

A GROSS STRUCTURE OF AN ACTIVATED FORM OF A SUBUNIT OF THE FIRST COMPONENT OF HUMAN COMPLEMENT. C1 \bar{r}

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1. Introduction

The classical complement reaction is known to be triggered by activation of C1, which is a Ca²⁺-dependent complex of C1q, C1r and C1s subunits [1]. C1q is a collagen-like protein having a binding site for immune complexes [2,3]. C1r and C1s are zymogens from which trypsin-like proteases, C1 \bar{r} and C1 \bar{s} , are activated, respectively [4]. The activation of C1r is a key reaction for initiation of the classical complement reaction, since the active form, C1 \bar{r} , is an intrinsic activator for C1s [5], and the activated C1 \bar{s} , in turn, attacks C4 and C2 to initiate immune reactions [6].

Recently, the activation of C1s by C1 \bar{r} has been shown to be a proteolytic process [7,9], as is the case of activation of other plasma zymogens in blood clotting and fibrinolysis systems [10]. However, extraordinary instability of C1r and C1 \bar{r} has made their purifications extremely difficult and, therefore, little is known on the molecular change accompanying the conversion of C1r into C1 \bar{r} .

In previous papers, we reported simple methods for isolation of C1 \bar{r} -C1 \bar{s} complex [11] and C1 \bar{s} [9]

from human plasma, using polyethylene glycol fractionation and affinity chromatography with IgG-Sepharose. In the present study, a simple method has been designed for isolation of C1 \bar{r} from human plasma and some polypeptide chain structure of C1 \bar{r} has been investigated.

It will be shown that the polypeptide chain structure of C1 \bar{r} is very similar to that of C1 \bar{s} ; C1 \bar{r} also consists of two chains, heavy and light chains, connected covalently by a disulfide bridge and the DFP-reactive active site of C1 \bar{r} locates on the light chain portion, which has Ile as its NH₂-terminal amino acid.

Based on the similarity of gross structure of C1 \bar{r} to that of C1 \bar{s} , it is proposed that the activation of C1 \bar{r} should also involve some proteolytic process, such as is observed with the activation of C1s.

2. Experimental

CDP-human plasma was obtained from the Blood Bank of the Japanese Red Cross, Hokkaido, Japan. Defibrinated C1 fraction was prepared with polyethylene glycol (#4000) from human plasma as described in a previous paper [11]. IgG-Sepharose 6B was prepared according to the modified method of Assimah et al. [12]. C1 \bar{r} activity was determined by its ability to hydrolyze synthetic amino acid ester or to convert C1s into C1 \bar{s} as described in a previous paper [11]. The esterase activity of C1 \bar{s} thus activated by C1 \bar{r} was determined with AGLME as the substrate by the hydroxamate method [13]. C1s used was purified by a slight modification [9] of the method of Sakai and Stroud [7] from human plasma.

Polyacrylamide gel electrophoresis was performed

Abbreviations: The symbols for complement components used in this paper conform to the recommendations of the World Health Organization Committee on Complement Nomenclature; *Immunochemistry*, (1970) 7, 137-142. Activated components were indicated by placing a bar over the numeral which refers to the active component or subunit. Other abbreviations used are: CPD, citrate-phosphate dextrose; AAME, *N*- α -acetyl-L-arginine methylester; AGLME, acetylglycyl-L-lysine methylester; ATEE, *N*- α -acetyl-L-tyrosine ethylester; DFP, diisopropylfluorophosphate; SDS, sodium dodecylsulfate; EDTA, ethylenediamine tetraacetate.

in the presence or absence of SDS, according to the methods of Weber and Osborn [14] and Davis [15], respectively. The molecular weights of polypeptide chains were estimated from their electrophoretic mobilities on SDS-gel with bovine serum albumin (monomer, dimer and trimer, Sigma Chemical Co., USA) and soybean trypsin inhibitor (Sigma Chemical Co., USA) as standard proteins of known molecular weight.

Preparation and NH_2 -terminal determination of two polypeptide chains of $\text{C1}\bar{\text{r}}$ were performed essentially according to the method of Weiner et al. [16]. Dansyl amino acids were identified by thin layer chromatography with polyamide sheet (Chen Chin Co.), using solvent systems reported [16,17].

DFP-sensitive active site of $\text{C1}\bar{\text{r}}$ was labelled with [^{32}P] DFP (Radiochemical Centre, Amersham, England) as follows; about 1 mg of $\text{C1}\bar{\text{r}}$ in 20 ml of 0.1 M Tris-HCl buffer, pH 8.5 and 0.4 mCi of [^{32}P] DFP (0.328 mCi/mg) in 0.4 ml of ethylene glycol were incubated at 37°C for 1 hr, dialyzed exhaustively against 0.1 M acetic acid and lyophilized. The lyophilized ^{32}P -labelled $\text{C1}\bar{\text{r}}$ was dissolved in 1 ml of 0.2 M phosphate buffer, pH 7.3 and electrophoresed on SDS-polyacrylamide gels before and after the treatment with 0.1 M 2-mercaptoethanol. The gels were stained with Coomassie blue and sectioned into 2mm width. The radioactivity in sectioned gels was determined with a liquid scintillation counter in toluene scintillator.

3. Results

3.1. Purification of $\text{C1}\bar{\text{r}}$

The various steps in purification of human $\text{C1}\bar{\text{r}}$ are shown in table 1. As reported in a previous paper [11], Ca^{2+} -dependent $\text{C1}\bar{\text{r}}-\text{C1}\bar{\text{s}}$ complex was isolated from human plasma by polyethyleneglycol fractionation followed by affinity chromatography on IgG-Sepharose 6B, and then chromatographed on DEAE-cellulose (DE-52) after the treatment with EDTA. As shown in fig.1, two protein peaks were eluted by increasing NaCl concentration; the $\text{C1}\bar{\text{s}}$ activator activity of $\text{C1}\bar{\text{r}}$ is associated with the first peak and ATEE esterase activity of $\text{C1}\bar{\text{s}}$ with the second peak.

The $\text{C1}\bar{\text{r}}$ fraction thus obtained gave a single band on polyacrylamide gel electrophoresis (fig.2a). The purification was about 160-fold with an overall yield of 36%, relative to the first precipitate with polyethylene glycol.

3.2. Polypeptide chains of $\text{C1}\bar{\text{r}}$

Non-reduced $\text{C1}\bar{\text{r}}$ gave a single stained band on SDS-polyacrylamide gel electrophoresis and its molecular weight was estimated to be 115 000 from its electrophoretic mobility (fig.2b). Upon the treatment with 0.1 M 2-mercaptoethanol, $\text{C1}\bar{\text{r}}$ dissociated into two components, the molecular weights of which were estimated to be 68 000 and 47 000, respectively (fig.2c).

Table 1
Purification of $\text{C1}\bar{\text{r}}$ from human plasma

Purification step	Total volume (ml)	Total protein ($A_{280\text{nm}}$)	Total activity ^a (units)	Specific activity (units/ $A_{280\text{nm}}$)
Plasma	300	22 000	nd	
Polyethylene glycol precipitate	60	840	100	0.12
Defibrinated fraction	60	210	92	0.44
IgG-Sepharose eluate	35	16.3	70	4.3
DEAE-cellulose eluate	60	2.00	32	19.0

^a One unit of $\text{C1}\bar{\text{r}}$ was tentatively defined at the amount of activity capable of activating 1 unit of $\text{C1}\bar{\text{s}}$ for 30 min. One unit of $\text{C1}\bar{\text{s}}$ esterase is the amount of enzyme capable of hydrolyzing 1 μmol of AGLME per min at 37°C . The value was corrected for the AGLME esterase activity of $\text{C1}\bar{\text{r}}$ fraction alone.

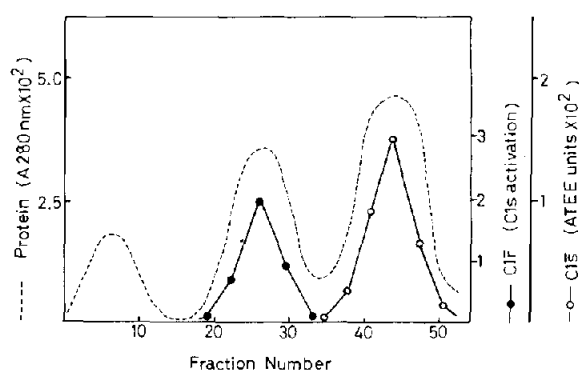


Fig. 1. Purification of C1f. C1f-C1s complex which was obtained by affinity chromatography with IgG-Sepharose 6B of polyethylene glycol-precipitate of human plasma [11] was dialyzed against 0.02 M phosphate buffer, pH 7.4, containing 5mM EDTA overnight and applied to a column (2.0 x 4.0 cm) of DEAE-cellulose (DF-52) which had been equilibrated with the dialysis buffer. After washing the column with the phosphate buffer, the column was eluted by linear gradient increase of NaCl concentration with each 200 ml of 0 and 0.25 M NaCl in the phosphate buffer. Fractions of each 6 ml were collected at the cold room. The C1f activity was determined by measuring C1s activator activity. Fractions 21-30 were pooled and used as the purified C1f.

These results indicated that C1f comprises two polypeptide chains, connected with one or more disulfide bridges, such as is the case of human C1s [7,9].

3.3. Incorporation of [³²P]DFP into C1f

The esterase activity of purified C1f was inhibited by incubation with DFP. As shown in fig.3, decrease of the esterase activity of C1f by DFP obeyed good first-order kinetics, suggesting that C1f belongs to a DFP-sensitive serine esterase. When C1f was incubated with ³²P-labelled DFP and electrophoresed on SDS-polyacrylamide gel, the radioactivity was found to be associated with the stained band of C1f (fig.4). When the radioactive C1f was treated with 0.1 M 2-mercaptoethanol and electrophoresed on SDS-polyacrylamide gel, the incorporated radioactivity was found to be associated with the stained band of light chain portion of C1f, indicating that the DFP-sensitive active site of C1f locates on the light chain portion, as is the case of human C1s [18].

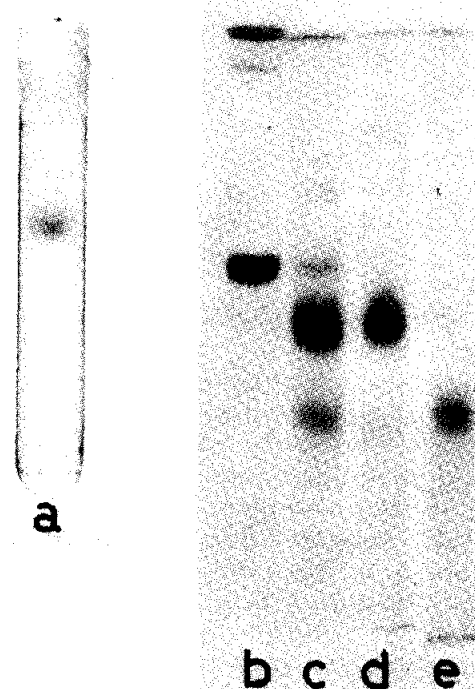


Fig. 2. Polyacrylamide gel electrophoresis. (a) Purified C1f in 5% polyacrylamide disc gel with Tris-glycine buffer, pH 8.6 [15]. (b) Purified C1f. (c) 0.1 M 2-mercaptoethanol-treated C1f and the isolated heavy (d) and light (e) chains of C1f in 5% polyacrylamide gels with 0.2% SDS and 0.2 M phosphate buffer, pH. 7.3 [14].

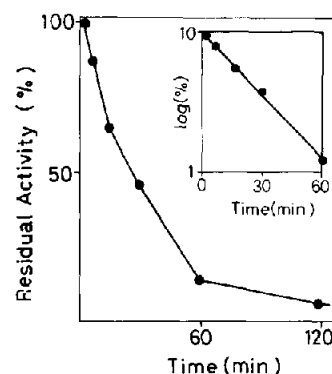


Fig. 3. Inhibition of C1f by DFP. C1f (about 0.2 mg) in 10 ml of 0.1 M Tris-HCl buffer, pH 8.5 was incubated with 0.25 mM of DFP at 37°C. After the time indicated, 1 ml of the reaction mixture was withdrawn and the remaining esterase activity of C1f was determined with 5 μmol of AAME as substrate. The remaining esterase activity was expressed as the per cent to the esterase activity of C1f not treated with DFP.

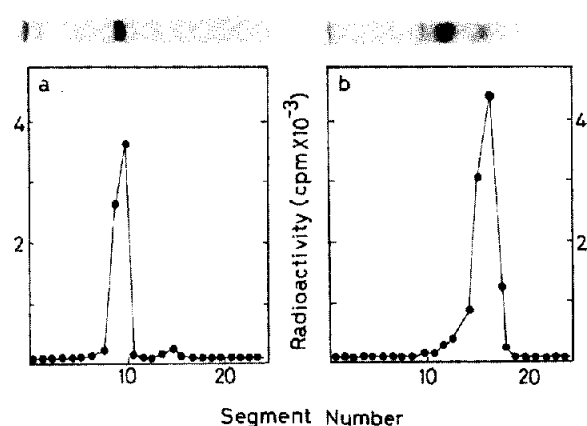


Fig.4. SDS-polyacrylamide gel electrophoresis of [^{32}P] DFP-treated C1r. [^{32}P] DFP-treated C1r was electrophoresed in 5% polyacrylamide gel with 0.2% SDS and 0.2 M phosphate buffer, pH 7.3 before (a) and after (b) treatment with 0.1 M 2-mercaptoethanol. The gels were stained with Coomassie blue, sectioned into each 2 mm width and counted for the distribution of radioactive [^{32}P] DFP as described in Experimental.

3.4. The NH_2 -terminal amino acids of C1r

The two polypeptide chains of C1r were isolated by analytical scale SDS-polyacrylamide gel electrophoresis of 2-mercaptoethanol-treated C1r (fig.2d and 2e).

By SDS-dansyl microtechnique [16], isoleucine was identified as the NH_2 -terminal amino acid of both heavy and light chains of human C1r.

4. Discussion

As mentioned by de Bracco and Stroud [9], the extraordinary instability of C1r has made its purification and structural studies extremely difficult. The present purification procedure is reproducible and can be done only in four days with a good recovery.

In a previous paper [9], we reported that human C1r cleaves C1s into heavy and light chains; the heavy chain of C1s is derived from the NH_2 -terminal portion of the zymogen molecule and the light chain from the COOH -terminal portion. In addition, the four NH_2 -terminal amino acid sequences of the light chain of C1s was found to be homologous to those of other serine proteases in plasma.

The two chain structure of C1r appears to be very similar to that of C1s; (1) the molecular weight of heavy chain of C1r is the same as that of heavy chain of C1s, (2) the DFP-sensitive active sites of C1r and C1s locate on the light chain portions of each enzyme and (3) Ile is the NH_2 -terminal amino acid of light chain of C1r as well as C1s. An apparent difference between C1r and C1s was that the molecular weight of light chain of C1r is larger than that of light chain of C1s by about 10 000.

These structural similarities between C1r and C1s may suggest that the mechanism of activation of C1r is similar to that of C1s; C1r is present as a single polypeptide chain and is proteolytically converted into active C1r composed of two chains.

Recently, Valet and Cooper [20] have reported the isolation of C1r zymogen from human serum, but it still remains to be solved whether C1r zymogen is composed of a single polypeptide chain or two polypeptide chains connected by disulfide bridge(s).

If the activation of C1r is a proteolytic process, as proposed in this paper, it is a subject of interest what is the activating enzyme for C1r. Recently, it is proposed that during zymogen activation, a latent enzymatic activity becomes very greatly enhanced rather than being generated de novo [21]. In supporting this model, McClintock and Bell presented evidence indicating that in the case of activation of plasminogen by streptokinase, streptokinase first induces a reversible conformational exposure of the latent active site in plasminogen molecule which may subsequently be permanently exposed by a spontaneous peptide bond cleavage [22].

So, it seems likely that binding of C1q subunit in C1 macromolecule to immune complex induces some conformational change in C1r molecule to recover the latent active site of C1r, and the activated state of C1r is finally stabilized by a spontaneous cleavage of X-Ile peptide bond in the zymogen molecule.

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