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The Molecular Mechanism of Dicarboxylic Acid Transport in Escherichia coli K 12

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It is the purpose of this communication to review the properties of the dicarboxylic acid transport system in Escherichia coli K12, in particular the role of various dicarboxylate transport proteins, and the disposition of these components in the cytoplasmic membrane. The dicarboxylate transport system is an active process and is responsible for the uptake of succinate, fumarate, and malate. Membrane vesicles prepared from the EDTA, lysozyme, and osmotic shock treatment take up the dicarboxylic acids in the presence of an electron donor. Genetic analysis of various transport mutants indicates that there is only one dicarboxylic acid transport system present in Escherichia coli K12, and that at least 3 genes, designated *cbt*, *dct A*, and *dct B*, are involved in this transport system. The products corresponding to the 3 genes are: a periplasmic binding protein (PBP) specified by *cbt*, and 2 membrane integral proteins, SBP 1 and SBP 2, specified by *dct B* and *dct A*, respectively. Components SBP 1 and SBP 2 appear to be exposed on both the inner and outer surfaces of the membrane, and lie in close proximity to each other. The substrate recognition sites of SBP 2 and SBP 1 are exposed on the outer and inner surfaces of the membrane respectively. The data presently available suggest that dicarboxylic acids may be translocated across the membrane via a transport channel. A tentative working model on the mechanism of translocation of dicarboxylic acids across the cell envelope by the periplasmic binding protein, and the 2 membrane carrier proteins is presented.

Key words: dicarboxylate transport, transport channel, membrane structure, membrane protein, periplasmic binding protein

In the past two decades considerable efforts have been devoted to the study of the relationship between membrane structure and function. In the area of membrane transport, most investigations have been limited to kinetic studies of uptake or the effect of environmental changes on the transport process. Only recently has significant progress been made in our understanding of the energy-coupling mechanisms for various transport processes. It has now been demonstrated by quite a number of workers that a membrane potential, and/or pH gradient are involved in a large number of active transport systems. Exactly

Abbreviations: EDTA – Ethylenediaminetetraacetic acid; DCCD – dicyclohexylcarbodiimide; CCCP – carbonyl cyanide-m-chlorophenylhydrazone; NEM – N-ethylmaleimide

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how this protonmotive force can affect the membrane structure, or more precisely how the membrane transport proteins respond to such changes, is still shrouded in darkness. Not until we have determined the number and spatial arrangement of membrane transport components involved in a transport process, can we begin investigating the mechanism(s) by which solute translocation is affected by the energized state of the membrane.

Generally speaking, in gram-negative bacteria, there are at least 2 different types of transport components. These are the periplasmic binding proteins and the intergral membrane transport proteins. Various periplasmic binding proteins have been isolated, purified, and characterized. Both biochemical and genetic evidence indicates that these proteins are indeed involved in the transport process. However, the disposition of these proteins in the periplasmic space or in the outer membrane is far from clear. It is not certain whether they are embedded in the outer membrane, or whether they exist in a free or bound form in the periplasmic space. Recently, Boos' laboratory indicated that some of these proteins may be exposed on the outer surface of the outer membrane of the cell envelope (1). Despite the enormous amount of work devoted to the characterization of the periplasmic binding proteins, the exact role of these proteins in the transport process is not known for certain. It has been suggested by Silhavy and Boos (2) that one of the functions of the periplasmic binding proteins may be the maintenance of a high concentration of substrate in the periplasmic space. On the other hand, G. Ames has demonstrated very elegantly by genetic means that specific physical interactions between the periplasmic binding protein, and specific membrane carrier protein(s) are required for the transport process (3). It was suggested by J. Singer (4) that the periplasmic binding proteins may actually be the loosely bound receptor proteins of the membrane transport proteins(s).

As far as the integral membrane transport components are concerned, even much less is known concerning their number, properties, and spatial arrangement in the membrane. Our ignorance in this area is mainly due to the inability to isolate "active membrane transport component(s)." The lactose transport component – the "M protein" – was ingeniously labeled with radioactive N-ethylmaleimide and isolated by Fox and Kennedy (5). However, since this protein is inactivated by the labeling procedure, not much information can be obtained on the mode of action of the M protein. Recently, Lo and Sanwal (6) have succeeded in isolating 2 active membrane-bound transport components which are involved in the translocation of dicarboxylic acids. More recent data (7) from this laboratory indicated that these 2 membrane transport components are transmembrane proteins and that they may form multimeric subunit aggregates traversing the entire thickness of the membrane. These findings suggest that dicarboxylic acids may be translocated across the membrane via a transport channel. In addition to these 2 membrane proteins, it was also found that a periplasmic binding protein is involved in dicarboxylate transport (8).

It is the purpose of this communication to review the properties of the dicarboxylic acid transport system, in particular the role of various dicarboxylate transport proteins, and the spatial arrangement of the membrane transport components. Finally, we will present a tentative working model for the translocation of dicarboxylic acids across the cell envelope by the periplasmic binding protein, and the 2 membrane carrier proteins.

METHODS

The methods used have been described in previous publications (6–12, 16) from this laboratory, and are referred to at the appropriate sections in this communication.

RESULTS

The Number of Dicarboxylate Transport Systems Present in Escherichia coli K12

In studying the transport of a given substrate, it is essential to establish the number of transport systems by which the organism can transport the substrate under a given experimental condition. This is very important in the interpretation of substrate specificity, transport kinetics, and in deciding the number of transport components which are involved in that particular transport system. The following biochemical and genetic studies on the transport system indicate that there is only one dicarboxylate transport system present. Using an Escherichia coli strain *(sdh, frd)* which cannot metabolize succinate, we found that there is only one K_m value (30 μM) for the transport of succinate, and no biphasic curve is observed when the data are plotted in the form of a double reciprocal plot. Transport studies with membrane vesicles also provide similar information (9, 10). These data indicate the presence of only one dicarboxylate transport system.

In the isolation of transport mutants, the frequency of spontaneous mutation is found to be around 2×10^{-6} (11). It is a well established fact that spontaneous mutations occur at frequencies around $10^{-5}-10^{-6}$. If there were 2 dicarboxylate transport systems present in the cell, transport defective mutants would be found only if mutations occur in both transport systems. The frequency of occurrence of such a double mutant would then be around 10^{-12}. The fact that we obtain a frequency of around 10^{-6} indicates that there is only one dicarboxylate transport system present.

Furthermore, as discussed later, at least 3 different genes are responsible for the transport process, and mutants defective in any one of these 3 genes are unable to transport the substrate (9). Again, this serves to indicate that only one transport system is present in the cell.

General Properties of the Dicarboxylic Acid Transport System

The dicarboxylic acid transport system is responsible for the uptake of succinate, fumarate, and malate. The uptake of succinate is competitively inhibited by fumarate and malate. Mutants defective in this transport system are unable to take up or to grow on succinate, fumarate, or malate (9). Membrane vesicles prepared from the EDTA, lysozyme, and osmotic shock treatment transport the 3 dicarboxylic acids in the presence of an electron donor, such as D-lactate. Transport studies carried out with these membrane vesicles show the same substrate specificity as the whole transport system (10).

Both whole cell and membrane vesicle studies indicate that dicarboxylic acids are transported against a concentration gradient. Using an *sdh, frd* mutant, we have demonstrated that at least 95% of the succinate taken up is not chemically modified (9). This would rule out the possibility of group translocation. Both uncouplers and inhibitors of the electron transport chain are found to inhibit succinate transport in membrane vesicles (12). Furthermore, membrane vesicles from mutants defective in lactate dehydrogenase are unable to utilize D-lactate as the electron donor for the transport process. These data suggest that the functioning of an electron transport chain is required for the generation of a proton gradient across the membrane before dicarboxylic acids can be translocated to the inside of the cell. Indeed, it has been demonstrated recently by Kaback that succinate transport is dependent on the protonmotive force (13) and by Rosenberg that approximately 2 protons enter the cell with each dicarboxylate molecule (14).

The dicarboxylate transport system appears to be more complicated than other active transport systems such as those for proline or lactose. Dicarboxylate uptake by membrane

vesicles is also dependent on the presence of a functioning Ca^{2+},Mg^{2+}-ATPase. The ATPase inhibitors, such as DCCD or pyrophosphate, are found to inhibit dicarboxylate transport in energized membrane vesicles, although there is no effect on proline uptake (12). The involvement of the Ca^{2+},Mg^{2+}-ATPase is further substantiated by the construction of a Ca^{2+},Mg^{2+}-ATPase negative mutant (12). Whole cells and membrane vesicles from this mutant are unable to take up succinate, even though they can transport proline normally. These findings suggest that the Ca^{2+},Mg^{2+}-ATPase may play an indirect role in dicarboxylate transport. The mechanism by which the Ca^{2+},Mg^{2+}-ATPase exerts its effects is currently being investigated in our laboratory.

Transport Components Involved in Dicarboxylic Acid Transport

a. Genetic dissection of the transport system. Indications of the number of components involved in dicarboxylate transport may be obtained by studying the genotypic and phenotypic properties of various transport mutants. Phenotypically the transport mutants can be divided into 2 classes, *cbt* and *dct* (Table I). Both types are unable to grow on the transport substrates, succinate, fumarate, or malate. The *cbt* mutants differ further from the *dct* mutants in that they are unable to grow on D-lactate (Table I). It turns out that the *cbt* mutants are also defective in the D-lactate transport system. The *dct* mutants can be divided genotypically into the *dct A* and the *dct B* mutants. Genetic analysis of these mutants indicates that the *dct A* and *dct B* genes map at 78 min and 16 min of the E. coli linkage map respectively (15, 16). The *cbt* gene is located at 16 min of the linkage map. The above findings suggest that at least 3 genes are involved in the dicarboxylate transport process (16).

b. Biochemical dissection of the transport system. At least 3 transport components are involved in dicarboxylate transport. They are comprised of a periplasmic binding protein (PBP), and 2 membrane transport proteins (SBP 1 and SBP 2). Active species of these molecules have been isolated through the use of aspartate-coupled Sepharose columns (6, 8).

i. The periplasmic binding protein (PBP). The involvement of PBP in the dicarboxylate transport process is based on the following findings: 1) The dicarboxylate transport system in whole cells is a shock-sensitive system. Transport activities are not observed after subjecting the cells to EDTA-osmotic shock treatment, and a periplasmic binding protein capable of binding with succinate can be isolated from the shock fluid (9). 2) PBP is found to have similar substrate specificity (i.e., same binding site for succinate, fumarate, and malate), and substrate affinity (K_d = 35 μM) as the whole cell transport system (8). 3) The whole cell transport system differs from the membrane vesicle transport system in that the former is inhibited by N-ethylmaleimide, whereas the latter is not. This suggests that an NEM-sensitive transport component may be present outside the cytoplasmic membrane. Indeed, it is found that the binding of succinate to PBP is inhibited by NEM (8–10). 4) Although the *cbt* mutant cannot transport dicarboxylic acids, membrane vesicles prepared from this mutant are able to do so (Table I). This means that the membrane components in this mutant are functioning normally, and it may be lacking an active periplasmic binding protein. 5) Biochemical analysis of the *cbt* mutant indicates that the PBP cannot be isolated from the osmotic shock fluid by the affinity column (Fig. 1) (8). This suggests that either the substrate recognition site of PBP is defective, or the protein is not synthesized. This serves as an important piece of evidence indicating that the *cbt* gene is responsible for the periplasmic binding protein. 6) It is found that D-lactate also binds to

TABLE I. Properties of Various Dicarboxylate Transport Mutants*

Strains	Phenotype			Relative rates of succinate transport		Genetic loci
	Acetate	D-Lactate	Succinate, malate, or fumarate	Whole cells	Membrane vesicles	
Wild type	+	+	+	+(710%)	+(52%)	
sdh, frd	–	+	(–)†	+(100%)	+(100%)	
cbt	+	–	–	–(8%)	+(58%)	16 min
dct A	+	+	–	–(9%)	–(2%)	78 min
dct B	+	+	–	–(12%)	–(5%)	16 min

*Under "PHENOTYPE", (+), (–) indicate growth or no growth respectively at 37°C for 48 h on minimal medium using the mentioned carboxylic acids as the sole carbon sources. Under "RELATIVE RATES OF SUCCINATE TRANSPORT",(+), (–) indicate the capabilities or incapabilities respectively of the whole cells or membrane vesicles to transport succinate. Transport studies with whole cells or membrane vesicles were carried out as described in Ref. 9 and 10. The initial rates of succinate uptake by the *sdh, frd* mutant were 0.9 nmoles/mg of cells (dry weight)/min for the whole cells, and 1.18 nmoles/mg protein/min for the membrane vesicles. These values were taken as 100% for the respective uptake systems. The mapping of various genes was described in Ref. 16. The genetic loci were revised according to the new map by Bachmann et al (15).

†The *sdh, frd* mutant is unable to grow on succinate or fumarate; however, it can grow on malate.

the substrate recognition site of PBP. If the *cbt* gene is responsible for PBP, then one would expect that the *cbt* mutant is unable to transport D-lactate. This indeed is the case. All the evidence presented above indicates that PBP is involved in the dicarboxylate transport process, and that the *cbt* gene is responsible for this protein. We will elaborate on the role of PBP in the "Discussion."

ii. The membrane transport components (SBP 1 and SBP 2). When membrane vesicles from an *sdh, frd* mutant or from a wild-type strain are treated with the nonionic detergent Lubrol 17A-10 (Imperial Chemical Industries Ltd., Blackley, Manchester, England), the succinate binding proteins can be solubilized. Fractionation of the solubilized membrane proteins on an aspartate-coupled Sepharose column using succinate elution yields 2 protein peaks (Fig. 2) (6). The following observations strongly suggest that these 2 components (SBP 1 and SBP 2) are involved in the translocation of the dicarboxylic acids across the membrane. 1) Treatment of the membrane vesicles with various detergents abolishes transport and releases succinate binding activity in the supernatant. 2) Both SBP 1 and SBP 2 are able to bind with succinate, fumarate, and malate. Malonate, which is a potent competitive inhibitor of succinate dehydrogenase, has no effect of the binding of succinate to these 2 proteins (6). 3) Like the whole cell transport system, SBP 1 has a K_d of 23 μM for succinate, and 47 μM for malate. However, it should be noted that SBP 2 has a K_d of 2 μM for succinate, and 7 μM for malate. No enzymatic activities can be detected in preparations of SBP 1 and SBP 2 (6). 4) Membrane vesicles from mutants defective in the *dct A* or *dct B* gene are unable to transport succinate in the

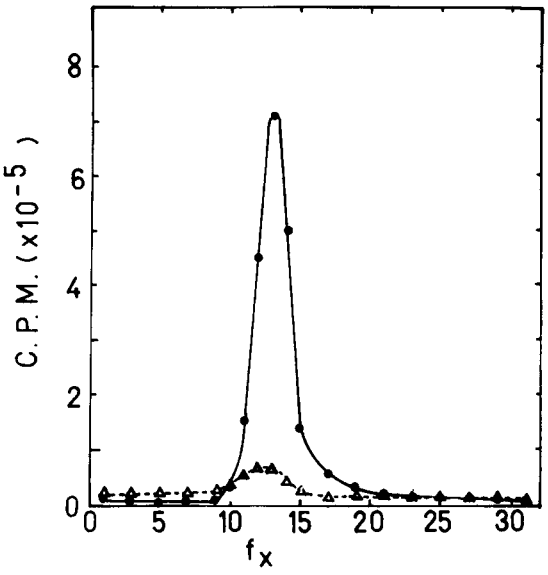

Fig. 1. Elution profile of PBP from the aspartate-coupled Sepharose. Cells were labeled with $^{35}SO_4^2$ for 12 h. After harvesting and washing, the cells were subjected to EDTA and osmotic shock treatment (8). The shock fluid was loaded onto the aspartate-coupled Sepharose column. After washing off the unbound proteins, 0.2 M succinate was then added to elute the bound proteins. (•) indicates elution profile from wild-type cells (*cbt*$^+$), (△) indicates elution profile from the *cbt* mutant.

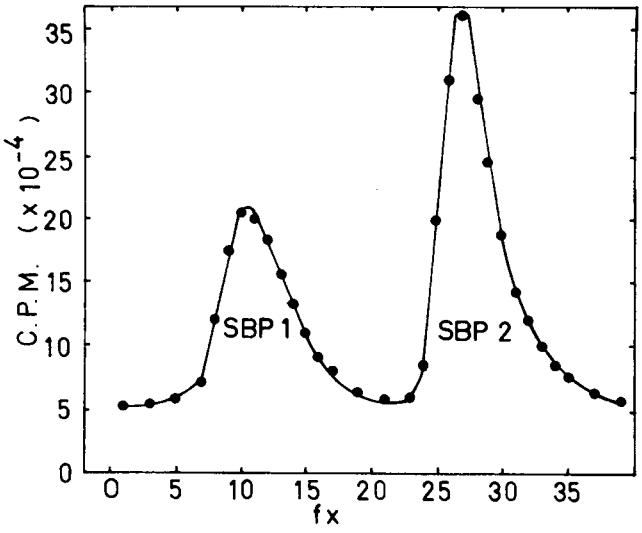

Fig. 2. Elution profile of membrane proteins from the aspartate-coupled Sepharose. $^{35}SO_4^2$-labeled membrane vesicles were prepared and solubilized according to Ref. 11. Fractionation of the solubilized proteins was carried out by affinity chromatography in the presence of 0.1% Lubrol-phosphate buffer, pH. 6.6.

presence of an electron donor (10). This points to the likelihood that the *dct* genes may be responsible for some membrane transport components. It will be shown later on in the membrane binding studies, that the *dct A* membrane vesicles do not possess the SBP 2 substrate recognition sites, and that the SBP 1 substrate recognition sites cannot be detected in the *dct B* membrane vesicles (Fig. 6) (11). 5) The properties of the *dct* membrane vesicles suggest that the SBP 1 and SBP 2 proteins may be altered or absent in the corresponding mutants. If so, one should be able to detect these changes using affinity chromatography. Figure 3 shows that the SBP 1 component cannot be detected when the solubilized membrane proteins from the *dct B* mutant are fractionated by affinity chromatography, and similarly SBP 2 protein cannot be detected in the *dct A* mutant (11). This is in agreement with the membrane binding studies with the mutant membranes. It should be noted that both *dct A* and *dct B* mutants contain a functioning succinate dehydrogenase. The fact that SBP 1 or SBP 2 is absent in the *dct* mutants again suggests that both of these proteins are different from succinate dehydrogenase. The above findings provide the essential evidence that both SBP 1 and SBP 2 are involved in dicarboxylate transport, and that the *dct A* and *dct B* genes are responsible for components SBP 2 and SBP 1 respectively.

The Orientation of the Substrate Recognition Sites of SBP 1 and SBP 2 in the Cytoplasmic Membrane

Having demonstrated that both SBP 1 and SBP 2 are involved in dicarboxylate transport, the next obvious question concerns the orientation of the substrate recognition sites on these 2 proteins. Formulation of reasonable molecular mechanisms for the membrane

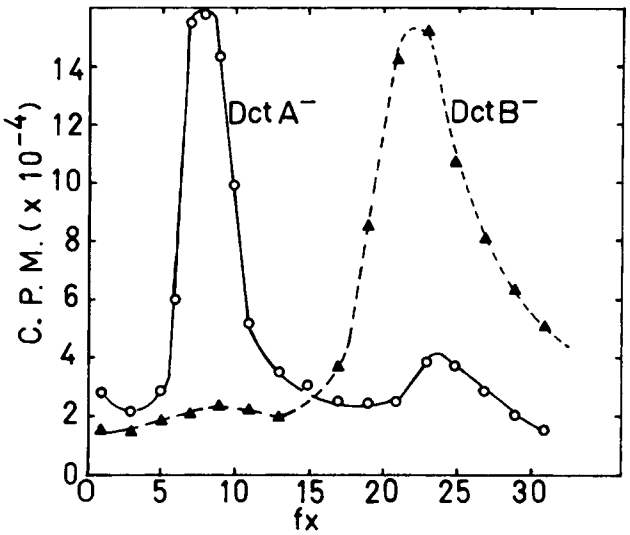

Fig. 3. Elution profiles of membrane proteins from the aspartate-coupled Sepharose. Membrane proteins were prepared according to procedure described in Fig. 2. ○) The elution profile of membrane preparations from the *dct A* mutant, Dct A^-; ▲) the elution profile of membrane preparations from the *dct B* mutant, Dct B^-.

translocation process would demand information on whether the substrate recognition site(s) of a protein can be exposed to both surfaces of the membrane by oscillation of the protein across the membrane as predicted by the "mobile carrier model (2)" (Fig. 4), or if these sites are fixed and accessible on only one surface of the membrane, as required by models (1), (3), and (4). If the latter is the case, then one would have to determine whether the substrate recognition sites of both SBP 1 and SBP 2 are exposed to the same surface of the membrane. The above information would certainly be very useful in deciphering the molecular mechanisms of the transport process.

As mentioned earlier, the K_d values of SBP 1 and SBP 2 are 47 μM and 7 μM respectively for malate. This difference in the K_d values can be used as a means of distinguishing the substrate recognition sites of SBP 1 and SBP 2. One may thus determine the disposition of the SBP 1 and SBP 2 substrate recognition sites on the cytoplasmic membrane by measuring the binding affinities of spheroplasts, right-side-out (R.S.O), and inside-out (I.S.O.) vesicles. It has now been established that R.S.O. vesicles can be prepared by subjecting the cells to EDTA, lysozyme; and osmotic shock treatment, and that I.S.O. vesicles can be obtained by subjecting the cells to French-press treatment.

Several precautions were taken in carrying out the membrane binding studies. Firstly, one must assure that there is no substrate transport during the binding process. The presence of any residual transport activity would certainly make the binding data difficult to interpret. We have shown previously that dicarboxylic acids are transported across the membrane only when a proton gradient is established across the membrane by the addition of an electron donor (10, 12); furthermore, this process is possible only if both SBP 1 and SBP 2 are functioning normally (10). We have also demonstrated that hardly any transport activity can be detected at 4°C. Therefore, in order to ensure that there is no transport

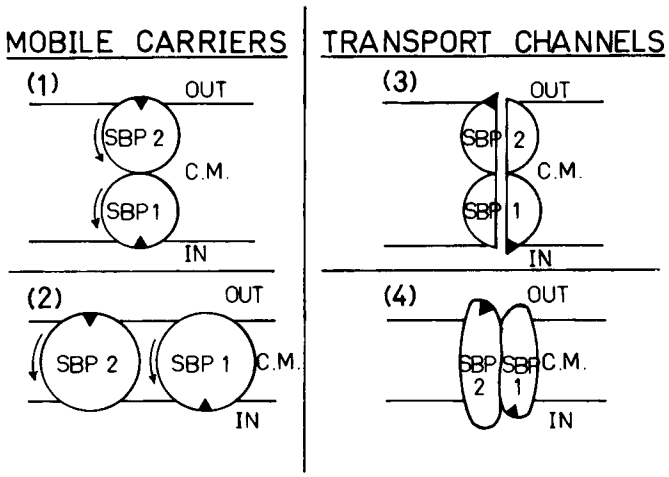

Fig. 4. Schematic diagram showing the various mechanisms by which dicarboxylic acids can be translocated across the cytoplasmic membrane. The disposition and mode of action of the 2 dicarboxylate membrane transport components (SBP 1 and SBP 2) in the cytoplasmic membrane are depicted in Models (1) to (4). Models (1) and (2) can be regarded as "mobile carrier" models; and Models (3) and (4) are referred to as "transport channel" models. In Models (2) and (4), both SBP 1 and SBP 2 are transmembrane proteins, whereas in Models (1) and (3), the membrane transport components are only exposed to one surface of the membrane. ▲) The substrate recognition sites(s) of the transport components; C.M.) cytoplasmic membrane.

activity, we carried out the binding studies in the absence of any electron donor and in the presence of an uncoupler, CCCP (which was shown to collapse to proton gradient). In the case of R.S.O. and I.S.O. vesicles, binding studies were carried out at 4°C; however, binding studies with spheroplasts were carried out at 23°C, so as to prevent lysis of spheroplasts at low temperatures (17). Results presented in Fig. 5 suggest that there is no detectable transport activity during the binding process. Our binding studies and previous report (10) from this laboratory indicate that the K_d value for binding to R.S.O. membrane preparations is 4 μM for malate, and the K_m value for transport is around 45 μM. If residual transport activity were occurring during the binding process, the resulting curve in a double reciprocal plot should show a biphasic behaviour. It is, however, clearly evident from Fig. 5 that the binding plot is linear and yields only one K_d value. This indicates that our procedure is capable of measuring exclusively the binding of the substrate to the membrane surface, and not the uptake of the substrate. This conclusion is strengthened by the results of binding studies with membrane transport mutant (*dct A* and *dct B*) vesicles (Fig. 6). These mutant membrane vesicles are unable to transport the substrate even after energization. Figure 6 indicates that essentially the same K_d values as those with the wild-type membrane vesicles are obtained for the normal components in these mutant membranes. Thus, one can eliminate the possibility of facilitated diffusion, or residual active transport of the substrate across the membrane during the binding process.

A second problem that arises in the interpretation of the binding data concerns the specificity of the binding to membrane preparations. It is well established that in Escherichia coli succinate dehydrogenase is a membrane-bound enzyme, whereas malate dehydrogenase

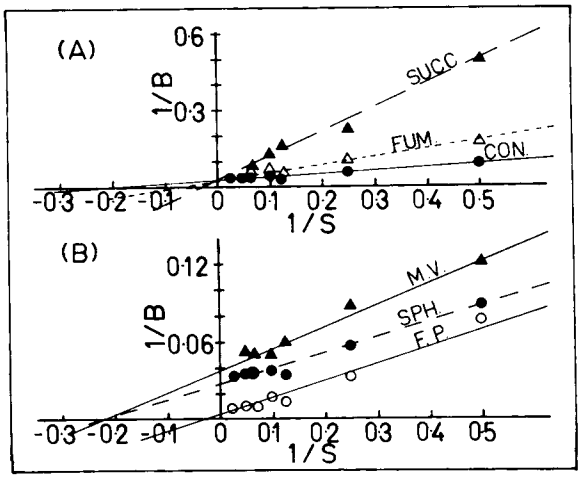

Fig. 5. The binding of [^{14}C] malate to membrane preparations from strain CBT 43 (*sdh,frd*). A) The binding of [^{14}C] malate to spheroplasts in the control (CON.) or in the presence of 0.1 mM succinate (SUCC.) or 0.1 mM fumarate (FUM.). Spheroplasts were kept in 20% sucrose, 0.05 M phosphate buffer, pH 6.6. They were preincubated with 10 μM CCCP, and binding studies were carried out as described in Ref. 11. Incubation was carried out at 23°C to prevent lysis of spheroplasts at low temperature. B) The binding of [^{14}C] malate to spheroplasts, right-side-out membrane vesicles (M.V.) and inside-out membrane vesicles (F.P.). Binding studies with R.S.O. and I.S.O. vesicles were carried out as in the case of spheroplasts (SPH.) (11) except that the binding studies were carried out in 0.05 M phosphate buffer, pH 6.6, at 4°C; the membrane preparations were preincubated with 10 μM CCCP as in the case of spheroplasts.

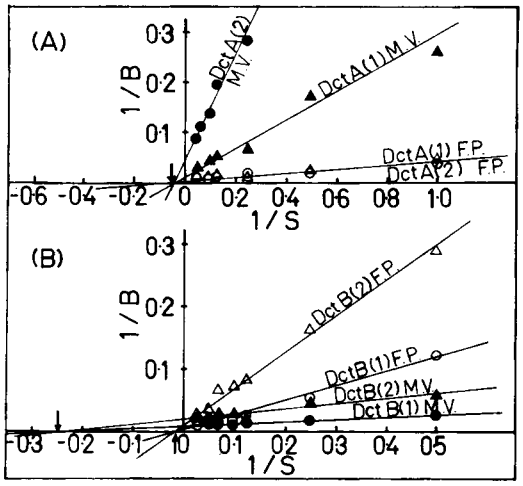

Fig. 6. The binding of [^{14}C] malate to membrane preparations from various *dct* mutants. Binding studies were carried out essentially as indicated in Fig. 5B. A) Malate binding by right-side-out membranes, (M.V.) and inside-out membrane vesicles (F.P.) with 2 independently isolated *dct A* mutants, Dct A (1) and Dct A (2). B) Similar binding studies with 2 independently isolated *dct B* mutants, Dct B (1) and Dct B (2).

is not (20). Although succinate dehydrogenase has quite a different optimum pH value and substrate specificity than the dicarboxylate transport components, it is still possible that succinate dehydrogenase may bind with succinate at pH 6.6; this would certainly complicate the interpretation of the binding data. Figure 5A shows that membrane vesicles have the same binding site for succinate, malate, and fumarate. Therefore in order to avoid the complications incurred with succinate binding, we carried out the binding studies using malate as the substrate. It will be indicated later on in the studies with various *dct* mutants, that the binding of malate to various membrane preparations is not due to the presence of malate dehydrogenase, or succinate dehydrogenase.

Binding studies with membrane preparations from "wild-type" cells indicate that both succinate and fumarate competitively inhibit the binding of malate to the spheroplasts (Fig. 5A) and membrane vesicles (results not presented). It is also found that both R.S.O. vesicles and spheroplasts have K_d values of around 4 μM for malate, and the I.S.O. vesicles have a value of around 30 μM (Fig. 5B). We have indicated earlier that SBP 1 and SBP 2 have K_d values of 47 μM, and 7 μM, respectively, for malate. This similarity between the binding affinities of the R.S.O. vesicles (or spheroplasts) and the SBP 2 component seems to indicate that the substrate recognition sites of SBP 2 are exposed to the outer surface of the membrane. Similarly, binding studies with the I.S.O. vesicles suggest that the substrate recognition sites of SBP 1 are exposed to the inner surface of the membrane. If this were the case, then one should not be able to detect any binding with the I.S.O. membrane vesicles from the *dct B* mutant (defective in SBP 1), and with the R.S.O. vesicles or spheroplasts from the *dct A* mutant (defective in SBP 2). Indeed this is what we found (Fig. 6). Although the R.S.O. membrane vesicles from the *dct B* mutants demonstrate the normal affinity of binding (i.e. 4 μM), the I.S.O. membrane vesicles can hardly bind with the substrate. This substantiates our findings that the substrate recognition sites of SBP 1 are exposed to the inner surface of the membrane. Binding studies with the *dct A* membrane vesicles show that the R.S.O. membrane vesicles have a much lower affinity for the

substrate as compared with the wild type, and I.S.O. membrane vesicles show the normal affinity of binding (Fig. 6A). Again, this confirms our findings that the substrate recognition sites of SBP 2 are exposed to the outer surface of the membrane, and this is altered in the *dct A* mutants. It should be pointed out here that spheroplasts prepared from the wild type and various *dct* mutants also provide the same results as the R.S.O. vesicles. This may serve as another indication that membrane vesicles prepared by the EDTA, lysozyme, and osmotic shock treatment are mainly R.S.O. vesicles and the amount of I.S.O. vesicles in the R.S.O. membrane preparation is negligible. Since membrane vesicles from the *dct* mutants are unable to transport malate even in the presence of an electron donor, and since binding studies with wild-type membrane vesicles agree with those from the transport mutant vesicles, we are quite confident that we are measuring the binding of the substrate to the surface of the membrane, and not some residual transport activities in the presence of CCCP; in fact, similar results are obtained even in the absence of CCCP, or in the presence of azide (11).

The above findings have several important implications for models of the molecular mechanisms of transport. If the membrane vesicles are not sealed, or if the substrate recognition sites of both SBP 1 and SBP 2 are present on the same surface of the membrane, then one would expect to obtain a biphasic curve on a double reciprocal plot giving binding constants representing those of SBP 1 and SBP 2. The fact that such curves are not observed with wild-type spheroplasts, R.S.O., and I.S.O. vesicles suggests that this is not likely to be the case. Using the appropriate mutant membrane vesicles, SBP 1 and SBP 2 substrate recognition sites cannot be detected on the R.S.O. and I.S.O. membrane vesicles respectively. This observation corroborates the above findings in that both R.S.O. and I.S.O. vesicles are sealed vesicles, and that both SBP 1 and SBP 2 substrate recognition sites cannot be exposed to the same surface of the membrane; and more important this suggests that the transport components are not likely to oscillate from one surface of the membrane to another -- as suggested by Model (2) (Fig. 4). Furthermore, one can conclude that the substrate recognition sites of SBP 1 and SBP 2 are only exposed to the inner and outer surfaces of the membrane respectively. It should be noted that these findings cannot be used to distinguish between Models (1), (3), or (4). Finally, binding studies with various membrane preparations indicate that the inner surface of the membrane has a much lower substrate binding affinity as compared with the outer surface.

It may be evident from Fig. 4 that the major difference between Models (1), (3), and (4) is that in Models (1) and (3), the transport proteins are not transmembrane proteins, whereas in Model (4) both proteins are exposed on both surfaces of the membrane. One should be able to distinguish between these 2 possibilities through the use of nonpenetrating covalent labeling reagents. Using R.S.O. or I.S.O. membrane vesicles, one should be able to label only one transport component in the case of Models (1), and (3). However, if Model (4) were applicable, then one should be able to label both transport components. By labeling spheroplasts or I.S.O. membrane vesicles with the lactoperoxidase system (Fig. 7A), or with the pyridoxal phosphate-sodium [^{3}H] borohydride system (Fig. 7B), we found that both components can be labeled on the inner or outer surface of the membrane (7). Similar findings are observed when R.S.O. membrane vesicles are used. Binding studies with various membrane preparations indicate that these are sealed vesicles and that they have the proper orientations. Since both lactoperoxidase and pyridoxal phosphate cannot penetrate the membrane, one can then conclude that both SBP 1 and SBP 2 are exposed on the 2 surfaces of the membrane. This would rule out the arrangements of the transport components as indicated in Models (1) and (3). As we have seen earlier, binding studies

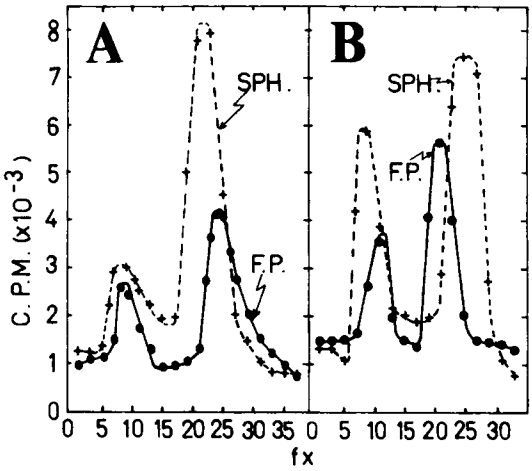

Fig. 7. Labeling the membrane transport components with nonpenetrating covalent labeling reagents. Spheroplasts (SPH.) and inside-out-membrane vesicles (F.P.) were prepared and labeled as described in Ref. 7. The labeled membrane preparations were then solubilized with 4% Lubrol 17A-10, and the solubilized proteins were fractionated by aspartate-coupled Sepharose as described in Fig. 2. A) The elution profiles of spheroplasts (+ – – – +) and I.S.O. vesicles (•—•) labeled with lactoperioxidase and ^{125}I; B) the elution profiles of spheroplasts (+ – – – +) and I.S.O. vesicles (•—•) labeled with pyridoxal phosphate and NaB^3H_4.

with membrane vesicles have eliminated Model (2), so it seems Model (4) may be the likely mechanism by which dicarboxylic acids are translocated across the membrane.

SBP 1 and SBP 2 Are Lying in Close Proximity to One Another

So far, our findings suggest that Model (4) seems to be the most feasible transport model. Let us explore the properties of this model one step further. This model predicts that the transport components may form multimeric subunit aggregates transversing the entire thickness of the membrane, thereby creating an aqueous transport channel. If this were the case, then one would expect that SBP 1 and SBP 2 should be in close proximity to each other. Hence one should be able to cross-link these 2 proteins. However, if the proteins are sitting one on top of the other, as depicted by Models (1) and (3), then one should not be able to cross-link these 2 proteins using nonpenetrating cross-linking reagents. We used a cleavable, nonpenetrating cross-linking reagent, tartaryl diazide, for such an experiment. This is a polar reagent, so it should not penetrate the membrane, and it has been demonstrated by Lutter (18) that the azide activated carbonyl groups react readily with amino groups to produce amide linkages. This tartaryl diazide is capable of cross-linking 2 protein amino groups which are 6 Å apart. Another advantage of this reagent is that the cross-linked proteins can be cleaved by mild treatment with periodic acid. Figure 8 shows that both SBP 1 and SBP 2 can be cross-linked with tartaryl diazide (7). However, when the cross-linked complex is cleaved with periodic acid, both SBP 1 and SBP 2 can be recovered. In the covalent labeling experiments, the validity of the results depends on the fact that the membranes vesicles are sealed structures, and we have demonstrated through binding studies that this is the case. However, in the cross-linking experiments, the reaction does not depend on the intactness of the membrane vesicles, it depends only on the

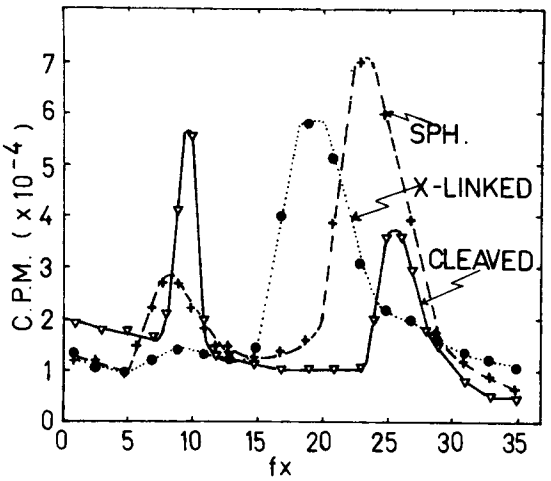

Fig. 8. Elution profile of cross-linked, cleaved, and un-cross-linked membrane transport components with tartaryl-diazide. Cross-linking experiments are carried out as indicated in the text. ^{35}S-labeled spheroplasts were cross-linked with freshly prepared 0.05 mmoles of tartaryl diazide. The cross-linked membranes were washed and solubilized with Lubrol 17A-10. Half of the solubilized proteins were fractionated by aspartate-Sepharose. The other half of the proteins were treated with periodic acid which was then removed by running through a Sephadex G-25 column. The protein peaks were then loaded onto an aspartate-sepharose column. The bound proteins were eluted with 0.2 M succinate. +) Elution profile of un-cross-linked spheroplasts; •) elution profile of cross-linked spheroplasts, and ▽) elution profile of the cleaved cross-linked complex.

fact that the transport components have to be in close proximity to each other on the membrane surface. Since we can cross-link these proteins with a nonpenetrating reagent, it indicates that both SBP 1 and SBP 2 must be exposed on the same surface of the membrane and that they are in close proximity to each other. Again this agrees with our previous postulation that Model (4) seems to be the most feasible transport mechanism.

Transport Studies With Cross-Linked Membrane Vesicles

According to the "mobile carrier model (2)," once the carrier protein has been cross-linked with other membrane surface components, transport should not be possible, as oscillation of the transport components across the membrane would no longer be possible. However, according to Model (4), the cross-linked protein may still be able to carry out the transport process – depending on the nature and the extent of the conformation changes (7). In order to test this hypothesis, we carried out transport studies with membrane vesicles that had been cross-linked with tartaryl-diazide under identical conditions as indicated earlier. Figure 9 indicates that membrane vesicles that have been cross-linked with tartaryl diazide can transport dicarboxylic acids to the same extent as the unmodified vesicles. This suggests that large conformational changes are not required for the transport process to occur. Consequently, it is unlikely that the substrate is translocated across the membrane through oscillation of the transport components across the membrane. Again, in agreement with the binding studies, one can eliminate the transport mechanisms depicted by Model (2).

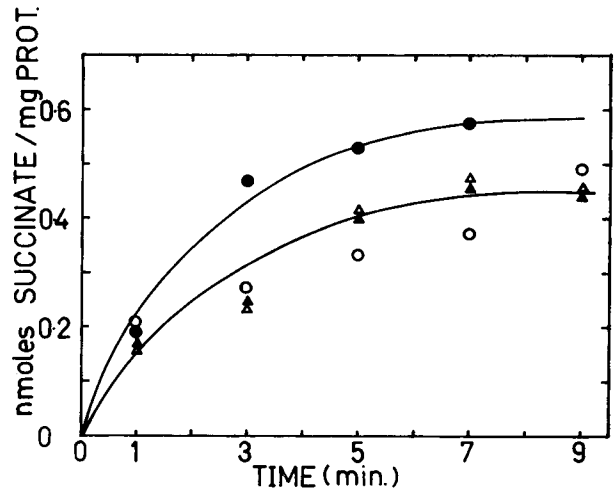

Fig. 9. Succinate transport by membrane vesicles cross-linked with different amounts of tartaryl diazide. Membrane vesicles from an *sdh, frd* strain (CBT 43) were prepared by the EDTA-lysozyme-osmotic shock method (10). Membrane vesicles were cross-linked under identical conditions as described in Fig. 8. Transport studies were carried out by the standard procedure using phenazine methosulfate and ascorbate as the electron donors (10). •) Succinate uptake by the normal membrane vesicles; ○) transport by membrane vesicles treated with the cross-linking buffer A (0.05 M triethanolamine-HCl pH 8.5, 0.005 M $MgCl_2$ and 0.1 M KCl); ▲ and △) transport by membrane vesicles treated with 0.025 mmoles and 0.05 mmoles of tartaryl diazide respectively in the cross-linking buffer A.

Role of Specific Phospholipids in the Transport Process

So far, we have been concentrating on the properties and the spatial arrangement of the membrane transport components; not much attention has been directed to the role of phospholipids in the transport process. Conventionally, most membrane functions have been attributed to the presence of certain specific proteins, and the role of phospholipids has often been ignored or underrated. Phospholipids play at least 2 conceivable roles in biological membranes. Firstly, they maintain the uniqueness and the integrity of the membrane, and they also serve as the matrix in which membrane proteins are embedded. Secondly, studies with various membrane-associated enzymes indicate that phospholipids are required for the normal functioning of these proteins. They generally play the role of physical cofactors, activating the enzyme system but not themselves participating in the reaction. They may activate the enzyme by inducing a conformational change in the protein. A well documented example of this kind is the very specific requirement of phosphatidylglycerol for both phosphorylation and transport of α-methylglucoside mediated by the phosphotransferase system of gram-negative bacteria. However, not all transport systems require phosphatidylglycerol for activities. It is therefore very important for our understanding of the molecular mechanism of the dicarboxylate transport process, and for our eventual reconstitution experiments, to determine whether any specific phospholipids are required for dicarboxylic acid transport.

A cursory examination of the phospholipid requirement for dicarboxylate transport was carried out by studying the effect of phospholipase on the transport process. Milner and Kaback (19) demonstrated quite clearly that phospholipase D (cabbage) acts specifically on phosphatidylglycerol of E. coli membranes releasing phosphatidic acid and glycerol.

Phosphatidylethanolamine, phosphatidylserine, cardiolipin, and lysophosphatidylethanolamine are not affected at all. The effect of phospholipase D (cabbage) on dicarboxylate transport is presented in this section.

Before using this commercially available enzyme, we ascertained that phospholipase D (cabbage) had no proteolytic activities by testing its effect on pyruvate kinase (rabbit muscle), and lactate dehydrogenase, and demonstrating that even after prolonged incubation with the phospholipase D preparation, these enzymes retained all of their activities. Figure 10 shows the effect of phospholipase D on the rates of uptake and efflux of succinate in membrane vesicles. Like the α-methylglucoside transport system, the dicarboxylate transport system is inhibited by phospholipase D (cabbage). Figure 10 also shows the rates of proline uptake and efflux. These results are similar to those reported by Milner and Kaback (19) who showed that proline uptake was only slightly affected by these concentrations of phospholipase D (cabbage) (7). Thus, these findings suggest that phosphatidylglycerol may be required for the transport of dicarboxylic acids. Further work is being carried out to determine the specificity of this requirement.

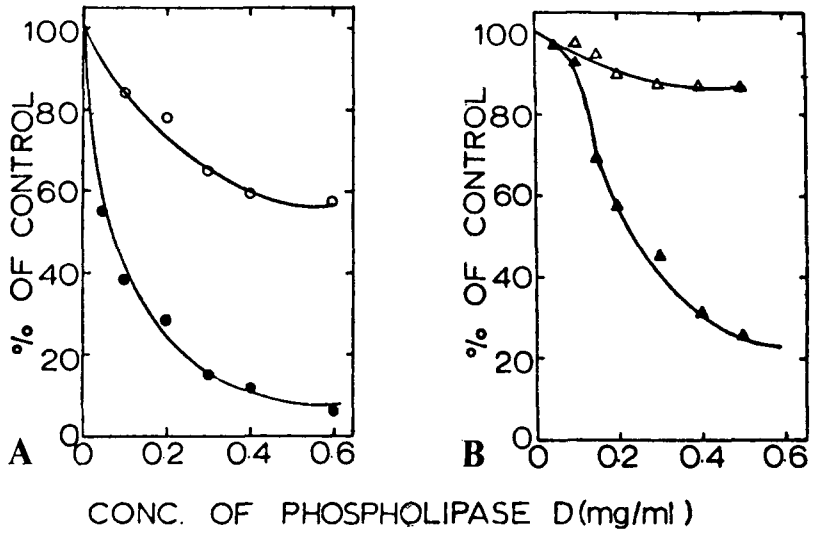

Fig. 10. Effect of phospholipase D (cabbage) on the initial rates of uptake and efflux of succinate and proline. Membrane vesicles prepared from strain CBT 43 at a concentration of 1.5 mg/ml were used. Phospholipase D (cabbage) (31 units/mg) was added to the membrane at zero time. The reaction was carried out at 23°C.

A) The effect of phospholipase D on the initial rates of uptake of succinate and proline. D-Lactate (20 mM) was used as the electron donor. Samples were taken at 20, 40, and 60 sec. •) The initial rate of uptake of succinate (2×10^{-5}M); ○) the initial rate of uptake of proline (2×10^{-5}M). In the absence of phospholipase D, the initial rates of succinate and proline uptake were 1.14 and 0.24 nmoles/mg protein/min, respectively. These values were taken as 100% for the respective uptake systems. B) The effect of phospholipase D on the initial rates of efflux of succinate and proline. The same concentrations of succinate (▲) and proline (△) were used as in Fig. 10A. Membrane vesicles were first preloaded with the respective radioactive ligands for 15 min using D-lactate as the electron donor, then phospholipase D was added at zero time. At zero time, the amount of succinate and proline accumulated in the vesicles were 2.03 nmoles/mg protein, and 0.37 nmoles/mg protein, respectively. These values were taken as 100% for the respective efflux systems. Percent of control indicates the percentage of radioactive ligands retained in the membrane vesicles after 20 sec.

DISCUSSION

At least 3 different transport components are found to be involved in dicarboxylate transport system of E. coli K12 – one periplasmic binding protein, and 2 membrane transport components. Transport of dicarboxylic acids across the cell envelope can best be described by the following tentative working model (Fig. 11). The substrate is first captured by PBP, which may be exposed on the outer surface of the outer membrane, or which may be located in the periplasmic space. After binding with PBP, the substrate is transferred to the substrate recognition site of SBP 2. It is quite possible that this process is carried out by direct specific physical interactions between PBP and the membrane carrier proteins, as found to be the case in the histidine transport system (3).

One may question the necessity of PBP in dicarboxylate transport, especially when it has been demonstrated that membrane vesicles are able to take up the substrate in the absence of PBP. Our findings indicate that in intact cells, PBP is required for delivering the substrate to the membrane transport components. As mentioned earlier, the whole cell transport system, but not the membrane vesicle transport system, is inhibited by N-ethylmaleimide. It is also found that the binding of succinate to PBP is inhibited by N-ethylmaleimide. This observation suggests that PBP is essential for the whole cell uptake system but not for the membrane system. This finding is corroborated by the properties of the *cbt* mutants. The *cbt* mutants are defective in PBP. As indicated earlier, although intact cells of the *cbt* mutant are unable to transport, *cbt* membrane vesicles take up the substrate normally. One may explain these observations by postulating that in the case of membrane vesicles, in which most of the cell wall materials are removed, the substrate is readily accessible to the membrane carrier proteins, and so PBP is not essential for the process.

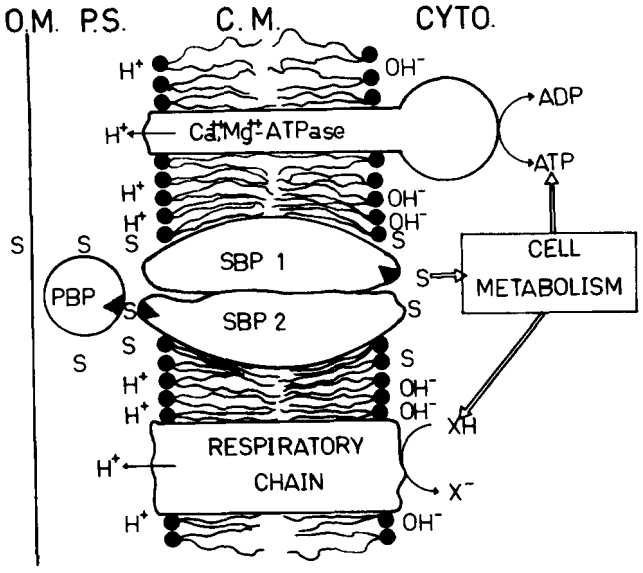

Fig. 11. Schematic diagram of the tentative working model for dicarboxylate transport system in Escherichia coli K12. S) Transport substrate; ▲) substrate recognition sites of the transport components; O.M.) outer membrane; P.S.) periplasmic space; C.M.) cytoplasmic membrane; CYTO.) cytoplasm.

However, in the case of intact cells, PBP is required to deliver the substrate across the cell wall to the membrane transport components. So far there is no indication that metabolic energy is required for this process.

The present evidence indicates that the substrate is translocated across the membrane via a transport channel formed by both SBP 1 and SBP 2. Both SBP 1 and SBP 2 are found to have the same substrate specificity as the transport system, i.e., they have the same binding site for succinate, fumarate, and malate. SBP 1 had a K_d of 23 μM for succinate and 47 μM for malate which are similar to the K_m values for transport. SBP 2 differs from SBP 1 in that it has a K_d of 2 μM for succinate and 7 μM for malate. Binding studies with spheroplasts and R.S.O. and I.S.O. vesicles indicate that the substrate recognition sites of only one membrane transport component are exposed on one surface of the membrane. The substrate recognition sites of SBP 2 and SBP 1 are exposed on the outer and inner surfaces of the membrane respectively. Of the 3 types of membrane preparations used, spheroplasts were subjected to only very mild treatment; therefore the binding capacity of spheroplasts for malate would likely be the best reflection of the number of binding sites in whole cells. Figure 5B indicates that spheroplasts have a binding capacity of 38 pmoles of malate per mg of cellular protein. In our laboratory, we found that 1 mg of cellular protein is equivalent to 7.83×10^9 cells, which is comparable to that reported by Jones and Kennedy (21). Assuming one molecule of substrate binds with one substrate recognition site on the transport component, we found that each bacterium contains around 3,000 binding sites on the outer surface of the membrane.

Both cross-linking and covalent-labeling experiments with nonpenetrating reagents point to the likelihood that both SBP 1 and SBP 2 are transmembrane proteins and that they lie in close proximity to each other. Transport studies with cross-linked membrane vesicles suggest that large conformational changes are not required for the translocation of the substrate across the membrane. This would tend to rule out the involvement of a mobile carrier mechanism. Therefore, the present available data points to the involvement of multimeric transport channels in the translocation of dicarboxylic acids across the membrane. This is depicted in Fig. 11. It is speculated that the transmembrane motion of the substrate is brought about by local conformational changes on the membrane transport components.

It should be noted that the transport channel model proposed is slightly different from that postulated by Singer for the shock-sensitive transport systems (4). In his model, the periplasmic binding protein is postulated as the loosely bound substrate recognition site of the transport components, and the integral proteins do not possess any substrate recognition site per se. However, in the present model, all 3 transport components have been demonstrated unequivocally to possess their own substrate recognition sites. Presumably the SBP 2 substrate recognition site is responsible for influx, and that of SBP 1 is responsible for both influx as well as efflux.

Cursory examination of the effects of phospholipases indicate that both the influx and efflux of dicarboxylic acids are inhibited after incubation of membrane vesicles with phospholipase D (cabbage). Thus, like the PEP-phosphotransferase system, phosphatidylglycerol seems to be required for the normal functioning of SBP 1 and SBP 2. It is quite possible that phosphatidylglycerol may play a role as a "physical cofactor," maintaining the transport components in the proper conformation.

It has been well established in our laboratory and by other workers, that a proton gradient is required for the translocation of dicarboxylic acids across the membrane. A proton gradient can be generated by the functioning of either the electron transport chain

or the Ca^{2+},Mg^{2+}-ATPase. How, at a molecular level, this proton gradient affects the functioning of the transport components is far from clear. Transport studies carried out with membrane vesicles in the presence of Ca^{2+},Mg^{2+}-ATPase inhibitors or with membrane vesicles prepared from a Ca^{2+},Mg^{2+}-ATPase mutant indicate that the functioning of Ca^{2+},Mg^{2+}-ATPase is required for the uptake of dicarboxylic acids by membrane vesicles. This is different from the proline transport system in which Ca^{2+},Mg^{2+}-ATPase is not required. It is possible that the Ca^{2+},Mg^{2+}-ATPase has a role in the transport of dicarboxylic acids other than maintaining a proton gradient in whole cells.

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