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INITIAL MEMBRANE REACTION IN PEPTIDOGLYCAN SYNTHESIS

INTERACTION OF LIPID WITH PHOSPHO-*N*-ACETYLMURAMYL-PENTAPEPTIDE TRANSLOCASE

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Summary

The initial membrane reaction in the biosynthesis of peptidoglycan is catalyzed by phospho-*N*-acetylmuramyl (MurNAc)-pentapeptide translocase (UDP-MurNAc-Ala-γ DGlu-Lys-DAla-DAla undecaprenyl phosphate phospho-MurNAc-pentapeptide transferase). In addition to the transfer reaction, the enzyme catalyzes the exchange of [^{3}H]uridine monophosphate with the uridine monophosphate moiety of UDP-MurNAc-pentapeptide. Two distinct discontinuities are observed in the slopes of the Arrhenius plots of the exchange and transfer activities at 22 and 30°C for the enzyme from *Staphylococcus aureus* Copenhagen. Anisotropy measurements of perylene fluorescence and electron spin resonance measurements of *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of 12- and 16-ketostearic acid intercalated into membranes from this organism define the lower (T_l = 16—22°C) and upper (T_h = 30°C) boundaries of a phase transition. These values correlate with the discontinuities observed for the activity measurements. Thus, it is proposed that the physical state of the lipid microenvironment of phospho-MurNAc-pentapeptide translocase has a significant effect on the catalytic activity of this enzyme.

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Unless stated, all abbreviations of residues denote the L-configuration. The omission of the hyphen, i.e. -D-Ala- for -D-Ala- conforms with the suggestion cited in the IUPAC-IUB, CBN, Recommendations (1966) Biochemistry 5, 2485 or J.Biol. Chem. 241, 2491. Although not stated, all D-glutamic acid residues are linked through the γ-carboxyl group to the diamino acid.

Abbreviations: MurNaC, *N*-acetylmuramyl; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ESR, electron spin resonance.

Introduction

Hydrophobic interactions with lipid play a dominant role in the association of proteins with membranes [1—4]. In the case of membrane-associated enzymes, these interactions may be reflected in a lipid requirement for catalytic activity [5,6]. Such enzymes require either a lipid environment for the diffusion of hydrophobic substrates or a specific conformation resulting from its interaction with lipid [5,7]. Since the lipids of biological membranes undergo thermotropic phase transitions, the catalytic activity of a membrane-bound enzyme may be affected by interaction with different physical states of the lipid [8,9]. Thus, these transitions provide the basis for investigating the interaction of lipid with a membrane protein catalyzing a specific reaction [10]. Examples of this interaction include the sodium and potassium-activated ATPase [11], the calcium-dependent ATPase [12], microsomal glucose-6-phosphatase and UDP-glucuronyl transferase [13], NADH oxidase and D-lactate oxidase [14], β-galactoside and β-glucoside transport [15,16], and undecaprenol phosphokinase [17]. Each of these examples suggests that the physical state of the lipid affects the catalytic activity of the enzyme or transport system.

The biosynthesis of peptidoglycan also includes examples of interaction with membrane lipid. The assembly of this glycan is catalyzed by a series of membrane-associated enzymes that require undecaprenyl phosphate as a lipid carrier [18]. In this system, cytoplasmic precursors are transferred to the membrane where intermediates covalently linked to this prenyl phosphate are assembled and translocated across the membrane to the sites of cell wall assembly. Pless and Neuhaus [19], Umbreit and Strominger [20], and Geis and Plapp [21] established that the initial enzyme in this series, phospho-*N*-acetylmuramyl (MurNAc)-pentapeptide translocase, requires lipid for reactivation of this enzyme in lipid-depleted membrane preparations. Several phospholipids reactivated this enzyme when added in the presence of undecaprenyl phosphate [19,20]. This observation suggests that the enzymatic activity reflects a general property of the lipid microenvironment rather than an absolute requirement for a specific phospholipid. This microenvironment has been defined with the fluorescently labeled product of the translocase, undecaprenyl diphosphate-MurNAc-(N^{ϵ}-dansyl)pentapeptide [22,23]. The fluorophore of this intermediate is immobilized within a hydrophobic environment close to the membrane surface and within 4—6 Å of the lipid matrix. The proximity of the fluorophore on this product to the lipid matrix suggests that the catalytic activity of the translocase could be influenced by lipid-translocase interactions.

It is the purpose of these experiments to demonstrate that the physical state of the membrane lipid affects the catalytic activity of phospho-MurNAc-pentapeptide translocase. Variations in the physical state of membrane lipid, as measured by fluorescence and electron spin resonance spectroscopy, correlate with the temperature-dependent variations in the two activities catalyzed by this membrane enzyme.

Materials and Methods

Perylene (gold label) was purchased from Aldrich. *N*-Oxyl-4′,4′-dimethyloxazolidine derivatives of 12-ketostearic acid and 16-ketostearic acid were

purchased from Syva. Paraffin oil (Saybolt viscosity at 100° F, 345—355) was obtained from J.T. Baker. UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla from *Staphylococcus aureus* Copenhagen was prepared by the method described by Hammes and Neuhaus [24]. UDP-MurNAc-Ala-DGlu-Lys-D[^{14}C]Ala-D[^{14}C]Ala was prepared from UDP-MurNAc-Ala-DGlu-Lys and D[^{14}C]Ala-D[^{14}C]Ala as described previously [25].

Membrane fragments from *S. aureus* Copenhagen were prepared as described by Struve et al. [26] and incubated with UMP in order to generate the highest concentration of undecaprenyl phosphate [24]. All membrane preparations were stored in 20 mM Tris-HCl (pH 7.8) containing 1 M KCl at −196° C.

The transfer assay, performed according to the method of Hammes and Neuhaus [24], measures the amount of phospho-MurNAc-[^{14}C]pentapeptide transferred from UDP-MurNAc-[^{14}C]pentapeptide to undecaprenyl phosphate by determining the amount of acid-precipitable radioactivity. The specific activity of UDP-MurNAc-[^{14}C]pentapeptide was 42 cpm/pmol. The exchange assay, measuring the exchange of [^{3}H]UMP with the unlabeled UMP moiety of UDP-MurNAc-pentapeptide, was performed by the method of Hammes and Neuhaus [24]. The specific activity of [^{3}H]UMP was 16 cpm/pmol. Exchange activity is calculated according to the first order rate equation reported by Struve et al. [26]. The rate of exchange, R, is presented as mol exchanged/l per min. In order to maintain pH 7.8 in the assay for the exchange or transfer activities of the translocase throughout the range of temperature employed, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (pH 7.8) was substituted for 50 mM Tris-HCl (pH 7.8).

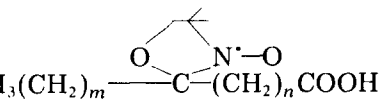
Fluorescence polarization measurements were made at an angle of 90° in a Farrand Mark I spectrofluorometer using Farrand polarizing filters in the excitation and emission beams. The temperature in the sample compartment was regulated by an Aminco refrigerated circulating water bath and monitored (±0.1° C) with a copper-constantan thermocouple attached to a Fluke digital voltmeter. For all measurements a Corning 3-73 interference filter was placed in the emission beam. Fluorescence anisotropy, r, is defined as

$$r = \frac{I_{\parallel} - I_{\perp}(G)}{I_{\parallel} + 2I_{\perp}(G)}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities observed with the analyzing polarizer oriented parallel and perpendicular to the polarization of the excitation beam, respectively. The unequal transmission of polarized light by a monochromator was corrected for by a factor, G (equal to $I'_{\perp}/I'_{\parallel}$) where the primes denote that the excitation beam was polarized in a horizontal direction [27]. The microviscosity within the membrane was evaluated by the procedure described by Shinitzky et al. [28] using perylene as a fluorescent probe. As shown by Hare and Lussan [29], the rotational relaxation rates of perylene in a variety of aliphatic oils of the same viscosity varies significantly. In our experiments, paraffin oil (Saybolt viscosity at 100° F, 345—355; J.T. Baker) was used as a reference for the determination of changes of relative microviscosity.

ESR spectra of membrane fragments containing *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of ketostearic acid were recorded on a Varian E-4 X-band spectrometer. The cavity temperature was regulated by a liquid nitrogen boil-

off variable temperature unit and monitored with a copper-constantan thermocouple. The *N*-oxyl-4',4'-dimethyloxazolidine derivatives of ketostearic acid have the general formula I(m, n):



They were dissolved in $CHCl_3$ at a concentration of 10 mM and stored under N_2 at −20° C. A standard aqueous flat cell purchased from Wilmad Glass Co. was used for the ESR measurements. A common method of analyzing the ESR spectra is to report $2T_{\parallel}$, the splitting between the low- and high-field extrema. There is little ambiguity in this measurement and, thus, it is an adequate reflection of the anisotropic motion of the nitroxide radical [30,31]. This measurement was used for I(5, 10). However, $2T_{\parallel}$ could not be determined from the spectra of I(1, 14). Thus, for this probe the empirical motion parameter for nearly isotropic motion, was used [32], where

$$\tau = 6.5 \cdot 10^{-10} W_0 [(h_0/h_{-1})^{1/2} - 1]$$

W_0 is the linewidth of the mid-field line, h_0 and h_{-1} are the heights of the mid- and high-field lines of the spectrum, respectively.

Protein was determined by the method of Lowry et al. [33] using bovine serum albumin as a standard. Radioactivity in aqueous samples was measured in the scintillation fluid described by Patterson and Greene [34].

Results

Phospho-MurNAc-pentapeptide translocase catalyzes the transfer of phospho-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide to membrane-associated undecaprenyl phosphate according to reaction 1,

$$\text{UDP-MurNAc-[}^{14}\text{C]pentapeptide + undecaprenyl phosphate} \xrightleftharpoons{K^+, Mg^{2+}}$$

$$\text{undecaprenyl-diphosphate-MurNAc-[}^{14}\text{C]pentapeptide + UMP} \qquad (1)$$

In addition, this enzyme catalyzes the exchange of UMP with the UMP moiety of UDP-MurNAc-pentapeptide according to reaction 2,

$$\text{UDP-MurNAc-pentapeptide + [}^{3}\text{H]UMP} \xrightleftharpoons{K^+, Mg^{2+}}$$

$$\text{[}^{3}\text{H]UDP-MurNAc-pentapeptide + UMP} \qquad (2)$$

Arrhenius plots of the initial rates of the transfer (reaction 1) and exchange (reaction 2) activities of phospho-MurNAc-pentapeptide translocase in membrane fragments from *S. aureus* Copenhagen are shown in Fig. 1. The initial velocity of each translocase activity is measured at a single substrate concentration and does not represent the enzyme activity at maximal velocity. Two distinct discontinuities are observed in the slopes of both the exchange and transfer activities at 22 and 30° C. Kumamoto et al. [35] have interpreted discontinuities in Arrhenius plots as resulting from two independent processes having different activation energies. At temperatures below the discontinuity,

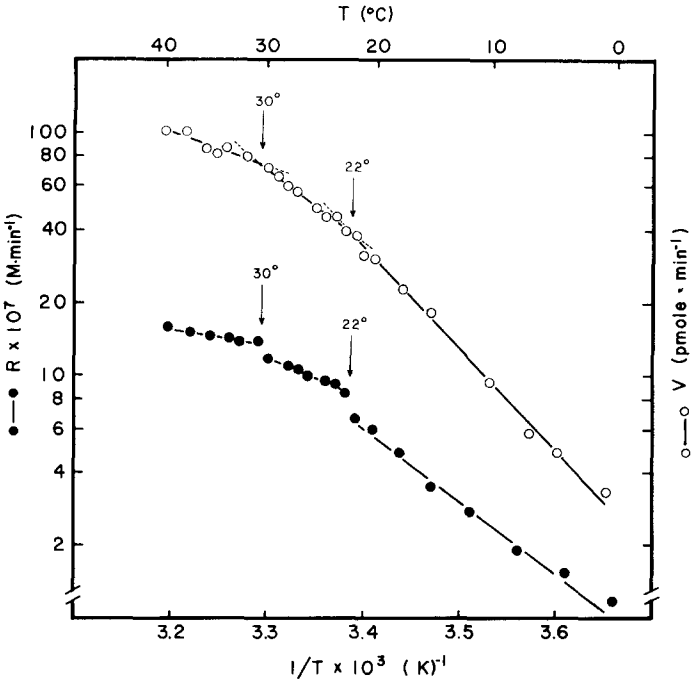


Fig. 1. Temperature dependence of the transfer and exchange activities of phospho-MurNAc-pentapeptide translocase. The transfer of phospho-MurNAc-[^{14}C]pentapeptide from UDP-MurNAc-[^{14}C]pentapeptide to undecaprenyl phosphate (○———○) was measured with membrane fragments from *S. aureus* Copenhagen (4.7 mg/ml of protein) and $2 \cdot 10^{-5}$ M UDP-MurNAc-[^{14}C]pentapeptide. The initial velocity was determined from the amount of phospho-MurNAc-[^{14}C]pentapeptide transferred to undecaprenyl phosphate in 1 min. The exchange of [^{3}H]UMP with the UMP moiety of UDP-MurNAc-pentapeptide (●———●) was measured with membrane fragments (0.53 mg/ml of protein), $3.3 \cdot 10^{-5}$ M UDP-MurNAc-pentapeptide, and $3.3 \cdot 10^{-5}$ M [^{3}H]UMP. The rate of exchange, R, was determined from the amount of [^{3}H]UMP exchanged after 10 min.

the process having the higher activation energy is operative. In the case of a membrane-associated enzyme, such discontinuities are interpreted in relation to the physical state of the membrane lipid [35]. Thus, the relationship between phase transitions in the physical state of the lipid and enzymatic activity provides a means for investigating the interaction of lipid with the translocase. However, for enzymes that utilize hydrophobic substrates, it is difficult to differentiate between an effect of the physical state of the lipid on the catalytic activity of the protein and the mobility of the hydrophobic substrate in the lipid matrix [10].

It is the purpose of these experiments to correlate the observed temperature-dependent variations in the catalytic activity of the translocase with the physical state of the membrane lipid. Changes in the physical state can be effected by increasing the thermal energy of the system [8]. The phase transitions which are observed during this process may be monitored by a variety of methods including fluorescence polarization and electron spin resonance [8]. These techniques measure only the average change in the physical state of the lipid bilayer. It is also recognized that the introduction of a bulky probe may itself affect the physical state of the bilayer [8]. However, by monitoring this

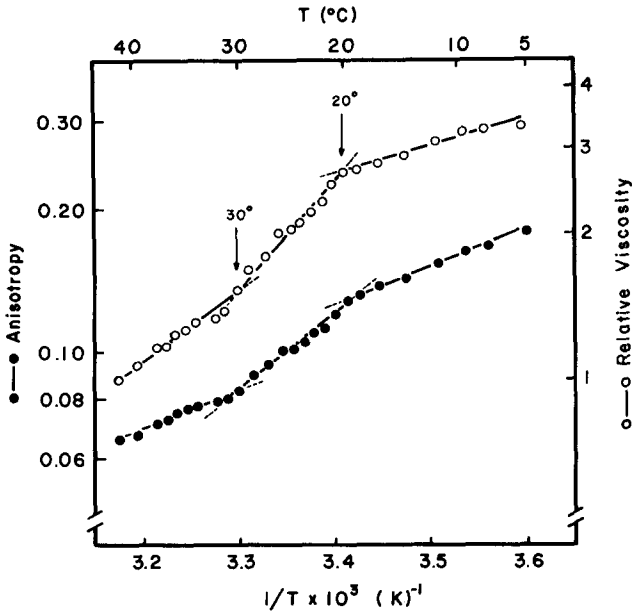


Fig. 2. Temperature dependence of the fluorescence anisotropy of perylene intercalated into membrane fragments from *S. aureus* Copenhagen. Perylene in ethanol was added to a final concentration of $2 \cdot 10^{-5}$ M to a membrane suspension that contained 330 μg/ml of membrane protein in 40 mM Tris-HCl (pH 7.8), 0.25 M KCl, and 42 mM $MgCl_2$. This suspension was stirred for 10 min at 37°C prior to the measurements of anisotropy (●———●). The fluorescence emission of perylene was measured at 470 nm (excitation, 410 nm). The relative viscosity of the membrane fragments (○———○) was determined as described in Materials and Methods.

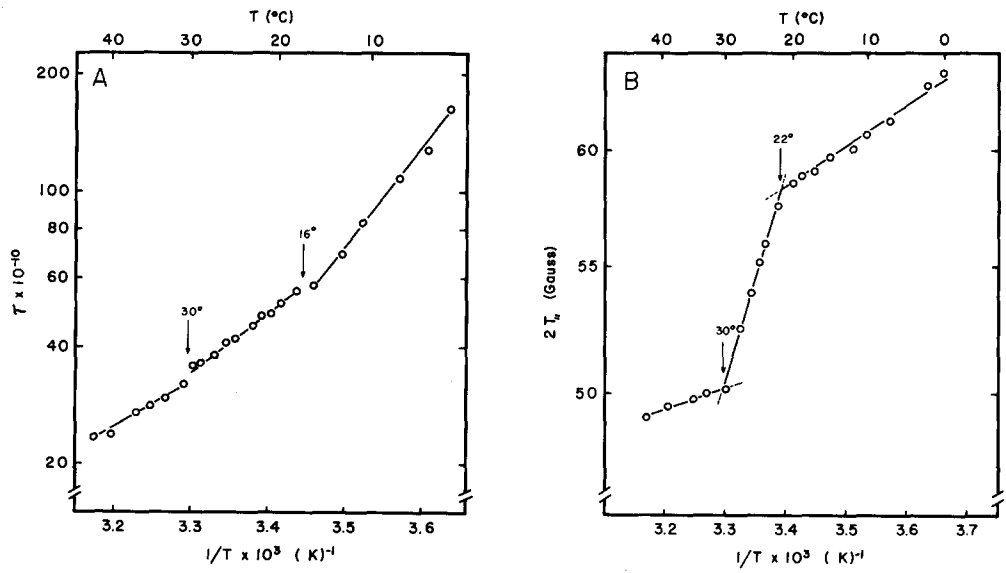


Fig. 3. Temperature dependence of the motion parameters of the nitroxyl-labeled stearic acid derivatives I(1, 14) (A) and I(5, 10) (B) intercalated into membrane fragments from *S. aureus* Copenhagen. The required amount of a chloroform solution of the nitroxyl stearate was taken to dryness under a stream of N_2. A suspension of membrane fragments (330 μg/ml of membrane protein) in 0.21 M KCl, 50 mM Tris-HCl (pH 7.8), and 42 mM $MgCl_2$ was added and intermittently vortexed for 5 min at 23°C. The nitroxyl stearates were added at a ratio corresponding to 2/100 molecules of membrane lipid. In (A) for I(1, 14), the motion parameter is τ and in (B) for I(5, 10) the parameter is $2T_{\parallel}$.

TABLE I

SUMMARY OF CHARACTERISTIC TEMPERATURES OF THE PHASE TRANSITION IN MEMBRANE FRAGMENTS FROM *S. AUREUS* COPENHAGEN

$\Delta T = T_h - T_l$, the width of the transition range. T_t, is the mid-transition temperature.

	Probe (°C)			Translocase activities (°C)	
	Perylene	I (1,14)	I (5,10)	Exchange	Transfer
T_h	30	30	30	30	30
T_l	20	16	22	22	22
ΔT	10	14	8	8	8
T_t	25	23	26	26	26

state with two different types of probes, we may provide an accurate analysis of the phase transition in the membrane from *S. aureus*.

The anisotropy of perylene fluorescence varies as a function of the viscosity of the medium surrounding the probe and has been used to monitor flexibility changes in the hydrocarbon region of both artificial [36] and biological [37] membranes. Increasing the fluidity of the membrane will result in the increased rotational diffusion of perylene and a decrease in its fluorescence anisotropy. Fig. 2 illustrates that the anisotropy of perylene fluorescence in membrane fragments from *S. aureus* Copenhagen decreases with increasing temperature. The average viscosity within the membrane opposing the rotation of perylene decreases 3.3 fold as the temperature is raised from 5 to 42° C. Two changes in slope occur in the Arrhenius plot at 20 and 30° C.

In a similar manner, two spin-labeled stearic acids, I(1, 14) and I(5, 10), were intercalated into membrane fragments from this organism to monitor the physical state of the lipid [38,39]. The degree of motion available to the spin-labeled fatty acid depends upon the flexibility of the neighboring lipid and the position of the doxyl group [38,40,41]. The specific parameters of the ESR spectra, τ or $2T_{\parallel}$, will indicate the effect of ordering of membrane lipids upon the motion of the nitroxyl label. As illustrated in Fig. 3, the effect of temperature on these motion parameters is presented for I(1, 14) and I(5, 10). Smaller values of either τ or $2T_{\parallel}$ indicate a greater freedom of motion for the nitroxyl group. Two slope changes in the Arrhenius plots occur for I(1, 14) at 16 and 30° C, and for I(5, 10) at 22 and 30° C. These values are summarized in Table I and compared with the characteristic temperatures established from the Arrhenius plots for the exchange and transfer activities.

Discussion

Temperature-dependent changes in the physical state of the lipid bilayer of *S. aureus* have been monitored with perylene, I(1, 14), and I(5, 10). In Table I, a comparison of the results with these probes reveals that the high temperature boundary (T_h) of the phase transition is 30° C. The low temperature boundary (T_l) varies among the different probes. For example, with perylene as the probe, T_l is 20° C and for I(1, 14) and I(5, 10) T_l is 16 and 22° C, respectively. The variation in T_l may reflect either the position within the lipid matrix

occupied by a particular probe [38] or the perturbation exerted by the probe on the flexibility of neighboring lipids. The major fatty acids of these lipids from this organism are *iso* and *anteiso* saturated branched methyl C_{15}, C_{17}, and C_{19} fatty acids [42—44]. Of this group C_{15_a} and C_{17_a} comprise 66% of the fatty acids. The phase equilibria expected from membranes with this type of lipid composition will have a transition phase in which both solid and liquid coexist [9,45]. The lower (T_l = 16—22°C) and upper (T_h = 30°C) boundaries of this transition define a temperature range of 8—14°C.

Differential scanning calorimetry of membranes from *S. aureus* shows that the T_l and T_h of the lipid phase transition are 4 and 31°C, respectively [42]. Although the lower boundary temperatures measured with the fluorescent and spin-labeled probes do not agree with that measured by differential scanning calorimetry, they do fall within the broad endothermic transition measured by this technique. For comparison, two enzymes, NADPH oxidase and succinate-dichlorophenolindophenol reductase, in membranes from *S. aureus* show slope changes at 18°C in the Arrhenius plots. This value correlates with T_l established in the present work and not with T_l established by differential scanning calorimetry [42].

An examination of the exchange and transfer activities as a function of temperature reveals discontinuities in the slope of both Arrhenius plots at 22 and 30°C. These values correlate well with T_l and T_h obtained with the three different probes. Thus, it is proposed that the physical state of the lipid in the bilayer has a significant effect on the catalytic activity of phospho-MurNAc-pentapeptide translocase. This proposal is also supported by studies of the fluorescently labeled product of the translocase, undecaprenyl diphosphate-MurNAc-(N^{ϵ}-dansyl)pentapeptide [22,23]. In contrast to perylene and the nitroxyl-labeled stearic acid derivatives, this product results in the specific introduction of a reporter group into the microenvironment of the translocase. Although the mobility of this reporter group does not change with temperature, an Arrhenius plot of fluorescence intensity shows slope changes at 18 and 30°C. Thus, the observed slope changes are the result of the temperature-induced alteration of the microenvironment immediate to the fluorophore of the dansylated lipid intermediate.

As mentioned in the Results, it is difficult to differentiate between an effect of the physical state of the lipid on the catalytic activity of the protein or the mobility/conformation of the hydrophobic substrate, undecaprenyl phosphate. In fact, both effects may be important in determining the activity of the translocase. Since this enzyme catalyzes both exchange and transfer activities, we may have an experimental approach for distinguishing between these effects in the reaction sequence proposed by Pless and Neuhaus [19] (reactions 3—7).

$$E + C_{55}P \rightleftharpoons E \cdot C_{55}P \tag{3}$$

$$E \cdot C_{55}P + UMPPMp \rightleftharpoons UMPPMp \cdot E \cdot C_{55}P \tag{4}$$

$$UMPPMp \cdot E \cdot C_{55}P \rightleftharpoons UMP \cdot E \cdot C_{55}PPMp \tag{5}$$

$$UMP \cdot E \cdot C_{55}P$$
$$|$$
$$PMp$$

$$UMP \cdot E \cdot C_{55}PPMp \rightleftharpoons E \cdot C_{55}PPMp + UMP \quad (6)$$

$$E \cdot C_{55}PPMp \rightleftharpoons E + C_{55}PPMp \quad (7)$$

The transfer of phospho-MurNAc-pentapeptide (PMp) to undecaprenyl phosphate ($C_{55}P$) would involve reactions 3—7 and the availability of undecaprenyl phosphate at substrate levels. Exchange activity, represented by reactions 4—6, requires catalytic amounts of $E \cdot C_{55}P$. Thus, the association of the lipid substrate ($C_{55}P$) and the dissociation of the lipid product ($C_{55}PPMp$) from the translocase are reflected only in the transfer activity. Such a model would predict that any condition which would alter the mobility of the lipid substrate or the lipid product could have different effects on the transfer and exchange activities. Since the exchange and transfer activities reflect the same T_h and T_l, we conclude that the physical state of the lipid matrix has a major effect on the catalytic activity of the translocase (presumably reaction 5).

Additional information on the nature of the interaction between phospho-MurNAc-pentapeptide translocase and the lipid matrix may be obtained by altering the fluidity of the lipid bilayer and correlating it with changes in enzymatic activity. Membrane perturbants, e.g. detergents and primary aliphatic alcohols, or specific phospholipid replacement in lipid-depleted membranes can be used to alter the fluidity. Incubation of these membrane fragments with several detergents has already been demonstrated to result in the concentration-dependent stimulation and inhibition of both the transfer [21,46,47] and exchange [19,21,46] activities of the translocase. Although the physical state of the lipid bilayer was not monitored in these experiments, these results could be correlated with the ability of such surfactants to alter the lipid microenvironment of the translocase. Similar investigations of the lipid requirements of other enzymes using undecaprenyl phosphate-linked intermediates in the synthesis of peptidoglycan may define a potential role for the physical state of lipid microenvironments in the mechanism of the intramembranal translocation of cell wall precursors.

Acknowledgements

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