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INACTIVATION BY DETERGENTS OF THE PROLINE TRANSPORT SYSTEM IN MEMBRANE VESICLES FROM *ESCHERICHIA COLI* AND ITS REACTIVATION BY BOVINE SERUM ALBUMIN

SHOJI MIZUSHIMA

Department of Agricultural Chemistry, Nagoya University, Chikusa-ku, Nagoya 464 (Japan)

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SUMMARY

The proline transport system of membrane vesicles from *Escherichia coli* was inactivated by a low concentration of detergents such as deoxycholate, dodecyl sulfate and Triton X-100 The addition of a large amount of bovine serum albumin to membrane vesicles which had been treated with one of these detergents resulted in the restoration of the proline transport activity The restoration of the transport activity by bovine serum albumin was most remarkable with the deoxycholate-inactivated membrane vesicle 80 % inactivation of the transport system with 0 005 % deoxycholate was completely overcome by the addition of albumin The degree of restoration was dependent on the concentration of albumin Although albumin stimulated the proline transport activity itself, the stimulatory effect could not account for the restoration of transport activity The binding of deoxy[^{14}C]cholate to the membrane vesicle was roughly proportional to the amount of detergent added Deoxycholate once bound to the membrane vesicle was removed almost completely by the incubation with albumin It is concluded that the removal of detergent from the membrane vesicle by bovine serum albumin results in the restoration of the proline transport activity.

INTRODUCTION

Many detergents are known to solubilize biological membranes Taking advantage of this property, many studies have been carried out to dissociate membranes to their molecular components and reassemble membranes by removal of detergents [1] However, it was often difficult to remove completely the detergent bound to membrane components and this made it difficult to reconstitute functionally active biological membranes During the course of studies on the role of membrane components on the reassembly of outer membranes in deoxycholate solution (Nakamura, K and Mizushima, S , unpublished) and on the reassembly of outer membranes on lipoprotein-peptidoglycan sacculus in dodecyl sulfate solution (Yamada, H and Mizushima, S , unpublished), we have also found difficulties in the complete removal of detergents

It has been well known that bovine serum albumin has an ability of binding a

considerable amount of detergents such as dodecyl sulfate, deoxycholate and Triton X-100 [2, 3] The protein has a higher binding affinity to deoxycholate than cytochrome b_5, a hydrophobic membrane protein, when the concentration of the detergent was lower than the critical micelle concentration [4]

Taking advantage of the property of bovine serum albumin, I have tried to reactivate a membrane function which had been destroyed by detergents When the membrane vesicles of *Escherichia coli* were treated with a small amount of either deoxycholate, dodecyl sulfate or Triton X-100, the ability of proline uptake was diminished almost completely The addition of a large amount of bovine serum albumin to the membrane vesicle which had been treated with a detergent resulted in the restoration of this ability During the incubation with albumin, the detergent once bound to the membrane vesicle was removed almost completely from the membrane These results are shown in this paper

MATERIALS AND METHODS

Chemicals

L-[U-^{14}C]Proline (spec act 163 Ci/mol), L-[4,5-$^{3}H_2$]leucine (spec act 32 Ci mmol) and [2-^{3}H]glycerol (spec act 500 Ci/mol) were purchased from Daiichi Pure Chemicals Co , Tokyo Deoxy[*carboxyl*-^{14}C]cholic acid (spec act 47 3 Ci/mol) was obtained from ICN, Irvine, California Sodium dodecyl sulfate and sodium deoxycholate were obtained from Yoneyama Chemicals Ind Ltd, Osaka, Triton X-100 was from Sigma Chemical Co , St Louis and bovine serum albumin (Cohn Fraction V) was from Daiichi Pure Chemicals Co , Tokyo

Bacterial growth and membrane preparations

Escherichia coli YA21 which has been used in a membrane research series was used The preparation of the purified cytoplasmic membrane was carried out by extensive dialysis of spheroplast membrane against EDTA solution followed by an isopycnic sucrose gradient centrifugation as described previously [5] The purified cytoplasmic membrane labelled with [^{3}H]leucine or [^{3}H]glycerol was prepared as described previously [5] Kaback's membrane vesicle was prepared by the repeated washing of spheroplast membrane in EDTA solution as described [6] Spheroplasts which had lost about a half of the cellular outer membrane were used as starting material [5] In this paper, Kaback's membrane vesicle and the purified cytoplasmic membrane will be called membrane vesicle I and II, respectively They were suspended in 100 mM potassium phosphate buffer (pH 6 6) to a final protein concentration of 5 mg per ml and stored at - 70 C

Transport studies

The method was basically the same as that developed by Kaback [6] In the present study, sodium DL-lactate was used as an energy source The termination of the reaction and the washing of membrane vesicles on Millipore filters were carried out with 100 mM LiCl/10 mM $MgCl_2$ solution The specific activity and final concentration of [^{14}C]proline were 163 Ci/mol and 9–10 μM, respectively Incubation was carried out for 10 min at 30 °C Radioactivity was counted in 7 ml of toluene scintillation fluid with a Packard 3320 Tri-Carb scintillation spectrometer

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and urea was performed as described previously [5]

Electron microscopy

Samples were fixed, embedded and sectioned according to the method of Silva and Sousa [7] Ethanol instead of acetone was used for dehydration Observations were made in a Hitachi HS-9 electron microscope

RESULTS

Gel electrophoretic analysis of membrane proteins

Fig 1 shows a gel electrophoretic profile of membrane vesicle I Several remarkable protein bands which cannot be found in that of the membrane vesicle II [5] were observed The position of these bands on a gel coincides with those of major outer membrane proteins as indicated by arrows in Fig 1

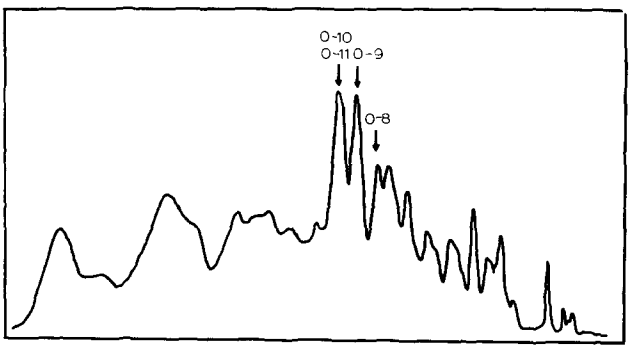

Fig 1 Scan of polyacrylamide gel electrophoresis of proteins of membrane vesicle I Sample had been preheated at 100 °C for 5 min in the presence of sodium dodecyl sulfate, mixed with solid urea and applied onto a gel containing 0 1 % dodecyl sulfate, 8 M urea and 100 mM sodium phosphate buffer (pH 7 2) The top of gel is to the right Arrows indicate peaks corresponding to major outer membrane proteins 0–8 through 0–11 (Uemura, J and Mizushima, S , unpublished)

Effect of bovine serum albumin on proline transport

As shown in Fig 2, bovine serum albumin stimulated the proline transport in both membrane preparations The transport reaction was lactate dependent and the stimulated activity by albumin was also lactate dependent Albumin alone without membrane preparation did not result in any retention of ^{14}C-proline on a Millipore filter Although the activity of proline transport was much higher with membrane vesicle I than with membrane vesicle II, a significant activity was also observed with the latter Previously it was reported that the purified cytoplasmic membrane (membrane vesicle II) had many open membranous structures when a negatively stained sample was examined under electron microscopy [5] However, a thin sectioned sample showed that the preparation was mainly composed of closed vesicles the size of which was comparable to an intact cell (average diameter, about 0 8 μm) (Fig 3)

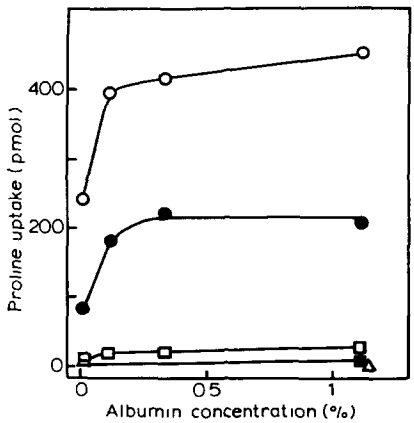

Fig 2 Effect of bovine serum albumin on proline transport activity of membrane vesicles 20 μl of membrane vesicle I (open symbols) or II (closed symbols) were mixed with 30 μl of 100 mM potassium phosphate buffer (pH 6 6), 50 μl of 20 mM magnesium sulfate and 15 μl of different concentration of bovine serum albumin, and incubated at 30 C for 10 min At this time, 10 μl of either 200 mM sodium DL-lactate (○, ●) or water (□, ■) and, immediately thereafter, 10 μl of [^{14}C]proline were added and incubation was continued for 10 min To terminate the reaction, each sample was rapidly diluted with 5 ml of 100 mM LiCl/10 mM $MgSO_4$ solution, immediately filtered through a Millipore HA filter and washed twice with an equal volume of the solution △, without membrane vesicle

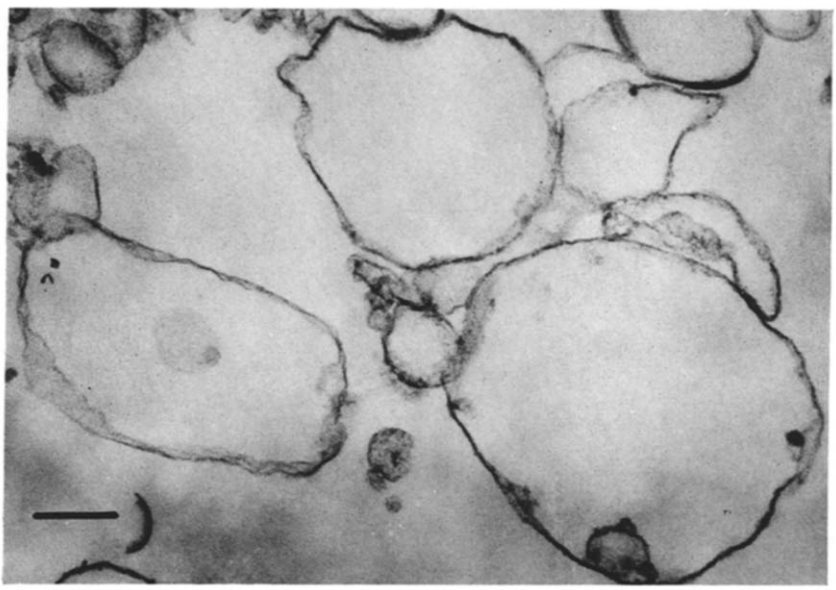

Fig 3 Electron micrograph of a thin section of membrane vesicle II Bar represents 0 2 μm

Effect of detergent on proline transport

The effects of deoxycholate, dodecyl sulfate and Triton X-100 on the transport of proline are shown in Tables I, II and III, respectively Proline transport in the absence of albumin was significantly inhibited by 0 005–0 01 % solution of these

TABLE I

INACTIVATION OF PROLINE TRANSPORT BY DEOXYCHOLATE AND REACTIVATION WITH BOVINE SERUM ALBUMIN

First incubation 20 μl of membrane vesicles were mixed with 30 μl of 100 mM potassium phosphate buffer (pH 6 6), 50 μl of 20 mM magnesium sulfate and 5 μl of different concentrations of detergent, and incubated at 30 °C for 10 min For studying the effect of detergent, 10 μl of 200 mM DL-lactate and 10 μl of [^{14}C]proline were added at this time and proline transport activity was assayed as described in Fig 2 Second incubation For studying the effect of albumin, 15 μl of 10 % (w/v) bovine serum albumin were added to reaction mixture after first incubation, and incubation was continued for 10 min Then, DL-lactate and [^{14}C]proline was added and transport activity was assayed as described in Fig 2

Membrane	Concn of deoxycholate in first incubation (%)	Second incubation with albumin	[^{14}C]Proline uptake (pmol)
Membrane vesicle I	0	−	230
	0	+	462
	0 005	−	50
	0 005	+	445
	0 010	−	6
	0 010	+	280
	0 015	−	1
	0 015	+	102
Membrane vesicle II	0	−	63
	0	+	94
	0 005	−	9
	0 005	+	75
	0 010	−	1 5
	0 015	−	0

TABLE II

INACTIVATION OF PROLINE TRANSPORT BY DODECYL SULFATE AND REACTIVATION WITH BOVINE SERUM ALBUMIN

Experimental methods were the same as those described in Table I

Membrane	Concn of dodecyl sulfate in first incubation (%)	Second incubation with albumin	[^{14}C]Proline uptake (pmol)
Membrane vesicle I	0	−	230
	0	+	462
	0 005	−	112
	0 005	+	464
	0 010	−	32
	0 010	+	206
	0 015	−	0
	0 015	+	13
Membrane vesicle II	0	−	84
	0	+	141
	0 005	−	11
	0 005	+	104
	0 010	−	1

TABLE III

INACTIVATION OF PROLINE TRANSPORT BY TRITON X-100 AND REACTIVATION WITH BOVINE SERUM ALBUMIN

Experimental methods were the same as those described in Table I

Membrane	Concn of Triton X-100 in first incubation (%)	Second incubation with albumin	[^{14}C]Proline uptake (pmol)
Membrane vesicle I	0	-	230
	0	+	462
	0.005	-	124
	0.005	+	360
	0.010	-	69
	0.010	+	266
	0.015	-	29
	0.015	+	159
Membrane vesicle II	0	-	64
	0	+	126
	0.005	-	18
	0.005	+	71
	0.050	+	0

detergents The effect was more remarkable with deoxycholate and dodecyl sulfate than with Triton X-100 The possibility that detergents caused the passing of membrane vesicles through Millipore filters was examined using [^{3}H]glycerol or [^{3}H]-leucine-labelled membrane vesicle II The addition of [^{3}H]glycerol or [^{3}H]leucine to the culture media resulted in the specific labelling of lipid and protein, respectively [5] The addition of deoxycholate ranging from 0.01 to 0.1 % did not cause any changes in the retention of membrane vesicles labeled with either [^{3}H]glycerol or [^{3}H]leucine on Millipore filters under the conditions for transport assay (data not shown)

Restoration of proline transport activity by bovine serum albumin

The effect of bovine serum albumin on the transport ability which had been inactivated by detergents was studied Membrane vesicles were preincubated with detergent at 30 °C to inactivate the transport activity Then they were incubated with a large amount of albumin for 10 min at 30 °C and subjected to the assay of transport activity The amount of albumin was fifteen times as much as that of protein in membrane vesicles Results are shown in Tables I, II and III The incubation with albumin resulted in a remarkable restoration of the transport activity The recovery from the inactivation caused by deoxycholate was most remarkable The restoration by albumin from the dodecyl sulfate inactivation was also significant However in the case of Triton X-100, the effect of albumin on the restoration was not remarkable when the stimulative effect of albumin on the transport activity itself was taken into consideration Albumin alone did not result in the retention of [^{14}C]proline on Millipore filters

Fig 4 shows the effect of albumin concentration on the recovery of transport activity from detergent inactivation The higher the concentration of detergent, the larger the amount of albumin required for the recovery Because of the

technical difficulty, the effect of a high concentration of albumin more than 1 1 % was not examined Therefore, it is uncertain whether the greater recovery can be achieved from the inactivation by 0 015 % deoxycholate or not Although albumin stimulated the proline transport activity as shown in Fig 2, the stimulatory effect could not account for the remarkable restoration of the transport activity, since the amount of albumin required was different in both cases and the considerable transport activity was restored on the detergent-inactivated membrane which had essentially no residual activity

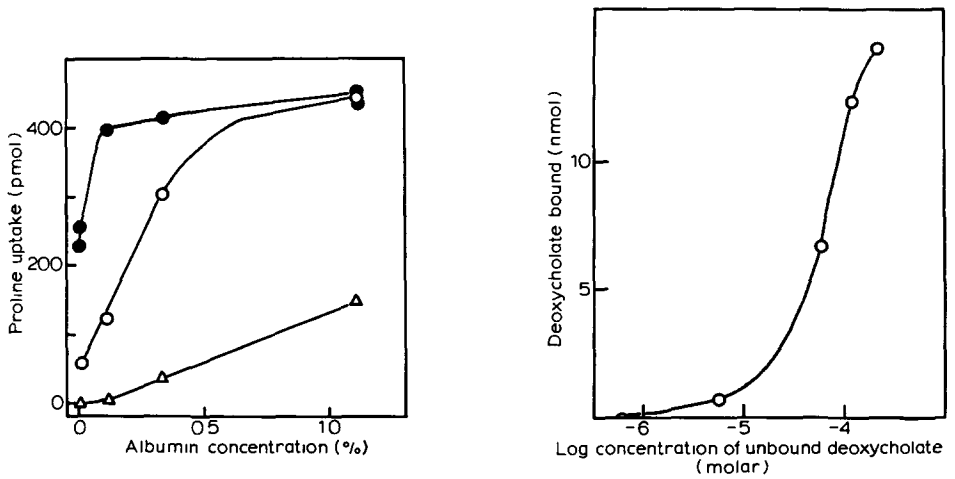

Fig 4 Effect of concentration of bovine serum albumin on the restoration of deoxycholate-inactivated proline transport Membrane vesicle I was used Conditions the same as those given in Table I except amount of albumin was variable in this experiment Concentration of deoxycholate in the first incubation was 0 % (●), 0 005 % (○) and 0 015 % (△), respectively

Fig 5 Binding of deoxycholate to membrane vesicles 40 μl of membrane vesicle I was mixed with 60 μl of 100 mM potassium phosphate buffer (pH 6 6), 100 μl of 20 mM magnesium sulfate and 10 μl of different concentrations of deoxy[^{14}C]cholate After incubation at 30 °C for 10 min, 50 μl of samples were taken out and remaining samples were centrifuged at 25 000 rev /min for 10 min at 20 °C to sediment the membrane vesicles Radioactivity in the whole sample and supernatant solution was counted with 10 ml of Bray's solution Amount of radioactivity bound to the membrane vesicle was estimated by subtracting the radioactivity in the supernatant solution from the total radioactivity

Binding of deoxycholate to membrane and its removal by bovine serum albumin

The binding of deoxycholate to the membrane vesicle was studied using deoxy-[^{14}C]cholate Membrane vesicle I was incubated with radioactive deoxycholate The mixture was then centrifuged to sediment membrane vesicles and the radioactivity in the supernatant was counted Results are shown in Fig 5 The amount of deoxycholate bound to the membrane vesicle was roughly proportional to the amount added The addition of albumin to the membrane vesicle treated with deoxycholate resulted in an almost complete removal of the detergent from the membrane vesicle over a fairly large range of deoxycholate concentration (Table IV) It should be noticed that although deoxycholate was almost completely removed by albumin from the membrane vesicle which had been pretreated with 0 015 % deoxycholate, the restora-

TABLE IV

REMOVAL OF DEOXYCHOLATE FROM MEMBRANE VESICLE BY BOVINE SERUM ALBUMIN

40 μl of membrane vesicle I were mixed with 60 μl of 100 mM potassium phosphate (pH 6 6) and 100 μl of 20 mM magnesium sulfate and 10 μl of different concentrations of deoxy[^{14}C]cholate After incubation at 30 C for 10 min, 30 μl of 10 % (w/v) bovine serum albumin or water were added and incubation was continued for 10 min Then 50 μl of samples were taken out and the remaining portions centrifuged at 25 000 rev /min for 10 min at 20 C to sediment membrane vesicle Radioactivity in whole sample and supernatant solution was counted in Bray s solution (10 ml)

Concn of deoxycholate in first incubation (%)	Albumin in second incubation	Deoxycholate (n mol)		
		Total (A)	Supernatant solution (B)	Membrane bound (A-B)
0 005	–	24 0	15 6	8 4
0 005	+	24 0	24 0	0 0
0 010	–	48 0	32 6	15 4
0 010	+	48 0	47 4	0 6
0 015	–	72 0	54 0	18 0
0 015	+	72 0	72 0	0 0

tion of the proline transport activity was only partial (see Table I) This fact indicates that the removal of detergent was not sufficient for the restoration of the transport function in heavily damaged membrane vesicles

DISCUSSION

In the present study, I have used two kinds of membrane preparations, that is, Kaback's membrane vesicle (membrane vesicle I) and the purified cytoplasmic membrane (membrane vesicle II) The membrane vesicle I preparation used showed high proline transport activity, while containing a considerable amount of the outer membrane protein as shown in Fig 1 On the other hand, although membrane vesicle II showed lower proline transport activity, it is essentially free from the outer membrane [5] Since outer membrane proteins are known to have a peculiar structure as membrane proteins [8, 9], the possibility that outer membrane components may interfere with the reorganization of membrane components required for the restoration of transport activity had to be taken into consideration Therefore, membrane vesicles I and II were used for comparison with each other Both preparations gave essentially the same results on the proline transport with respect to the inactivation by detergent and the reactivation with albumin In connection with this point, I am interested in a purified cytoplasmic membrane preparation which was reported to possess a high proline transport activity [10]

Membrane vesicles are fairly sensitive to detergents in their proline transport activity For example, a significant inactivation was observed with 0 005–0 01 % deoxycholate (Table I) Under the conditions used more than 30 % of detergent was found to be associated with the membrane vesicle (Fig 5) This means that 2–4 molecules of the detergent per molecule of membrane protein as an average was enough to inactivate the transport activity, providing that an average molecular weight of mem-

brane proteins is 40 000 At present, it is unknown whether the detergent primarily attacks the transport machinery, the energy providing system or the membrane structure itself

It has been established that native bovine serum albumin has high affinity binding site for many detergents including deoxycholate, dodecyl sulfate and Triton X-100 The almost complete restoration of the transport activity which had been inactivated by 0 005 % deoxycholate was observed with a large amount of albumin In the reaction mixture (140 μl), the amount of albumin and deoxycholate were 22 nmol and 12 nmol, respectively Then using results obtained by Makino et al [3] and those in Fig 5, one can estimate the amount of deoxycholate bound to albumin and the membrane vesicles and that in solution (unbound) to be approximately 11 6, 0 2 and 0 3 nmol, respectively Since the amount of detergent bound to the membrane vesicle was about 4 2 nmol in the absence of albumin (from Table IV), one can conclude that more than 95 % of the detergent which had been bound to the membrane vesicle should be removed by the addition of albumin, providing that the interaction between the detergent and both albumin and membrane vesicle is reversible Data throughout the present paper clearly support this conclusion The extent of deoxycholate removal in Table IV was even better Although I have not studied the binding of dodecyl sulfate to membrane vesicles, the restoration by albumin of the dodecyl sulfate-inactivated transport system may be explained in the same way, since the detergent also shows high binding affinity to bovine serum albumin [2] The restoration by albumin of the Triton X-100 inactivated transport system was rather poor This can be explained by its relatively weak interaction with albumin [3]

Under the conditions from which the restoration of deoxycholate-inhibited activity can be restored, the release of protein from membrane vesicles was insignificant and the supernatant solution separated from the membrane vesicle after the detergent treatment was not required for the reactivation of the transport system (data not shown) This indicates that the restoration of transport activity by albumin is not a result of the reconstitution of membrane structure but may be a result of the reorganization of the membrane structure including the transport machinery by the removal of detergent under the conditions used However, when membranes had been treated with a large amount of detergent, the removal of detergent from membranes was not sufficient for the restoration

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