MEMBRANE BOUND SUBSTRATE RECOGNITION COMPONENTS OF THE DICARBOXYLATE TRANSPORT SYSTEM IN ESCHERICHIA COLI

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SUMMARY: Membranes isolated from Escherichia coli and solubilized with Lubrol 17A-10 contain two proteins, designated SBP 1 and SBP 2 which can be separated and purified on aspartate-coupled Sepharose 4B. Both of the proteins bind succinate, malate and fumarate but not aspartate, malonate, tartarate and acetate. The dissociation constants of SBP 1 and SBP 2 for succinate are 23 μM and 2.3 μM, respectively. The binding of succinate to these components is inhibited by sulfhydryl reagents. A mutant of Escherichia coli (dct A) which is unable to transport succinate and other dicarboxylic acids has very small amounts of the membrane bound SBP 2 protein. Genetic and biochemical evidence strongly suggests that SBP 1 and SBP 2 cooperate in some unknown way in the transport of succinate in vivo.

INTRODUCTION: In previous studies (1,2) on the dicarboxylate transport system of Escherichia coli, we demonstrated that the entry of succinate and other dicarboxylic acids, malate and fumarate, inside the cell occurred by means of a permease, or a "carrier-mediated" process. Analysis of mutants unable to transport the dicarboxylic acids indicated (1,2) that at least three genetic loci were involved in transport. Two of these genes termed dct A and dct B possibly determined the structure or regulation of membrane bound components. This surmise was based on the observation that membrane vesicles prepared from wild type cells (2) transported succinate in the presence of electron donors while vesicles prepared from dct A or dct B cells failed to do so. The third genetic locus, termed cbt possibly specified the structure of an extra-membranal component (3). Whole cells harbouring this mutation were unable to take up the dicarboxylic acid but membrane vesicles obtained from these cells transported succinate normally in the presence of an electron donor (2). Some of the cbt mutants were shown to lack a soluble, periplasmic protein involved in the recognition and transport of succinate (3). In addition to the three genetic loci mentioned above, mutants lacking a Ca^{2+}, Mg^{2+}-stimulated ATPase were also deficient in the transport of succinate (and, other dicarboxylic acids) in whole cells as well as in membrane vesicle preparations (4).

In recent years, considerable progress has been made in the isolation and characterization of the easily accessible periplasmic binding proteins for diverse transport systems in E. coli (5,6) including the dicarboxylate system (3). In contrast, however, reports of successful isolation and study of recognition elements firmly embedded in the cytoplasmic membrane matrix are few and far between. Kennedy and his associates (5,7,8) have succeeded in purifying the M protein (a component of the lactose transport system) and the membrane bound components of the phosphotransferase system of E. coli have been purified by Kundig and Roseman (9). A report has also appeared (10) on the solubilization of some amino acid binding components from the membranes of E. coli by the non-ionic detergent Brij 36-T.

In the following communication we present results obtained in an attempt to isolate and to characterize the membrane bound dicarboxylate transport components from various E. coli strains.

MATERIALS AND METHODS: E. coli K12 strains were used. The relevant genetic characteristics of the various strains are listed in Table I. The cells were grown exactly as described earlier (2). Membrane vesicles from various strains were prepared by a lysozyme EDTA method (11). The transport of succinate in membrane vesicles was measured by an isotopic procedure (2). Aspartate - coupled Sepharose 4B was prepared according to procedures described earlier (3). The gel was stored at 4° before use. Equilibrium dialysis was performed in chambers (0.2 ml) designed according to Myer and Schellman (12) at $23^{\circ}C$. The radioactive ligand (0.1 ml) was introduced in one chamber, and the protein solution (0.1 ml) in the other. To achieve rapid equilibration the contents of the chambers were agitated by rotation in a multipurpose rotator. After equilibration was achieved, 0.04 ml of a sample was removed from each chamber and counted in Aquasol. Binding assays with the solubilized membrane proteins were done in the presence of 0.05 M phosphate buffer (pH 6.6) containing 0.5% Lubrol 17A-10 and 50 mM arsenate. Lubrol 17A-10 was obtained from the Imperial Chemical Industries Ltd. All other chemicals were obtained from commercial sources.

RESULTS: *Purification of the Membrane Bound Components:* Preliminary results obtained with the use of various non-ionic detergents (lysolecithin, deoxycholate, Brij 58, Lubrol PX, WX and 17A-10 and Triton X 100) showed that Lubrol 17A-10 was most effective in releasing succinate binding material from membranes. This detergent was

TABLE 1

The Genetic Characteristics of Strains Used in this Work

The abbreviations are: sdh, succinate dehydrogenase; frd, fumarate reductase; cbt, carboxylate transport; pro and met, requirement for proline and methionine, respectively.

Strain Designation	Genetic Markers	Origin
CB11	sdh, frd	Laboratory stock
CB20	dct A	Laboratory stock
CBT312	sdh, frd, dct A	This work
CB22	pro, met, cbt	Laboratory stock

therefore used routinely. In a typical purification procedure, to 50 ml of membrane suspension (6 mg membrane protein per ml) obtained from strain CB11, 4% (final concentration) Lubrol 17A-10 was added and the mixture was stirred for 30 min at room temperature. The membranes were spun down, and the supernatant was applied to an aspartate-coupled Sepharose column (1.5 x 30 cm) equilibrated with 0.5% detergent, 50 mM arsenate and 0.05 M phosphate buffer, pH 6.6. The column was then washed with the above buffer until no more protein came out. Then 0.2 M succinate (dissolved in the above buffer) was used to elute the bound proteins. A typical protein elution profile is illustrated in Fig. 1 It shows that the membrane proteins bound to the affinity column can be fractionated into two separate and distinct peaks, SBP 1 and SBP 2.

Binding of Dicarboxylic Acids to Membrane Proteins: Pooled fractions 12 and 13 in case of SBP 1 protein and fractions 24 and 25 for SBP 2 component (Fig. 1) are capable of binding succinate. Table 2 shows that the binding of succinate by both membrane proteins SBP 1 and SBP 2 is inhibited by malate and fumarate. Unlike the periplasmic binding protein described elsewhere (3), D-lactate has virtually no effect on succinate binding by these two proteins. Other dicarboxylic acids such as citrate, acetate, tartarate, and malonate are also without any significant effect on succinate binding. These data are

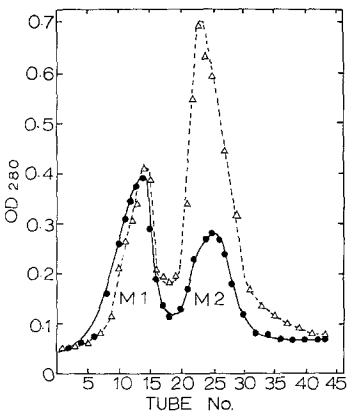

Fig. 1. A representative elution profile of solubilized membrane proteins from aspartate-coupled Sepharose 4B column. Details of solubilization of the membrane, loading of the column and elution are given in the text. Fractions of 2 ml were collected after the start of the elution by 0.2 M succinate. OD_{280} refers to absorbance measurements at 280 nm. The symbols are, (Δ), elution profile from strain CB11 and, (●), elution profile from strain CBT 312.

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in agreement with previous findings on the transport kinetics of the membrane vesicles (2). The fact that malonate does not affect succinate binding indicates that these membrane proteins are not likely to be part of succinate dehydrogenase.

Fig. 2 and 3 show that fumarate and malate competitively inhibit the binding of succinate to both membrane proteins, SBP 1 and SBP 2, exactly as they inhibit the transport system in membrane vesicles. In a separate study, we were able to show that succinate dehydrogenase activity is not inhibited by fumarate and malate nor do these compounds serve as substrates for the enzyme; these data serve again to show that these two fractions are possibly not part of succinate dehydrogenase. The binding constants of these two membrane proteins for succinate are quite interesting. SBP 1 has a binding constant of 23 μM, whereas SBP 2 has a constant of 2.3 μM. The binding constants of SBP 1 for malate and fumarate are around 47 μM; the binding constants of SBP 2 for these two dicarboxylic acids are around 7 μM. Thus SBP 2 has a much higher affinity for the dicarboxylic acids than SBP 1. It may be recalled that the K_m of succinate transport through membrane vesicles (2) is of the order of 20 μM, and there is no genetic (1) or biochemical evidence that there is more than one dicarboxylate transport system in *E. coli* (1,2). It, therefore, seems likely that SBP 1 and SBP 2 proteins

TABLE 2

Effect of Various Carboxylic Acids on the Binding of Succinate to Membrane Components, SBP 1 and SBP 2

1×10^{-4} M (2,3-^{14}C) succinate and about 50 µg of purified membrane transport components were used in each binding assay. Equilibrium dialysis was carried out at 23° in binding cells as described in the text. The binding assay was done in the presence of 0.05 M phosphate buffer, pH 6.6, containing 0.5% Lubrol 17A-10, and 50 mM of arsenate. When required the cold ligands were used at a final concentration of 1 mM. In the absence of any addition, SBP 1 and SBP 2 bound 6.6 and 7.58 nanomoles of succinate, respectively. These values are considered to be 100% binding in respective cases.

Additions	Amount of Succinate bound % of Control	
	SBP 1	SBP 2
None	100	100
Acetate	92	77
Citrate	99	104
Fumarate	10	11
D-lactate	84	76
Malate	28	12
Malonate	88	104
Tartrate	89	98
N-Hydroxy-succinimide	94	97

are involved in the same transport system. Although one of the proteins (SBP 2) has a much higher affinity for succinate, the K_m for transport will obviously be limited to the value of the component with lower affinity (i.e. around 20 µM).

The binding of succinate to both SBP 1 and SBP 2 is inhibited by p-CMB and NEM. At a concentration of 0.05 mM to p-CMB for example binding to SBP 1 and SBP 2 is inhibited to the extent of 45 and 92 percent, respectively. This inhibition is overcome to a large extent by 0.25 mM dithiothreitol.

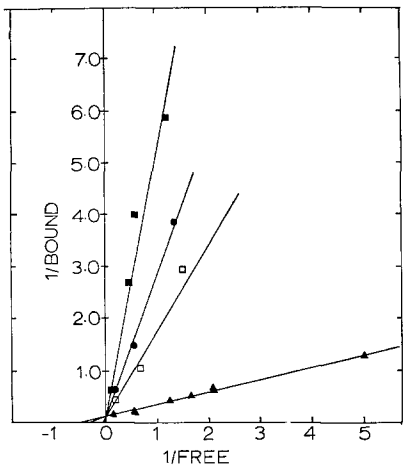

Fig. 2. Competitive inhibition of succinate binding by fumarate and malate to the membrane component SBP 1. A fixed amount (about 50 µg) of purified SBP 1 was used in all assays. Free and Bound refer to the amount of succinate in nanomoles. The concentrations of various inhibitors are, fumarate (●), 1 mM; malate (□), 0.5 mM; malate (■), 1 mM. The symbol (▲) denotes succinate binding in the absence of inhibitors.

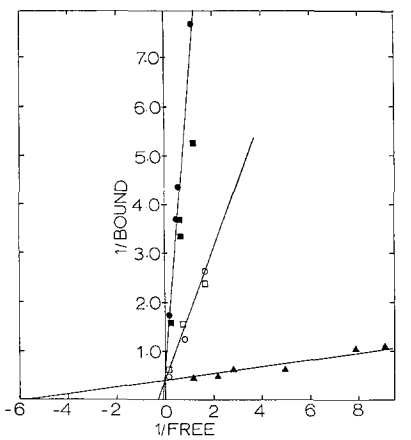

Fig. 3. Competitive inhibition of succinate binding by fumarate and malate to the purified membrane component SBP 2. The experimental details are exactly as described in the legend to Fig. 2. The concentrations of various inhibitors are: fumarate (●), 1 mM; fumarate (□), 0.5 mM; malate (■), 1 mM. The lowermost line represent binding of succinate in the absence of inhibitors.

SBP 1 and SBP 2 Components in Various Mutants: In earlier studies (2) we had demonstrated that membrane vesicles prepared from two types of mutants, viz., dct A and dct B, were defective in the

transport of succinate. This study had provided evidence that there were at least 2 macromolecular components in the membranes which were somehow responsible for the uptake of succinate in vesicles. To test the surmise that SBP 1 and SBP 2 were the products of dct genes various mutants were cursorily tested for the presence of these proteins by using affinity chromatography. Only in one strain (CBT 312, Table 1) out of several tested was there a much lower quantity of SBP 2 (Fig. 1). It is clear, however, that this decrease in the level of SBP 2 cannot be construed to mean that the dct A locus is responsible for the synthesis of SBP 2. Indeed, if some of the dct mutants we have tested produce a subtly altered SBP 1 or SBP 2 protein, our method which only demands integrity of the substrate binding site for detection, may not be the most desirable one for the recognition of components altered in other ways.

DISCUSSION: The question of major interest posed by our studies is whether SBP 1 and SBP 2 are the components involved in the transport of dicarboxylic acids in intact cells. In the present state of our knowledge this seems likely but is not proven unequivocally. Apart from the genetic evidence presented before (2) which suggested that there were possibly two membrane bound components involved in the transport of succinate, the only other evidence that implicates SBP 1 and SBP 2 in the transport process is the similarity of substrate specificity and affinity of the isolated components to the specificity and affinity characteristics of the uptake process in intact membrane vesicles. Thus, both the isolated and vesicle systems, as judged by competition experiments, are specific for the dicarboxylic acids, succinate, fumarate and malate. Furthermore, the binding affinity of the isolated component SBP 1 (23 μM) is similar to the K_m for succinate uptake in membrane vesicles.

Another question of interest with regard to the dicarboxylate transport system is whether the periplasmic succinate binding protein we isolated earlier (3) and shown by genetic methods to be involved in transport, is different from SBP 1 and SBP 2. That this may be so is suggested by two experimental findings. First, cbt mutants which lack the periplasmic protein retain SBP 1 and SBP 2 components. Second, while D-lactate competes for the succinate binding site of the soluble, periplasmic protein, it has no effect on the binding of succinate by SBP 1 and SBP 2. We are currently attempting to make nonsense dct type of mutants to clarify the role of SBP 1 and SBP 2 in transport.

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