# Assignment of the Functional Loci in Colicin E2 and E3 Molecules by the Characterization of Their Proteolytic Fragments†

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ABSTRACT: Plasmin split the protein A molecule of colicin E3 ($M_r$ 61 000) into two fragments, P1 and P2, with molecular weights of 47 000 and 14 000, respectively. Characterization of these fragments revealed that P1 is derived from the N-terminal part of protein A and has receptor-binding activity, while P2 assigned to the C-terminal part of protein A has full ribosome inactivation activity. Thus, it was suggested that protein A consists of P1 and P2 domains, in charge of receptor binding and ribosome inactivation, respectively. Bromelain removed a short peptide from the N-terminal part of protein A, yielding the Br-A fragment ($M_r$ 55 000). This fragment accounts for receptor binding and ribosome inactivation but not bacteriocidal activity. Thus, it was suggested that this short peptide is responsible for the transportation of the colicin molecule through cell membranes. On treatment with plasmin the P2 fragment was removed from Br-A, yielding the BP fragment ($M_r$ 41 000) which had the same receptor-binding activity as the P1 fragment. Thus, RNase, receptor-binding, and transmembrane activities of colicin E3 were assigned to the C-terminal, central and N-terminal parts of protein A, respectively. P1, Br-A, and BP fragments were also obtained from colicin E2. E2-BP and E3-BP are serologically identical, which shows that colicins E2 and E3 share a common receptor. With the results of analysis of CD spectra of these fragments the structure–function relationship of colicin E3 is discussed.

The mode of action of colicin E2 or E3 can be divided into three stages: (I) specific binding to the receptor of the sensitive cell, (II) penetration through the cell membranes, and (III) digestion of DNA (E2)[1] (Nomura, 1963; Saxe, 1975a,b; Schaller & Nomura, 1976) or inactivation of ribosomes (E3) (Bowman et al., 1971a,b; Senior & Holland, 1971; Boon, 1971). Recent studies on the structure of the colicin E2 and E3 molecules have revealed the mechanism of stage III. Although colicin E3 is composed of two components (Jakes & Zinder, 1974a; Hirose et al., 1976), only the A component is responsible for ribosome inactivation while component B inhibits this activity. The protein B corresponds to the immunity proteins which have been discovered and characterized by previous workers (E3 immunity protein, Jakes & Zinder, 1974b; Sidikaro & Nomura, 1974; E2 immunity protein, Schaller & Nomura, 1976). Likewise, protein A of E2 accounts for its DNase activity (Schaller & Nomura, 1976). Moreover, we have shown that protein A of E3 cleaves not only 16S RNA of 70S ribosomes but also that of the isolated 30S subunits (Ohno-Iwashita & Imahori, 1977) and even isolated 16S RNA itself (Ohno & Imahori, 1978), although the cleavage in the last case was not as specific as in the case of the former two.

Tryptic digestion of E2 and E3 split each protein A into two fragments, T1 and T2A (Ohno et al., 1977; Yamamoto et al., 1978). The T2A fragment of E2 and E3 showed full DNase or RNase activity, respectively, and the activity was neutralized by the corresponding protein B. Thus, T2A is a real active fragment and the amino acid sequence of E3-T2A was determined (Suzuki & Imahori, 1978a,b). By comparing the sequences around the C termini, it was confirmed that T2A is derived from the C-terminal part of protein A. Thus, the nuclease activity was assigned to the C-terminal region of protein A. However, the function of the rest of the protein A molecule is still unclear. The machineries necessary for the functioning of stages I and II might be in this part. Beyond doubt the T1 fragment is derived from this part of the molecule.

The T1 fragments of E2 and E3 were almost identical in their amino acid compositions and antigenicities (Ohno-Iwashita & Imahori, 1979), reflecting that E2 and E3 share a common receptor. Thus, the T1 region was a good candidate for that accounting for the receptor binding. However, this was not so since the T1 fragment failed to inhibit the infection by colicin E2 or E3 of the sensitive cells. It is conceivable that functional groups necessary for the above two stages might be lost during trypsinolysis. Thus, we searched for better conditions for limited proteolysis and found out that cleavage with plasmin yields a fragment which is larger than T1 and has receptor-binding activity. We also obtained another fragment by bromelain digestion which has receptor-binding activity but lacks the ability to penetrate the membranes.

In this paper we describe the isolation and characterization of these fragments. We determined the locations of these fragments in the original molecule and assigned three functions of the colicin molecule to three different parts of the molecule.

## Experimental Procedures

*Materials.* Fibrinolysin (plasmin) from porcine blood was purchased from Sigma. Bromelain, phage PM2 DNA, and marker proteins for analytical $NaDodSO_4$ gels were from Boehringer Mannheim. Trypsin (TRTPCK) and carboxypeptidases A and B (COADFP and COBDFP) were products of Worthington Biochemical Corp. The labeled compound [$^3$H]phenylalanine was obtained from New England Nuclear Corp. $^3$H-Labeled E3 was prepared according to Lau & Richards (1976) and was the kind gift from Dr. K. Suzuki of our laboratory. Polyamide sheets were from Cheng Chin Trading Co. CM-Sephadex (C-50) and Sephadex G-100 were

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; K, $\times 10^3$; E2 and E3, colicin E2 and colicin E3; E3-T1 and E3-T2A, T1 and T2A fragments isolated from E3; E2-T1 and E2-T2A, T1 and T2A fragments isolated from E2; E3-A, E3-B, E2-A, E2-B, E3-Br, E2-Br, E3-Br-A, E2-Br-A, E3-P1, E3-P2, E2-P1, E3-BP, and E2-BP, see Chart I.

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the products of Pharmacia Fine Chemicals.

*Bacterial Strains.* *Escherichia coli* W3110(E3) and W3110(E2) were used for the preparation of colicins E3 and E2, respectively. *E. coli* K12-A19(Hfr, met$^-$, RNase I$^-$) was used as a colicin-sensitive strain (Gesteland, 1966).

*Preparation of Colicins, Proteins A and B, and Their Tryptic Fragments.* Colicins E2 and E3 were prepared according to Herschman & Helinsky (1967) with some modifications as described by Konisky & Nomura (1967). Proteins A and B were prepared as described previously (Ohno et al., 1977), except that buffer B (10 mM potassium phosphate, pH 6.0, containing 0.1 M NaCl, 8 M urea, and 1 mM EDTA) was used for the separation of E2-A and E2-B instead of buffer A (50 mM potassium phosphate, pH 6.0, containing 6 M urea and 1 mM EDTA). Tryptic fragments, T1 and T2A, were prepared as described previously (Ohno-Iwashita & Imahori, 1979). Although three species of E2-T2A were observed when E2 was digested for a short period (see Figure 2, gel 5), the larger ones were converted to the smallest one (15.5K) by further digestion. Therefore, the smallest one was used for further analyses.

*Digestion of Colicins with Plasmin and Bromelain.* Plasmin was dissolved in buffer C (50 mM Tris-HCl, pH 9.0, containing 0.1 M NaCl, 0.02 M lysine, and 1 mM EDTA) at a concentration of 1 mg/mL and was added to a solution of protein A in buffer C (E3-A/plasmin = 200:1 w/w and E2-A/plasmin = 25:1 w/w). The mixture was incubated for 4 (for E3-A) or 12 h (for E2-A) at 30 °C. Plasmin digestions of E3-Br-A and E2-Br-A were carried out under similar conditions.

For bromelain digestion, E2 or E3 was dissolved in buffer D (10 mM potassium phosphate, pH 7.0, 6 mM 2-mercaptoethanol, and 1 mM EDTA) and incubated with bromelain (colicin/bromelain = 1000:1 w/w) for 2.5 h at 30 °C.

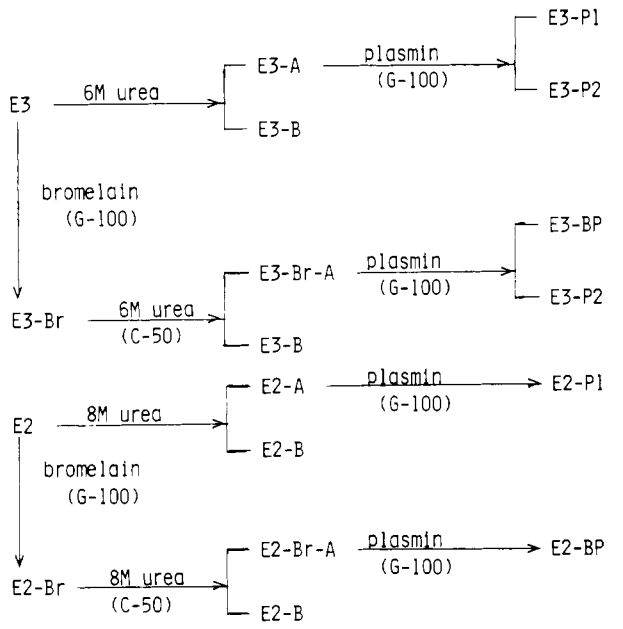
*Isolation of the Fragments Produced by Plasmin and Bromelain Digestions.* Each digestion mixture was applied to a Sephadex G-100 column equilibrated with buffer E (10 mM potassium phosphate, pH 6.0, and 0.1 M NaCl), and the column was developed with the same buffer. Two protein fractions, designated as P1 and P2 were obtained from the plasmin digest of E3-A; however, only one fraction (P1) was obtained in the case of E2-A. E3-Br and E2-Br were obtained as the firstly eluted main peaks from the bromelain digests of E3 and E2, respectively, and the plasmin digestion of E3-Br-A and E2-Br-A yielded E3-BP and E2-BP, respectively.

E3-Br-A and E2-Br-A were prepared from E3-Br and E2-Br, respectively. The solution of E3-Br or E2-Br was freshly prepared in buffer A or buffer B, respectively, and applied to a CM-Sephadex column equilibrated with the same buffer. Each column was developed with a linear gradient of NaCl in the same buffer. Both E3-Br-A and E2-Br-A were eluted at 0.2 M NaCl in one large peak, although a small peak was sometimes observed as a shoulder on the main peak. The isolation procedures are summarized in Chart I.

*Assay Methods for the in Vivo and in Vitro Activities of Colicins.* The spot test was used for the assay of bacteriocidal activity of colicins and their fragments. A nutrient agar plate was seeded with $10^8$ sensitive cells, and 10 $\mu$L each of serial threefold dilutions of a colicin sample was spotted on the plate. The highest dilution of colicin which still gave a visible zone of inhibition of growth was used as the measure of killing units of the colicin.

Binding of the fragments to the sensitive cells was assayed by measuring (1) the degree of inhibition of bacteriocidal activity of native colicins and (2) inhibition of binding of $^3$H-labeled E3 to the sensitive cells. With method 1 a fixed amount of cells ($5 \times 10^8$/mL), preincubated with 2,4-dinitrophenol for 20 min at 37 °C, was incubated with various amounts of a fragment for 5 min at 37 °C, and 30 ng/mL colicin E3 was then added. After the mixture was incubated further for 10 min at 37 °C, the cells were plated out and the number of colonies was counted. Method 2 was carried out according to de Graaf et al. (1978) except that the $^3$H-labeled E3 and competitors were simultaneously added to the cell suspension.

Chart I




The in vitro nuclease activities of colicins E2 and E3 were measured as described previously (Yamamoto et al., 1978; Ohsumi & Imahori, 1974).

*Polyacrylamide Gel Electrophoresis.* Analytical NaDodSO$_4$ gels were prepared and run according to Laemmli (1970) or Weber & Osborn (1969).

*Terminal Amino Acid Analyses.* N-Terminal amino acid analysis by dansylation was carried out according to Gray (1972). Dns-amino acids were identified on polyamide sheets.

Carboxypeptidases A and B were used to analyze amino acids in the C-terminal region as described previously (Suzuki & Imahori, 1978a). Digestion was carried out for 5 h at 30 °C (enzyme/substrate ratio 1:50 mol/mol) unless otherwise specified.

*Amino Acid Analysis.* Proteins were hydrolyzed in vacuo at 110 °C with 6 N HCl for 24–72 h, and the hydrolysates were analyzed on a Hitachi 835-50 amino acid analyzer.

*Preparation of Antisera.* Preparation of antisera and the analyses of antigen–antibody reactions by the double-diffusion technique were carried out as described previously (Ohno-Iwashita & Imahori, 1979).

*Circular Dichroism.* Circular dichorism spectra were recorded on a Jasco J40 spectropolarimeter. For calculation of mean residue ellipticity ([$\theta$] in deg cm$^2$ dmol$^{-1}$), the following mean residue weights of the respective proteins were used: for E3-A, 104.9; for E3-P1, 102.8; for E3-BP, 106.3; for E3-T1, 100.0; for E3-P2, 108.9.

In this work the concentrations of E3-A, E2-A, E3-Br-A, E2-Br-A, E3-P1, E3-P2, E3-BP, E3-T1, and E2-T2A were calculated by assuming their molecular weights to be 61K, 62K, 55K, 56K, 47K, 14K, 41K, 35K, and 15.5K and the

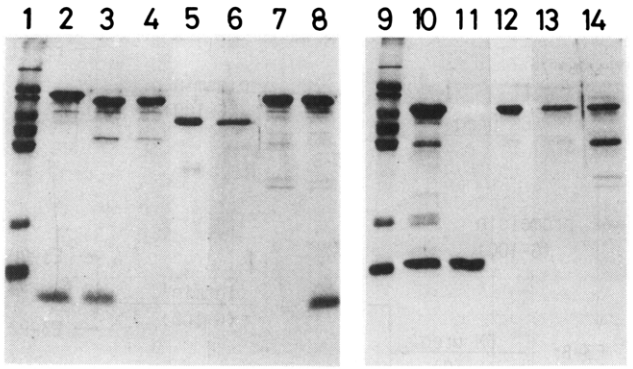



FIGURE 1: $NaDodSO_4$ gel electrophoresis (14%) of digestion mixtures and isolated fragments. Gel 2, E3; gel 3, E3-Br; gel 4, E3-Br-A; gel 5, E3-BP; gel 6, E2-BP; gel 7, E2-Br-A; gel 8, E2-Br; gel 10, mixture of plasmin digest of E3-A; gel 11, E3-P2; gel 12, E3-P1; gel 13, E2-P1; gel 14, mixture of plasmin digest of E2-A. Gels 1 and 9 are standard markers: phosphorylase *a* (97K), bovine serum albumin (68K), pyruvate kinase (57K), ovalbumin (43K), aldolase (40K), lactate dehydrogenase (36K), trypsin inhibitor (21.5K), and lysozyme (14.3K).

$E_{280nm}^{0.1\%}$ values to be 1.1, 0.9, 1.0, 0.9, 1.0, 2.5, 0.7, 1.0, and 1.2, respectively.

## Results

*Digestion and Isolation of Fragments.* When the proteolytic product of E3 or E2 with bromelain was chromatographed on a Sephadex column, a major peak, E3-Br or E2-Br, appeared. Each is a complex of two polypeptides judging from $NaDodSO_4$ gel electrophoresis (Figure 1, gels 3 and 8). The larger one is slightly smaller than protein A and is probably derived from protein A. The smaller one is probably protein B itself or a derivative of it, since it comigrated with protein B on $NaDodSO_4$ gel electrophoresis and can neutralize the activity of protein A to the same extent as protein B. The complex was dissociated in the buffer containing urea, and the larger component (E3-Br-A or E2-Br-A) was separated from the smaller one by CM-Sephadex column chromatography as described under Experimental Procedures. Gels 4 and 7 in Figure 1 show the $NaDodSO_4$ gel patterns of isolated E3-Br-A and E2-Br-A, respectively. The molecular weights of E3-Br-A and E2-Br-A were estimated to be 55K and 56K, respectively, from the results of $NaDodSO_4$ gel electrophoresis in the Laemmli (1970) system (10% gel) and in the Weber & Osborn (1969) system (7.5% gel). Protein A was more susceptible to bromelain digestion than E2 or E3, and no fragment as large as E3-Br-A or E2-Br-A was obtained after the digestion. This suggested that protein B protects the C-terminal region against bromelain digestion.

Digestion of E3-A with plasmin produced mainly two peptides, E3-P1 and E3-P2, as shown in Figure 1, gel 10. E3-P1 and E3-P2 were separated by Sephadex G-100 column chromatography without any denaturants (Figure 1, gels 11 and 12). This suggests that no strong interaction exists between P1 and P2. In fact, isolated P1 and P2 failed to make a complex judging from disc gel electrophoresis (data not shown). The molecular weights of P1 and P2 were estimated to be 47K and 14K, respectively. Digestion of E2-A with plasmin produced a peptide of a molecular weight of 47K, E2-P1, but did not produce a peptide corresponding to P2 (Figure 1, gels 13 and 14). E3 and E2 were resistant to plasmin digestion.

When E3-Br-A was digested with plasmin in a similar manner as for E3-A, two peptides were obtained. The smaller one could not be distinguished from E3-P2, in molecular weight, amino acid composition, or in vitro ribosome-inactivating activity. The molecular weight of the larger one (E3-BP) is 41K, which is smaller than that of E3-P1. Thus, it seems that plasmin attacks both E3-Br-A and E3-A at the same position(s). On plasmin digestion of E2-Br-A, a peptide (E2-BP) of a molecular weight of 41K was obtained. These fragments were isolated as described under Experimental Procedures (Figure 1, gels 5 and 6).

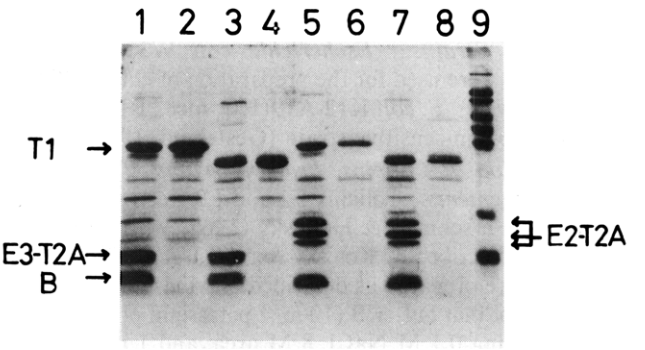



FIGURE 2: $NaDodSO_4$ gel electrophoresis (14%) of trypsin digests of colicin fragments. Fragments were digested with trypsin for 30 min at 37 °C (enzyme/substrate ratio 1:25 w/w). Gels 1–8: trypsin digests of E3 (1), E3-P1 (2), E3-Br (3), E3-BP (4), E2 (5), E2-P1 (6), E2-Br (7), and E2-BP (8), respectively. Gel 9, standard markers (see legend to Figure 1).

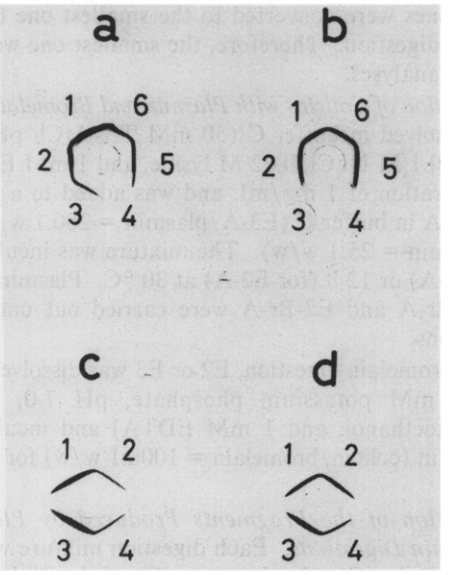



FIGURE 3: Immunochemical analyses of colicin fragments. (a) (1) E3-T1 (0.70 $A_{280}$); (2) E3-P1 (0.50 $A_{280}$); (3) E2-P1 (0.20 $A_{280}$); (4) E3-P2 (0.25 $A_{280}$); (5) E3-BP (0.54 $A_{280}$); (6) E3-Br-A (0.47 $A_{280}$). Anti-E3-T1 serum was placed in the central hole. (b) (1) E3-T2A (0.037 $A_{280}$); (2) E3-P2 (0.063 $A_{280}$); (3) E3-BP (0.54 $A_{280}$); (4) E3-P1 (0.50 $A_{280}$); (5) E3-Br-A (0.47 $A_{280}$); (6) E3-A (0.37 $A_{280}$). Anti-E3-T2A serum was placed in the central hole. (c and d) (1) E3-P1 (0.50 $A_{280}$); (2) E2-P1 (0.20 $A_{280}$); (3) E3-BP (0.54 $A_{280}$); (4) E2-BP (0.20 $A_{280}$). Anti-E3-A (c) and anti-E2-A (d) sera were placed in the central holes, respectively. Antisera were undiluted. The antibody (20 $\mu$L) and antigens (20 $\mu$L, each) were allowed to diffuse for 24–48 h at 4 °C before fixing and staining. The values in parentheses show extinctions of the antigen solutions.

*Assignment of the Fragments by Serological Reactions.* As reported previously (Ohno et al., 1977), trypsinolysis of E3 gave three fragments, T1, T2A, and T2B (Figure 2, gel 1). T2B was protein B itself. T2A originated from the C-terminal region of protein A. T1 was derived from the rest of the protein A molecule, but T1 plus T2A could not account for the whole of protein A. Since we have obtained other fragments, we tried to assign their locations in the protein A molecule by serological reactions (Figure 3). When reacted

Table I: C-Terminal Amino Acids Released from the Fragments by Carboxypeptidase Digestion

| fragment | condition | amino acids released (mol/mol) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Leu | Tyr | Lys | Ile | Asn | Arg |
| E3-Br-A | CPA[a] | 1.42 | 1.28 | | | | |
| | CPB[b] | | | | | | |
| | CPA + CPB | 1.11 | 1.22 | 1.70 | 0.80 | 1.11 | 1.11 |
| E3-P2 | CPA | 1.21 | 0.99 | | | | |
| | CPB | | | | | | |
| | CPA + CPB | 1.21 | 1.02 | 2.07 | 0.93 | 1.21 | 0.77 |

| fragment | condition | amino acids released (mol/mol) | | | |
|---|---|---|---|---|---|
| | | Lys | Arg | Ala | Phe |
| E3-P1 | CPA | | | | |
| | CPB | 2.55 | 1.22 | | |
| | CPA + CPB | 2.18 | 1.80 | 0.63 | 0.51 |

| fragment | condition | amino acids released (mol/mol) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Lys | Gly | Arg | His | Ile | Asp |
| E2-T2A | CPB → CPA[c] (1 h) | 1.01 | 0.12 | 0.21 | | | |
| | CPB → CPA[c] (3 h) | 1.07 | 0.23 | 0.37 | 0.17 | | |
| | CPB → CPA[c] (4 h) | 0.97 | 0.62 | 0.79 | 0.43 | | |
| | CPB → CPA[c] (6 h) | 1.10 | 0.89 | 1.05 | 0.92 | | |
| E2-A | CPA in $NaDodSO_4$ | | | | | | |
| | CPB in 2.5 M urea | 0.99 | 0.18 | 0.20 | | | |
| | CPB in $NaDodSO_4$ | 1.14 | 1.06 | 0.64 | 0.18 | | |
| | CPA + CPB in $NaDodSO_4$ | 1.11 | 1.15 | 1.20 | 1.23 | 1.01 | 0.59 |
| E2-Br-A | CPA in $NaDodSO_4$ | | | | | | |
| | CPB in $NaDodSO_4$ | 0.87 | 0.12 | | | | |
| | CPA + CPB in $NaDodSO_4$ | 1.14 | 1.29 | 1.16 | 0.82 | 0.57 | 0.42 |

[a] CPA = carboxypeptidase A. [b] CPB = carboxypeptidase B. [c] E2-T2A was digested with CPB (enzyme/substrate ratio 1:150 mol/mol) for 3 h, and then CPA was added for further digestion.

with anti-T1 serum, each of P1, Br-A, and BP gave a single precipitation line but E3-P2 did not (Figure 3a). On the contrary, E3-P2 or E3-Br-A but not P1 or BP gave a precipitation line with anti-E3-T2A serum (Figure 3b). These results led us to the following conclusion. P1 and BP include the T1 region, at least partly, but not the T2A region. P2 includes the T2A region and E3-Br-A includes both the T1 and T2A regions, at least partly.

In fact, trypsinolysis of E3-P1 gave a peptide equivalent to T1 (Figure 2, gel 2). However, trypsinolysis of E3-Br or E3-BP gave a slightly smaller peptide ($M_r \sim 30$ K) than T1. This would mean that the E3-Br-A and E3-BP fragments do not contain the T1 region as a whole, although the determination site of antigenicity of T1 is in these fragments. A peptide equivalent to T2A was obtained by trypsinolysis of E3-Br.

From these results, the following is suggested. P1 and P2 are derived from the N-terminal and C-terminal regions of protein A, respectively. Besides, P1 plus P2 cover almost the whole of protein A as judged by their molecular weights. E3-Br-A is derived from protein A probably by loss of a short fragment from the N terminus of the latter. BP is missing the P2 fragment from the C-terminal part of E3-Br-A.

*Comparative Studies on the Fragments of E2 and E3.* It was reported that the T1 and T2A fragments were obtained by trypsinolysis of colicin E2 (Yamamoto et al., 1978). Thus, we compared E2-T1 and E2-T2A with the corresponding fragments of E3 and the following results were obtained (Ohno-Iwashita & Imahori, 1979). E3-T1 and E2-T1 are almost identical in molecular size, amino acid composition, and antigenicity. However, little similarity was observed between E3-T2A and E2-T2A, in any respect. Thus, we conducted similar comparative studies on the fragments mentioned above. Fragments corresponding to E3-P1, E3-Br, E3-Br-A, and E3-BP were obtained from E2 in similar ways as those from E3 were, except that the P2 fragment is missing from E2. E2-T1 was also obtainable from E2-P1 while E2-Br and E2-BP gave a slightly smaller peptide than T1 on trypsinolysis (Figure 2, gels 6–8). Thus, the fragments obtained from E2 are completely analogous to the corresponding fragments of E3. Moreover, E2-P1 gave a single precipitation line when reacted with anti-E3-T1 serum (Figure 3a). This is understandable since E2-P1 includes the E2-T1 region which is serologically identical with E3-T1. In addition, as shown in parts c and d of Figure 3, E2-P1 and E3-P1 or E2-BP and E3-BP gave fused precipitation lines when reacted with anti-E2-A or anti-E3-A. These results indicated that the P1 or BP fragment of E2 is serologically identical with the counterpart of E3.

*End-Group Determinations.* Table I shows the C-terminal amino acids released by carboxypeptidase digestions. The structure near the C terminus of both E3-Br-A and E3-P2 was -[Arg,Asn,Ile,$(Lys)_2$]-(Tyr,Leu)-COOH, which is in good agreement with the C-terminal sequence of E3-A or E3-T2A, reported as -Pro-Lys-Arg-Asn-Ile-Lys-Lys-Tyr-Leu-COOH (Suzuki & Imahori, 1978b). In the primary structure of T2A, there is no sequence which fits the cleavage products mentioned above, except that of the C-terminal region. In contrast, the C-terminal amino acid of E3-P1 was determined as Lys or Arg, which supports the view that a new C terminus appeared in E3-P1 on plasmin digestion. E2-Br-A has the same C-terminal amino acids as those of E2-A and E2-T2A, determined as -(His,Arg,Gly)-Lys-COOH.

No N-terminal amino acid could be obtained for E3-P1 as well as for E3-A by the dansylation method, while the N-terminal amino acids of E3-P2 (Asx) and E2-T2A (Gly) were determined.

These results support the conclusion of the previous section.

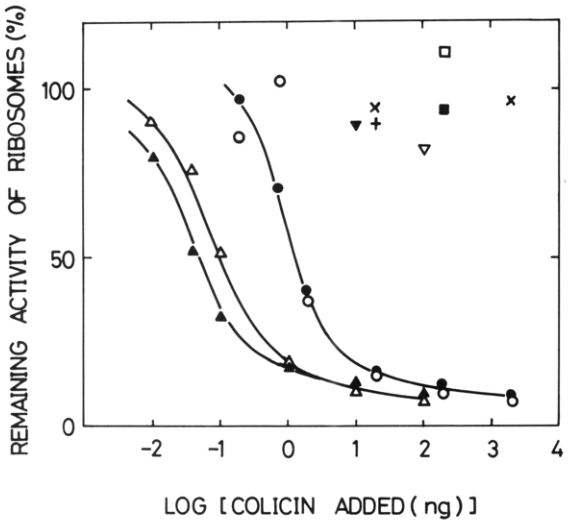



FIGURE 4: In vitro ribosome inactivation by fragments derived from E3. Ribosomes (1.0 $A_{260}$ unit) were incubated with various amounts of colicin samples in 30 $\mu$L of 50 mM Tris-acetate buffer, pH 7.8, containing 10 mM $Mg^{2+}$ and 50 mM $NH_4Cl$, for 15 min at 37 °C. The remaining activity of the incubated ribosomes was measured by using a poly(uridylic acid)-directed poly(phenylalanine)-synthesizing system. The activity of ribosomes incubated without colicin was taken as 100% activity. (○) E3-A; (●) E3-Br-A; (△) E3-T2A; (▲) E3-P2; (×) E3-P1; (+) E3-BP; (□, ■, ▽, and ▼) E3-A, E3-Br-A, E3-T2A, and E3-P2 which were preincubated with 0.4 $\mu$g of E3-B for 5 min at 37 °C, respectively.

P1 and P2 are derived from the N-terminal and the C-terminal parts of protein A, respectively. Br-A includes the C-terminal part of protein A.

*Amino Acid Compositions.* Table II shows the amino acid compositions of the fragments. E3-P2 is rich in basic amino acids, and its amino acid composition is very similar to that of E3-T2A. Judging from the molecular sizes and the C-terminal amino acids, it is concluded that E3-P2 has a stretch of a short peptide beyond the N terminus of E3-T2A. The sum of the amino acid compositions of E3-P1 and E3-P2 is very similar to that of E3-A. Probably plasmin caused a single cut in protein A without releasing an appreciable number of amino acids.

From the results described in the previous section, the Br-A fragment should lack the N-terminal region of protein A, ~6K in molecular weight. Therefore, the difference of amino acid compositions between protein A and Br-A should correspond to that of the missing N-terminal region. It is worthy to note that the N-terminal regions of both E2-A and E3-A are very rich in glycine residues; they account for 40–50% of the 69 residues. The difference of amino acid compositions between P1 and BP shows that BP also lacks the glycine-rich N-terminal region.

*Activities of the Fragments.* Figure 4 shows the in vitro ribosome-inactivating activity of the fragments derived from E3. E3-Br-A and E3-P2 have the same in vitro activity as E3-A and E3-T2A, respectively. The activity of these fragments was neutralized by E3-B. These data also support the results obtained in the previous section that E3-Br-A and E3-P2 have the same C terminus as that of E3-A since the removal of two residues (Tyr and Leu) from the C terminus of E3-A or E3-T2A with carboxypeptidase A greatly reduces the in vitro activity of the resulting peptide (Ohno et al., unpublished experiments). E3-P1 and E3-BP have no in vitro activity.

E2-Br-A has the same in vitro DNase activity as E2-A, while E2-P1 and E2-BP have no activity (Figure 5). The DNase activity of E2-Br-A was neutralized by E2-B.

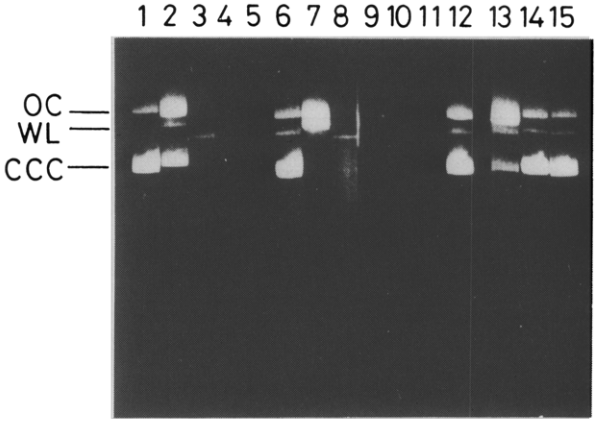



FIGURE 5: In vitro DNase activity of fragments of E2. Phage PM2 DNA (1 $\mu$g) was incubated for 1 h at 37 °C with E2-A or its fragments. The resulting samples were analyzed by agarose gel electrophoresis. Incubation products without (1) colicin; with (2) 0.15 $\mu$g, (3) 0.76 $\mu$g, (4) 2.3 $\mu$g, and (5) 7.6 $\mu$g of E2-A and (6) 2.3 $\mu$g of E2-A preincubated with 3.2 $\mu$g of E2-B; with (7) 0.05 $\mu$g, (8) 0.15 $\mu$g, (9) 0.81 $\mu$g, (10) 2.2 $\mu$g, and (11) 5.4 $\mu$g of E2-Br-A and (12) 2.2 $\mu$g of E2-Br-A preincubated with 3.2 $\mu$g of E2-B; with (13) 4.0 $\mu$g of E2-P1, (14) 2.0 $\mu$g of E2-BP, and (15) 13 $\mu$g of E2-T1. OC, open-circular DNA; WL, whole linear DNA; CCC, covalently closed-circular DNA.

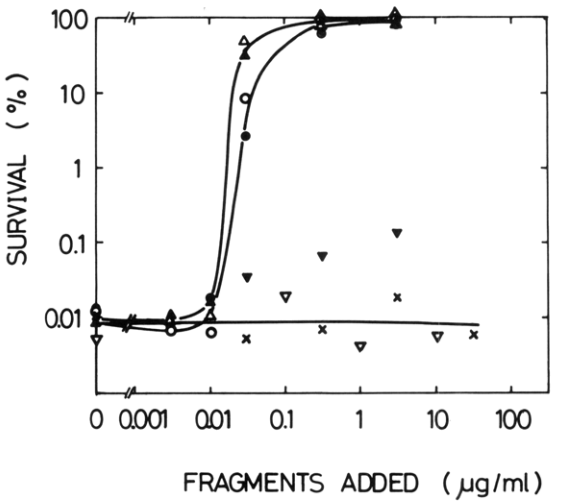



FIGURE 6: Protection of the sensitive cells against E3 action by colicin fragments. The cells ($5 \times 10^8$/mL) were incubated for 5 min with the indicated amounts of fragments, and then colicin E3 (30 ng/mL) was added. Each sample was assayed for viable cells. Details are given under Experimental Procedures. (○) E3-Br; (●) E3-Br-A; (△) E3-P1; (▲) E3-BP; (×) E3-T1; (▼) E3-P2; (▽) E3-P2 preincubated with an equimolar amount of E3-B.

Since none of the fragments obtained had in vivo killing activity, assayed by the spot test, we next examined the receptor-binding activity of the fragments. Figure 6 shows the activity of the fragments to bind to the sensitive cells judged from the degree of protection against the killing action of E3. E3-BP, E3-P1, E3-Br, and E3-Br-A can fully protect the cells against the action of E3. In contrast, E3-T1 and E3-P2 cannot protect the cells and the addition of protein B has no effect. Similar results were obtained in experiments using the fragments derived from E2. As shown in Figure 6, 30 ng/mL P1 or BP molecules is just enough to saturate almost all of the receptors. This corresponds to 800–900 molecules/cell, which is comparable to the number of E3 receptors (220 copies/cell) reported by Sabet & Schnaitman (1973). This suggested the strong binding of the fragment molecules to the sensitive cells. In addition, P1 and BP inhibit the binding of $^3H$-labeled E3 to the sensitive cells with a similar dose response as observed

Table II: Amino Acid Compositions of Fragments of E2 and E3[a]

| amino acid residue | no. of residues | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | E3-BP | E3-P1 | E3-P2 | P1 + P2 | E3-A | E3-Br-A | E3-A − E3-Br-A | E2-A | E2-Br-A | E3-P1 − E3-T1 |
| Asp | 55.3 | 65.4 | 15.9 | 81.3 | 81.8 | 71.6 | 10.2 | 86.0 | 76.5 | 18.1 |
| Thr[b] | 15.7 | 19.2 | 5.7 | 24.9 | 24.7 | 22.1 | 2.6 | 23.7 | 21.2 | 0.2 |
| Ser[b] | 28.7 | 37.9 | 7.9 | 45.8 | 43.0 | 34.5 | 8.5 | 43.0 | 34.5 | 1.2 |
| Glu | 39.6 | 42.3 | 13.2 | 55.5 | 54.7 | 53.7 | 1.0 | 55.2 | 53.7 | 18.9 |
| Pro | 24.6 | 28.2 | 10.1 | 38.3 | 39.3 | 38.9 | 0.4 | 35.7 | 34.8 | |
| Gly | 24.6 | 57.3 | 23.3 | 80.6 | 76.0 | 41.6 | 34.4 | 69.7 | 43.0 | 0.8 |
| Ala | 55.7 | 57.3 | 2.1 | 59.4 | 61.9 | 61.1 | 0.8 | 65.5 | 63.3 | 22.0 |
| Val[c] | 37.0 | 37.9 | 0.7 | 38.6 | 39.8 | 38.9 | 0.9 | 44.4 | 44.1 | 0.1 |
| Met | 7.6 | 7.6 | 0.0 | 7.6 | 8.4 | 8.4 | | 8.1 | 7.6 | 3.3 |
| Ile[c] | 12.1 | 14.3 | 5.7 | 20.0 | 19.8 | 17.7 | 2.1 | 22.0 | 19.3 | 1.4 |
| Leu[c] | 20.2 | 21.7 | 8.3 | 30.0 | 29.2 | 27.7 | 1.5 | 26.5 | 23.7 | 4.2 |
| Tyr | 3.6 | 3.7 | 6.0 | 9.7 | 8.8 | 8.8 | | 4.8 | 4.4 | 1.7 |
| Phe | 10.6 | 10.9 | 1.5 | 12.4 | 12.7 | 12.7 | | 18.0 | 17.0 | 2.9 |
| Lys | 26.4 | 24.2 | 17.0 | 41.2 | 40.0 | 38.6 | 1.4 | 44.4 | 42.0 | 12.3 |
| His | 3.8 | 7.6 | 5.1 | 12.7 | 12.6 | 8.4 | 4.2 | 13.5 | 7.9 | 1.0 |
| Arg | 21.0 | 22.0 | 6.1 | 28.1 | 29.0 | 28.5 | 0.5 | 31.5 | 30.2 | 10.4 |
| total | 386.5 | 457.5 | 128.6 | 586.1 | 581.7 | 513.2 | 68.5 | 592.0 | 523.2 | 98.5 |
| $M_r$[d] × $10^{-3}$ | 41 | 47 | 14 | | 61 | 55 | | 62 | 56 | |

[a] The values are the numbers of residues in one molecule calculated on the basis of the molecular weights given on the bottom row. [b] Extrapolated value to zero time of hydrolysis. [c] Average of 72-h values. [d] Estimated by $NaDodSO_4$ gel electrophoresis.

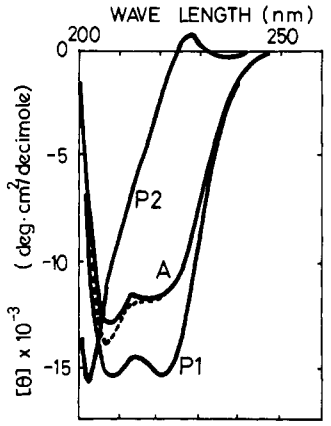


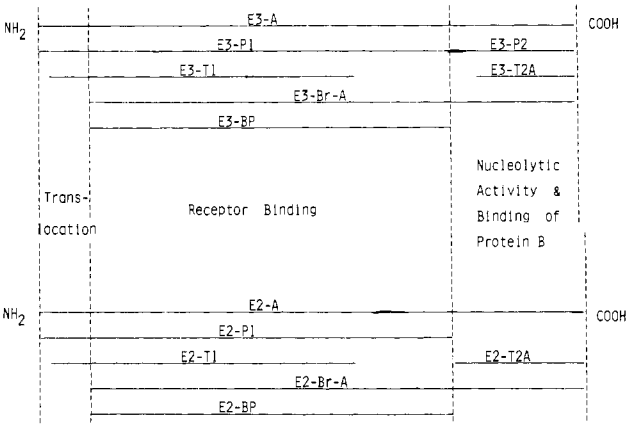

FIGURE 7: CD spectra of fragments derived from E3. Spectra were measured in 50 mM Tris-HCl buffer, pH 7.4, containing 50 mM NaCl. The dotted line shows the graphical sum of the P1 and P2 spectra.

for the inhibition by cold E3 (data not shown). Thus, the binding constant of P1 and BP to the sensitive cells is probably comparable to that of E3 itself.

Although both E3-Br-A and E2-Br-A have both receptor-binding activity and in vitro nuclease activity, they have no bacteriocidal activity. This suggested that these fragments could not reach the cell interior. The missing N-terminal region of protein A probably has an important role in penetration of the membranes.

*CD Measurement.* Figure 7 shows the CD spectra of E3-A, E3-P1, and E3-P2. Although the spectrum of E3-BP is not shown, it shows a very similar pattern to that of E3-P1. Thus, the N-terminal region of E3-A, which is deleted in E3-Br-A, has a slightly ordered structure. The spectra of E3-P1 and E3-BP are typical ones for proteins rich in the $\alpha$ helix, while P2 is rather disordered. The approximate contents of $\alpha$ helix in E3-A, E3-P1, and E3-BP were 30.7, 39.4, and 42.8%, respectively, when calculation was based on the $[\theta]$ values at 208 nm with the formula proposed by Greenfield & Fasman (1969). This suggested that the $\alpha$-helix region of E3-A mainly resides in BP. In contrast, the $\alpha$-helix content of E3-T1 was greatly reduced, to 14.6% (spectrum not shown). Both the arithmetical and experimental spectral sums of P1 and P2 fitted the spectrum of E3-A well. Thus, even from a secondary structural point of view the P1 and P2 regions probably exist as independent regions and the sum of them accounts for the secondary structure of the whole E3-A molecule.

Scheme I




## Discussion

We have obtained several new fragments from both colicin E2 and E3. From the studies on their molecular sizes, antigenicities, amino acid compositions, and terminal analyses we can assign their locations in protein A, which are summarized in Scheme I. Moreover, the studies on the functions of these fragments allowed us to assign several functions of the colicins to different domains of protein A, as will be discussed below.

Protein A of colicin E3 is composed of two domains, P1 and P2, which are separated as fragments P1 and P2, respectively, by plasminolysis. This was suggested by the additivity of their molecular weights or CD spectra. The P2 fragment is somewhat larger than the T2A fragment, and the C-terminal analysis indicated that P2 has a short extra peptide beyond the N terminus of T2A. Both P2 and T2A originate from the C-terminal part (P2 domain) of protein A, and all machineries necessary for ribosome inactivation are in this domain. Protein B, which inhibits this activity, binds specifically to this domain.

By plasminolysis of the E2-A protein, we could obtain the E2-P1 fragment with the same molecular size as E3-P1, but

we failed to obtain the E2-P2 fragment. However, E2-P1 plus E2-T2A is just enough to account for the size of E2-A since E2-T2A is somewhat larger than E3-T2A. Thus, we may assume that the E2-A protein consists of P1 and T2A domains. All machineries necessary for DNase activity of E2 are in the T2A domain. As reported previously (Ohno-Iwashita & Imahori, 1979), E2-T2A and E3-T2A are different in their amino acid compositions and antigenic properties. Protein B of E2 binds specifically to the T2A region.

On the other hand, E3-P1 has a strong receptor-binding activity, comparable with native E3. Thus, the receptor recognition site of E3 is probably in the P1 region. However, our studies on several fragments allowed us to divide the P1 domain into two regions: the N-terminal head region and the BP region. As described above, the BP fragment is derived from the C-terminal part of P1. Thus, the region corresponding to this fragment is called the BP region. The rest of P1 is called the N-terminal head region, which is lost from protein A or P1 on the treatment with bromelain.

Since the BP fragment can inhibit the infection by colicin E3 or E2 of the sensitive cells as strongly as P1, it is suggested that the receptor-binding activity is located in the BP region. The same can be said for the structural analysis of the E2-P1 domain. The P1 or BP fragment of colicin E2 inhibits the infection by colicin E2 or E3. Moreover, as shown before, P1's of E2 and E3 or BP's of E2 and E3 are serologically identical. This agrees very well with the fact that colicins E2 and E3 share a common receptor.

As for the function of the N-terminal head region, we can assume that it is related to the transportation of colicins through the cell membranes. The Br-A fragment of E2 or E3, which carries both BP and T2A regions, has naturally nucleolytic and receptor-binding activities. However, it has no bacteriocidal activity. Probably this fragment can attach to the receptor but cannot penetrate the membranes because of the lack of the N-terminal head region. As described above, this N-terminal head region is quite rich in glycine and supposedly has a unique conformation. This conformation may have something to do with the penetration through the cell membranes. Tilby et al. (1978) suggested that the step of penetration through the cell wall of *E. coli* can be bypassed by osmotic shock treatment although the step of penetration through the cytoplasmic membrane remains normal. If the bromelain fragment can kill the cells under the conditions of osmotic shock, the function of the N-terminal head region will be more clearly seen.

Although P1 fragments can bind to the receptor normally, the T1 fragment, derived from P1, has completely lost this function. Thus, we may determine the nature of receptor recognition in more detail from the structural differnces between P1 and T1. Although we do not know the exact location of T1 in the P1 domain, T1 is probably very close to the N terminus for the following reasons. As shown in Table II, nearly all the glycine residues of P1 are involved in T1. We mentioned above that the N-terminal head region of P1 is quite rich in glycine. Thus, if an appreciable part of this head region is missing in T1, its glycine content should be lower. From these facts we can assume that the C-terminal portion of P1 (designated here as the P1 tail) is missing in T1 and this P1 tail plays a significant role in receptor binding. As shown in Table II (the last column), the P1 tail is quite rich in Asx, Glx, Ala, Lys, and Arg. It is reasonable to say that this portion will be easily lost on trypsinolysis. As mentioned above, the helical contents of P1 and T1 are 39 and 15%, respectively. Thus, it is conceivable that the P1 tail has a very high $\alpha$-helix content. It is true, according to Chou & Fasman (1977), that Ala, Lys, and Glx residues which are abundant in the P1 tail are strong $\alpha$-helix formers. Probably this ordered structure existing in the P1 tail region is important for the recognition of the receptor. However, it is also possible that the existence of the P1 tail transforms the conformation of the T1 region so as to be complementary to the receptor.

The fragments obtained here are useful tools to clarify the reaction steps of colicin action, further and in more detail.

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## References

Boon, T. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2421–2425.
Bowman, C. M., Dahlberg, J. E., Ikemura, T., Konisky, J., & Nomura, M. (1971a) *Proc. Natl. Acad. Sci. U.S.A. 68*, 964–968.
Bowman, C. M., Sidikaro, J., & Nomura, M. (1971b) *Nature (London), New Biol. 234*, 133–137.
Chou, P. Y., & Fasman, G. D. (1977) *Trends Biochem. Sci. 2*, 128–131.
De Graaf, F. K., Stukart, M. J., Boogerd, F. C., & Metselaar, K. (1978) *Biochemistry 17*, 1137–1142.
Gesteland, R. F. (1966) *J. Mol. Biol. 16*, 67–84.
Gray, W. R. (1972) *Methods Enzymol. 25*, 121–138.
Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108–4116.
Herschman, H. R., & Helinsky, D. R. (1967) *J. Biol. Chem. 242*, 5360–5368.
Hirose, A., Kumagai, J., & Imahori, K. (1976) *J. Biochem. (Tokyo) 79*, 305–311.
Jakes, K. S., & Zinder, N. D. (1974a) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3380–3384.
Jakes, K. S., & Zinder, N. D. (1974b) *J. Biol. Chem. 249*, 438–444.
Konisky, J., & Nomura, M. (1967) *J. Mol. Biol. 26*, 181–195.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lau, C., & Richards, F. M. (1976) *Biochemistry 15*, 3856–3863.
Nomura, M. (1963) *Cold Spring Harbor Symp. Quant. Biol. 28*, 315–324.
Ohno, S., & Imahori, K. (1978) *J. Biochem. (Tokyo) 84*, 1637–1640.
Ohno, S., Ohno-Iwashita, Y., Suzuki, K., & Imahori, K. (1977) *J. Biochem. (Tokyo) 82*, 1045–1053.
Ohno-Iwashita, Y., & Imahori, K. (1977) *J. Biochem. (Tokyo) 82*, 919–922.
Ohno-Iwashita, Y., & Imahori, K. (1979) *FEBS Lett. 100*, 249–252.
Ohsumi, Y., & Imahori, K. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4062–4066.
Sabet, S. F., & Schnaitman, C. A. (1973) *J. Biol. Chem. 248*, 1797–1806.
Saxe, L. S. (1975a) *Biochemistry 14*, 2051–2057.
Saxe, L. S. (1975b) *Biochemistry 14*, 2058–2063.
Schaller, K., & Nomura, M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3989–3993.
Senior, B. W., & Holland, I. B. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 959–963.
Sidikaro, J., & Nomura, M. (1974) *J. Biol. Chem. 249*, 445–453.
Suzuki, K., & Imahori, K. (1978a) *J. Biochem. (Tokyo) 84*, 1021–1029.

Suzuki, K., & Imahori, K. (1978b) J. Biochem. (Tokyo) 84, 1031–1039.
Tilby, M., Hindennach, I., & Henning, U. (1978) J. Bacteriol. 136, 1189–1191.
Weber, K., & Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
Yamamoto, H., Nishida, K., Beppu, T., & Arima, K. (1978) J. Biochem. (Tokyo) 83, 827–834.

# Amino Acid Sequence of the Light Chain of Bovine Factor $X_1$ (Stuart Factor)†

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ABSTRACT: The detailed proof of the amino acid sequence of the 140 residues (16 193 daltons) of the light chain of bovine factor $X_1$ (Stuart factor) is presented. Sequence analyses were performed on fragments obtained after chemical cleavage of asparagine–glycine and tryptophanyl peptide bonds and after various enzymatic digestion procedures. Twelve γ-carboxyglutamyl residues are clustered in the amino-terminal 39 residues and 13 half-cystine residues are found in the carboxyl-terminal 91 residues, suggesting two domains in the light chain, one exceptionally anionic and the other extensively cross-linked by disulfides.

Factor X (Stuart factor) is the zymogen of a protease which participates in the middle phase of a series of activation reactions which regulate blood coagulation (Davie & Fujikawa, 1975). The zymogen is activated by factor $IX_a$ in the presence of calcium, phospholipid, and factor VIII (Lundblad & Davie, 1964, 1965; Biggs & MacFarlane, 1965; Hougie et al., 1967; Barton, 1967; Osterud & Rapaport, 1970; Fujikawa et al., 1974b) in the intrinsic pathway or by a tissue factor and activated factor VII (Williams & Norris, 1966; Nemerson & Pitlick, 1970; Osterud et al., 1972; Jesty & Nemerson, 1974; Fujikawa et al., 1974b) in the extrinsic pathway. It can also be activated under nonphysiological conditions by various proteases such as trypsin and a protease from Russell's viper venom (RVV-X) (Fujikawa et al., 1972b). Together with factor V, calcium, and phospholipid, factor $X_a$ catalyzes the conversion of prothrombin to thrombin (Papahadjopoulos & Hanahan, 1964; Barton et al., 1967; Jobin & Esnouf, 1967; Jesty & Esnouf, 1973; Suttie & Jackson, 1977).

Factor X is a glycoprotein of molecular weight 55 000, consisting of a light chain having a molecular weight of 17 000 and a heavy chain having a molecular weight of 38 000 (Fujikawa et al., 1972a; Jackson, 1972). These chains are linked by a single disulfide bond (Titani et al., 1975). Bovine factor X can be fractionated chromatographically into two components (factors $X_1$ and $X_2$) possessing similar chemical and biological properties (Jackson & Hanahan, 1968). The protein contains ~10% carbohydrate associated with the heavy chain. During activation in the absence of phospholipid, a single peptide bond is cleaved from the amino terminus of the heavy chain, releasing a large glycopeptide (Fujikawa et al., 1972b, 1974a; Radcliffe & Barton, 1973; Jesty et al., 1974). During activation in the presence of phospholipid, another glycopeptide is also released from the carboxyl terminus of the heavy chain (Jesty et al., 1974), but the cleavage of the second peptide bond is unrelated to the activation process (Fujikawa et al., 1975). The heavy chain of activated factor $X_{1a}$ is homologous to trypsin and other mammalian serine proteases, including the B chain of thrombin and the heavy chain of factor $IX_a$ (Titani et al., 1972, 1975; Enfield et al., 1974).

The light chain of factor X has no counterpart in the zymogens of the pancreatic serine proteases. A preliminary analysis of its structure revealed that the amino-terminal region is homologous to the corresponding regions of prothrombin, factors VII and IX, protein C and protein S, the other vitamin K dependent plasma proteins (Fujikawa et al., 1974a; Kisiel & Davie, 1975; Fernlund et al., 1978; Stenflo & Jonsson, 1979). The 12 residues of γ-carboxyglutamic acid, an amino acid first identified in prothrombin (Stenflo et al., 1974; Nelsestuen et al., 1974; Magnusson et al., 1974), are located in the amino-terminal portion of the light chain of factor X (Bucher et al., 1976; Thorgersen et al., 1978).

This communication presents the detailed proof of the amino acid sequence of the light chain of factor $X_1$, as determined largely by automated Edman degradation of fragments in a sequenator. A preliminary account of this work has been published (Enfield et al., 1975).

## Materials and Methods

TPCK-trypsin, α-chymotrypsin, and carboxypeptidases A and B were obtained from Worthington. Before use, chymotrypsin was treated with α-*N*-tosyllysine chloromethyl ketone to inactivate trypsin. Thermolysin was a product of Calbiochem. Aminopeptidase M was purchased from Rohm and Haas. A glutamyl-specific protease from *Staphylococcus aureus* was a gift from Dr. Gabriel Drapeau (University of Montreal). 4-Vinylpyridine monomer (practical grade) from J. T. Baker or Aldrich was further purified by vacuum distillation and stored at −20 °C in the dark. Reagent grade pyridine was redistilled from ninhydrin. Glycinamide hydrochloride was purchased from Sigma, and its purity was confirmed by high-voltage paper electrophoresis at pH 3.75. Hydroxylamine hydrochloride was from Merck and guanidine

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