FORMATION OF A CONFORMATIONALLY CHANGED C1r, A SUBCOMPONENT OF THE FIRST COMPONENT OF HUMAN COMPLEMENT, AS AN INTERMEDIATE OF ITS AUTOACTIVATION REACTION

Yoshinori KASAHARA, Kazuhiko TAKAHASHI, Shigeharu NAGASAWA and Jiro KOYAMA
Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, 060, Japan

Received 26 February 1982

1. Introduction

The first component of complement, C1, is a Ca^{2+}-dependent complex of 3 subunits, C1q, C1r and C1s [1]. When C1 binds to immune complexes via C1q, a single chain zymogen C1r is proteolytically activated into two-chain $C1\bar{r}$, which subsequently catalyzes a proteolytic activation of C1s [2]. Since the activation of C1r is a triggering reaction for the sequential activation of the classical pathway of complement, how C1r becomes activated upon binding of C1 to immune complexes has been of interest.

Although there remain inconsistencies about details of the activation mechanism, the activation of C1r has been accepted to be an autocatalytic reaction [3–6] and the proteolytic site for autoactivation of C1r has been proposed to be generated in a zymogen C1r through a conformational change [4,5]. However, no report directly indicates that a conformational change occurs in a zymogen C1r prior to the proteolytic activation of C1r.

Here, we examined a possible conformational change occurring during the autoactivation of isolated C1r, using a hydrophobic fluorescent probe, 7-(*p*-methoxybenzylamino)-4-nitrobenzoxadiazole (MBD), which is strongly fluorescent upon binding to a hydrophobic area of a macromolecule [7]. We show that upon incubation of isolated C1r at 37°C, a conformational change, as judged by the MBD binding assay, occurs rapidly and reversibly and after a time lag, a progressive limited proteolysis occurs to produce an active $C1\bar{r}$. The implication of the present results for autocatalytic activation of C1r is discussed.

Abbreviations: MBD, 7-(*p*-methoxybenzylamino)-4-nitrobenzoxadiazole; NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoate; DFP, diisopropylfluorophosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; M_r, relative molecular mass

Nomenclature: Components of complement follow that recommended by the World Health Organization (1968); a bar indicates the activated state of a component

2. Experimental

2.1. *Complement components*

C1r was purified from human serum as follows: 300 ml human serum, containing 1 mM diisopropylfluorophosphate (DFP, Kishida Chemicals) and 0.5 mM *p*-nitrophenyl-*p'*-guanidinobenzoate (NPGB, United States Biochemical Corp.) and 200 ml IgG–Sepharose (10 mg IgG/ml Sepharose 6B, Pharmacia, Uppsala) pre-equilibrated with 2.5 mM veronal buffer (pH 7.4) containing 0.15 M NaCl and 1 mM $CaCl_2$ were mixed under stirring for 60 min at 0°C. The IgG–Sepharose layer was poured on a filter funnel and quickly washed with 6 l starting buffer at 0°C. The washed IgG–Sepharose was then packed into a column (4 × 17 cm) and eluted with 0.2 M diaminobutane (pH 7.4) containing 0.15 M NaCl. C1r fraction thus eluted was mixed with 2 M DFP to give 1 mM final conc. and dialyzed against 20 mM phosphate buffer (pH 7.4) containing 10 mM EDTA and 0.5 mM NPGB. The dialyzed C1r fraction was applied to a column (1.5 × 4.0 cm) of DE-52 (Whatman), pre-equilibrated with 20 mM phosphate buffer (pH 7.4) containing 10 mM EDTA and eluted by a linear increase of [NaCl] from 0–0.25 M with a total 200 ml of the starting buffer. C1r fraction thus obtained was

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

concentrated and finally purified by gel filtration through a column (2.5 × 60 cm) of Sephadex G-200 in 50 mM acetate buffer (pH 5.5) containing 0.1 M NaCl and 10 mM EDTA. About 1.5 mg purified C1r was obtained by these procedures and kept at 0°C before use. For detection of C1r fractions, 10–50 μl of each fraction and 5–10 μg C1s were incubated for 30 min at 37°C and the esterase activity of $C1\bar{s}$ thus activated was determined with 5 mM acetyl-glycyl-lysine methyl ester (Peptide Institute, Japan) as a substrate [8]. $C1\bar{r}$ and C1s were purified from human plasma according to [8] and [9], respectively. Reduced C1r showed a single band of 100 000 M_r upon SDS–PAGE, while reduced $C1\bar{r}$ showed two bands of 60 000 and 40 000 M_r upon SDS–PAGE (fig.1).

2.2. *Fluorescent measurement*

Measurements of binding of MBD (Dojin Labs, Japan) to C1r and $C1\bar{r}$ were made at ~40 μg protein/ml. Binding experiments were conducted at 20°C and pH 7.4, utilizing a 20 mM Tris–HCl buffer, containing 4 μM MBD and 0.15 M NaCl. Fluorescent emission spectra were recorded in a Hitachi fluorescent photometer over 500–600 nm under excitation at 490 nm.

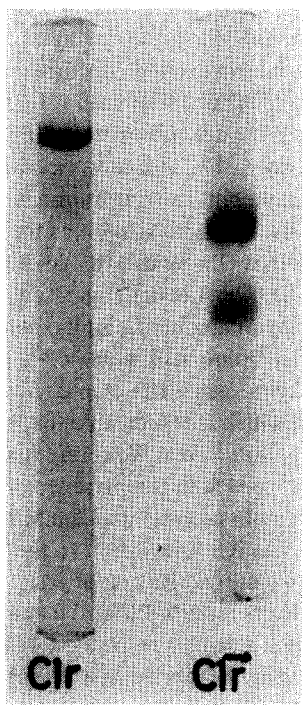

Fig.1. SDS -PAGE of C1r and $C1\bar{r}$: C1r and $C1\bar{r}$ were reduced and subjected to SDS–PAGE according to [10].

2.3. *SDS–PAGE*

Electrophoresis was done in 5.6% polyacrylamide gel in buffer containing 0.1% SDS as in [10]. The applied proteins were heated for 5 min at 100°C with 1% SDS and 2% 2-mercaptoethanol. The gels were stained with Coomassie blue R-250 and scanned using a dual-wavelength TLC scanner.

3. Results

3.1. *Binding of MBD to C1r and $C1\bar{r}$*

Fig.2 shows the fluorescent spectra of free MBD, MBD–C1r, and MBD–$C1\bar{r}$ solutions. Free MBD shows the maximum fluorescent emission at 560 nm when excited at 490 nm. When C1r was added to MBD, there was a marked increase in fluorescent intensity with a corresponding decrease in wavelength of maximum emission of ~20 nm, suggesting binding of MBD to a hydrophobic area of C1r. On the contrary, addition of $C1\bar{r}$ to MBD did not induce any significant change in the fluorescent spectrum of MBD, suggesting that the MBD-binding hydrophobic area in a zymogen C1r is disrupted upon proteolytic activation of C1r.

3.2. *Relationship between a conformational change and proteolytic activation of C1r*

The above results suggested that MBD was a useful

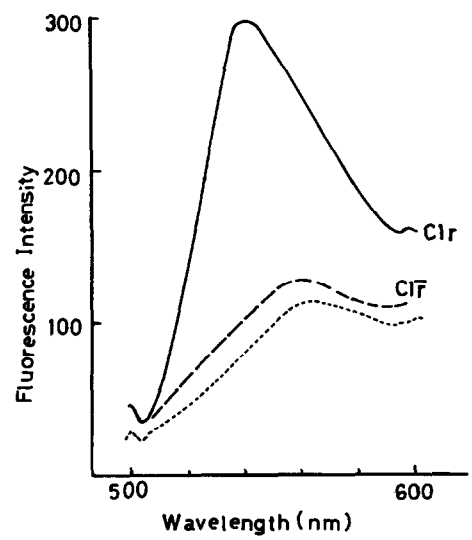

Fig.2. Fluorescent emission spectra of MBD, MBD–C1r and MBD–$C1\bar{r}$ solutions: (- - - -) MBD (4 μM) alone; (——) MBD–C1r; (– – –) MBD–$C1\bar{r}$. Excitation was at 490 nm. C1r and $C1\bar{r}$ were 40 μg protein/ml in 20 mM Tris–HCl buffer (pH 7.4).

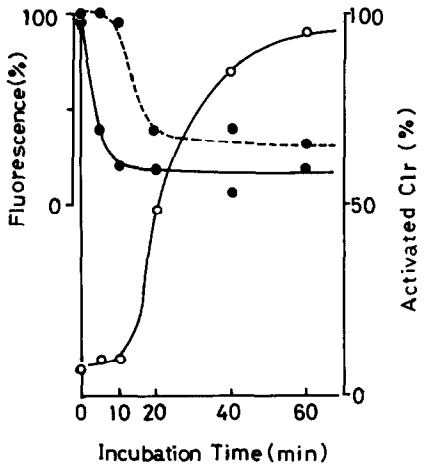

Fig.3. Time courses of a conformational change and proteolytic activation of C1r upon incubation at 37°C. C1r (40 μg/ml) was incubated for different periods at 37°C and determined for conformational change (•——•) by MBD-binding assay and proteolytic activation (o——o) by SDS–PAGE after reduction. The fluorescent data are expressed as % fluorescent intensity which was calculated by assuming that the fluorescent intensities of MBD–C1r and MBD alone are 100% and 0%, respectively; % Activation of C1r was determined by SDS–PAGE after reduction and scanning the gels after staining with Coomassie blue. The reversibility of a conformational change of C1r (•– – –•) was determined by measuring the recovery of MBD-binding activity after chilling the incubated C1r in ice bath, as described in the text.

tool for analysis of a possible conformational change occurring during autoactivation of C1r. Then, we attempted to follow autoactivation of C1r both by measuring the limited proteolysis and a conformational change of C1r by SDS–PAGE and the MBD-binding assay, respectively.

The cleavage of C1r into two-chain $C1\bar{r}$ proceeded showing a sigmoidal curve with a lag phase of ~10 min, suggesting an autocatalytic process (fig.3). However, the MBD-binding assay revealed that a conformational change rapidly occurred without any lag phase and reached its plateau during the first 10 min of incubation at 37°C. These results suggest that autoactivation of C1r is a two-step reaction; the first rapid conformational change and the subsequent slow limited proteolysis of an altered C1r.

Neither conformational change nor limited proteolysis of C1r occurred when autoactivation of C1r was attempted at 20°C, suggesting that the conformational change of C1r is a reaction of thermal dependency, as proposed in [5]. Then it was interesting whether the conformational change of C1r is a reversible or irreversible reaction. To make clear this problem, C1r pre-incubated for different periods at 37°C, was chilled at 0°C and measured for the recovery of MBD-binding ability. Fig.3 (– – –) shows that if the incubation period of C1r at 37°C is <10 min (i.e., during the lag phase of autoactivation) the disrupted hydrophobic area of C1r appears to be fully refolded upon chilling at 0°C. However, as the proteolytic activation of C1r progressed, there was an approximately proportional decrease in the refolding of MBD-binding area. These results suggest that the conformational change to produce altered C1r is a reversible reaction and that once altered, C1r is cleaved into two-chain $C1\bar{r}$, the MBD-binding hydrophobic area is not refolded even chilling at 0°C.

4. Discussion

Using MBD as a hydrophobic probe, it is now possible to detect a conformational change of C1r which occurs prior to the autocatalytic cleavage of C1r. Considering that the conformational change of C1r is induced at 37°C but not at 20°C and is reversed upon chilling at 0°C, a high energy seems to be required both for initiation of a conformational change and holding the resulting excited conformation of a zymogen C1r. MBD was first used for analysis of a conformational change occurring during autoactivation of trypsinogen [7]. A hydrophobic area able to bind MBD was present in trypsinogen and disrupted proportionately with activation into trypsin. Under the condition that C1r binds MBD, neither C1s nor $C1\bar{s}$ binds MBD (not shown), suggesting that although C1r and C1s are similar in M_r, amino acid composition and partial amino acid sequence [2], the two homologous zymogens differ characteristically in a conformation which is responsible for the MBD-binding hydrophobic area. We found that autoactivation of C1r proceeded in the presence of MBD (not shown). This result suggests that the MBD-binding hydrophobic area has no relation to the potential catalytic site for autoactivation of C1r.

Several authors reported that the autoactivation of C1r was catalyzed not by activated $C1\bar{r}$ but by an active site possibly generated in a zymogen C1r [4,5]. The intermediary C1r having an autocatalytic site was termed C1r*, which was proposed to be reversibly inhibited by NPGB [4]. The catalytic site in C1r* was

supposed to be generated by a conformational change which is slow and gives rise to the time lag in the autoactivation of C1r [4]. MBD-binding assay revealed that a conformational change of C1r rapidly occurred upon incubation at 37°C, while the proteolytic activation of C1r proceeded after a time lag for 10 min. So, it seems unlikely that the putative C1r* is generated in parallel to the rapid conformational change of C1r. It may be that following the rapid conformational change of C1r, a slow conformational change subsequently occurs and produces the putative C1r* capable of activating C1r. An alternative possibility is that a proteolytic site is rapidly generated in parallel to the conformational change of C1r but catalyzes only slowly the activation of altered C1r into active $C1\bar{r}$, which once formed, catalyzes rapidly proteolytic activation of altered C1r. Although this mechanism may well explain the kinetics of autoactivation of C1r, there are several reports suggesting that $C1\bar{r}$ by itself has no ability to activate C1r [4,5]. However, it was reported in an abstract [11] that autoactivation of C1r involved a double mechanism: the first intramolecular catalysis due to C1r itself; the second intermolecular reaction due to activated $C1\bar{r}$ itself.

Further studies, such as active site titration with NPGB or conformational analysis with different kinds of fluorescent hydrophobic probes will be required to more clearly define which mechanism is responsible for the autoactivation of C1r.

References

[1] Lepow, I. H., Naff, G. B., Todd, E. W., Pensky, J. and Hinz, C. F. (1963) J. Exp. Med. 117, 983–1008.
[2] Porter, R. R. and Reid, K. B. M. (1979) Adv. Protein Chem. 33, 1–71.
[3] Takahashi, K., Nagasawa, S. and Koyama, J. (1976) FEBS Lett. 65, 20–23.
[4] Dodds, A. W., Sim, R. B., Porter, R. R. and Kerr, M. A. (1978) Biochem. J. 175, 383–390.
[5] Arlaud, G. J., Villiers, C. L., Chesne, S. and Colomb, M. G. (1980) Biochim. Biophys. Acta 616, 116–129.
[6] Assimeh, S. N., Chapuis, R. M. and Isliker, H. (1978) Immunochemistry 15, 13–17.
[7] Kenner, R. A. and Aboderin, A. A. (1977) Biochemistry 10, 4433–4440.
[8] Takahashi, K., Nagasawa, S. and Koyama, J. (1975) FEBS Lett. 55, 156–160.
[9] Takahashi, K., Nagasawa, S. and Koyama, J. (1975) FEBS Lett. 50, 330–333.
[10] Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617.
[11] Villiers, C. L., Arlaud, G. J., Duplaa, A. M. and Colomb, M. G. (1981) IXth International complement workshop abstract.