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PHOSPHO-*N*-ACETYLMURAMOYL-PENTAPEPTIDE-TRANSFERASE OF *ESCHERICHIA COLI* K12

PROPERTIES OF THE MEMBRANE-BOUND AND THE EXTRACTED AND PARTIALLY PURIFIED ENZYME

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Summary

Phospho-*N*-acetylmuramoyl-pentapeptide-transferase (UDP-*N*-acetyl-muramoyl-L-alanyl-D-γ-glutamyl-L-lysyl-D-alanyl-D-alanine:undecaprenoid-alcohol-phosphate-phospho-*N*-acetylmuramoyl-pentapeptide-transferase, EC 2.7.8.13) was solubilized by repeated freezing and thawing of crude envelopes of *Escherichia coli* K12. The solubilized enzyme was partially purified by gel filtration and ion-exchange chromatography. This preparation contained small amounts of phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol but no endogenous lipid substrate, C_{55}-isoprenyl phosphate, could be detected. Some catalytic properties (exchange reaction) of the solubilized enzyme were compared to those of membrane-bound transferase. The transfer activity of the partially purified transferase was restored by the addition of an aqueous lipid dispersion. All the transferase activity was found to become incorporated into the liposomes. Preincubation of the transferase preparation with phospholipase A_2 or D strongly reduce both exchange and transfer activity. This suggests that phospholipids sensitive to phospholipases are necessary for the enzymatic reaction. Different effects of some neutral detergents on the exchange activity were reported.

Introduction

Solubilization and purification of membrane-bound enzymes are tools to study the properties of components of the macromolecular organization of bio-

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logical membranes. Phospho-*N*-acetylmuramoyl-pentapeptide-transferase (UDP-*N*-acetylmuramoyl-L-alanyl-D-γ-glutamyl-L-lysyl-D-alanyl-D-alanine:undecaprenoid-alcohol-phosphate-phospho-*N*-acetylmuramoyl-pentapeptide-transferase EC 2.7.8.13) is part of this membrane structure. The enzyme is involved in the biosynthesis of bacterial peptidoglycan. It is of particular interest because it catalyzes the transfer of a hydrophobic substrate into the lipophilic environment of the membrane. The reaction catalyzed by the transferase is shown in the following equation [1,2].

$$\text{UDP-MurNAc-pentapeptide} + C_{55}\text{-isoprenylphosphate} \rightleftharpoons C_{55}\text{-isoprenylphosphate-}P\text{-}P\text{-MurNAc-pentapeptide} + \text{UMP} \quad (1)$$

The enzyme also catalyzes an exchange reaction according to the equation [1]

$$[^{14}\text{C}]\text{UMP} + \text{UDP-MurNAc-pentapeptide} \rightleftharpoons [^{14}\text{C}]\text{UMP-}P\text{-MurNAc-pentapeptide} + \text{UMP} \quad (2)$$

As yet the work concerned with this enzyme was mainly done with crude particulate enzyme preparations from Gram positive bacteria [1]. Solubilization and subsequent purification of the enzyme is complicated by strong inhibition of the transferase reaction after addition of detergents normally used for the solubilization [3]. The transferase could be extracted by repeated freezing and thawing of *Escherichia coli* crude envelopes. Although the exchange activity of Triton X-100-solubilized transferase, which is partially depleted of phospholipids was reported to be highly dependent on the addition of C_{55}-isoprenylphosphate [4] this dependence was not observed by Umbreit and Strominger [5], who found that the exchange activity of Triton X-100-solubilized transferase from *Micrococcus luteus* was not stimulated by the addition of lipid substrate, but by some other polar phospholipids. Our results are in agreement with the latter findings.

Materials and Methods

Organism and growth condition. E. coli K12 (ATCC 23811) was obtained from American Type Culture Collection.

Cultures were grown in complex medium containing 0.5% yeast extract, 1% peptone, 0.5% glucose and 0.2% NaCl. The pH was adjusted to 7.5. Organisms were cultured at 37°C under aeration.

Preparation of crude envelopes. E. coli K12 cells (late logarithmic phase of growth) were harvested by centrifugation at 3000 × *g* for 10 min at 4°C. The wet weight yield of cells was 4—4.5 g/l. 40—45 cells were washed once with 0.01 M Tris-HCl buffer (pH 7.8), mixed with two volumes of 0.18 mm glass beads and shaken in a Bühler cell homogenizer for 5—6 min at about 10°C in the presence of 0.1 mg/ml DNAase and RNAase. The filtrate was centrifuged at 3000 × *g* to remove large particles. The supernatant was centrifuged at 50 000 × *g* for 60 min at 4°C. The resulting pellet was washed thoroughly with 0.05 M Tris-HCl buffer (pH 7.8).

Extraction and partial purification of phospho-N-acetylmuramoyl pentapeptide-transferase. A suspension of crude envelopes in 0.05 M Tris-HCl (pH 7.8)

with a protein concentration of 10—15 mg/ml was rapidly frozen in liquid N_2 and thawed in a water bath at 37°C (3X). After the final thawing, the suspension was centrifuged at 200 000 X *g* for 90 min at 4°C. The supernatant fraction was carefully removed, centrifuged once more under the same conditions and then concentrated by ultrafiltration (Amicon PM-10 membrane) to 10—15 mg protein/ml. The concentrated fraction was then applied to a Sephadex G-200 column (5 X 50 cm) equilibrated and eluted with 0.05 M Tris-HCl (pH 7.8) (flow rate, 30 ml/h). Exchange activity was only eluted in the void volume of the column. The active fractions were pooled and applied to a DEAE-cellulose column. Transferase activity was eluted as a single peak at 0.2—0.3 M NaCl during elution with a linear gradient of 0—0.6 M NaCl in 0.05 M Tris-HCl (pH 7.8) between 0.2 and 0.3 M NaCl. The fractions containing transferase activity were pooled and concentrated by ultrafiltration (Amicon PM-10 membrane). The concentrated material (5—10 mg protein/ml) could be stored for several weeks without any loss of activity when frozen at —30°C.

Enzyme assays. The activity of the phospho-*N*-acetylmuramoyl-pentapeptide-transferase was assayed by two methods. (a) Exchange assay: The exchange assay allows determination of the rate of exchange of [^{14}C]UMP with the unlabeled UMP moiety of UDP-*N*-acetylmuramoyl-pentapeptide. The standard incubation mixture contains $4.8 \cdot 10^{-6}$ M [U-^{14}C]uridine 5′-monophosphate (519 Ci/mol), $4.5 \cdot 10^{-4}$ M UDP-*N*-acetylmuramoyl-pentapeptide, 12.5 mM $MgCl_2$, 25 mM Tris-HCl (pH 7.8) and enzyme in a total volume of 40 µl. At this concentration the reaction was linear for at least 15 min. After incubation at 37°C, the reaction was stopped by the addition of 20 µl 0.1 M Tris (pH 10). To remove the excess [^{14}C]UMP, 2 µl (0.04 unit) alkaline phosphatase was added. The reaction mixture was further incubated at 37°C for 15 min and then spotted on Whatman DE-81 filter paper discs. The [^{14}C]uridine was removed by washing the filters with water. The filters were dried, and the remaining radioactivity was counted in a toluene-based scintillation cocktail. The first-order rate equation described by Dunfield and Calvin [6] was used to calculate the rate of exchange [3].

(b) Transfer assay: The standard assay contains 0.7 mM ^{32}P-labeled UDP-*N*-acetylmuramoyl-pentapeptide (15 Ci/mol), 25 mM $MgCl_2$, 25 mM Tris-HCl (pH 7.8), 0.04 unit alkaline phosphatase and a mixture of partially purified transferase and lipid dispersion or buffer in a total volume of 70 µl. After incubation the reaction was stopped by the addition of 10 µl isobutyric acid. The incubation mixture was spotted on Whatman 3 MM paper and subjected to chromatography in isobutyric acid/1 M ammonia (5 : 3, v/v). The radioactive reaction product (R_F 0.8—0.9) was located by autoradiography, cut out and counted.

Succinate dehydrogenase (EC 1.3.99.1) and NADH oxidase (EC 1.6.3.1) were tested as described by Sekizawa and Fukui [7].

UDP-*N*-acetylmuramoyl-pentapeptide was prepared from *Bacillus cereus* as described previously [8]. Phospholipids were extracted and fractionated by the method described by Ames [9]. Phospholipid phosphate was determined according to Ames [10]. The phospholipid content was calculated assuming an average molecular weight of 700. C_{55}-isoprenylphosphate was prepared from crude lipid extracts (*E. coli* K12). After performing mild alkaline hydrolysis

twice according to Kennedy and Tarlov [11], purification was achieved by preparative thin-layer chromatography on silica gel 60 (Merck) [8]. Lipid dispersion was prepared from crude lipid extracts according to Morrisett et al. [12].

Sucrose density gradient centrifugations were carried out in linear sucrose gradients containing 0.05 M Tris-HCl (pH 7.8), 1 mM EDTA for 18—24 h at 25 000 rev./min at 4°C. The gradients were collected from the bottom.

Protein concentration was determined by the method of Lowry et al. [13] using hen egg white lysozyme as standard.

Chemicals. Lysozyme (hen egg white), DNAase I, RNAase (bovine pancreas), phospholipase A_2 (snake venom), D (cabbage) and alkaline phosphatase (*E. coli*) were from Boehringer-Mannheim. Detergents were from Serva, Feinbiochemica, Heidelberg. All reagents used were of analytical grade.

Results

Extraction and partial purification of phospho-N-acetylmuramoyl-pentapeptide-transferase

6% of the membrane-bound protein can be extracted from *E. coli* K 12 crude envelopes by repeated freezing and thawing. After this treatment transferase activity is found in the 200 000 × *g* supernatant (S200 fraction). The specific activity of the soluble enzyme was 15% of the activity of the crude envelopes. Succinate dehydrogenase and NADH oxidase were not detected in the soluble fraction. The solubilized material is distinct in sedimentation behaviour from membranes as can be shown by centrifugation in sucrose density gradients. The S200 fraction was found in the same position on the gradients as the alkaline phosphatase of *E. coli* and this fraction contained all the exchange activity (Fig. 1).

As well as proteins, 0.5% of the phospholipids was extracted by the freezing and thawing. C_{55}-isoprenylphosphate could not be detected in the soluble fraction (Table I). Due to these phospholipids, reaggregation occurred during the purification of the transferase. Therefore, only a partial purification (8-fold) could be achieved.

Properties of the membrane-bound and the solubilized transferase (exchange reaction)

Both the particulate and the solubilized transferase have an absolute requirement for Mg^{2+}. The crude envelopes show an activity maximum between 30 and 40 mM, whereas the partially purified enzyme is stimulated in a biphasic manner (Fig. 2). Mn^{2+} can substitute for Mg^{2+}, giving, at 12.5 mM Mn^{2+}, activities of 26% (crude envelopes) and 7% (partially purified enzyme) of those with Mg^{2+}. The K_m for UDP-MurNAc-pentapeptide were $5.6 \cdot 10^{-6}$ M for the crude envelopes and $3.7 \cdot 10^{-5}$ M for the solubilized enzyme. These K_m values are apparent values, since the concentration of [^{14}C]UMP was not saturating [14]. The corresponding values for 5'-UMP were $8.7 \cdot 10^{-5}$ and $6.7 \cdot 10^{-5}$ M, respectively. The apparent values for the maximal exchange rate, R_{max}, were $0.83 \cdot 10^{-6}$ M/min for the crude evelopes and $0.66 \cdot 10^{-7}$ M/min for the partially purified enzyme at various concentrations of UMP. At various concentrations of

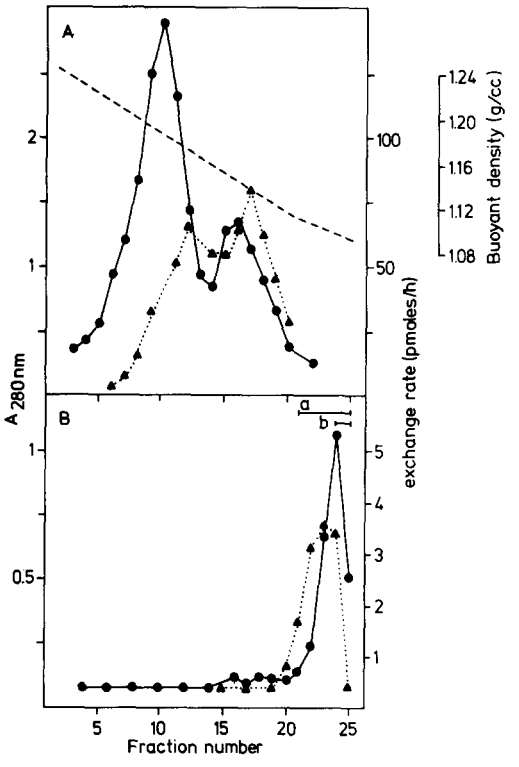

Fig. 1. Sucrose density gradient centrifugation of total membranes (A) and S200 fraction (B*). The total membrane fraction was prepared according to the method of Osborn et al. [25]. S200 fraction was prepared from crude envelopes. Isopynic sucrose gradient centrifugation was carried out in a SW 27 rotor. 0.7-ml fractions were collected. Refractive indices and absorbance at 280 nm were measured. Exchange activity was determined with 20 μl of each fraction. ●———●, absorbance at 280 nm; ▲———▲, exchange rate (pmol/h); - - - - - -, buoyant density. The bars in B represent the position of soluble marker proteins (a = alkaline phosphatase from *E. coli;* b = cytochrome *c*). * The condition used here only allowed isopynic equilibrium banding for the particles of the membrane preparation but not for the soluble material of the S200 fraction and the soluble marker proteins. Due to the small particle size of the soluble material a shift from equilibrium banding to velocity sedimentation occurred.

TABLE I

PHOSPHOLIPID COMPOSITION OF CRUDE ENVELOPES AND S200 FRACTION

From cells grown in [^{32}P]orthophosphate-supplemented complex medium (0.1 mCi/l) crude envelopes and S200 fraction were prepared. Lipids were extracted and separated by thin-layer chromatography on silica gel [9]. C_{55}-isoprenylphosphate content was determined after mild alkaline hydrolysis (2N) [11] and analysis of the non-saponificable fraction by two-dimensional thin-layer chromatography [8]. It was localized by autoradiography and the use of anisaldehyde reagent [24]. PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; C_{55}-P, C_{55}-isoprenylphosphate.

Fraction	Phospholipid/ protein (μg/mg)	Phospholipids (percent of total)			
		PE + LPE	PG	DPG	C_{55}-P *
Crude envelopes	400	79	12	1.5	0.3
S200 fraction	35	76.5	20	2.5	n.d. *

* Not detected (amounts less than 1 pmol could be detected by this method).

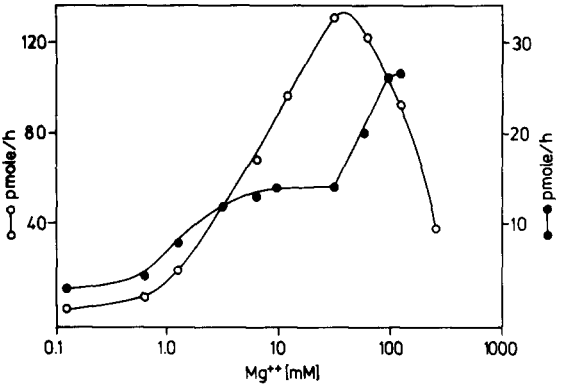

Fig. 2. Effect of Mg^{2+} concentration on the exchange rate. The incubation mixture contained $4.8 \cdot 10^{-6}$ M [U-^{14}C]uridine 5'-monophosphate (519 Ci/mol), $4.5 \cdot 10^{-4}$ M UDP-*N*-acetylmuramoyl-pentapeptide, 25 mM Tris-HCl (pH 7.8), various concentrations of $MgCl_2$ and enzyme in a total volume of 40 µl. ○———○, crude envelopes; ●———● G200 fraction.

UDP-MurNAc-pentapeptide, R_{max} were $0.13 \cdot 10^{-6}$ and $0.28 \cdot 10^{-7}$ M/min, respectively.

Crude envelopes as well as the partially purified enzyme showed a pH optimum at pH 7.8 in 0.1 M Tris-HCl buffer. Moenomycin, a surface-active antibiotic [15], showed different effects on the exchange rate of the particulate and solubilized transferase. The exchange reaction catalyzed by the crude envelopes was partially inhibited by the antibiotic. The extend of inhibition of about 50% is the same as reported for the transfer reaction in Gaffkya monari [16]. The partially purified enzyme, however, was activated by the antibiotic with a maximum activation at about 1 mg moenomycin/mg protein.

Effect of phospholipases on the exchange reaction

The partially purified enzyme fraction contains phospholipids. To examine whether these phospholipids have any influence on the exchange activity, the enzyme preparations were preincubated with phospholipase A_2 or D (0.1 mg phospholipase/mg protein). Incubation of the partially purified enzyme with phospholipase A_2 for 30 min at 25°C resulted in a decrease of the exchange rate to about half of the original rate. Further incubation for 1 h had no additional effect. After treatment of the crude envelopes with phospholipase A_2, only a slight loss of activity was observed (14% inhibition). Phospholipase D treatment of the solubilized enzyme results in a complete inhibition, whereas in the case of the crude envelopes about 40% of the activity was retained.

Restoration of the transfer activity by addition of lipid dispersion

With the envelope fraction, the transfer of the pentapeptide moiety from the nucleotide to C_{55}-isoprenylphosphate could be easily measured, since this fraction contained endogenous lipid substrate. In the partially purified enzyme fraction, however, no C_{55}-isoprenylphosphate could be detected. In delipidated envelope preparations from *M. luteus* and *Staphylococcus aureus*, the transfer activity could be restored by the addition of lipids in the presence of deoxycholate or Cutscum [17,18]. In contrast to these results, all our efforts to

restore the transfer activity of the partially purified enzyme by the addition of C_{55}-isoprenylphosphate in the presence of a variety of detergents (deoxycholate, Triton X-100, Tween 20 and Myrj 52) were unsuccessful. Only after addition of an aqueous lipid dispersion from crude lipid extracts transfer activity could be measured (Table II). For this purpose, the enzyme suspension was thoroughly mixed with lipid dispersion on a Vortex mixer. Brief sonication did not give better results and was, therefore, omitted. The transfer activity restored by this procedure was dependent on the amount of added lipid dispersion (Table III). Preincubation of the lipid dispersion/enzyme/mixture with phospholipase A_2 or D for 30 min at 25°C (0.1 mg phospholipase/mg protein) reduced the transfer activity to about 10% of the activity of an untreated sample. The restored transfer reaction was strongly dependent on bivalent cations, with Mg^{2+} giving the best activation. The optimal Mg^{2+} concentration was about 15 mM. Replacement of Mg^{2+} by Mn^{2+} (15 mM) restored only 10% of the activity obtained with Mg^{2+}. Cu^{2+}, Ca^{2+} and Zn^{2+} were without effect. The apparent Michaelis-Menten constant, K_m, for UDP-MurNAc-pentapeptide was $8.7 \cdot 10^{-5}$ M at $1.6 \cdot 10^{-5}$ M C_{55}-isoprenylphosphate.

Physical state of the restored transferase

In order to examine the interference of protein and lipid in the restoration process an isopynic density gradient centrifugation in linear sucrose gradients (2—18% sucrose) was performed. The liposomes of the dispersion banded at a density of 1.055 g/ml. Lipid dispersion and partially purified enzyme were mixed at a phospholipid to protein ratio from 10 : 1 (w/w). After centrifugation all the protein was found in the liposome fraction which now banded at the somewhat higher density of 1.063 g/ml. Both exchange and transfer activity could be detected at this position.

Effect of some neutral detergents on the exchange and the transfer reaction

In the course of our first experiments to restore the transfer activity, some neutral detergents were used to solubilize the added C_{55}-isoprenylphosphate. In these experiments, we found very different influences of these compounds

TABLE II

RESTORATION OF THE TRANSFER ACTIVITY OF PARTIALLY PURIFIED TRANSFERASE BY AQUEOUS LIPID DISPERSION

The enzyme-lipid dispersion mixture contained 5 mg phospholipid and 0.5 mg protein per ml and was prepared as described in the text. The C_{55}-isoprenylphosphate concentration was $1.6 \cdot 10^{-5}$ M. The final protein concentration was 0.36 mg/ml. The samples were incubated for 4 h at the indicated temperature.

Incubation temperature (°C)	Phospholipid concentration (mg/ml)	^{32}P incorporated in lipid intermediate (pmol)
20, 30, 37	0	0
20	3.6	65
30	3.6	79
37	3.6	119
30	3.6 minus enzyme	0

TABLE III

DEPENDENCE OF THE TRANSFER ACTIVITY ON THE AMOUNT OF LIPID DISPERSION

Various amounts of lipid dispersion were used in the restoration procedure. The final protein concentration was 0.36 mg/ml. The number in parentheses is the concentration of lipid substrate ($\times 10^{-6}$ M).

Phospholipid concentration (mg/ml)	^{32}P incorporated in lipid intermediate (pmol/h)
0 (0)	0
0.6 (2.9)	11
1.4 (6.5)	15
2.4 (11)	20
3.6 (16)	25

on the exchange reaction in the range of total inactivation to strong stimulation. Table IV shows the effects of various detergents on the exchange rate of the partially purified enzyme fraction. All the detergents so far tested which had hydrophilic-lipophilic balance numbers above 15 stimulate the exchange reaction, whereas the more hydrophobic detergents strongly inactivate the enzyme. Inactivation could be prevented by the addition of lipid dispersion to the incubation mixture (Table V). Additional lipid dispersion, however, had almost no effect on the activity of the untreated and the Tween-solubilized enzyme.

Some preliminary experiments to stimulate the transfer reaction with detergents showed that detergents which activated the exchange reaction strongly inhibit the transfer reaction. Tween 20, up to now the best activator of the exchange reaction, completely inhibits the transfer reaction at 0.5% (v/v). Span 20 and Triton X-100, inhibitors of the exchange reaction had almost no effect on the transfer activity.

TABLE IV

EFFECT OF SOME NEUTRAL DETERGENTS ON EXCHANGE ACTIVITY OF PHOSPHO-*N*-ACETYL-MUTAMOYL-PENTAPEPTIDE-TRANSFERASE

Aliquots of a suspension of partially purified enzyme (1 mg protein/ml) were solubilized with the indicated detergent (1%, v/v) for 30 min at room temperature and the exchange rate was then determined. HLB, hydrophilic-lipophilic balance number.

Detergent added	HLB number of detergent	Exchange rate	
		pmol/h	Percent of control
No addition (control)	—	11.3	100
Tween 20	16.7	22	200
Tween 40	15.6	16.8	149
Tween 60	14.9	14.7	130
Tween 80	15.5	15.5	137
Brij 35	16.9	14.6	129
Triton X-100	13.5	0	0
Span 20	8.6	0	0
Sucrose palmitate stearate 7	7.0	2	18

TABLE V

EFFECT OF ADDITION OF LIPID DISPERSION TO DETERGENT-SOLUBILIZED TRANSFERASE

The enzyme preparation was pretreated with detergents. The exchange rate was determined with and without addition of 10 µl lipid dispersion (15 µmol phosphate/ml).

Detergent added	Exchange rate (pmol/h) Addition of lipid dispersion	
	—	+
Span 20	0	6.2
Triton X-100	0	10
Sucrose palmitate stearate 7	2	9
Tween 40	16.8	16.6
No detergent	11.3	13

Discussion

Membrane-bound enzyme could be solubilized by a great variation of methods [19]. We have extracted *P*-MurNAc-pentapeptide transferase, originally tightly bound to the cytoplasmic membrane, by repeated freezing and thawing of *E. coli* crude envelopes. Some properties of the solubilized material, as its sedimentation behaviour in sucrose density gradients, its composition and the absence of succinate dehydrogenase and NADH oxidase activities show that not only a fragmentation to non-sedimentable membrane particles had occurred.

Besides protein, a small amount of phospholipids is extracted by this treatment. It was shown that a minor part of the phospholipids is strongly associated with intrinsic membrane proteins, whereas the bulk forms a bilayer [20, 21]. These boundary lipids seem to remain attached to protein during our extraction procedure. These lipids are purified together with the transferase, suggesting that the enzyme is liberated from the membrane in form of a lipid-protein complex.

Compared with the corresponding membrane-bound form, solubilized enzymes often show differences in their properties such as kinetic parameters or pH optimum. In the case of *P*-MurNAc-pentapeptide transferase differences in the stimulation of the exchange activity by Mg^{2+} were found. The partially purified enzyme shows a biphasic stimulation. The K_m for the first activation step was significantly lower (1.3 mM) compared with the value for the particulate enzyme (10.5 mM). At Mg^{2+} concentrations above 30 mM, the solubilized enzyme has a second activation step, whereas the membrane-bound form is inactivated. The reasons for this different response are not yet clear. In the exchange reaction both preparations of the transferase show the same K_m for UMP, whereas the value for the second substrate, UDP-MurNAc-pentapeptide was about 10 times higher for the partially purified enzyme. In the non-membranous from the transferase is stimulated by moenomycin, a surface-active antibiotic, which inhibits the exchange reaction catalysed by the membrane-bound form to about 50%. This stimulation might be the result of the detergent-like nature of the antibiotic. The partially purified enzyme,

which reaggregated during the purification procedure, seems to become better accessible for the substrate after the addition of the antibiotic. This change in the accessibility of the substrate to the enzyme interferes with the inhibitory effect of the antibiotic and gives a net stimulation.

After resolubilization of the partially purified enzyme with the non-ionic detergent Tween 20, the K_m values for both substrates were diminished by half. The membrane-bound form shows nearly unaltered substrate affinities after an identical treatment. It is therefore probable that the observed differences in the K_m values for UDP-MurNAc-pentapeptide are the result of the reaggregation and not of an altered substrate affinity. The need of phospholipids for activity of an increasing number of solubilized membrane-bound enzymes was reported [19]. Triton X-100-solubilized transferase, which is partially depleted of phospholipids, could be activated by the addition of phospholipids [5]. After preincubation of our partially purified enzyme preparation with phospholipase A_2 or D, exchange and transfer activity are strongly inhibited. This suggests that phospholipids sensitive to phospholipase A_2 and D are necessary for the enzyme activity. From this finding and the similarities in the kinetic data of both forms of the transferase it can be assumed that the extracted enzyme has the same microenvironment as the membrane-bound form. Little is known about the microenvironment of intrinsic enzymes in its original state in the membrane. It might be that acidic phospholipids are those involved in the interaction with the membrane proteins [22]. The enrichment of these phospholipids in the fraction solubilized by the freezing and thawing is in agreement with this assumption. The composition of the extracted phospholipids may therefore reflect the proper native lipid environment of the transferase in the membrane.

We failed to restore the transfer activity by the addition of pure C_{55}-isoprenylphosphate solubilized by different detergents. It may be that the lipid substrate is not accessible for the enzyme in this form of mixed lipid-detergent micelles. After addition of lipid dispersion to the partially purified transferase all the transfer activity is associated with the liposome fraction. This suggests that an incorporation of the protein occurred into the lipid phase of the liposomes. The observed incorporation of mitochondrial membrane proteins into liposomes, recently reported by Eytan and Racker [23], supports this assumption. The method used in their work is very similar to that reported here. In this form of lipid dispersion the lipid substrate is available for the enzyme. It cannot be excluded, however, that the incorporation of the enzyme into the liposomes also restores the proper conformation of the transferase which is necessary for the catalysis of the overall reaction. The inhibition of the transferase activity by phospholipase treatment also agrees with this idea. Upon treatment of the partially purified enzyme with neutral detergents different influences on the rate of exchange and the transfer reaction has been observed. It is assumed that the activation of the exchange reaction by some detergents, as these of the Tween series is due to a resolubilization of the aggregated enzyme, resulting in a better accessibility of the substrates to the enzyme. Other detergents, such as Triton X-100 and Span 20, however, strongly inhibit the exchange reaction. These detergents seem to extract the phospholipids which are essential for the enzymatic reaction. The reactivation of the enzyme

by addition of phospholipids supports this idea.

Surprisingly, detergents which stimulate the exchange reaction are inhibitors of the transfer reaction. The detergents which inhibit the exchange reaction had almost no effect on the transfer activity. These differences in the effects of detergents are not yet understood. It should be noted that all detergents, activating the exchange and inhibiting the transfer reaction are rather hydrophilic (hydrophilic-lipophilic balance numbers between 15 and 17). Detergents with an opposite effect on the exchange rate all have hydrophilic-lipophilic balance numbers of 13.5 and below and are, therefore, more lipophilic.

In a first reaction mechanism proposed by Neuhaus [1], an initial transphosphorylation to a nucleophilic center on the enzyme occurs according the following equation:

$$\text{Enzyme} + \text{UDP-MurNAc-pentapeptide} \rightleftharpoons \text{Enzyme-}P\text{-MurNAc-pentapeptide} + \text{UMP}$$

An alternative mechanism was later supported which should explain the stimulation of the exchange reaction by either the lipid substrate or the lipid product involved in the transfer reaction [4]. In this mechanism exchange activity would require the initial formation of a complex between the transferase and C_{55}-isoprenylphosphate. With the partially purified transferase we found no stimulation of the exchange activity after the addition of lipid substrate.

It could also be shown that this enzyme preparation contains no detectable amounts of endogenous C_{55}-isoprenylphosphate. From these results we suggest that at least in *E. coli* the transfer reaction is catalyzed according to the proposal of Neuhaus [1].

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