STUDIES ON THE $C\bar{1}r$-BINDING SITE OF RABBIT $C\bar{1}s$ BY PLASMIN FRAGMENTATION

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1. Introduction

The first component of complement (C1) is a macromolecular complex consisting of C1q, C1r and C1s which link together in the presence of Ca^{2+} [1]. When C1 binds to immune complex via C1q, C1r is activated autocatalytically to its active form, $C\bar{1}r$, and converts C1s to $C\bar{1}s$. Much information on the biochemical properties of C1s and $C\bar{1}s$, and on the molecular mechanism of activation of C1s by $C\bar{1}r$ is available from many intensive studies on human components. The structure of C1s and $C\bar{1}s$ involved in C1 complex formation, however, has remained to be elucidated.

We have reported [2] that rabbit $C\bar{1}s$ consisted of H and L chains with relative molecular masses (M_r) of 70 000 and 36 000, respectively, as is the case with its human counterpart, and also that its active serine residue was located in the L chain. Furthermore, the H chain was found to be susceptible to proteolytic cleavage by plasmin, resulting in the formation of active $C\bar{1}s$ fragments with an M_r of 72 000. This conversion was not accompanied by any loss of the $C\bar{1}s$ activity, but by complete loss of the ability to form $C\bar{1}r$–$C\bar{1}s$ complex [3]. These results suggest that the site of $C\bar{1}s$ involved in the binding to $C\bar{1}r$ may be located in the H chain, especially in the portion which is deleted by the action of plasmin and which does not contribute appreciably to the structural integrity of the active site.

To clarify the $C\bar{1}r$-binding site of the H chain, it is desirable to study the fragments of the H chain capable of binding to $C\bar{1}r$ which may be released from the $C\bar{1}s$ molecule by limited plasmin hydrolysis. Therefore, we attempted to isolate the $C\bar{1}r$-binding fragments from the digested products by disc gel electrophoresis. Here, isolation of the $C\bar{1}r$-binding fragments with M_r-values of 40 000 and 36 000 is reported.

2. Materials and methods

Rabbit $C\bar{1}r$ and $C\bar{1}s$ were highly purified from freshly drawn serum by successive applications of fractionation with polyethylene glycol, affinity chromatography on a column of IgG–Sepharose 6B, and QAE–Sephadex A-50 and CM–Sephadex C-50 column chromatographies, as in [3]. Purified $C\bar{1}r$ and $C\bar{1}s$ were estimated from their absorbance at 280 nm using $E^{1\%}_{1\,cm}$ of 11.5 and 9.5, respectively [4].

Fragmentation of rabbit $C\bar{1}s$ by homologous plasmin was performed as in [3]; $C\bar{1}s$ was reacted with the enzyme at a molar ratio of 1:50 in 0.01 M phosphate buffer (pH 7.5) at 37°C for varying incubation periods. Analysis of the digested products was performed by disc polyacrylamide gel electrophoresis in the absence or presence of sodium dodecyl sulfate (SDS). Various $C\bar{1}s$ fragments were estimated from their absorbance at 280 nm using $E^{1\%}_{1\,cm} = 9.5$ as in the case of intact $C\bar{1}s$ [4].

Disc gel electrophoresis on 7.5% gel was performed in Tris buffer (pH 7.5) [5]. SDS disc gel electrophoresis was performed in Tris buffer (pH 8.8) containing 1% SDS, by the Laemmli method [6] as modified in [7]. In all the cases, Coomassie blue was used for staining the protein bands on the gel. M_r-Values of polypeptide chains were determined by measuring their mobilities on SDS disc gel electrophoresis [7]. The following standard proteins with known M_r-values were used for the determination; phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase from bovine erythrocytes, soybean trypsin inhibitor and lactoalbumin from bovine milk (Pharmacia Fine Chemicals, Uppsala).

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3. Results

3.1. *Fragmentation of rabbit C1s with plasmin*

Rabbit C1s was digested with plasmin and the reaction was followed by SDS disc gel electrophoresis. Three fragments were found to be formed at the initial stage of the digestion (fig.1): C1s(p), fragment 1 and 2, each with an M_r of 61 000, 40 000 or 36 000. When incubation was prolonged, fragment 1 and 2 disappeared, indicating that they were further fragmented to small peptides. In particular, fragment 1 was found to be more susceptible to digestion.

On the other hand, C1s(p) seems to be resistant to the action of plasmin. Gel filtration on a Sephadex G-200 column of the products obtained by incubation with plasmin for 16 h gave C1s(p) alone. This fragment again was found to consist of an H chain fragment with an M_r of 28 000 and the L chain with an M_r of 32 000. In addition, it was unable to form C1 complex with homologous C1q and C1r even in the presence of Ca^{2+}, when estimated as in [7].

SDS disc gel electrophoresis of products reduced with 2-mercaptoethanol also demonstrated the release of fragments 1 and 2 each with an M_r of 40 000 or 36 000 (fig.2). The other two bands, which were resistant to plasmin digestion, were identified as the L chain and the H chain fragment connected covalently with each other, respectively, based on their M_r-values. These results indicate that C1s is initially converted to C1s(p) by proteolytic cleavage of the H chain by the action of plasmin, and that this conversion is accompanied by the release of fragment 1 and 2.

C1s(p) thus formed, however, is not homogeneous since it gave two bands moving slower than intact C1s on disc gel electrophoresis (fig.3). It appeared

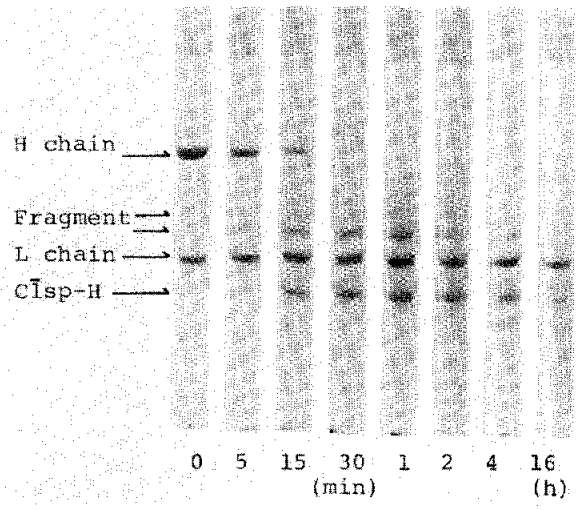

Fig.2. SDS disc gel electrophoresis of digested C1s after reduction with 2-mercaptoethanol. C1s was digested, as described in fig.1, and SDS gel electrophoresed after reduction with 0.4 M 2-mercaptoethanol.

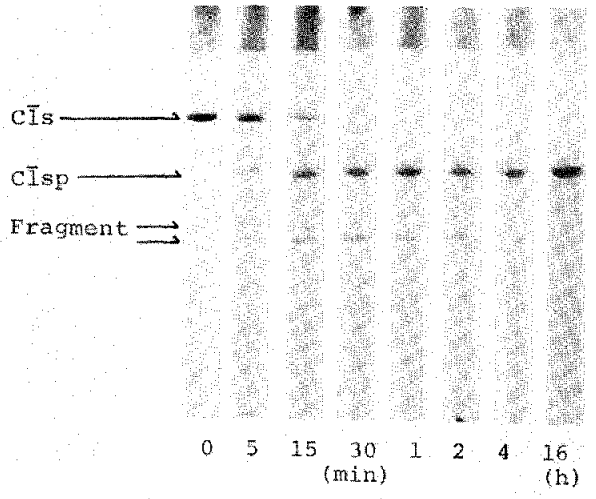

Fig.1. SDS disc gel electrophoresis of C1s fragmented by plasmin. C1s (153 μg) was digested with plasmin for varying incubation periods, and the digested products were SDS disc gel electrophoresed on 12% gel.

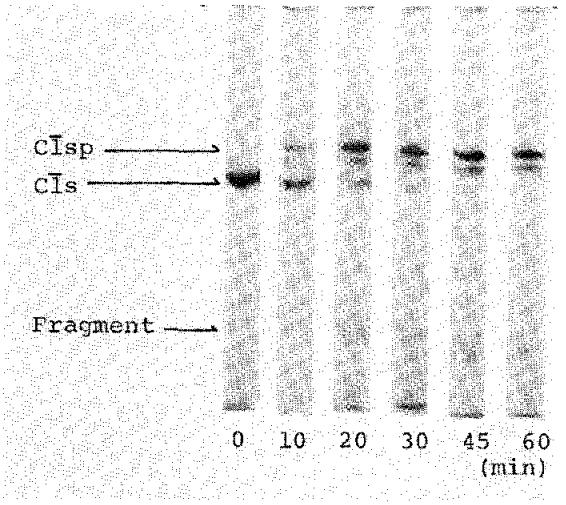

Fig.3. Disc gel electrophoresis of C1s fragmented by plasmin. C1s (77 μg) was digested with plasmin for varying incubation periods. After addition of soybean trypsin inhibitor (Sigma Chemicals) to terminate the reaction, the digested products were disc gel electrophoresed on 7.5% gel.

that plasmin cleaves the H chain of $C\bar{1}s$ at two different positions, resulting in the formation of 2 different molecules of $C\bar{1}s(p)$. Both of these $C\bar{1}s(p)$ molecules seems to retain $C\bar{1}s$ activity since esterolytic activities of $C\bar{1}s$ toward *N*-α-acetyl-L-arginine methyester, acetylglycyl–L-lysine methylester and *N*-α-acetyl-L-tyrosine ethylester were almost completely retained even after digestion with plasmin for 2 h, when estimated as in [3]; after the incubation, 93%, 90% and 89% of $C\bar{1}s$ activities were found with *N*-α-acetyl-L-arginine methylester, acetylglycyl–L-lysine methylester and *N*-α-acetyl-L-tyrosine ethylester, respectively.

3.2. *Identification of H chain fragments of binding to $C\bar{1}r$*

To investigate certain structures of $C\bar{1}s$ involved in the binding to $C\bar{1}r$, H chain fragments of $C\bar{1}s$ were isolated from digested products of $C\bar{1}s$ obtained by incubation with plasmin for 60 min. For this purpose, the products were electrophoresed on disc gel, and the H chain fragments were extracted from the unstained gel column. In another experiment, the electrophoresis was performed using a gel column attached by a Visking tube at its cathode, and the H chain fragments moving into the tube were taken out and used for experiments. The fraction thus isolated was found to contain fragment 1 and fragment 2, when analyzed by SDS disc gel electrophoresis.

When the H chain fragments were disc gel electrophoresed after incubation with purified $C\bar{1}r$ in the presence of Ca^{2+}, their bands disappeared, indicating that they bound to the $C\bar{1}r$, as in the case with intact $C\bar{1}s$ [3] (fig.4). The complex formation was also shown by a change in the mobility of $C\bar{1}r$. Apart from the complex formation, Ca^{2+} was found to affect the mobilities of the H chain fragments and also $C\bar{1}s$; these proteins began to move slower in the presence of Ca^{2+} (fig.5). No similar effect of Ca^{2+}, on the other hand, was observed with the mobility of $C\bar{1}s(p)$ (not shown). Therefore, Ca^{2+} seems to bind to the H chain fragments and $C\bar{1}s$, but not to $C\bar{1}s(p)$, and causes certain conformational changes in these two proteins, which may be reflected in the changes of their mobili-

Fig.4. Binding of H chain fragments to $C\bar{1}r$. About 5 μg of the H chain fragments was incubated alone or with 6.3 μg of $C\bar{1}r$ in the presence of 7.5 mM Ca^{2+}, then disc-gel electrophoresed on 7.5% gel: (1) H chain fragments in the presence of 5 mM EDTA; (2) H chain fragments in the presence of 7.5 mM Ca^{2+}; (3) H chain fragments after incubation with $C\bar{1}r$ in 7.5 mM Ca^{2+}; (4) 8.6 μg $C\bar{1}r$ only, in the presence of Ca^{2+}; (5) 8.6 μg $C\bar{1}r$ only, in EDTA.

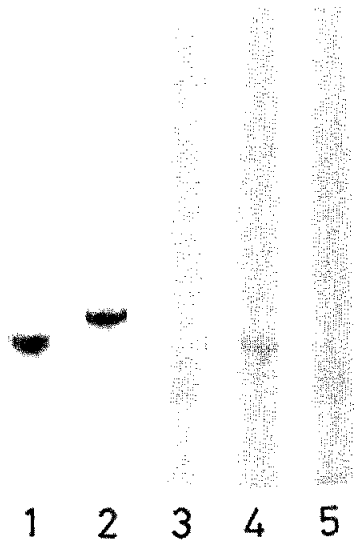

Fig.5. Effects of divalent cations on mobilities of H chain fragments and $C\bar{1}s$. $C\bar{1}s$ (12 μg) and H chain fragments (~5 μg) were disc-gel electrophoresed on 5% gel in the absence or presence of divalent cations: (1) $C\bar{1}s$ in the presence of 5 mM EDTA; (2) $C\bar{1}s$ in the presence of 20 mM Ca^{2+}; (3) H chain fragments in the presence of 5 mM EDTA; (4) H chain fragments in the presence of 20 mM Ca^{2+}; (5) H chain fragments in the presence of 20 mM Mg^{2+}.

ties. Although the nature of these induced conformational changes is unknown, they may be required for the development of the abilities of the H chain fragments and $\overline{C1s}$ for complex formation with C1r. In fact, Mg^{2+}, which fails to mediate the formation of $\overline{C1r}$–$\overline{C1s}$ complex, could not induce any change in the mobilities of $\overline{C1s}$ as well as the H chain fragments (fig.5).

4. Discussion

We have reported [3] that plasmin digestion of rabbit $\overline{C1s}$ resulted in the formation of an active $\overline{C1s}$ fragment, $\overline{C1s}$(p), which consisted of the L chain and a fragment of the H chain each with an M_r of 37 000. These results also confirm the formation of $\overline{C1s}$(p), though an M_r of 28 000 differing from a previous value of 37 000, was obtained with the H chain fragment. This value may be a more accurate estimate of the M_r of the H chain fragment since SDS disc gel electrophoresis is a more exact method for estimating M_r-values of protein than the SDS gel electrophoresis employed in [3].

The electrophoretic heterogeneity of $\overline{C1s}$(p) seems to indicate that plasmin cleaves primarily H chain of $\overline{C1s}$ at 2 different positions, though 2 different molecules of $\overline{C1s}$(p) were found to be indistinguishable from each other with regard to the M_r of their constituent H chain fragments. Fragments 1 and 2, released on the conversion of $\overline{C1s}$ to $\overline{C1s}$(p), on the other hand, were distinguishable with regard to M_r. This suggests that fragment 2 is not a primary product of plasmin action and may be derived from further fragmentation of fragment 1, and that fragment 1 consists of at least two different portions of the H chain though they are indistinguishable by SDS disc gel electrophoresis.

As suggested in [3], the finding that C1s(p) loses the ability to form $\overline{C1r}$–$\overline{C1s}$ complex serves to identify the location of a site which is involved in the binding of $\overline{C1s}$ to $\overline{C1r}$. In fact, this site of $\overline{C1s}$ was demonstrated to be located in the H chain since the H chain fragments released by action of plasmin were found to retain the site for binding to $\overline{C1r}$.

Some Ca^{2+}-binding proteins in serum, such as prothrombin, bind the ion via γ-carboxyglutamic acid residues on their polypeptide chains [8]. Human $\overline{C1s}$, however, is found to contain no γ-carboxyglutamic acid residue [4]. Although the structure of Ca^{2+}-binding site of $\overline{C1s}$ is still unknown, $\overline{C1s}$ and C1s were found to bind Ca^{2+} with equivalent valency and with comparable affinities [9]. In addition, this binding results in a dimerization of C1s monomer [9]. The effect of Ca^{2+} on the electrophoretic mobilities of C1s as well as fragment 1 and fragment 2, observed here, suggests that certain conformational changes may be induced in these proteins by the binding of Ca^{2+} and these changes may cause dimerization of these proteins as well as the development of the ability in them to bind to $\overline{C1r}$. In [10], $\overline{C1s}$ monomer and C1r dimer interacted even in the absence of Ca^{2+} to form a $\overline{C1r}_2$–$\overline{C1s}$ complex, whereas in the presence of Ca^{2+}, the two subcomponents were associated into a $\overline{C1r}_2$–$\overline{C1s}_2$ complex. Therefore, it is also probable that fragment 1 and 2 interact with $\overline{C1r}$ even in the absence of Ca^{2+}. Further studies on the effect of Ca^{2+} on the binding of fragments 1 and 2 to $\overline{C1r}$, and the molecular mechanism of $\overline{C1}$ complex formation from C1q, $\overline{C1r}$ and $\overline{C1s}$ are underway.

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