Length of Hydrocarbon Chain and Antimicrobial Action to Gram-Negative Bacteria of Fatty Acylated Lysozyme

Hisham Radwan Ibrahim, Kunihiko Kobayashi, and Akio Kato*

Department of Biochemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753, Japan

We have previously shown that lysozyme can be converted into a potent bactericidal protein against *Escherichia coli* by covalent attachment of one of two palmitic acids to its lysyl residues. Here we report the antimicrobial behavior against *E. coli* K-12 3301 of lysozyme covalently modified with myristic and stearic acids to determine the effect of each type of fatty acid on the bactericidal action of lysozyme. All types of attached fatty acids, including palmitic acid, were able to transform lysozyme into a bactericidal molecule against *E. coli*, depending on the length and number of each type of fatty acid. Among the monoacylated lysozyme derivatives, the longer the carbon chain of the attached fatty acid, the more potent the bactericidal lysozyme type, providing a good linear correlation. On the other hand, among the diacylated lysozyme derivatives, palmitoylated lysozyme was found to be the most potent bactericidal agent. The binding capacity of purified *E. coli* lipopolysaccharide to the most potent bactericidal acylated lysozymes was stronger than to nonmodified lysozyme, whereas myristoylated lysozyme exhibited the strongest affinity. Thus, the results indicate that the bactericidal activity of the modified lysozymes could be attributed to their moderately enhanced membrane fusion capabilities.

INTRODUCTION

Invasive Gram-negative bacteria, which cause about one-third of all bacteremic infections, are resistant to the lytic action of lysozyme. Lysozyme, a basic protein of M_r = 14 400, is bactericidal to certain Gram-positive bacteria, whereas it catalyzes the hydrolysis of the β-1,4-glycosidic linkage of the peptidoglycan in the bacterial cell wall. The peptidoglycan layer in Gram-negative bacteria is protected from the lytic action of lysozyme, as the outermost surface (outer membrane) of the bacteria functions as a permeability barrier. Hence, for lysozyme to be effective against Gram-negative bacteria, it must overcome the outer membrane permeability barrier. We assumed that this could be circumvented if the lysozyme molecule was equipped with a hydrophobic carrier, which can mediate its interaction and insertion into membrane to facilitate its delivery to the site of action (the peptidoglycan). We have recently developed an effective lysozyme in killing wild-type *Escherichia coli* 3301 by covalent attachment of one or two palmitic acids to lysyl residues of the molecule (Ibrahim et al., 1991). It has been shown that increasing the number of attached palmitoyl residues strengthened the insertion of modified lysozyme into the purified outer membrane lipopolysaccharide (LPS) and subsequently reduced its bactericidal activity to *E. coli*.

Stearic, palmitic, and myristic acid, which are all saturated fatty acids, are the predominant fatty acids found attached to proteins in living cells to mediate their membrane interaction and translocation and to stabilize protein–protein interactions (McIlhinney et al., 1987). Accordingly, characterization of the antimicrobial behavior of acylated lysozyme with these three different types of fatty acids would provide much needed and interesting information about the optimum length as well as the number of attached fatty acid residues required to achieve maximal antibacterial potency to Gram-negative bacteria.

In the present study, we modified hen egg-white lysozyme with one or two myristic, palmitic, or stearic acids. Antimicrobial assay revealed that lysozyme derivatives incorporating one stearate or two palmitate residues are the most potent lysozyme type against *E. coli* K-12 3301 due to their moderately enhanced binding affinity to the outer membrane LPS, while myristoylated lysozyme was less effective in killing *E. coli* as its affinity to LPS was greater. Furthermore, a good correlation between the length of the hydrocarbon chain of the attached fatty acid and bactericidal activity of monoacylated lysozyme was obtained.

MATERIALS AND METHODS

Lysozyme was purified from fresh hen egg white at pH 9.5 in the presence of 5% NaCl and recrystallized five times. Microbial substrate of lysozyme, *Micrococcus lysodeikticus* cells, was purchased from Sigma Chemical Co. (St. Louis, MO). LPS was prepared from *E. coli* WT-3301 by extraction according to a modified phenol–water procedure as reported earlier (Morrison and Leive, 1975). Unless otherwise stated, all reagents used in this study were of reagent grade.

Modification of Lysozyme. Fatty acids were esterified with *N*-hydroxysuccinimide as reported previously (Haque and Kito, 1983). The fatty acids were covalently attached to the lysyl residue of lysozyme by base-catalyzed ester exchange using the method of Haque et al. (1982). The degree of incorporation was controlled by adjusting the molar ratio of *N*-hydroxysuccinimide ester of fatty acid to lysozyme as reported earlier (Ibrahim et al., 1991). The mole content of lysine in lysozyme was considered to be seven residues (six side chains of lysine plus the amino terminus). Thus, the preparations were performed at a mole ratio of the *N*-hydroxysuccinimide ester of the respective fatty acid to lysozyme at 1, 2, 3, or 4 to obtain stearoylated (S-Lz), palmitoylated (P-Lz), and myristoylated (M-Lz) lysozyme with various degrees of modification. Ten milliliters of tetrahydrofuran (THF) containing the appropriate amount of *N*-hydroxysuccinimide ester of the respective fatty acid (0.7, 1.4, 2.1, and 2.8 mM final concentration in the reaction mixture) was gradually added to 40 mL of 50 mM potassium phosphate buffer (pH 7.4) containing a single concentration of lysozyme (0.7 mM). With agitation at 25 °C, the pH was then brought to 9.0 with NaOH (1 N). The reaction mixture was incubated for 1 h with gentle agitation. The pH was then adjusted back to 7.0 with HCl (1 N), and the mixture was subsequently dialyzed for 2 days at 4 °C against buffer containing 1 M NaCl. Thereafter, the reaction mixture was washed five times with ice-cold diethyl ether and

* Author to whom correspondence should be addressed.

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was then dialyzed exhaustively for 3 days against three daily exchanges of distilled water. The lysozyme derivatives were then lyophilized and used for the following tests. A control sample was taken through all steps of preparation without addition of *N*-hydroxysuccinimide ester of fatty acids in THF.

Protein Quantitation. Protein concentration of the lysozyme derivatives was assessed using the modified Lowry method (Miller, 1959). The degree of modification was determined by measuring the free amino groups of control and modified lysozyme, using TNBS reagent, as reported earlier (Kato et al., 1989).

Antimicrobial Action. The lytic action of lysozyme derivatives against *M. lysodeikticus* cells was determined according to turbidometric methods (Muraki et al., 1988) based on the decrease in turbidity of a cell suspension following the addition of lysozyme derivatives. The lysis of cells in 50 mM potassium phosphate buffer (pH 8.0) was monitored at 600 nm. A 100-μL portion of the lysozyme derivatives solution (final concentration, 1.67 μg/mL) was added into 1.9 mL of *M. lysodeikticus* cell suspension (final concentration, 250 μg/mL; dry weight in the same buffer). This lysis mixture gave initial OD of 0.8–0.85 at the onset of the experiment. The decrease in absorbance at 600 nm (25 °C) was monitored using a Hitachi U-2000 recording spectrophotometer.

To assess the antimicrobial action of lysozyme derivatives against *E. coli* K-12 3301, a 4.5-mL *E. coli* cell suspension (10^5 cells/mL in 50 mM potassium phosphate buffer, pH 7.0) was mixed with a 0.5-mL solution of the lysozyme derivatives (final concentration, 50 μg/mL). The mixture was kept at room temperature for 10 min. A 100-μL portion was plated out onto MacConkey agar. Colonies were counted after incubation at 35 °C overnight. Percentage survival was represented with respect to control mixture (no protein added).

LPS Binding Assay. Lytic action of lysozyme derivatives against *M. lysodeikticus* cells was monitored in the presence of purified LPS (lipopolysaccharide from *E. coli*) essentially as above except that the concentration of LPS in buffer was varied against a single concentration of the lysozyme derivatives (final concentration, 1.67 μg/mL). The mixtures were incubated at 37 °C for 10 min before tempering and mixing with the *M. lysodeikticus* cell suspension (250 μg/mL, OD of 0.85). The final volume of the lysis mixture was always kept at 2 mL.

Trypsin Digestion. The usual procedure involved suspension of reduced *S*-2-aminoethylated native or dipalmitoylated lysozyme, at 1% concentration in distilled water as described by Okazaki et al. (1985). The enzymatic digestion was allowed to proceed for 120 min.

Reversed-Phase High-Performance Chromatography. A TSKgel ODS-120T column (4 × 200 mm, TOSOH, Japan) was attached to a Hitachi 655A-11 liquid chromatograph equipped with a Hitachi 655-A LC detector. The column was equilibrated with 1% acetonitrile containing 0.1% concentrated HCl. Twenty microliters of tryptic hydrolysates containing 50 μg of peptides was injected, and the column was eluted with a gradient of 40 mL of 1% acetonitrile containing 0.1% concentrated HCl and 40 mL of 40% acetonitrile containing 0.1% concentrated HCl at a flow rate of 0.4 mL/min for 200 min. Peptide elution was monitored by absorbance of effluent at 210 nm. Peptide peaks were manually collected, concentrated under vacuum, and used for TLC analysis.

TLC Analysis. To identify the palmitoylated peptide, peptide peaks were developed alone with parallel references on silica gel TLC sheets (0.2 mm, Merck) by using a solvent system of chloroform–methanol–acetic acid (65:25:8 v/v/v). Peptide-rich sections corresponding to the ninhydrin-visualized parallel references were sprayed faintly with ethanolic dichlorofluorescein (DCF) to locate palmitoylated peptides and viewed under a UV lamp as bright yellow patches against a light orange background as described earlier (Haque and Kito, 1983). DCF-positive staining peptides (palmitoylated peptides) were assigned as described by Canfield (1963).

RESULTS AND DISCUSSION

The stoichiometry of modification was determined by measuring the TNBS-nonavailable ε-amino groups of lysozyme derivatives, since the mole content of free amino groups of lysozyme has been considered to be 7. Figure 1 shows the number of modified (masked) lysyl residues with stearic, palmitic, and myristic acid as a function of increasing mole ratio of *N*-hydroxysuccinimide ester of the respective fatty acid to lysozyme in the reaction mixture. The degree of modification with palmitic acid displayed approximately a linear correlation with increasing mole ratio of palmitic acid to lysozyme. Incorporation of myristic acid exhibited a sigmoidal curve, and the rate of incorporation was considerably slower than that of palmitic acid. On the other hand, incorporation of stearic acid into lysozyme showed a progressive increase with an increase in the mole ratio of stearic acid to lysozyme. The results suggest that the rate of incorporation of these different three fatty acids was in the order of palmitic > stearic > myristic acid. However, lysozyme modified with one or two residues of respective fatty acid was obtained. Here, it should be noted that the stoichiometries of acylation of the considered mono- and diacylated lysozymes S1-Lz and S2-Lz are 1.1 and 1.9, where P1–Lz and P2-Lz are 1.0 and 1.7, while M1-Lz and M2-Lz are 0.7 and 2.0, respectively.

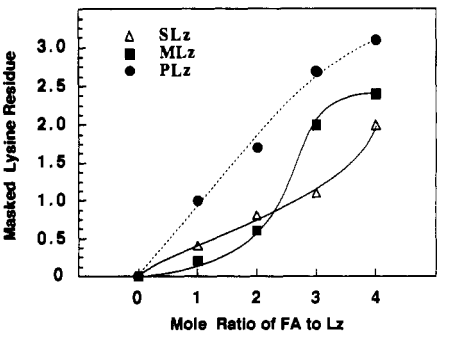

Figure 1. Number of modified lysine residues of lysozyme as a function of increasing molar ratio of *N*-hydroxysuccinimide ester of fatty acid (FA) to lysozyme (Lz) in the reaction mixture. (△) S-Lz, stearoylated; (●) P-Lz, palmitoylated; (■) M-Lz, myristoylated lysozyme.

Figure 2 shows the reversed-phase high-performance liquid chromatography elution patterns of tryptic peptides from nonmodified (N-Lz) and dipalmitoylated (P2-Lz) lysozymes (parts A and B, respectively), and the TLC patterns of tryptic peptides of P2-Lz visualized by DCF (Figure 2C). The elution pattern of tryptic peptides of dipalmitoylated lysozyme was similar to that of nonmodified lysozyme. The mapping of peptide fragments was carried out according to the methods of Okazaki et al. (1985). As shown in the figure, the positive DCF peptides (Figure 2C) were eluted relatively slower than the corresponding peptides of nonmodified lysozyme (Figure 2B), because the hydrophobicity was increased. The palmitoylated peptides (DCF-positive) were found to represent the segments 22–33 (T6) and 97–112 (T12+13), indicating that Lys-33 and -97 are the most susceptible nucleophiles. From the TLC analysis it is apparent that Lys-33 is much more susceptible than Lys-97.

Figure 3 shows the lytic activities of modified lysozyme to various extents with different fatty acids. There was a gradual decrease in the enzymatic activity of stearoylated and palmitoylated lysozymes as the degree of incorporation increased. However, a sharp decrease in the activity of lysozyme incorporating 0.7 mol of myristic acid (M1-Lz) was observed, where it reached a constant. The activities of P1-Lz and S1-Lz were 96 and 87%, respectively, while the residual activities of P2-Lz and S2-Lz were similar (73% of nonmodified lysozyme). In parallel, the activities of myristoylated lysozymes were similar (60%), despite

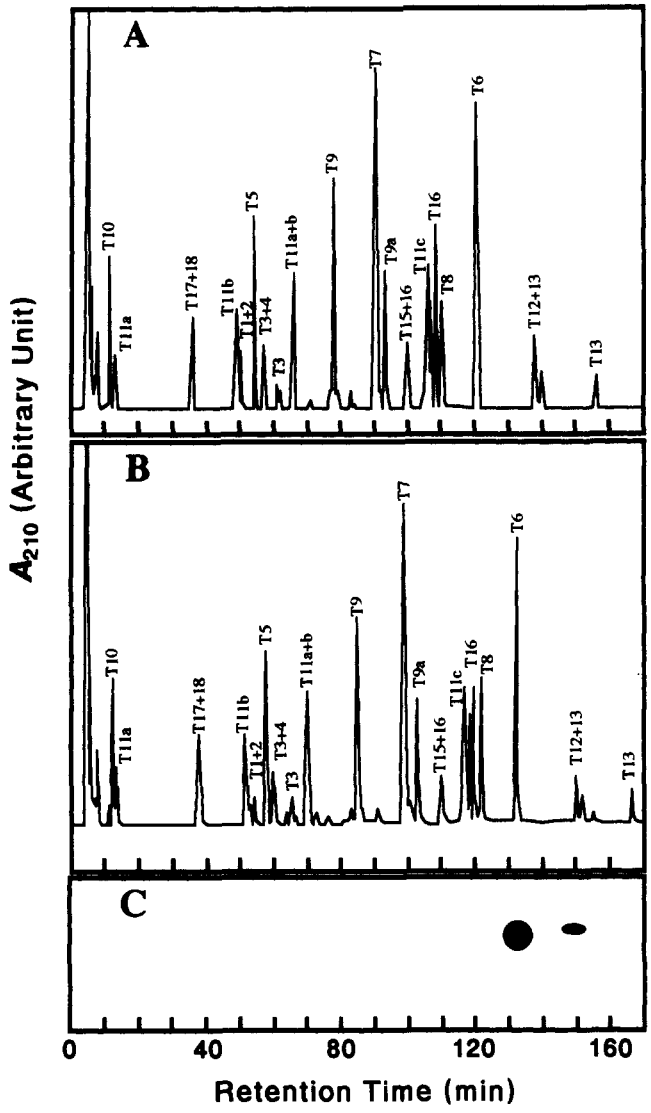

Figure 2. Reversed-phase HPLC elution patterns of tryptic peptides of nonmodified (A) and dipalmitoylated lysozymes (B) and TLC analysis of palmitoylated peptides visualized with DCF (C). T refers to Canfield's nomenclature (Canfield, 1963).

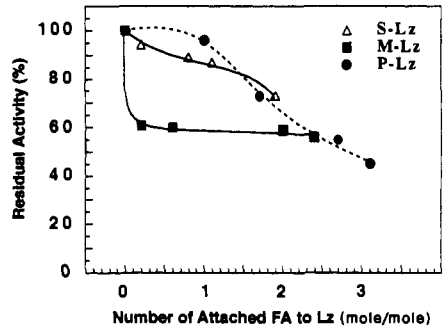

Figure 3. Lytic activity of modified lysozyme with different fatty acids as a function of the degree of modification. Symbols are the same as in Figure 1.

the increase in the degree of modification. It seems probable that this short fatty acid (14 carbon atoms) facilitated the formation of a large and tight micelle in which the active site of lysozyme is shielded. This assumption was evident by measuring the absorbance at 500 nm of acylated lysozyme solution, whereas the myristoylated lysozyme exhibited extremely high turbidity compared with that of stearoylated or palmitoylated lysozymes (data not shown). In general, the observed

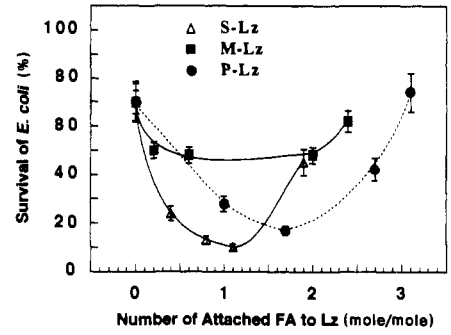

Figure 4. Antibacterial activity of modified lysozyme with different fatty acids against *E. coli* K-12 3301 as a function of the degree of modification. Symbols are the same as in Figure 1. Each point represents the mean of four trials, and standard deviations are shown as bars.

decrease in the residual activities of modified lysozyme derivatives may be attributed to the decreased surface positive charge (Frieden, 1956; Yamasaki et al., 1968; Davies and Neuberger, 1969), as the attachment method used in this study is specific to ϵ-amino groups of lysyl residues of lysozyme. It is noteworthy that modification with approximately one or two fatty acids per molecule of lysozyme with any type of fatty acids restored a reasonable enzymatic activity (more than 60%).

To assess the capability of modified lysozyme with fatty acids having different lengths of hydrocarbon chain to kill Gram-negative bacteria, we incubated *E. coli* K-12 3301 (as a representative microorganism) with fatty acylated lysozymes for 10 min at 25 °C, and the viable cell numbers were determined on MacConkey agar plates. Figure 4 shows the antimicrobial activities of modified lysozymes with various fatty acids as a function of the degree of modification. A significant decrease in the viable cell numbers was observed with stearoylated and palmitoylated lysozymes with one residue of fatty acid. The bactericidal activity of monostearoylated lysozyme was more pronounced than that of monopalmitoylated lysozyme. On the other hand, monomyristoylated lysozyme exhibited remarkable antibacterial activity but was less effective than stearoylated and palmitoylated lysozymes. A further increase in the degree of modification to two residues of fatty acids per molecule of lysozyme fairly promoted the antibacterial action of palmitoylated lysozyme but decreased that of stearoylated lysozyme. In contrast, dimyristoylated lysozyme showed a marginal increase in the antibacterial action. It can be seen that a high degree of modification was accompanied by a dramatic loss in the antibacterial activity of lysozyme modified with any type of these three fatty acids. It seems probable, therefore, that the longer the attached hydrocarbon chain, the stronger the bactericidal action of modified lysozyme to *E. coli*.

To gain insight into the possible mechanism of fatty acid-mediated penetration of lysozyme into the outer membrane permeability barrier of bacteria, we tested the interaction of the most potent bactericidal lysozyme types (S1-Lz, P2-Lz, and M2-Lz) with a purified lipopolysaccharide (LPS), the most abundant component in the outer membrane of Gram-negative bacteria. Penetration of the molecule into the LPS vesicles can be followed by monitoring the residual lytic activity of lysozyme on *M. lysodeikticus* (Ohno and Morrison, 1989). As shown in Figure 5, incubation of either nonmodified (control) or acylated lysozyme derivatives with *E. coli* LPS decreased the residual activity in a dose-dependent manner. However, the decrease in activity of acylated lysozymes was

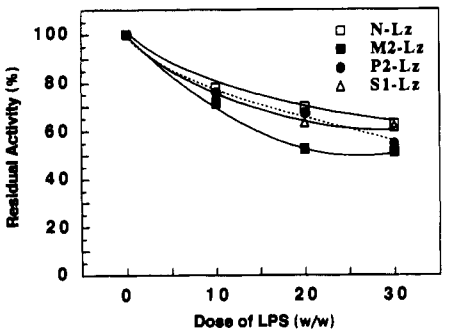

Figure 5. Effect of LPS vesicles on the lytic activity of the potent bactericidal lysozyme derivatives. (□) NLz, nonmodified; (■) M2-Lz, dimyristoylated; (●) P2-Lz, dipalmitoylated; (△) S1-Lz, monostearoylated lysozyme.

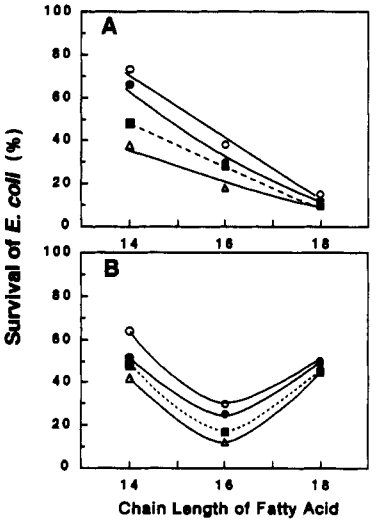

Figure 6. Relationship between the chain length of the attached fatty acid and antibacterial activity to *E. coli* K-12 of modified lysozyme with one (A) and two (B) residues of the fatty acid as a function of protein concentration. Protein was tested at (○) 10, (●) 30, (■) 50, and (△) 100 μg/mL of cell suspension (10^5 cells/mL).

more prominent, implying the insertion of protein into the LPS vesicles. The binding capacity of *E. coli* LPS to dimyristoylated lysozyme was found to be stronger than that to either monostearoylated or dipalmitoylated lysozymes at any LPS concentration examined. At high LPS concentration (30-fold excess) the binding of LPS vesicles to lysozyme derivatives was increased in the order M2-Lz > P2-Lz > S1-Lz. It appears, therefore, that the moderate affinity of lysozyme to LPS is preferable to achieve stronger bactericidal action, where the potency of bactericidal activity was inversely correlated as it was increased in the order S1-Lz > P2-Lz > M2-Lz. Taken together, the data demonstrate that the binding of lysozyme to bacterial outer membrane LPS could be promoted by coupling hydrocarbon chain to the molecule, thus providing the initial force for membrane fusion into the permeability barrier. However, the strong binding is not favorable in terms of impeding the consequent penetration steps upon interaction with the membrane. In other words, intensive binding affinity may lead to the entrapment of lysozyme into the outer membrane and hence would hamper its delivery to the site of action (peptidoglycan).

It is of general interest to know the relationship between the length of attached fatty acid and the antibacterial activity to *E. coli* of modified lysozyme. Figure 6 shows the plots of the length of attached hydrocarbon chain vs bactericidal activity of modified lysozyme. At any protein concentration tested, a linear correlation was observed between the decrease in the survival of *E. coli* and the length of carbon chain (from 14 to 18 carbon atoms) for modified lysozyme with a single residue of a respective fatty acid (Figure 6A). The plots were displaced to a higher antibacterial activity as the protein concentration increased (from 10 to 100 μg/mL of cell suspension, 10^5 cells/mL). Even though the lytic activity of monopalmitoylated lysozyme was greater (97%) than that of monostearoylated (87%) and monomyristoylated (60%) lysozymes, their antibacterial activities against *E. coli* K-12 were independently related to the length of attached fatty acid. Further inspection of Figure 6A suggests that the protein concentration dependency of antibacterial action was very prominent for monomyristoylated lysozyme compared with that of monopalmitoylated lysozyme, while it became undetectable for monostearoylated lysozyme. The results suggest that the rate of killing as well as insertion of stearoylated lysozyme is greatly higher than the rate of palmitoylated and myristoylated lysozymes. Also, the data indicate that the most potent bactericidal lysozyme type to *E. coli* is the monostearoylated lysozyme, and thus it may provide a useful tool for food and drug applications. Figure 6B shows the plots of viable numbers of *E. coli* vs chain length of diacylated lysozymes as a function of protein concentration. Among the diacylated lysozyme types, palmitoylated lysozyme was found to be the most potent bactericidal agent. A linear correlation could not be obtained with such a degree of modification at any protein concentration tested.

The literature data reveal that the *in vivo* modification of proteins by fatty acids, in particular myristic, palmitic, and stearic acid, is a widespread method of protein anchoring and fusion into membranes (McIlhinney, 1990). Lines of evidence were derived from work on the two transforming proteins $p60^{src}$ and $p21^{ras}$, whereas fatty acid attachment was proven necessary for the full expression of the transforming ability of these proteins (Kamps et al., 1985). More recently, direct evidence has been found for viral capsid protein VP4 (Chow et al., 1987) and neuronal protein GAP-43 (O'Dowd et al., 1989). Our results demonstrate that attachment of fatty acids to lysozyme is important for mediating its interaction with and subsequent insertion into the outer membrane of *E. coli*.

In the present study, it has been shown that lysozyme can be converted into an effective agent in killing *E. coli* K-12, a Gram-negative strain, via its modification with one or two residues of saturated fatty acids, in particular, stearic and palmitic acid rather than myristic acid. In this respect, it should be remembered that *in vivo* acylated proteins contain exactly the same number of attached saturated fatty acids (McIlhinney, 1990). Also, it has been reported that stearate and palmitate mediate a number of functions, including membrane anchorage and transport, and promote protein–protein interaction in the living cells (Schlesinger and Malfer, 1982; James and Olson, 1989). *In vivo* myristoylation of proteins was found to stabilize the protein–protein interaction, not to imply membrane translocation (Chow et al., 1987). The results obtained in the present study are consistent with the above-mentioned literature in terms of elucidating the role of each of these three different fatty acids on imparting membrane fusion properties to lysozyme. The myristoylated lysozyme was a less effective antibacterial agent against *E. coli* as the protein aggregation prevailed, thus leading to the reduced lytic activity (Figure 3) and the entrapment of lysozyme

molecule into the outer membrane of *E. coli* (Figure 5). By contrast, stearoylation and palmitoylation have been regarded as directly mediating the interaction and penetration of lysozyme into the membrane to perform its action on the peptidoglycan. Thus, imparting transmembrane properties to the catalytic function of lysozyme via its acylation with palmitic and stearic acid can be potentially used for the development of safe drugs against the envasive bacteria, or even can be generalized to involve the design of essentially new drugs capable of penetrating target cells. Furthermore, our data permit us to emphasize that a protein can be tailored to achieve a particular function by introducing a proper functional domain, such as membrane binding domain, to its intrinsic catalytic function (e.g., bacteriolytic activity of lysozyme), as has been already elaborated for molecules *in vivo*.

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Received for review November 16, 1992. Accepted April 13, 1993.