude as the affinities for them in the binding reaction. Thus the kinetic parameters of the transport reaction shown above were derived from the binding parameters directly without further assumption than that the Na^+ affecting the binding is the coupling Na^+. This suggests that the binding properties of the carrier are essential parts of the whole transport cycle of the carrier and that this secondary active transport system is driven by coupling with the cation via an affinity change of the carrier for substrate. This type of coupling may guarantee tight coupling (1:1) without any leakage (loose coupling) of Na^+, or substrate flow alone.

From the observation that $\Delta\psi$ enhanced the transport reaction without any appreciable affect on the apparent affinity of the carrier for the substrate, $\Delta\psi$ has been postulated to enhance the translocation steps by changing k_c, k_{-c}, k_s, or k_{-s} [7,20].

The binding and transport properties of the proline carrier dependent on H^+ and Na^+ were also studied in detail and it was found that Na^+ affecting the binding affinity for proline ($K_{d_{Na^+}}$=10 mM) also affects the Michaelis constant Kt for the transport reaction of proline ($K_{t_{Na^+}}$=30 μM) [12]. Then a Na^+/proline symport model as in the case of *gltS* carrier was constructed. The ordered binding model for proline carrier explained most of the binding and transport properties

of the carrier [12] The very high affinity for Na^+ in the transport reaction ($K_{t_{Na^+}}$=30 μM) was explained by assuming the asymmetry of the affinities of the carrier for Na^+ on the two sides of the membrane [12], which has not yet been demonstrated experimentally. $\Delta\psi$ was also postulated to enhance the translocation velocity of the carrier. Furthermore, for support of this model, several transport mutants having altered affinities for the coupling cation, Na^+, were isolated and shown to have altered affinities for Na^+ in the binding reaction in parallel to the transport properties [42,43]. Kinetic parameters were also derived according to the affinity model. And this formulation could explain well the observed parameters for binding and transport activities [12].

It was similarly pointed out that the properties of substrate binding depending on pH of the lactose carrier could well explain the transport properties of the *lacY* carrier [11]. Thus the kinetic parameters were also formulated. Furthermore, Yamato and Anraku postulated that, as in the case of the *gltS* carrier, $\Delta\psi$ enhances the transport velocity of the translocation of the H^+/substrate/carrier ternary complex from one side of the membrane to the other. Then it was demonstrated [11] that the high pK_a for the coupling proton explained the difference [4,44] between the K_d, dissociation constant of lactose from the carrier, and the K_t, Michaelis constant for lactose of transport in the active transport mode at neutral pH and that the absence of enhancement of translocation velocity in the absence of $\Delta\psi$ explained the similar K_d and K_t values for lactose in the facilitated diffusion mode of the carrier (for details, see discussion section of the previous paper [11]) On the other hand, the slow translocation velocity of the active transport cycle for certain substrates such as thiodigalactoside explained the observed similar values [44] of K_d and K_t for even the active transport mode of the carrier [11]. Thus this model did not necessitate additional postulations, such as conformational change induced by $\Delta\psi$ to change the affinity for the substrate [4] or different binding sites for two substrates, where one (lactose) has a lower K_t than the K_d value in the active transport mode and the other (thio-digalactoside) has similar K_t and K_d values [4]

DISCUSSION

As I have pointed out in the Introduction section, several secondary active transport systems have been postulated to operate according to the ordered binding mechanism on the basis of the observation that the affinity for the substrate in the transport reaction apparently increases with the higher concentrations of the coupling cation. However, mere demonstration of the dependence of the affinity of the transport reaction on the concentration of the coupling cation does not necessarily exclude other mechanistic mechanims than the ordered binding mechanism as the secondary active transport mechanism [6]. And so far, the measurement of the binding activity of the substrate to carrier has been difficult, because the carrier concentration in membranes was not high enough Recently, the DNA technique enabled us to amplify the carrier protein in membranes, and as I have described in the above section, binding properties of several carrier proteins in *E. coli*, especially the dependences on the concentrations of the coupling cations, were examined. On the basis of these binding properties, I have discussed in this review that these secondary active transport systems operate with the ordered binding mechanism.

Then in this section, I propose that the secondary active transport mechanism is generally understood to be the ordered binding model shown in Fig 3, and especially for the four cases described in this review, the ordered binding models other than that shown in Fig 3A are excluded. According to this model it is considered that there is a conformational change at the step of binding of the coupling cation enabling the carrier to bind the substrate, and then the cation/substrate/carrier ternary complex can be translocated inside.

It will be interesting to see how the charge (proton) relay mechanism postulated by Kaback [37–39] can be reconciled with this ordered binding model of transport I am also interested in looking for, or making a carrier operating according to the random binding model, which may reveal the physiological significance of the ordered binding mechanism for energy transduction. Possibly its significance is to ensure a tight coupling process, or to economize in protein design in nature

Even within the limit of Mitchell's chemiosmotic theory with tight coupling (Fig. 1), it is possible that the imposition of $\Delta\psi$ affects the apparent affinity (K_t) and, or velocity (V_{max}) of the transport activity: if $\Delta\psi$ changes the ratio of the amounts of carrier oriented inside and outside, it may alter the K_t and if it changes the translocation rate constants of the carrier, it may change the V_{max}. Furthermore, $\Delta\psi$ may exert its effect on the conformation of the carrier with consequent change in affinities for ligands. Wright et al. [4] proposed this possibility to explain the discrepancy between the K_t (apparent Michaelis constant for substrate in transport in the presence of $\Delta\psi$) and the K_d (dissociation constant of the substrate in the binding reaction without $\Delta\psi$) for lactose transport. But at present there is no evidence for this possibility and even without such a special conformational change by $\Delta\psi$ of the LacY carrier, the K_t/K_d change could be explained by the ordered binding model assuming that $\Delta\psi$ enhances the translocation rate constants. This assumption seems likely for most of the transport activities of the *putP*, *lacY* and *gltS* carriers. The mechanism and the physiological significance of this effect of $\Delta\psi$, however, must be elucidated to understand the whole cycle of the secondary active transport system.

PROSPECT

I have shown that several secondary active transport processes in bacteria may be understood by an ordered binding mechanism which involves conformational change induced by a ligand that alters the affinity of the carrier for its substrate by direct demonstration of the binding properties. Proof is needed for the assumption that the cation affecting the binding is the coupling cation. This will be difficult to obtain, but supporting evidence has been obtained from binding and transport studies of several cation coupling mutants [11,42,43] and also should be obtained from detailed studies of the structure/function relationships of carrier proteins. Further studies are also required on the effect of $\Delta\psi$ on the transport reaction for full understanding of the involvement of $\Delta\psi$ in transport.

Since many possible mechanisms for secondary active transport systems seem in general to be ruled out by the ordered binding mechanism, as I have discussed in this review by the direct demonstration of the cation dependent binding properties, this mechanism probably has some biological significance, such as to guarantee tight coupling without leakage. Therefore, by the same reason, other possible energy transducing machineries may also be considered to be ruled by this ordered binding mechanism. The binding properties of several ATPases such as Ca^{2+}- and Na^+,K^+-ATPases [18,19] have been studied extensively and the transport reactions seem to be explained well by ordered binding mechanisms. However, the mechanisms of energy transduction in systems such as F_1F_o-ATPase, the respiratory H^+ pump and even actomyosin ATPase in muscle contraction, are not well understood, because the binding properties of substrates (H^+) to these molecular machineries have not been characterized. For support of the above idea, information is required about whether the properties of ligand-binding of these machineries are similar to those of the carriers.

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