Physical, Chemical and Physiological Properties of S-CM Subunits of Ricin D[†]

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Received April 8, 1975

Some physical, chemical and physiological properties of two S-CM subunits (S-CM Ile and S-CM Ala chain) of ricin D were studied. Physical properties of subunits are summarized in Table I. The S-CM subunits consisted of 244 (S-CM Ile chain) and 254 (S-CM Ala chain) amino acid residues. By the specific cleavage of the single intermolecular disulfide bond of ricin D, no remarkable change in conformation of polypeptide chains of ricin D was detected, but the toxicity was markedly decreased. The toxicity of ricin D is due to the quaternary structure of the ricin D molecule.

In a preceding paper,¹⁾ ricin D, a toxic glycoprotein from castor bean seeds (*Ricinus communis* L.), was shown to consist of two polypeptide chains, linked by a single disulfide bond. These polypeptide chains have been denominated as isoleucyl (Ile) and alanyl (Ala) chains based on the fact that one polypeptide chain has isoleucine and the other alanine as its amino terminal amino acid, respectively. The specific cleavage of the single disulfide bond in the absence of urea by reduction with β -mercaptoethanol is accomplished followed by the masking of the resulting sulfhydryl groups with monoiodoacetic acid. The separation of the reduced and carboxymethylated subunits by DEAE-cellulose column chromatography have been reported.¹⁾

The present paper deals with physical, chemical and physiological properties of isolated subunits.

MATERIALS AND METHODS

Materials. Ricin D used throughout this study was purified as described in the preceding paper.²⁾ S-Carboxymethylated isoleucyl (S-CM-Ile) and S-

carboxymethylated alanyl (S-CM-Ala) chains were prepared according to the procedures described in the previous paper.¹⁾ Furthermore S-CM-Ile and S-CM-Ala chains were rechromatographed separately through DEAE-cellulose column equilibrated with 0.005 M Tris-HCl buffer of pH 8.5 and developed by linear gradient elution with the same buffer using increasing sodium chloride concentration from zero to 0.2 M. All the reagents used in the present work were of analytical grade.

Polyacrylamide gel disc electrophoresis. The lyophilized preparations were subjected to disc electrophoresis in polyacrylamide gels prepared according to the method of Orstein.³⁾ Protein bands were detected by staining with Amide Black 10 B, followed by removing unbound dye with several changes of 7% acetic acid (v/v).

Ampholine electrophoresis. Isoelectric points, as well as homogeneity, were determined by the electrofocusing technique⁴⁾ with a LKB 8101 electrofocusing column. A lyophilized S-CM-Ile chain (10 mg) was applied to ampholine electrophoresis in 0.8% carrier ampholytes (pH 5~8) and S-CM-Ala chain (10 mg) was subjected to ampholine electrophoresis in 0.8% carrier ampholytes (pH 3~6). The other procedures employed in the present work were the same as those described in a preceding paper.²⁾

Sedimentation analysis. Sedimentation experiments were performed with a Spinco Model E Ultracentrifuge at 20°C. All runs were done at a speed of 56,100 rpm. The protein concentration utilized were 0.5% for S-CM-Ile chain and 1.0% for S-CM-Ala chain.

[†] Biochemical Studies on Ricin. Part VI. Previous paper: K. Hara, M. Ishiguro, G. Funatsu and M. Funatsu, *Agr. Biol. Chem.*, **38**, 65 (1974).

Determination of molecular weight. Determination of molecular weight of subunits were carried out by Archibald's method⁵⁾ and polyacrylamide gel sodium dodecyl sulfate (SDS) disc electrophoresis.⁶⁾ For Archibald's method, 0.5% solution of S-CM-Ile chain was dialyzed against 0.01 M acetate buffer of pH 4.5 and 1.0% solution of S-CM-Ala chain was dialyzed against 0.01 M phosphate buffer of pH 6.5. Molecular weight was calculated as reported earlier.⁶⁾ The procedures of polyacrylamide gel SDS electrophoresis for the determination of molecular weight were the same as those described in a preceding report.²⁾ The marker proteins used for the determination of molecular weight were purchased from Boeringer Mannheim GmbH (Germany).

Ultraviolet absorption. The ultraviolet absorption spectra were determined with a Cary Model 14 Recording Spectrophotometer. The protein concentrations were 0.23% S-CM-Ile chain and 0.05% S-CM-Ala chain in 0.005 M Tris-HCl buffer of pH 8.5.

Optical rotatory dispersion measurement. Optical rotatory dispersion was measured with JASCO ORD/UV-5 spectropolarimeter between 600 nm and 300 nm. Parameters, a_0 and b_0 , as well as helical content were calculated according to a Moffit-Yang plot⁷⁾ as reported earlier.²⁾

Amino acid analysis. All procedures were as described in the previous paper.⁸⁾ For the amino acid analysis, 1.6 mg freeze dried protein sample was hydrolyzed with 5.8 N HCl in an evacuated sealed glass tube at 106°C for 24, 48, and 72 hr, respectively, and was analyzed with a Hitachi Amino Acid Analyzer Model KLA-3B. Additionally, the tryptophan content of the protein was determined spectrophotometrically by Goodwin-Morton's method.⁹⁾

Amino-terminal amino acid analysis. Dinitrophenylation of the protein of 6.0 mg (0.2 μ moles) was carried out for 5 hr under the conditions reported by Sanger.¹⁰⁾ All procedures were as described in the previous paper.⁸⁾ After the DNP-protein was collected, it was hydrolyzed with 2.0 ml of 5.8 N HCl at 105°C for 14 hr in a sealed glass tube. The ethylether soluble fraction of the hydrolyzate was identified by means of two-dimensional chromatography on Toyo Roshi No. 51 paper in an upper layer of *n*-butanol, 0.2% ammonia water (1:1) and 1.5 M phosphate buffer at pH 6.0. Each spot on a chromatogram was cut out, and DNP-derivatives were extracted with 4 ml of 1% sodium bicarbonate for 15 min at 55°C. The optical densities of the extracts were measured at 360 and 390 nm. The water-soluble fraction of the hydrolyzate was applied to a talc column and DNP-amino acids adsorbed on the column were eluted with an ethylalcohol-1 N HCl (4:1) mixture.¹¹⁾ DNP-Amino acids were identified by one-way ascend-

ing chromatography on Toyo Roshi No. 51 paper in an upper layer of *n*-butanol-acetic acid-water (4:1:5). After development, DNP-arginine was detected on the paper with the Sakaguchi spray reagent.¹²⁾

Determination of the lethal toxicity. Pure-bred female mice (ddN) weighing 20 grams were used to determine the lethal toxicity. A sample solution, diluted appropriately with physiological saline, was injected intraperitoneally and the results were observed at intervals of 24 hr. The minimum lethal dose at 48 hr (MLD₄₈) was adopted as a measure of toxicity and was expressed as μ g protein nitrogen per gram body weight of mouse.

RESULTS AND DISCUSSION

A. Physical properties

Polyacrylamide gel disc electrophoresis.

When S-CM-Ile and S-CM-Ala chains were subjected to disc electrophoresis in 7.5% polyacrylamide gel at pH 8.3, it migrated as a defined band, as shown in Fig. 1. The band of S-CM-Ala chain moved faster than that of S-CM-Ile chain.

Ampholine electrophoresis. The purified S-CM-Ile and S-CM-Ala chains were sub-

FIG. 1. Polyacrylamide Gel Disc Electrophoretic Patterns of S-CM-Ile and S-CM-Ala Chains.

Electrophoresis was performed with 2 mA per tube for 2.5 hr.

FIG. 2. Ampholine Electrophoresis of S-CM-Ile (A) and S-CM-Ala Chains (B). The dotted line represents the pH gradient. The solid line represents absorbance at 280 nm (1 cm cell).

FIG. 3. Sedimentation Patterns of S-CM-Ile (A) and S-CM-Ala Chains (B).

Sedimentation is from right to left in photographs taken at indicated times after reaching 56,100 rpm.

jected to ampholine electrophoresis for the determination of isoelectric points. A single peak was obtained at pH 7.42 (S-CM-Ile chain) and pH 5.17 (S-CM-Ala chain) as shown in Fig. 2. The experimental results obtained indicate that the S-CM-Ile and S-CM-Ala chains are homogeneous. The S-CM-Ile chain is a basic protein with an isoelectric

point of 7.42 and the S-CM-Ala chain is an acidic protein with an isoelectric point of 5.17.

Sedimentation analysis. The protein solution was ultracentrifuged with a Spinco Model E Ultracentrifuge operating at 56,100 rpm at 20.0°C. The sedimentation patterns are shown in Fig. 3. S-CM-Ile and S-CM-

Ala chains were homogeneous with a symmetric sedimenting boundary. The sedimentation coefficients, $s_{20,w}$ of S-CM-Ile and S-CM-Ala chains were calculated to be 3.45S and 3.52S according to the methods of Schachman¹³⁾ and Svedberg.¹⁴⁾ Both chains have very similar values.

Determination of molecular weight. The values of $(dc/dx)m/Xm \cdot Cm$ and $(dc/dx)b/Xb \cdot Cb$, which were estimated from boundary photographs, were plotted against time after reaching constant revolution and extrapolated to zero time. From the ordinate intercept, the values of $\{(dc/dx)m/Xm \cdot Cm\}t=0$ and $\{(dc/dx)b/Xb \cdot Cb\}t=0$ were found to be 0.672 and 0.562 for the S-CM-Ile chain and 0.655 and 0.582 for the S-CM-Ala chain, respectively. The molecular weight of S-CM-Ile chain was calculated to be 3.32×10^4 at the meniscus and 2.77×10^4 at the bottom of the cell with an average value of 30,400. The molecular weight of S-CM-Ala chain was calculated to be 3.24×10^4 at the meniscus and 2.88×10^4 at the bottom of the cell with an average value of 30,600.

S-CM-Ile and S-CM-Ala chains were

also subjected to 10% cross-linked gel SDS disc electrophoresis for molecular weight determination. The marker proteins were cytochrome c, chymotrypsinogen A, aldolase, hen egg albumin and bovine serum albumin. The molecular weights of S-CM-Ile and S-CM-Ala chains were determined to be 30,000 (Fig. 4). From the mobility, the molecular weight of S-CM-Ala chain was slightly larger than that of S-CM-Ile chain. These results agree with those of S. Olsnes and A. Pihl.¹⁵⁾ The molecular weights determined by SDS disc electrophoresis were in good agreement with the molecular weights determined by Archibald's method.

Ultraviolet absorption. Typical absorption spectra of S-CM-Ile and S-CM-Ala chains are shown in Fig. 5; the maximum and the minimum absorptions are found at 280 nm and 251 nm for the S-CM-Ile chain and at 280 nm and 250 nm for the S-CM-Ala chain, respectively.

Optical rotatory dispersion. ORD constant, λ_c , was estimated from the slope of the Yang plot shown in Fig. 6. A Moffitt-Yang plot for calculating the Moffitt-Yang parameters, a_0 and b_0 is shown in Fig. 7. In this plot, the value of a_0 is obtained from the inter-

FIG. 4. Determination of the Molecular Weight of S-CM-Ile and S-CM-Ala Chains by Disc Electrophoresis in Sodium Dodecyl Sulfate.

FIG. 5. Ultraviolet Absorption Spectra of S-CM-Ile and S-CM-Ala Chains (pH 8.5, 0.005 M Tris-HCl buffer).

—, S-CM-Ile chain; ----, S-CM-Ala chain.

FIG. 6. Yang Plot of S-CM-Ile and S-CM-Ala Chain.

The concentration of the S-CM-Ile chain was 0.5% in 0.005M Tris-HCl buffer at pH 8.5 and the S-CM-Ala chain was 1.0% in deionized water. The temperature was 13.0°C.

-----, S-CM-Ile chain; —, S-CM-Ala chain.

FIG. 7. Moffit-Yang Plot of S-CM-Ile and S-CM-Ala Chains.

The protein concentrations were as described in Fig. 6.

-----, S-CM-Ile chain; —, S-CM-Ala chain.

cept and the value of b_0 from the slope. In the case of S-CM-Ile chain, the value of λ_c , a_0 and b_0 were 249 nm, -134 and -2, respectively and for the S-CM-Ala chain were 204 nm, -133 and -158, respectively. The α -helix content of the S-CM-Ile and S-CM-Ala chains were calculated to be around 0.3 and 25.0% from the values of λ_c and b_0 . The value of b_0 was markedly different between the two subunits. This may be mainly attributable to the difference in helical content. The helical content

of ricin D was 10.5%.²⁾ In addition, the values of a_0 for ricin D and the subunits were almost identical, indicating that the conformation of polypeptide chains in ricin D molecule was not markedly affected by dissociation to subunits.

Physical properties of the subunits compared with ricin D^{2,6)} are summarized in Table I.

TABLE I. PHYSICAL PROPERTIES OF RICIN D AND S-CM-SUBUNITS OF RICIN D

	Ricin D ^{2,6)}	S-CM-Ile chain	S-CM-Ala chain
UV-absorption spectra			
max. nm	280	280	280
min. nm	251	251	250
E_{max}/E_{min}	2.32	2.09	2.38
Optical rotation			
a_0 (deg)	-138	-134	-133
b_0 (deg)	-66	-2	-158
Helical content assumed (%)			
(by b_0)	10.5	0.3	25.0
(by λ_c)	16.4	0.7	26.4
Ultracentrifugation			
Sedimentation			
constant	4.64S	3.45S	3.52S
Molecular weight			
	60,000	30,000	30,000
Molecular weight by SDS			
polyacrylamide gel electrophoresis		30,000	30,000
Ampholine electrophoresis			
Isoelectric point	7.34	7.42	5.17

B. Chemical properties

Amino acid composition. The amino acid compositions of S-CM-Ile and S-CM-Ala chains are given in Table II. The measured values of threonine, serine and methionine were plotted against time of hydrolysis and extrapolated to zero time to give the corrected values. The values of 72 hr hydrolyzates were adopted for valine and isoleucine. Tryptophan was determined spectrophotometrically. The average values of other amino acids are also listed. The amino acid recovery of the S-CM-Ile and S-CM-Ala chains were estimated to be 95.37 and 93.80%, respectively.

Since the calculated molecular weight from histidine, S-CM-Ile chain (28,457) and S-CM-Ala chain (31,201), approximated the molecular weight of 30,000 determined according to Archibald's method and polyacrylamide gel SDS disc electrophoresis. The total numbers of amino acid residues in the S-CM-Ile and S-CM-Ala chains were estimated to

be 244 and 254, respectively.

The amino-terminal amino acid residues. The results of two dimensional paper chromatography, for S-CM-Ile chain, DNP-Ile and one minor DNP-amino acids are shown in Fig. 8 (A). In the case of the S-CM-Ala chain, DNP-Ala was found as shown in Fig. 8 (B). DNP-Arg, however, was not detected on paper with the Sakaguchi spray reagent. The results of amino-terminal amino acid analyses are summarized in Table III. From these results, isoleucine and alanine were concluded to be the amino-terminal amino acids of S-CM-Ile and S-CM-Ala chains, respectively.

TABLE II. AMINO ACID COMPOSITION OF S-CM-Ile AND S-CM-Ala CHAINS

Amino acid residues	Number of amino acid residues	
	S-CM-Ile chain	S-CM-Ala chain
Aspartic acid	24	35
Threonine	16	19
Serine	17	19
Glutamic acid	27	22
Proline	14	13
Glycine	16	21
Alanine	22	18
Half cystine	+	4
Valine	13	15
Methionine	3	3
Isoleucine	20	17
Leucine	21	24
Tyrosine	12	9
Phenylalanine	12	6
Lysine	3	7
Histidine	3	3
Arginine	18	14
Tryptophan	2	6
S-CM-Cysteine	1	1

TABLE III. AMINO-TERMINAL AMINO ACIDS ANALYSIS OF S-CM-Ile AND S-CM-Ala CHAINS

Sample	DNP-amino acid	Total O.D. ₃₆₀	μ moles per 0.2 μ moles ^{a1} of protein	Recovery (%)
S-CM-Ile chain	DNP-Ile	0.460	0.104	52.0
	minor spot	0.156		
S-CM-Ala chain	DNP-Ala	0.500	0.116	58.0

^{a1} Calculated from molecular weight 30,000.

FIG. 8. Paper Chromatograms of DNP-Amino Acids for Determination of Amino-terminal Amino Acids of S-CM-Ile (A) and S-CM-Ala Chains (B).

Intensity of spots: ○, medium; ◌, weak.

TABLE IV. TOXICITY OF S-CM-Ile AND S-CM-Ala CHAINS

Sample	Dose ($\mu\text{g}\cdot\text{N/g}$)	24 hr	48 hr	72 hr	MLD ₄₈ ($\mu\text{g}\cdot\text{N/g}$)
S-CM-Ile chain	0.7591	10/10 ^{a)}			0.076
	0.0759	0/10	5/10	7/10	
	0.0380	0/10	0/10	0/10	
S-CM-Ala chain	0.4048	0/10	9/10	10/10	0.300
	0.2024	0/10	3/10	7/10	
	0.0810	0/10	0/10	2/10	
0.85% NaCl		0/10	0/10	0/10	

^{a)} X/Y : X and Y represent the number of mouse that died and mouse used for experiment, respectively.

C. Physiological properties

The minimal lethal dose of the S-CM-Ile and S-CM-Ala chains are shown in Table IV. The MLD₄₈ of the separated subunits were found to be about 80 times as large as that of ricin D (MLD₄₈ of ricin D is 0.001 μg protein nitrogen per gram body weight of mouse.⁶⁾). The relative toxicities of ricin D, S-CM-Ile and S-CM-Ala chains were estimated to be 100, 1.5 and 0.3, respectively. It was also found that the separated subunits could be easily digested by trypsin although ricin D is not easily attacked by this proteolytic enzyme.¹⁶⁾ These observations, decrease in toxicity and increase in susceptibility toward trypsin by the cleavage of the single disulfide bond, indicate that the toxicity of ricin D can be ascribed to the quaternary structure of the ricin D molecule. Namely, a certain conformational change should occur with the combination of two subunits forming a quaternary structure of ricin D molecule.

Acknowledgements. The authors are indebted to Dr. Victor G. Glushko for his comments.

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