SOLUBILIZATION AND PARTIAL PURIFICATION OF ALANINE CARRIER FROM MEMBRANES OF A THERMOPHILIC BACTERIUM AND ITS RECONSTITUTION INTO FUNCTIONAL VESICLES

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SUMMARY

An alanine transport carrier was solubilized from membranes of the thermophilic bacterium PS3 with cholate-deoxycholate mixture. It was then partially purified by diethyl aminoethyl cellulose column chromatography and gel filtration. For assay of alanine carrier activity it was reconstituted into vesicles with P-lipids and the transport energy was supplied as a membrane potential introduced by K^+-diffusion *via* valinomycin. The partially purified carrier had no ATPase or NADH dehydrogenase activity. Active transport of alanine driven by the membrane potential was completely abolished by an uncoupler.

Bacterial membrane transport of nutrients has been studied intensively using isolated membrane vesicles (1). Recent results showed that coupling of metabolic energy to transport can best be interpreted by Mitchell's chemiosmotic theory (2 - 5). However, to obtain more convincing results a much purer system is required. Unfortunately, no transport carrier has yet been isolated from bacterial membranes. This may be because all known transport carriers are too unstable to be solubilized from membranes. Thus for further studies a more stable material is required.

It seemed possible that this could be obtained from the membranes of the thermophilic bacterium PS3, which are resistant to various drugs, organic solvents and detergents (6). Indeed recently a dicyclohexyl carbodiimide sensitive ATPase complex was purified from this bacterium using ionic and noninonic detergents (7).

This communication describes the solubilization and partial purification of an alanine carrier from this bacterium and its reconstitution with P-lipids into vesicles capable of active transport of alanine.

MATERIALS AND METHODS

Materials —— The thermophilic bacterium PS3 (kindly donated by Dr. T. Oshima) and preparation of membranes were described previously (6). Soybean P-lipids (asolectin) were obtained from Associated Concentrates, Woodside, New York, and partially purified as described previously (7). The preparation of lipids of PS3 was also as described previously (7). L-[U-^{14}C]alanine was purchased from Daiichi Radiochemicals. Cholic acid was recrystallized as described by Kagawa (8). Other compounds were commercial products.

P-Lipid-detergent Mixture —— P-Lipids (10 mg) were suspended in 2 ml of 40 mM Tris-SO_4 (pH 8) containing 2% Na-cholate, 1% Na-deoxycholate and 1 mM dithiothreitol, and sonicated in a Tomy probe sonic oscillator, Model UR-150P, at 20 KHz and 150 watts in an ice bath for 5 min.

Solubilization and Partial Purification of Alanine Carrier —— Membranes (1 g of protein) were suspended in a solution containing Na-cholate, 2%; Na-deoxycholate, 1%; Na_2SO_4, 0.2 M; Tris-SO_4 (pH 8), 30 mM; and dithiothreitol, 0.5 mM in a final volume of 100 ml. The mixture was sonicated in an ice bath for 10 min and then centrifuged at 140,000 × *g* for 1 hr. The supernatant was concentrated to approximately 20 ml in a Diaflo apparatus with a UM10 filter and dialyzed against 2 liters of 50 mM Tris-SO_4 (pH 8) containing 0.25 mM dithiothreitol for 17 hrs at room temperature. The contents in the dialysis bag were then centrifuged at 140,000 × *g* for 1 hr and the resulting precipitate was suspended in 50 mM Tris-SO_4 (pH 8). This preparation is named CDE-P. CDE-P was treated with Triton X-100 (2% final concentration) in the presence of 0.2 M Na_2SO_4, sonicated for 1 min and centrifuged at 140,000 × *g* for 1 hr. The supernatant was diluted 20-fold with distilled water and applied on a DEAE cellulose column equilibrated with 25 mM Tris-SO_4 (pH 8) containing 0.1% Triton X-100 (Tris-Triton). The column was washed with 10 bed volume of Tris-Triton, and then eluted with Tris-Triton containing 50 mM Na_2SO_4. Fractions with UV-absorption were combined and mixed with Na-cholate at a final concentration of 1%. Then 210 mg of solid ammonium sulfate were added. The precipitate was collected by centrifugation (20,000 × *g*, 10 min) and suspended in 50 mM Tris-SO_4 (pH 8) (DE-1). DE-1 was purified further by gel filtration. For this it was dissolved in a minimal volume of 2% Triton X-100 and applied on a Sepharose 6B column equilibrated with Tris-Triton. The column was eluted with the same buffer and the eluate with UV-absorption (one broad peak) was collected and divided into three fractions (Fr. 1, 2 and 3).

Reconstitution of Vesicles and K^+-Loading —— Samples of the preparations obtained at each step (10 to 100 µg protein) were added to the P-lipid-detergent mixture (2 ml) described above and sonicated for 30 sec. Then the mixtures were dialyzed against 50 mM Tris-SO_4 (pH 8) containing 2.5 mM $MgSO_4$, 0.2 mM EDTA and 0.25 mM dithiothreitol for 17 to 20 hrs at 40°. The reconstituted vesicles were incubated in 0.5 M K-phosphate buffer (pH 8) for 30 min at 55° and then

transferred to an ice bath. Then $MgSO_4$ was added to a final concentration of 10 mM and incubation was continued for 30 min in the ice bath. The reconstituted-K^+-loaded vesicles were collected by centrifugation at 140,000 × *g* for 60 min, washed with 0.4 M sucrose containing 10 mM $MgSO_4$ and suspended in 0.4 M sucrose-10 mM $MgSO_4$.

Assay of Alanine Carrier Activity —— Alanine carrier was defined as protein(s) catalyzing active transport of radioactive alanine into the reconstituted vesicles depending on an artificial membrane potential (negative inside). The reconstituted-K^+-loaded vesicles were suspended in 1 ml of solution containing final concentrations of 0.1 M Tris-maleate, pH 7.0, 0.013 M $MgSO_4$, 0.28 M sucrose, and 10 μM L-[U-^{14}C]alanine, (1 μCi/ml). After incubation for 5 min at 40°, 2 μg of valinomycin in 2 μl of methanol were added. At intervals 0.1 ml samples were filtered through membrane filters (Sartorius, 0.45 μm pore size) and washed with 0.4 M sucrose-10 mM $MgSO_4$. The filters were then rapidly removed, dried and their radioactivities were assayed in a gas-flow counter (Aloka LBC-451).

Protein was determined as described by Lowry et al. (9).

RESULTS AND DISCUSSION

A transport carrier protein(s) must be determined as protein catalyzing movement of nutrient across a membrane against a concentration gradient dependent on some sort of energy. However, in a simplified system containing only carrier protein(s), energy must be supplied in other than a metabolic form. In our previous work alanine uptake by isolated membrane vesicles of PS3 was driven by an artificial membrane potential introduced by K^+ diffusion mediated by valinomycin (manuscript in preparation) similar to that achieved with *Escherichia coli* vesicles (4). In this work we applied this method for assay of alanine carrier activity after reconstitution of vesicles.

Figure 1 illustrates results on alanine uptakes by K^+-loaded vesicles reconstituted from cholate-deoxycholate soluble and insoluble proteins. On addition of valinomycin, vesicles reconstituted from the detergent-soluble proteins showed rapid uptake of alanine which reached a maximum in 2 min. Vesicles reconstituted from the insoluble proteins showed less active uptake.

The validity of this assay method is confirmed by the results in Fig. 2. The initial rate of alanine uptake was proportional to

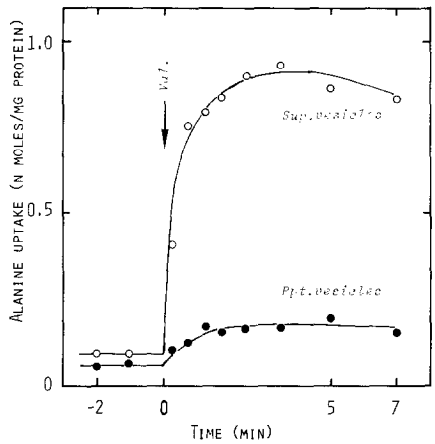

Fig. 1 : Alanine uptake by reconstituted, K^+-loaded vesicles. Vesicles containing the detergent-soluble (O) or -insoluble (●) proteins were reconstituted and loaded with K-phosphate (pH 8) as described in the text. The reaction mixture (1 ml) contained final concentrations of 0.1 M Tris-maleate, pH 7.0, 0.013 M $MgSO_4$, 0.28 M sucrose, and 10 µM L-[U-^{14}C]alanine, (1 µCi/ml). After incubation for 5 min at 40° (arrow) 2 µg of valinomycin in 2 µl of methanol were added. At intervals, 0.1 ml aliquots were filtered and washed.

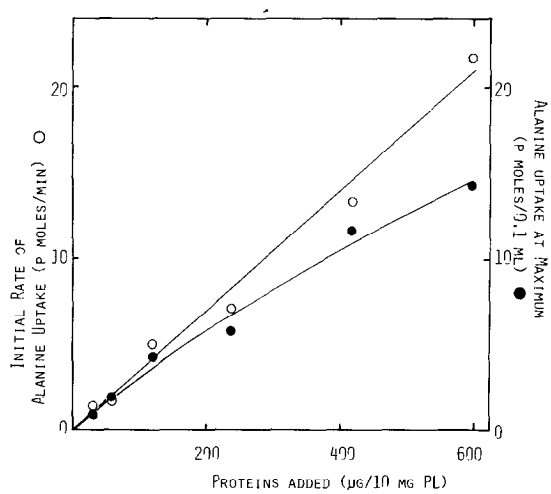

Fig. 2 : Alanine uptake by reconstituted-K^+-loaded besicles as a function of amount of detergent-soluble proteins added. Reconstituted, K^+-loaded vesicles containing various amounts of detergent-soluble proteins and a fixed amount of PS3 P-lipids (10 mg) were prepared as described in the text. The initial velocities (O) and maximal uptakes (●) were calculated from the values observed 15 sec and 2.5 min after addition of valinomycin.

the amount of protein (CDE-P), over the range of 20 to 600 μg, added to a fixed amount of P-lipids (10 mg). The maximal level, on the other hand, tended to reach a definite level. This could be explained by supposing that the initial rate of uptake was proportional to both the size of the population of vesicles containing carrier protein(s) and the amount of carrier protein(s) in the vesicles, while the maximal level was related to the former only and thus reached a definite level when most of the vesicles contain at least one carrier protein(s).

The uptake by these reconstituted vesicles, which was dependent on an artificial membrane potential (negative inside), was completely blocked by the uncoupler, carbonylcyanide *p*-trifluoromethoxyphenyl-hydrazone, but not by dicyclohexyl carbodiimide (data not shown).

Various P-lipid preparations were tested, including PS3 P-lipids, soy bean P-lipids, those from other thermophilic bacterial strain *Thermus thermophilus* HB8, ATCC 27634, and synthetic dipalmitoyl phosphatidyl glycerol; only PS3 P-lipids formed vesicles with alanine carrier showing uptake activity (data not shown). Thus specific P-lipids are required for functional reconstitution of the carrier protein. The main components of PS3 P-lipids are phosphatidyl ethanolamine, phosphatidyl glycerol and cardiolipin (11), and the fatty acyl groups of which are almost completely saturated (7).

The alanine carrier was purified as described in the "MATERIALS AND METHODS" and results of a typical purification are summarized in Table I. The process of extraction of the membranes with cholate-deoxycholate mixture increased the specific activity 2.4-fold. The alanine carrier activity was eluted from a DEAE cellulose column with rather a low salt concentration, sometimes as low

Table I

Purification of Alanine Carrier

	Protein (mg)	Sp. Act. n moles/min·mg
Membrane vesicles	1,096	0.76
Cholate-DOC ext.	96.3	1.85
Triton ext. sup	49.7	1.89
Triton ext. ppt	37.8	0.50
DEAE 50 mM Na_2SO_4	2.5	5.12
DEAE 200 mM Na_2SO_4	16.0	0.77
Sepharose Fr. 1	0.65	9.93
Sepharose Fr. 2	0.64	5.88
Sepharose Fr. 3	0.18	1.84

as 25 mM Na_2SO_4. This step removed ATPase and NADH dehydrogenase but small amounts of cytochromes were still present in the preparation eluted (data not shown). On Sepharose 6B column, the highest activity was found in Fr. 1 of Table I. Thus the molecular weight of the alanine carrier must be between 100,000 and 200,000. The specific activity of the final preparation was about 13-fold that of the original membranes and the preparation was almost colorless, suggesting that it contained little if any cytochrome. More effective procedures are required to increase the yield.

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