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Isolation of the Alanine Carrier From the Membranes of a Thermophilic Bacterium and Its Reconstitution Into Vesicles Capable of Transport

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A carrier protein mediating alanine transport was purified from the membranes of the thermophilic bacterium PS3, by ion exchange chromatography in the presence of both Triton X-100 and urea.

The alanine carrier was recovered in the nonadsorbed fraction from either DEAE- or CM-cellulose columns, suggesting that its isoelectric point was in the neutral pH region.

The final preparation contained virtually no electron transfer components, ATPase, or NADH dehydrogenase. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed that the final preparation consisted of two major protein components with molecular weights of 36,000 and 9,400.

Active transport of alanine after incorporation of the alanine carrier into reconstituted proteoliposomes was driven not only by an artificial membrane potential generated by potassium ion diffusion via valinomycin but also by mitochondrial cytochrome oxidase incorporated into the same liposomes and supplemented with both cytochrome c and ascorbic acid.

The membrane-integrated portion (TF_0) of the ATPase complex uncoupled alanine transport by conducting protons across the membrane.

Key words: thermophilic bacterium, transporting proteoliposome, proteoliposome reconstitution, alanine carrier

Isolated bacterial membranes have been widely used for studies on various energy coupling mechanisms including oxidative phosphorylation and active transport (1, 2). Since they are devoid of the many other complicated biochemical reactions occurring in the cytoplasm, such preparations are suitable for studying membrane-bound reactions. However, a much more simplified system is obviously required for detailed studies on the molecular mechanisms of these energy transformation processes. Recently components necessary for oxidative phosphorylation have been isolated and reconstituted into proteoliposomes capable of energy transformation (3–6). On the other hand, carrier proteins mediating movement of substrates across the permeability barrier have not been solubilized, except in a few cases, including the glucose carrier of erythrocytes (7), that from small intestine (8) or the proline carrier from Bacillus subtilis (9).

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In our earlier reports (10), we described the solubilization and partial purification of an alanine carrier from the membranes of the thermophilic bacterium PS3 and its reconstitution into proteoliposomes capable of active transport of alanine dependent on a membrane potential induced by K^+ diffusion mediated by valinomycin. However, those preparations were contaminated by minute quantities of cytochromes which might have introduced unnecessary complications of interpretation. The present report describes more satisfactory purification procedures and a further characterization of the active transport of alanine in reconstituted proteoliposomes.

MATERIALS AND METHODS

Materials

The thermophilic bacterium PS3 (kindly donated by Dr. T. Oshima) and preparation of membranes were described previously (11). The preparation of PS3 phospholipids was also described previously (3). Phospholipid fractionation was carried out by silicic acid column chromatography using Unisil (100–200 mesh, Clarkson Chemical Company, Inc., Pennsylvania). We used L-[U-^{14}C]-alanine purchased from Daiichi Radiochemicals. Cholic acid (Sigma Chemical Company) was recrystallized as described by Kagawa (12). Cytochrome oxidase of beef heart mitochondria was prepared according to the method of Yonetani (13). Horse heart cytochrome c was purchased from Sigma Chemical Company and the other compounds used were commercial preparations.

Phospholipid-Detergent Mixture

The phospholipid-detergent mixture was prepared as described elsewhere (14).

Reconstitution of Proteoliposomes

Reconstitution of proteoliposomes and K^+-loading were carried out by either the dialysis method or the dilution method as described elsewhere (14).

Proteoliposomes containing both the alanine carrier and cytochrome oxidase were prepared by the method of Hinkle et al. (15), except that the carrier and deoxycholate were added to the phospholipid, oxidase, and cholate as described in the Figure legends.

Assay of Alanine Carrier Activity

The alanine carrier activity was assayed at 40°C as described elsewhere (10, 14).

Other Analytical Methods

Gel electrophoresis was carried out on 7.5% polyacrylamide gel in the presence of 0.05% sodium dodecyl sulfate under the conditions described by Weber and Osborn (16). Before application to the gels, proteins were incubated at 95°C for 5 min in a solution containing 1% sodium dodecyl sulfate, 5% 2-mercaptoethanol, and 25 mM potassium phosphate buffer, pH 7.0.

Other analytical methods were described previously (3).

Purification of Alanine Carrier

The extraction of membranes by cholate-deoxycholate (Step 1) and the first DEAE-cellulose column chromatography in the presence of 0.25% Triton X-100 (Step 2) have been described in detail elsewhere (14). After removal of Triton X-100 from the DEAE eluate by ammonium sulfate fractionation in the presence of 1% Na-cholate (10, 14), the

resulting precipitates were collected by centrifugation (20,000 × g, 10 min), washed twice with 50 mM Tris-SO_4 (pH 8) and resuspended in the same buffer. This preparation was named DE-1.

Step 3: DEAE-cellulose column chromatography in the presence of Triton and urea. The DE-1 preparation was diluted fivefold with distilled water to which Triton X-100 and solid urea were added to final concentrations of 0.5% and 6 M, respectively. The solution was centrifuged for 10 min at 140,000 × g, and the supernatant liquid was applied to a DEAE-cellulose column equilibrated with 10 mM Tris-SO_4 (pH 8) containing 0.25% Triton X-100 and 4 M urea. The nonadsorbed fractions were collected, combined, and immediately dialyzed overnight at room temperature against 25 mM Tris-SO_4 (pH 8) containing 0.25 mM EDTA. The dialysate was subjected to ammonium sulfate fractionation in the presence of 1% Na-cholate. The resulting precipitates were collected by centrifugation (20,000 × g, 10 min), washed twice with 50 mM Tris-SO_4 (pH 8), and resuspended in the same buffer. This preparation was named UDE-1.

Step 4: CM-cellulose column chromatography in the presence of Triton and urea. The UDE-1 preparation was diluted 2.5-fold with distilled water and treated with Triton X-100 and urea as described for Step 3. After centrifugation the supernatant liquid was applied to a CM-cellulose column equilibrated with 20 mM Tris-Cl (pH 7.4) containing 0.25% Triton X-100 and 4 M urea. The nonadsorbed fractions were collected and treated as described for Step 3. The final preparation was named UDE-CM-1.

RESULTS AND DISCUSSION

Purification of Alanine Carrier

In our earlier reports (10) we described the solubilization of the alanine carrier from membranes by the use of a cholate-deoxycholate mixture and its partial purification by DEAE-cellulose column chromatography and gel filtration in the presence of Triton X-100. However, the final preparation contained small amounts of cytochromes and numerous other protein contaminants detectable by gel electrophoresis in the presence of sodium dodecyl sulfate. Furthermore, the prolonged exposure of the alanine carrier to a high concentration of Triton X-100 (more than 1%), which happened at the gel filtration step, resulted in considerable inactivation. After various efforts to remove the contaminants, we found that ion exchange cellulose column chromatography in the presence of both Triton X-100 and urea was most effective. Results of a typical purification of the alanine carrier are summarized in Table I. In this particular case, CDE-P preparations (cholate-deoxycholate extracts, see Ref. 10 for details) from several batches were combined and used as the starting material. The specific activity of alanine transport by the original membranes was around 1 nmole per min per mg of protein. Since the alanine transport activity of the reconstituted vesicles was dependent on the species of phospholipids used to form them (10), meaningful comparison of the specific activities with those of the original membrane was not possible. However, the specific activity of the final preparation was more than 10-fold that of the original membranes.

In Table II the amounts of the various contaminants in the final preparation are shown. The preparation contained virtually no electron transfer components and no ATPase. During the first DEAE-cellulose column chromatography, most of the ATPase and NADH dehydrogenase activities were removed. However, a considerable amount of

TABLE I. Summary of Purification of Alanine Carrier

Preparation[a]	Protein mg	Alanine carrier activity[b] Specific U/mg	Total U	Yield %
CDE-P	326	4.29	1,399	100
DE-1	35.1	7.51	263.6	18.8
UDE-1	14.7	14.35	210.9	15.1
UDE-CM-1	3.0	18.00	53.8	3.8

[a]Abbreviations are as in text.
[b]One unit of alanine carrier activity is defined as the amount transporting 1 nmole of alanine under the standard assay condition (10, 14).

TABLE II. Contaminants in UDE-CM-1 Preparation

Component	Membranes	CDE-P[a]	UDE-CM-1[a]
		units/mg. protein	
ATPase	1.19	3.78	< 0.01
NADH dehydrogenase	3.24	0.10	< 0.01
NADH oxidase	1.12	0.10	0.00
		nmoles/mg protein	
Cytochrome b	4.0	0.89	0.00
Cytochrome c + c_1	2.6	0.93	0.00
Cytochrome a	0.6	0.24	0.00
Flavin	3.1	0.21	0.00
		mg/mg protein	
Phospholipids	0.11	0.21	< 0.01

[a]Abbreviations are as in text.

cytochromes b and c + c_1 and approximately one-third of the phospholipids remained in the DE-1 preparation (data not shown). Removal of the phospholipids resulted in aggregation of the proteins, which were hard to dissolve in the Triton solution. Thus, for solubilization of these aggregates, more than 6 M urea was required in addition to Triton. However, since the exposure of proteins to high concentrations of urea inactivated the activity (data not shown), a rather rapid manipulation was necessary during the urea treatment. At the second DEAE-cellulose column chromatography in the presence of both Triton and urea, the activity appeared in the nonadsorbed fractions, which were completely free of phospholipids and electron transfer components except for a minute quantity of cytochrome b (measureable only by its absorbance in the Soret region). The final preparation after CM-cellulose column chromatography, however, was completely devoid of cytochrome. The fact that the activity adsorbed on neither DEAE- nor CM-cellulose columns in the presence of Triton and urea indicated that the alanine carrier had a neutral isoelectric point.

Gel Electrophoresis

Figure 1A shows the electrophoretic pattern of the final preparation on polyacrylamide gel containing 0.05% sodium dodecyl sulfate, pH 7.0. There were 2 major

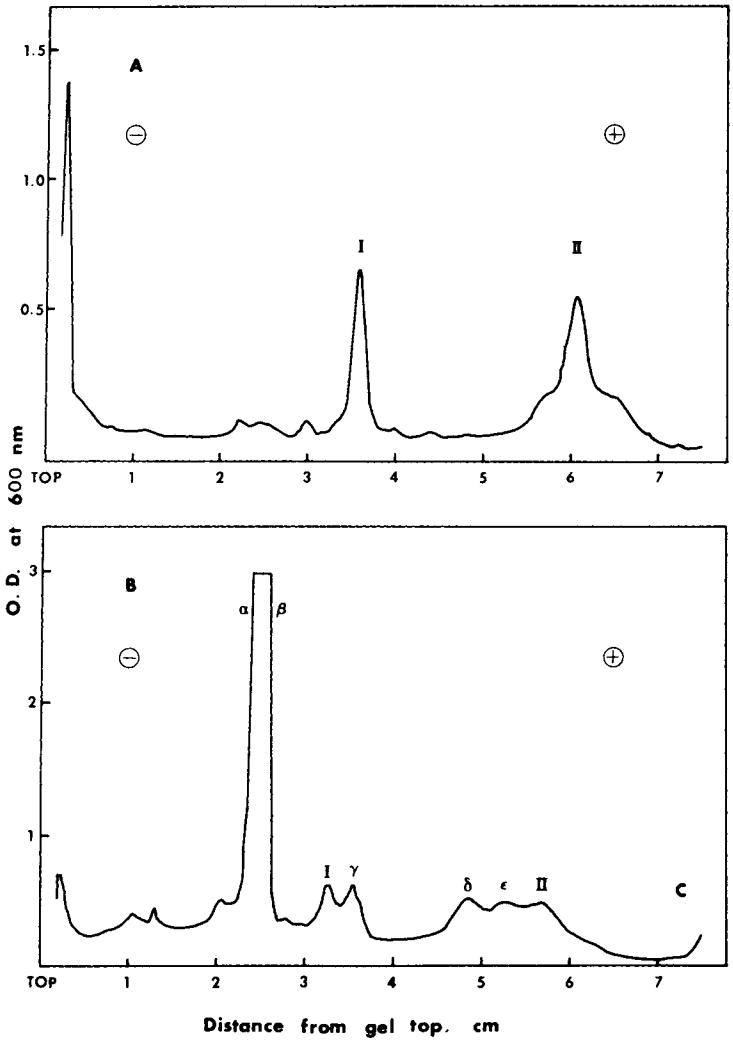

Fig. 1. Electrophoresis of the final preparation (UDE-CM-1) in sodium dodecyl sulfate-polyacrylamide gels. The gels were loaded with A) UDE-CM-1, 20 μg or B) UDE-CM-1, 20 μg plus TF_1 30 μg. The staining was carried out using Coomassie Brilliant Blue. α, β, γ, δ, and ε correspond to subunits of TF_1 (19).

bands (I and II) and a small number of minor bands. The latter seem to be due to contaminants since their relative intensities varied in different preparations. In Fig. 1B the electrophoretic pattern of the final preparation mixed with purified TF_1, so as to provide subunits as molecular weight markers, is shown. The α, β, γ, δ, and ε subunits of TF_1 have molecular weights of 56,000, 53,000, 32,000, 15,500 and 11,000, respectively. From these results, the molecular weights of bands I and II were estimated to be 36,000 and 9,400, respectively. Although we have no evidence at the moment whether these 2 major bands are the essential components (or subunits) of the alanine carrier protein, these 2 bands appear to relate to its activity since they can be observed in every preparation so far tested. In our earlier reports, we described the apparent molecular weight of the alanine carrier to be 150,000, as determined by gel filtration in the presence of 0.5%

Triton X-100 (10). Possible explanations for the discrepancy in the two values for the molecular weight are: a) the protein may be in its aggregated form when only Triton X-100 is present, b) the protein may be a large oligomer, or c) bound surfactant may increase the apparent molecular weight of the protein when estimated by gel filtration.

Alanine Transport in Proteoliposomes With Integral Cytochrome Oxidase

As described previously, alanine transport by the reconstituted proteoliposomes is dependent on a membrane potential generated by K^+ diffusion mediated by valinomycin (10). However, this uptake is transient and dependent on the magnitude of the K^+ concentration gradient across the membrane. Thus, the extent of loading of K^+ into the proteoliposomes was the limiting factor in the alanine transport. For better analysis of

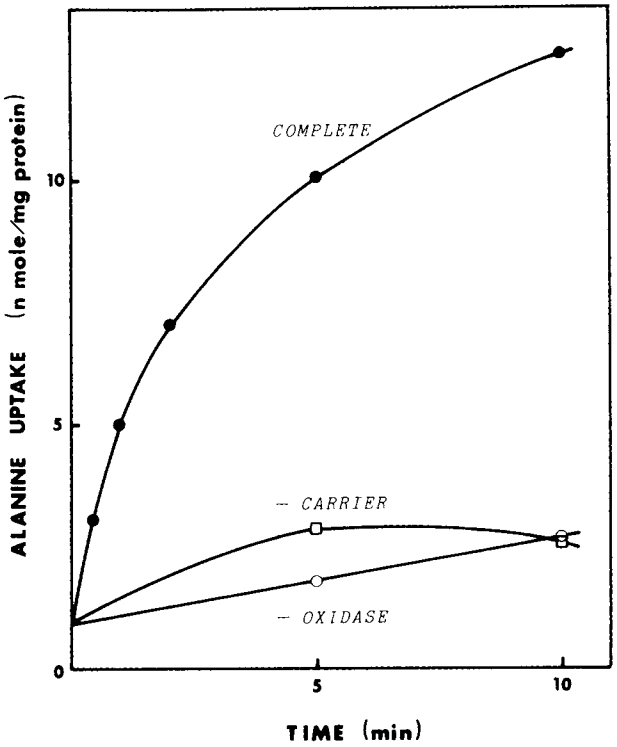

Fig. 2. Uptake of alanine by proteoliposomes containing both the alanine carrier and cytochrome oxidase. To 0.2 ml of PS3 phosphatidylethanolamine-detergent mixture (50 mg of phosphatidylethanolamine, 20 mg Na-cholate, and 10 mg deoxycholate per ml) was added 20 μg of protein of UDE-CM-1 and 0.4 mg protein of purified cytochrome oxidase and the mixture dialyzed against 10 mM Tricine-NaOH (pH 8) containing 150 mM KCl and 5 mM $MgSO_4$ for 20 hr at 4°C. The reconstituted proteoliposomes were collected by centrifugation (140,000 × g, 60 min), washed once with 50 mM Tricine-NaOH (pH 8) containing 5 mM $MgSO_4$, and resuspended in the same buffer. The assay mixture (0.1 ml) contained proteoliposomes, 1.7 μg of UDE-CM-1 proteins; cytochrome c, 0.375 mg; Tris-maleate, pH 7.0, 0.188 M; $MgSO_4$, 0.019 M; Na-ascorbate, 0.025 M; and ^{14}C-alanine, 0.021 mM, 1.25 μCi/ml. The reaction was started by the addition of ^{14}C-alanine and ascorbate with vigorous shaking at 37°C, and at an appropriate time it was terminated by dilution with 2 ml of 0.05 M LiCl. Then the mixture was filtered and washed with 2 ml of 0.05 M LiCl. ●) proteoliposomes containing both the alanine carrier and cytochrome oxidase; ○) proteoliposomes without cytochrome oxidase; □) proteoliposomes without the alanine carrier.

the molecular mechanisms of active alanine transport, a continuous supply of electrochemical energy might be desirable.

Mitochondrial cytochrome oxidase has been known to generate a proton-motive force when reconstituted into proteoliposomes and supplemented with cytochrome c and ascorbic acid (15, 17). The direction of generation of the proton-motive force is dependent on the side to which these electron donors are added; an interior negative potential is obtained when cytochrome c and ascorbic acid are added to the external medium.

Figure 2 shows the alanine uptake driven by ascorbic acid oxidation by the reconstituted proteoliposomes containing both the alanine carrier and the purified cytochrome oxidase of beef heart mitochondria. On addition of ascorbic acid, an accumulation of alanine by the reconstituted proteoliposomes was observed. Control experiments with proteoliposomes lacking either the carrier or cytochrome oxidase showed no uptake of alanine. The presence of KCN or carbonylcyanide p-trifluoromethoxyphenylhydrazone completely abolished the uptake (data not shown). The results indicated that energization of the alanine transport can be accomplished by an electrical generator of a quite different nature, animal mitochondrial cytochrome oxidase.

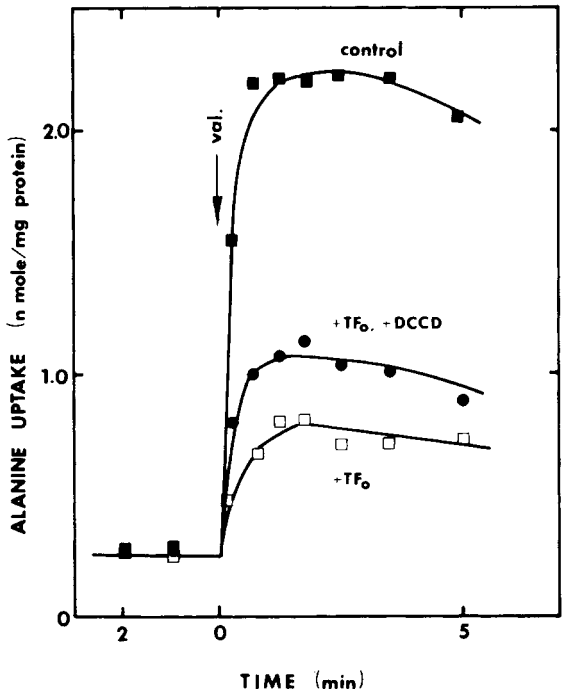

Fig. 3. Alanine uptake by proteoliposomes containing both the alanine carrier and TF_0. Proteoliposomes were reconstituted by the dialysis method and loaded with 0.5 M potassium phosphate buffer (pH 8), as described alsewhere (14). In the case of preparations of proteoliposomes containing TF_0, 50 μg of TF_0 proteins and 40 μg of UDE-1 proteins were reconstituted with 10 mg of PS3 phospholipids. Assay methods and conditions were described previously (10, 14). ■) proteoliposomes containing only the alanine carrier; □ and ●) proteoliposomes containing both the alanine carrier and TF_0. Dicyclohexylcarbodiimide (0.1 mM) was added as indicated. An arrow indicates the addition of valinomycin (2 μg per ml).

TF_0 Integrated Proteoliposomes

The membrane-integrated portion (TF_0) of the proton-translocating ATPase complex of the thermophilic bacterium PS3 has been shown to be a specific proton conductor (18). As shown on Fig. 3, proteoliposomes containing both the alanine carrier and TF_0 show reduced alanine accumulation driven by a membrane potential, which is partially restored by the addition of dicyclohexylcarbodiimide. These results are consistent with the fact that the proton-conducting activity of TF_0 is known to be inhibited by dicyclohexylcarbodiimide (18). Thus TF_0 uncouples the active transport of alanine by conducting protons across the membranes.

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