Accumulation of the Prolipoprotein of the *Escherichia coli* Outer Membrane Caused by Benzoyloxy carbonylalanine Chloromethyl Ketone*

(Received for publication, January 14, 1981, and in revised form, February 25, 1981)

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Benzoyloxy carbonylalanine chloromethyl ketone (Z-Ala-CH₂Cl) was found to inhibit the processing of the prolipoprotein, a secretory precursor of the lipoprotein of the *Escherichia coli* outer membrane. The accumulation of the prolipoprotein in the presence of Z-Ala-CH₂Cl was observed both in vivo and in vitro. Z-Ala-CH₂Cl did not inhibit the processing of precursors of the other major outer membrane proteins. A preliminary experiment to identify the processing enzyme(s) of the prolipoprotein was carried out with use of Z-[¹⁴C]Ala-CH₂Cl and revealed that two cytoplasmic membrane proteins with *M*₅₇, 80,000 and 46,000 were predominantly labeled.

In *Escherichia coli*, many proteins are secreted across the cytoplasmic membrane and these secretory proteins have been shown to be produced from their precursors with signal peptides, NH₂-terminal extensions (see review in Refs. 1 and 2). On the basis of the structural analysis of the amino acid sequences of the signal peptides, a model (loop model) has been proposed to explain the function of the signal peptide in protein secretion across the membrane. One of the important aspects in the molecular mechanism of protein secretion is to identify signal peptides.

In the present report, we have attempted to develop a specific inhibitor for signal peptides which can irreversibly bind to the enzymes. We found that benzoyloxy carbonylalanine chloromethyl ketone inhibits the processing of the outer membrane prolipoprotein of *E. coli*. With use of [¹⁴C]Z-Ala-CH₂Cl, only a few cytoplasmic membrane proteins were labeled which suggests some degree of specificity for the inhibition. This method provides a new approach to identify and characterize signal peptidases.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strain**—*E. coli* K12 strain W1485 was used. Cells were grown in M9 medium supplemented with 2 μg/ml of glucose and 2 μg/ml of vitamin B1. For labeling experiments, 4 μg/ml of 1-methionine were also added to the medium.

**Labeling Experiments**—Cells were labeled in the presence and the absence of Z-Ala-CH₂Cl ([¹⁴C]methionine as follows: 50 μCi of [¹⁴C]-methionine were added with 10 ml of the culture at about 10⁶ cells/ml. The culture was incubated at 37 °C for 20 min. After incubation, the membrane fraction was prepared as previously described (4).

Labeling of cells with Z-[¹⁴C]Ala-CH₂Cl was carried out as follows; cells from a 90-ml culture of 2 × 10⁸ cells/ml were centrifuged and resuspended in 3 ml of M9 culture medium. Of 1 ml of Z-[¹⁴C]Ala-CH₂Cl (5 Ci/mmoll, 120 μCi) were added to the cell suspension. The mixture was then incubated at 37 °C for 1 h. After incubation, the membrane fraction was prepared as previously described (4).

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The membrane-bound polypeptides were prepared as described by Randall and Hardy (6). The membrane-bound polypeptides were separated in 10 mM Tris-acetate, pH 8.2, 15 mM magnesium acetate, 60 mM NH₄Cl, 1 mM diithiothreitol. The suspension was kept at -70 °C until used. The membrane suspension was pretreated with Z-Ala-CH₂Cl (0, 100, and 400 μg/ml) in 0.5% ethanol for 10 min at room temperature. The cell-free protein synthesis was carried out in a 50-ml reaction mixture, as described by Zubay et al. (6) except for methionine, UTP, CTP, 3':5'-AMP, p-aminobenzoic acid, calcium chloride, and φ80dHac DNA. The reaction mixture also contained 20 μCi of [¹⁴C]methionine and the Z-Ala-CH₂Cl-treated membrane fraction (10 μg). The reaction was carried out for 30 min at 37 °C. The product was immunoprecipitated with antiprotein serum.

**Immunoprecipitation**—To 50 μl of the reaction mixtures or the membrane fractions were added 10 μl of 10% sodium dodecyl sulfate solution. The mixture was boiled for 2 min and 0.95 ml of 10 mM Tris-HCl, pH 7.2, containing 150 mM NaCl, 5 mM EDTA, and 100 units/ml of aprotinin (Buffer A) and 10 μl of immunoglobulin fraction containing antilipoprotein serum were added to the boiled mixture. After overnight incubation at 4 °C, 30 μl of packed protein A-Sepharose (Pharmacia) was added to the mixture. The mixture was incubated in a shaker at 4 °C and the Sepharose was collected by centrifugation, and washed three times with Buffer A containing 1% Triton X-100. Proteins were solubilized from the Sepharose fraction by boiling it in 2% SDS with 5.4 ml of 0.1 M Tris-HCl, pH 7.4, 0.5 ml of 1 M sodium dodecyl sulfate, 0.1 ml of 0.5 M mercaptoethanol, and 0.3 ml of 1% SDS. The mixture was then boiled for 2 min and 0.95 ml of 10 mM Tris-HCl, pH 7.2, containing 150 mM NaCl, 5 mM EDTA, and 100 units/ml of aprotinin (Buffer A) and 10 μl of immunoglobulin fraction containing antilipoprotein serum were added to the boiled mixture. After overnight incubation at 4 °C, 30 μl of packed protein A-Sepharose (Pharmacia) was added to the mixture. The mixture was incubated in a shaker at 4 °C and the Sepharose was collected by centrifugation, and washed three times with Buffer A containing 1% Triton X-100. Proteins were solubilized from the Sepharose fraction by boiling it in 2% SDS.

**Gel Electrophoresis**—SDS-polyacrylamide gel electrophoresis was carried out with 7.5% acrylamide disc gels as previously described (4).

**Synthesis of Z-Ala-CH₂Cl**—Unlabeled Z-Ala-CH₂Cl (7) was prepared via the corresponding diazomethyl ketone which was, in turn prepared by acylation of diazomethane using the mixed anhydride route. Tritium-labeled Z-Ala-CH₂Cl was obtained by the same synthetic route using benzoyloxy carbonylalanine which was prepared from 5 mCi of [2,3-³H]alanine (0.5 Ci/mmoll). The labeled product was obtained in 20% overall yield based on alanine. Of the radioactivity, 96% co-migrated with authentic Z-Ala-CH₂Cl on Silica Gel G plates developed in the solvent system of ethyl acetate/toluene/acetic acid (10:10:1).

**RESULTS**

**Effects of Z-Ala-CH₂Cl in Vivo**—In order to examine the effect of Z-Ala-CH₂Cl on growth of *E. coli*, Z-Ala-CH₂Cl was added to cultures at various concentrations. At 80 μg/ml of Z-Ala-CH₂Cl, growth was completely inhibited. At lower concentrations of Z-Ala-CH₂Cl, no severe effects were observed for the first 30 min. However, at 27 μg/ml of Z-Ala-CH₂Cl
growth was completely stopped after 30 min, whereas at 9 \( \mu g/\) ml of Z-Ala-CH2Cl severe growth inhibition was observed after 1 h (data not shown).

In spite of severe inhibition of growth at 80 \( \mu g/\) ml of Z-Ala-CH2Cl, considerable synthesis of membrane proteins was observed. When the immunoprecipitate with antilipoprotein was analyzed with disc gel electrophoresis, some fractions moved more slowly than the fully processed lipoprotein (Fig. 1B) indicating that the processing of the prolipoprotein was partially inhibited. At 120 \( \mu g/\) ml of Z-Ala-CH2Cl, a peak in the position corresponding to prolipoprotein appeared clearly (Fig. 1C). From the size of the peaks and the contents of methionine residues in the prolipoprotein (3 residues; Ref. 3) and the lipoprotein (2 residues; Ref. 3), the rate of the processing was calculated to be inhibited by about 35% At 250 \( \mu g/\) ml of Z-Ala-CH2Cl, the inhibition increased to about 55% (Fig. 1D).

No accumulation of precursors of other major outer membrane proteins, pro-matrix protein and pro-ompA protein, was observed under the same condition.

Effects of Z-Ala-CH2Cl in Vitro—The processed lipoprotein was produced in a cell-free system directed by the membrane-bound polysomes as shown in Fig. 2A. However, when the membrane-bound polysomes were pretreated with Z-Ala-CH2Cl, prolipoprotein was accumulated (Fig. 2, B and C). At 400 \( \mu g/\) ml of Z-Ala-CH2Cl, the processing of the prolipoprotein was completely inhibited. When 10 \( \mu g/\) ml of globomycin, a specific inhibitor for the prolipoprotein signal peptidase (10), were added to the same cell-free system, the prolipoprotein appeared at the same position as shown in Fig. 2C (data not shown).

Labeling of Membrane Proteins with [3H]Z-Ala-CH2Cl—The membrane fraction labeled with [3H]Z-Ala-CH2Cl was further fractionated into the sarkosyl-soluble cytoplasmic membrane and the sarkosyl-insoluble outer membrane fraction (11). Almost all radioactivity (90%) was found in the cytoplasmic membrane. When the [3H]Z-Ala-CH2Cl-labeled membrane was analyzed by SDS-gel electrophoresis as described under "Experimental Procedures." The gel was run with internal molecular weights standards (9): a, dimer; b, monomer of Dns-bovine serum albumin; c, dimer; d, monomer of Dns-egg white lysozyme; e, cytochrome c; f, Dns-insulin.

**Fig. 1. Effects of Z-Ala-CH2Cl on in vivo synthesis of the lipoprotein.** Cells were labeled with [3S]methionine in the presence of various concentrations of Z-Ala-CH2Cl. The lipoprotein was immunoprecipitated as described under "Experimental Procedures" and analyzed by SDS-polyacrylamide gel electrophoresis. The lipoprotein labeled with [3H]arginine was also run together with samples as an internal standard. Only the portions between cytochrome c and Dns-insulin as internal molecular weight standards (9) were sliced and counted. A, 0 \( \mu g/\) mg; B, 80 \( \mu g/\) mg; C, 120 \( \mu g/\) mg; and D, 250 \( \mu g/\) ml of Z-Ala-CH2Cl; •, [3S]methionine and •—•, [3H]arginine. a and b indicate the positions of cytochrome c and Dns-insulin, respectively.

**Fig. 2. Effects of Z-Ala-CH2Cl on in vitro synthesis of the lipoprotein.** The lipoprotein was labeled with [3S]methionine in a cell-free system directed by the membrane-bound polysomes which were pretreated with Z-Ala-CH2Cl as described under "Experimental Procedures." The products were immunoprecipitated with antilipoprotein serum and analyzed by SDS-polyacrylamide gel electrophoresis. [3H]Arginine-labeled lipoprotein was run together with the same samples as an internal standard. Only the portions between cytochrome c and Dns-insulin as internal molecular weight standards (9) were sliced and counted. The membrane-bound polysomes were pretreated with (A) 0 \( \mu g/\) ml, (B) 100 \( \mu g/\) ml, and (C) 400 \( \mu g/\) ml of Z-Ala-CH2Cl. •, [3S]methionine; —•—•, [3H]arginine. a and b indicate the positions of cytochrome c and Dns-insulin, respectively. Arrows indicate the position of the prolipoprotein accumulated in the presence of 10 \( \mu g/\) ml of globomycin.

**Fig. 3. SDS-polyacrylamide gel electrophoresis of Z-[3H]-Ala-CH2Cl-labeled membrane fraction.** The membrane fractions were prepared and analyzed by SDS-gel electrophoresis as described under "Experimental Procedures." The gel was run with internal molecular weights standards (9): a, dimer; b, monomer of Dns-bovine serum albumin; c, dimer; d, monomer of Dns-egg white lysozyme; e, cytochrome c; f, Dns-insulin.

**DISCUSSION**

Chloromethyl ketone derivatives of protease substrates have been shown to be very effective inhibitors for proteases, because they covalently bind to the active centers of proteases...
(12). Signal peptidases required for protein secretion across
the membrane are also considered to be proteases, which have
extremely high specificity at the cleavage site. In the loop
model for protein secretion, we have pointed out that bacterial
signal peptidases usually cleave at the carboxyl end of glycine
or alanine residue (1, 2). On the basis of this finding, we have
examined Z-Ala-CH$_2$Cl as a possible inhibitor for bacterial
signal peptidases.

Indeed, we have found that Z-Ala-CH$_2$Cl inhibited the
processing of the prolipoprotein. However, it is rather surpris-
ing that Z-Ala-CH$_2$Cl inhibited specifically the processing of
the prolipoprotein, of which the cleavage site is a glycine
residue, but did not inhibit the processing of the pro-matrix
protein and the pro-ompA protein, of which the cleavage site
is an alanine residue (13). Since the processing of prolipopro-
tin to lipoprotein involves modification of a cysteine residue
as well as cleavage of a peptide bond (2), the possibility must
be considered that Z-Ala-CH$_2$Cl inhibits the modification
step(s) rather than the peptidase which is sensitive to glo-
bo-mycin. It is interesting to note that a few cytoplasmic mem-
brane proteins ($M_r$ = 78,000 and 46,000) were specifically
labeled with Z-[3H]Ala-CH$_2$Cl. A signal peptidase for M13
procoat protein ($M_r$ = 39,000) has been purified (14). It is
possible that one of these proteins may be the signal peptidase
or modification enzyme for the prolipoprotein. Purification
and characterization of these proteins are now in progress.

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Accumulation of the prolipoprotein of the Escherichia coli outer membrane caused by benzyloxy carbonylalanine chloromethyl ketone.

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