The Target Sites of Glycerol Monocaprates against *Escherichia Coli*

(Antibacterial Action of Monoglycerides. I)

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The target sites of glycerol monocaprates (MC₁₀) against *Escherichia coli* JE 1011 (*E. coli* JE) were elucidated by using succinate dehydrogenase (SDH), a membrane-bound enzyme of the cytoplasmic membrane. Succinate dehydrogenase of *E. coli* JE was activated *in vivo* by MC₁₀ but that of the cell envelopes prepared from the bacterium was inhibited by it. In addition, we used the NS-mutants (NS-3, NS-4) which were found to have incomplete LPS and carried out similar experiments. *In vivo*, SDH of NS-3 was activated by MC₁₀ but that of NS-4 was inhibited by it. However, SDH activities of the cell envelopes prepared from these NS-mutants were inhibited by MC₁₀.

From these results, we reduce that MC₁₀ may act on the outer membrane of *E. coli* JE, stimulating the permeation of substrates and apparently activating the SDH of the cell *in vivo*. Thus, it is likely that the first target site of MC₁₀ is on the outer membrane of *E. coli* JE.

Key words: target site; glycerol monocaprates; antibacterial action; *Escherichia coli*; monoglyceride; succinate dehydrogenase; outer membrane; cell envelope; NS-mutant

Introduction

The antibacterial action of monoglycerides on film yeasts was determined by Ueda *et al.*¹⁾ and Koga *et al.*^{2),3)}. We also revealed that glycerol monocaprates (MC₁₀), a kind of monoglyceride, showed antibiotic activity against *Staphylococcus aureus*, but not *Escherichia coli*.

On the other hand, though the addition of monoglycerides to food is permitted, it is not intended as a preservative against microbial food decay, but as an emulsifier. Accordingly, we have tried using monoglycerides as a preservative. However, we were concerned about the ineffectiveness of monoglycerides against gram-negative bacteria. Shortly afterward, Kato *et al.*^{4)~6)} and we⁷⁾ overcame this difficulty by the combined use of monoglycerides and polyphosphates (permitted as a food additive), and have been

working to identify the mode of the combined effect. Since that work was unsuccessful, we have tried investigating the action of MC₁₀ or hexametaphosphate (HP) alone against *Escherichia coli* in further detail. These experiments proved the target site of HP to be the outer membrane of *Escherichia coli*; in addition, in the case of the combined effect of MC₁₀ and HP, it was found that HP acted on the outer membrane of *Escherichia coli* first and the damage induced by HP facilitated the subsequent action of MC₁₀*. However, we consider that MC₁₀ must also act on the outer membrane of *Escherichia coli*, though the mode of its action may differ from that of HP in some respects. In order to check this by experiment, succinate dehydrogenase (SDH), a membrane-bound enzyme of the cytoplasmic membrane was used

* Suda, I.: Doctoral Thesis, 156~157, (1981).

as a marker enzyme and the effect of MC₁₀ on SDH of *Escherichia coli* was investigated *in vivo*. As described above, the purpose of the present study was to elucidate the action of MC₁₀ on the outer membrane of *Escherichia coli* by using SDH as a marker enzyme in order to obtain clues to elucidate the mode of synergistic action between MC₁₀ and HP on *Escherichia coli*.

Materials and Methods

1. Strains:

Escherichia coli JE 1011 (*E. coli* JE) and its NS-mutants (NS-3, NS-4) were generously provided by Dr. Matsuhashi, Institute of Applied Microbiology, Tokyo University. Some characteristics of these strains were reported by Tamaki *et al.*⁸⁾ and Suda *et al.*⁹⁾.

2. Chemicals:

Monoglycerides, phospholipase C (EC 3.1.4.3., from *Bacillus cereus*) and papain (EC 3.4.22.2, from *Carica papaya*) were obtained from Taiyo Kagaku K. K., Boehringer-Mannheim Yamanouchi K. K. and Nakarai Kagaku K. K., respectively. All other reagents (analytical grade reagent) were of commercial origin.

3. Media and culture conditions:

E. coli JE and its NS-mutants were grown to mid-exponential phase with shaking on a reciprocal shaker at 30°C in 1% nutrient broth which consisted of 10 g of Ehrlich meat extract (Kyokuto Kagaku K. K.), 10 g of polypeptone (Daigo Eiyo Kagaku K. K.) and 1 g of sodium chloride per liter of distilled water, adjusted to pH 7. *E. coli* JE grown to mid-exponential phase in 1% MC₁₀ and in GSHS medium¹⁰⁾ under the same conditions was used in experiments 4 and 5 in "Results", respectively.

4. Preparation of *E. coli* JE and its NS-mutant suspensions:

A mixture of 8 ml of 1% nutrient broth and 2 ml of overnight culture of bacteria growing in the same broth was incubated at 30°C for 4 hours with shaking. The bacteria were harvested by centrifugation, washed with 0.1 M phosphate buffer at pH 7.4 and re-suspended in the same buffer. The optical density at 660 nm (OD₆₆₀) of the suspension

was adjusted to 2~3. This suspension was used as the source of enzyme for SDH assay.

5. SDH assay:

Succinate dehydrogenase activity was determined spectrophotometrically by a modification of the method of Slater *et al.*¹¹⁾. The reaction mixture contained 1.5 ml of 0.5 M sodium succinate, 1 ml of 0.1 M phosphate buffer, pH 7.4, 0.5 ml of 0.15 M potassium cyanide, 1 ml of 0.01 M potassium ferricyanide, 0.5 ml of the bacterial suspension and 0.5 ml of distilled water.

The reaction was started by addition of the bacterial suspension at 30°C and terminated by adding 1 ml of 1 N hydrochloric acid after 30 min of incubation, then 5 ml of ethanol was introduced into the reaction mixture. After the precipitates produced had been removed by centrifugation, the absorbance at 400 nm of the supernatant was measured. The effects of monoglycerides on SDH activity were measured by adding 0.5 ml of monoglyceride instead of distilled water. The enzyme activity was measured in terms of the difference (ΔE_{400}) in the optical density at 400 nm of ferricyanide and ferrocyanide. The data in the present paper are expressed as the magnitude of reduction ($\Delta E_{400} \times 10^4$) per mg protein per minute. Protein was determined according to the method of Lowry *et al.*¹²⁾, using dry bovine serum albumin as a standard.

6. Preparation of cell envelopes:

The cell envelope fractions were prepared from *E. coli* JE and its NS-mutants by the method of Mutoh *et al.*¹³⁾. The envelopes were suspended in 10 mM Tris-hydrochloric acid buffer at pH 7.4 and the suspension was used at the source of enzyme for SDH assay.

7. Pretreatment of *E. coli* JE cells with chemicals:

1). MC₁₀: To the suspension (9 ml) prepared according to section 4 above, 1 ml of 1% MC₁₀ was added and the mixture was incubated for 30 min at 30°C, then harvested, washed with the phosphate buffer and re-suspended in 9 ml of the buffer.

2). Phospholipase C: After being harvested in mid-exponential phase, *E. coli* JE was suspended in 10 mM Hepes buffer at pH 7.4 and OD₆₆₀ of the suspension was adjusted to 2.5.

This suspension (5 ml) was incubated for 30 min with 0.125 ml of phospholipase C (800 U/ml) at 37°C. The suspension was cooled in ice water, then the cells were harvested, washed with the same buffer and resuspended in 5 ml of the buffer.

3). Papain: The suspension (5 ml) prepared according to section 4 above was incubated with 20 mg of papain at 37°C for 30 min then added to 45 ml of the phosphate buffer cooled in ice water. The treated cells were harvested, washed with the same buffer and resuspended in 5 ml of the buffer.

4). EDTA: The suspension (8 ml) prepared according to section 4 above was incubated with 2 ml of 10 mM EDTA—0.1 M phosphate buffer (pH 7.4) at 30°C for 30 min. The treated cells were harvested, washed with the phosphate buffer and resuspended in 8 ml of the buffer.

Results

1. Effects of monoglycerides on SDH activity of *E. coli* JE

The effects of monoglycerides (MC₆~MC₁₄) on SDH activity of *E. coli* JE were measured *in vivo*.

As shown in Table 1, the SDH activities of *E. coli* JE were activated by the addition of monoglycerides. Moreover, within the limits of C₆ to C₁₄, the data show that the longer the carbon chain length of fatty acid

Table 1. SDH Activities of *E. coli* JE in the Presence of Monoglycerides

Monoglyceride	SDH activity
Control	33.3
MC ₆	85.0
MC ₈	102.0
MC ₁₀	113.3
MC ₁₂	124.3
MC ₁₄	153.0

Monoglyceride

MC₆: Glycerol monocaproate

MC₈: Glycerol monocaprylate

MC₁₀: Glycerol monocaprinate

MC₁₂: Glycerol monolaurate

MC₁₄: Glycerol monomyristate

Concentration of monoglycerides=4 mM

Fig. 1. SDH activities of *E. coli* JE and its NS-mutants in the presence of MC₁₀

*: 4 mM MC₁₀

Fig. 2. Effect of MC₁₀ on the SDH activities of cell envelopes prepared from each bacterium

*: 4 mM MC₁₀

constituting the monoglycerides, the more the SDH activity is stimulated *in vivo*.

2. Effect of MC₁₀ on *E. coli* JE and its NS-mutants

Monoglycerides appear to act on the outer membrane of *E. coli* JE¹⁴⁾. Therefore the effect of MC₁₀ on SDH activity was determined *in vivo* by using *E. coli* JE and the NS-mutants, which have incomplete lipopolysaccharides (LPS).

Succinate dehydrogenase activities of *E. coli* JE and NS-3 were activated by MC₁₀ *in vivo*, though that of NS-4 was inhibited as shown in Fig. 1; differences in SDH activities also existed between NS-4 and either *E. coli* JE or NS-3 in the absence of MC₁₀. From the above results, it is deduced that the variation of SDH activities may be dependent

Table 2. SDH Activity of *E. coli* JE Grown in MC₁₀-containing Medium

Strain	SDH activity	
	Control	MC ₁₀
<i>E. coli</i> JE	34.0	108.8

Concentration of MC₁₀=4 mM

on the structure and components in the outer membrane of these bacteria.

3. Effect of MC₁₀ on SDH activities of the cell envelopes prepared from *E. coli* JE and its NS-mutants

The cell envelopes were prepared from *E. coli* JE and its NS-mutants¹³⁾ and the effect of MC₁₀ on the SDH activities of the cell envelopes was measured.

The SDH activities of the cell envelopes prepared from each bacterium were strongly inhibited by MC₁₀. This results suggest that SDH itself may be inhibited by MC₁₀.

4. SDH activity of *E. coli* JE grown in medium containing MC₁₀

Good growth of *E. coli* JE was observed in a medium containing 0.1% MC₁₀ (data not shown). However, it is thought that the structure and components in the outer membrane of *E. coli* JE grown in MC₁₀-containing medium differ from those of the cells grown in control medium (without MC₁₀). Table 2 shows the SDH activities of the cells grown in both the presence and absence of MC₁₀. MC₁₀ was not added to the reaction mixture for measurement of the enzyme activity.

The cells grown in MC₁₀-containing medium exhibited greater SDH activity than the normal cells.

5. SDH activities of *E. coli* JE lacking 0-9 protein in the outer membrane

E. coli JE lacking 0-9 protein in the outer membrane was obtained according to the method of Hasegawa *et al.*¹⁰⁾, and the SDH activities of the cells lacking 0-9 protein and normal cells were measured. The effects of MC₁₀ on those cells were also determined.

As shown in Fig. 3, the SDH activities of the cells lacking 0-9 protein were lower than those of the normal cells and were not greatly activated by the addition of MC₁₀ to the reaction mixture.

Table 3. SDH Activities of *E. coli* JE Pretreated with Chemicals

Chemicals	SDH activity
Water (Control)	33.3
MC ₁₀ (4 mM)	134.0
Phospholipase C (800 U/ml)	73.7
EDTA (1 mM)	34.0
Papain (0.4%)	33.2

Fig. 3. SDH activities of *E. coli* JE lacking 0-9 protein
*: 4 mM MC₁₀

6. Pretreatment of *E. coli* JE with chemicals

Succinate dehydrogenase activity of *E. coli* JE pretreated with chemicals was measured in the absence of MC₁₀.

As shown in Table 3, the SDH of *E. coli* JE was activated by pretreatment with MC₁₀ or phospholipase C, though no activation of SDH by EDTA or papain was observed.

Discussion

In general, it is said that gram-negative bacteria are more resistant to antibacterial substances than gram-positive bacteria. We have confirmed that antibacterial substances such as polyphosphates, monoglycerides and sodium cholate are effective against most gram-positive bacteria but are inactive against most gram-negative bacteria¹⁵⁻¹⁹⁾. The outer membrane of gram-negative bacteria may cause the resistance of these bacteria to the action of antibacterial substances. However, the growth of gram-negative bacteria is strongly inhibited by the combined use of

polyphosphates and cholate, or polyphosphates and monoglycerides, though the mode of the combined effect of these substances is obscure. Therefore we have tried investigating the modes of action of these substances separately as a means of elucidating that of the combined effect. However, it is difficult to elucidate the target site by making use of the antibacterial action of these substances, because they do not all show antibacterial action against gram-negative bacteria. Accordingly, we tried elucidating the action of antibacterial substances on the outer membrane by measuring the activity of SDH, which is bound to the cytoplasmic membrane of *E. coli*. The principle of this method is based on the fact that the SDH reaction is started by the binding to SDH of substrates which permeate the outer membrane. Thus, if monoglycerides act on the outer membrane and alter the nature of it, the permeability of the outer membrane to substrates may change and variation of SDH activity may be observed. As we expected, the SDH activity of *E. coli* was modified by the action of monoglycerides or cholate¹⁴⁾.

In the range of C₆ to C₁₄ carbon chain number of the fatty acid constituting the monoglycerides, SDH was activated by monoglycerides *in vivo*. Two reasons for this activation of SDH can be considered; *viz.* (1) activation of SDH itself by monoglycerides, (2) stimulation of the permeability of the outer membrane to substrates. To determine which is the case, the cell envelopes of *E. coli* JE were fractionated and the enzyme activities of the fraction were measured with and without MC₁₀. It was found that the enzyme activities of the cell envelopes were strongly inhibited by MC₁₀. From these results, we deduced that the activation of SDH in the whole cells by MC₁₀ was due to the latter effect. In addition, we carried out similar experiments on NS-mutants, which have incomplete LPS. *In vivo*, SDH of NS-3 was activated by MC₁₀, while that of NS-4 was inhibited by it. However, the SDH activities of the cell envelopes prepared from these NS-mutants were inhibited by MC₁₀.

Since the nature of the outer membrane

of *E. coli* is dependent on the culture conditions, we measured the SDH activities of the altered cells *in vivo* under the same conditions. The SDH activities of the cells grown in a medium containing MC₁₀ were greater than those of normal cells, whereas the SDH activities of the cells lacking O-9 protein in the outer membrane were lower than those of normal cells and were not activated by the addition of MC₁₀ to the reaction mixture. These results confirm that the progress of the SDH reaction is dependent on the nature of the outer membrane of *E. coli*.

The pretreatment of *E. coli* JE cells with chemicals was carried out in order to elucidate the relationship between the permeability of the outer membrane to substrates and the components or structure of the membrane. The SDH activity of *E. coli* JE was activated by pretreatment of the cells with MC₁₀ or phospholipase C though no effect was observed on pretreating the cells with EDTA or papain. Glycerol monocaprates (MC₁₀) is a kind of detergent and phospholipase C acts on phospholipids, that is, the fact that SDH of *E. coli* JE was activated *in vivo* by pretreating the cells with MC₁₀ or phospholipase C suggests that there is a relationship between the permeability of the outer membrane to substrates and phospholipids in the membrane. Though 30~50% LPS is lost on pretreatment of *E. coli* with 1 mM EDTA, no effect on the SDH activity of the bacterium was observed *in vivo*. This suggests that there is little causal connection between the variation of SDH activity and the nature of LPS in the outer membrane. Papain pretreatment was also ineffective; but this does not indicate that there is no relationship between the variation of SDH activity and the nature of proteins in the outer membrane, because the SDH activity of the cells that lacked O-9 protein (a major protein of the outer membrane) was lower than that of normal cells. It is possible that papain was unable to digest the protein affecting the permeation of the substrates through the outer membrane.

It is not clear what kinds of components in the outer membrane affect the permea-

tion of the substrates and the enzymatic reaction, but proteins and phospholipids in the membrane are probably important, and glycerol monocaprate (MC₁₀) may act on certain components to stimulate (or retard) the permeation of substrates and apparently activate (or inhibit) the SDH of *E. coli in vivo*. Furthermore, these results suggest that the first target site of MC₁₀ is on the outer membrane of *E. coli*. However, MC₁₀ may act not only on the first target site but also on a second or third target site in the presence of polyphosphates. More work is needed to elucidate the nature of these target sites.

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