REVISTA ESPAÑOLA DE FISIOLOGIA, 44 (2), 169-178, 1988

Immunochemical Recognition of the Binding of C-Reactive Protein to Solid-Phase Phosphorylethanolamine

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(Received on November 23, 1987)

J. M. COLL. *Immunochemical Recognition of the Binding of C-Reactive Protein to Solid-Phase Phosphorylethanolamine.* Rev. esp. Fisiol., 44 (2), 169-178, 1988.

The influence of polyclonal and monoclonal antibodies, trypsin digestion and mercaptoethanol treatment of C-reactive protein (CRP) in the CRP binding to solid-phase phosphorylethanolamine (PE) has been investigated. Nine monoclonal antibodies reacting with CRP could be divided into at least 2 well-defined groups: one group of 6-7 monoclonals interfering with the binding of CRP to PE (mainly represented by monoclonal 2) and the not interfering with the binding of CRP to PE (mainly represented by monoclonal 5). Trypsin digestion resulted in sequence identified CRP fragments still able to bind to PE and detectable by monoclonal 5 but not by monoclonal 2. On the other hand, binding of CRP to PE was abolished by mercaptoethanol treatment. These results, together with the estimation of the extent of the antigenicity of the PE binding site and the characteristics of the hydrophobicity profile of CRP, suggest that most of the hydrophilic sequences contribute to the PE binding region except a non-overlapping region defined by monoclonal 5. Most probably, some of these sequences are located inside or around the internal bisulphide bridge of each monomer of the pentameric CRP.

Key words: C-reactive protein, Monoclonal antibodies, Phosphorylethanolamine, Binding-region.

The c-reactive protein (CRP) molecule has a molecular weight of about 107500 (5). It is made up of 5 identical non-glucosylated polypeptide monomers, noncovalently associated in a disclike configuration with cyclic pentameric symmetry and containing one internal bisulphide bridge per monomer (5, 6). CRP undergoes calcium dependent binding (26) to phosphorylamine compounds such as phosphorycholine (PC) and phosphorylethanolamine (PE). All of the biological *in vitro* effects that have been assigned to CRP seem to be initiated following the binding of CRP to phosphorylcholine containing substances (25). Once complexed via its calcium dependent binding site, it becomes a potent activator of the classical complement pathway starting with C_{1q} (11), and it may bind to lymphocytes bearing Fc receptors with yet unknown *in vivo* functions (16, 29).

The structure of CRP binding and location in the sequence of the phosphorylcholine binding site(s) has not been determined yet although crystallization of CRP has long since been achieved (22, 23). However X ray studies on mouse PC binding immunoglobulins from induced myelomas revealed that the PC binding site contained the sequence Phe^{32}-Tyr^{33}-Met^{34}-Glu^{35} (10, 13, 20, 21, 30). This sequence was also unique among the heavy chains of 89 % phosphorylcholine-binding immunoglobulins from myeloma proteins (13). By examining the partial sequences of human and rabbit CRP (15, 18, 19) similar sequences are found in their structure: Phe^{39}-Tyr^{40}-Thr^{41}-Glu^{42} for human CRP and Phe^{38}-Tyr^{39}-Thr^{40}-Asx^{41} for rabbit CRP. In both cases this structure is located inside the bisulphide bridge (11, 17). Although other residues probably are involved in the binding, this conserved 4 amino acid sequence seems to be common to all the phosphorylcholine binding proteins (13).

Binding of CRP to phosphorylcholine (but not of immunoglobulins) is dependent on the presence of Ca^{++}. It has been suggested that Ca^{++} acts as an allosteric effector of CRP by inducing the proper conformation of the phosphorylcholine binding site (12, 27, 28).

Experiments were made on the CRP binding to immobilized PE by using a set of monoclonal antibodies, chemical modification, and trypsin treatments. The results suggest the existence of 2 regions on the CRP molecule: one highly antigenic region containing the PE binding site and another, less antigenic region which seems to be independent of this binding.

Materials and Methods

CRP purification. — The method of PONTET *et al.* (24), modified (4), was used to purify CRP. Immunodiffusion and polyacrylamide gel electrophoresis were performed as described (4).

Preparation of conjugates. — The conjugates used for most experiments were obtained by the one step glutaraldehyde method (2, 7).

When the conjugates were used in blotting experiments, only one band comigratting with CRP was obtained. Purified CRP (5 μg/well) was separated by 5-10 % polyacrylamide gel electrophoresis without SDS, in 100 × 80 × 1 mm slab gels (14). Once the gel was run it was transferred to nitrocellulose paper by electrotransfer in 25 mM Tris, 400 mM glycine, pH 8 by using an apparatus from Biorad at 60 V for 1 hour. After transfer, the nitrocellulose paper was incubated with 20 mM phosphate 150 mM NaCl, pH 7.4, 1 % bovine serum albumin, 0.5 % milk (Molico) for 30 min at 37° C for blocking. Then it was incubated with conjugate in the same buffer with 0.05 % Tween 20 for 30 min at 37° C and afterwards washed thoroughly with 20 mM Tris, 150 mM NaCl, pH 7.4. The bands were stained by immersion of the nitrocellulose paper in 10 ml of Tris buffered saline pH 7.4 with 3 mg/ml of 4-chloro-naphtol in 2 ml of methanol and 1 % H_2O_2.

Production of monoclonal antibodies to CRP. — The method was modified from the one previously described (3, 9). Non secreting myeloma X63 Ag 8653 was used as parental cell line, maintained in DMEM, 15 % fetal calf serum, 15 mM sodium bicarbonate in a CO_2 incubator (Flow).

Immunoenzymatic assays. — Immunoenzymatic assays were performed in polystyrene microtiter plates, either 96 microwell module F-16 medium binding capacity (Nunc, Kamstrup, Denmark) or 96 microwell plates (Costar, Cambridge, MA.). The wells were coated either with CRP, PE or anti-CRP. Plates coated with

CRP (native or denatured) were used for the assay of anti-CRP antibodies in mouse ascites together with rabbit anti-mouse IgG conjugated to peroxidase. Plates coated with PE were used for the assay of antibodies against CRP bound to PE in mouse ascites together with either rabbit anti-mouse IgG or polyclonal anti-CRP conjugated to peroxidase. Plates coates with anti-CRP antibodies (polyclonal or monoclonal) were used for the assay of CRP in human fluids together with either mouse anti-CRP and rabbit anti-mouse IgG conjugated to peroxidase or anti-CRP (polyclonal or monoclonal) conjugated to peroxidase.

The solid phase for the assay of anti-CRP antibodies was made by dissolving 0.23 μg of either native or denatured CRP (CRP exposed to pH 10.5 during 3 min and then adjusted to pH 6) in 100 μl of 2.5 mM $CaCl_2$, 0.25 mM Hepes, pH 7 per well and then drying it at 37° C overnight (Costar plates). Other details as described (9).

The CRP enzymeimmunoassay for the assay of anti-CRP (bound to PE) antibodies was the one described by MARTÍNEZ and COLL (15) for the quantitative determination of CRP. Plates that had been incubated with saturating amounts of CRP and washed, were incubated with 100 μl of mouse ascitic fluids diluted in dilution buffer for 60 minutes at room temperature. After they had been washed twice, 100 μl/well of rabbit anti-mouse IgG conjugated to peroxidase (1,000-fold diluted in dilution buffer) was added and incubated for 30 minutes at room temperature. After washing 4 times colour was developed.

The solid-phase for the assay of CRP was made by dissolving in 100 μl of H_2O either polyclonal or monoclonal anti-CRP (2 μg) and drying at 37° C overnight in Costar polysterene wells. Before use they were washed once in washing buffer. Then 50 μl of the purified CRP samples in the dilution buffer were added per well followed by another 50 μl of diluted ascitic fluids containing the monoclonal antibodies. The plates were agitated and incubated for 60 minutes at room temperature. After washing twice, 100 μl/well of rabbit anti-mouse IgG conjugated to peroxidase (Nordic, Tilburg, The Netherlands) diluted 1/1,000 in dilution buffer was added and incubated for 30 min. After washing 4 times, substrate was added and colour was developed as described (4, 15).

Results

CRP binding to solid-phase PE. — Incubation of CRP with phosphorylethanolamine (PE) or phosphorycholine (PC) prior to their addition to solid-phase phosphorylethanolamine completely inhibited CRP binding to solid-phase PE when concentrations were high enough. The concentrations required for half the maximum inhibition were about 1.6×10^{-4} M for PE and 6×10^{-6} M for PC. The binding of CRP to solid-phase PE increased with time, approaching plateau at 60 minutes both at 22° C and at 37° C. The binding was 1.6 fold higher at 37° C than at 22° C. To show that the binding of CRP to immobilized PE is also Ca^{++} dependent, the assay was carried out in the presence of several concentrations of Ca^{++}. The concentration of Ca^{++} required to get half the maximum reaction was about 5.6×10^{-4} M. The optimal pH of the binding was found to be between 7.5 to 8.5. An increase of pH above 8.5 caused a strong decrease in the binding of CRP to PE. The optimal pH for binding of the conjugate anti-CRP was 7.5.

To study whether the reaction was dependent on protein structure, 30 μg/l of CRP in 0.1 M ammonium bicarbonate, pH 8 was digested with proteinase K (Worthington) or subtilisin (Sigma) by incubation at 37° C overnight. The digestion was performed in the absence and in

the presence of 10 mM $CaCl_2$. Gel electrophoresis under denaturing conditions showed disappearance of the Coomassie stained band at 21 K in both cases. In either case, the percentage of remaining binding was lower than 1 %. On the other hand, the addition of an excess of CRP (2 μg/well) during the conjugate incubation step, inhibited the reaction 99 %.

Titration of monoclonal antibodies. — Mouse monoclonal antibodies against CRP belonged to IgG_1 class, except number 8 that belonged to IgG_{2a} class, as shown by immunodiffusion against specific antimouse immunoglobulins (Nordic).

The monoclonals were titrated by the CRP-(native and denatured) and by the anti-CRP-PE assays (fig. 1). In the CRP assay (method also used to screen the hybridomas), monoclonals were allowed to react at several dilutions with CRP coated plates. The strongest relative titres were for monoclonals, 2, 3, 7 and 9. All the rest were very similar and 10-100 times lower. By using a PE solid-phase fully saturated with CRP (anti-CRP-PE assay), monoclonals were allowed to react at several dilutions with CRP bound to immobilized PE. The strongest relative titre was for monoclonal 5. Monoclonals 4 and 6 were at about 10 fold and 100 fold less titre, respectively.

Monoclonals 2 and 9 had about 1,000 fold less titre than monoclonal 5 (fig. 1). When monoclonal antibody containing ascitic fluids was used at a constant dilution (dilution obtaining 1.5 A492-620 nm in the above mentioned test) and CRP concentrations were varied, only monoclonals 5, 6 and 7 and polyclonals gave a linear relationship between absorbance and CRP concentrations, highest maximal absorbances and lower than 25 % backgrounds (fig. 2). In the denatured-CRP assay, monoclonals were allowed to react at several dilutions with high-pH

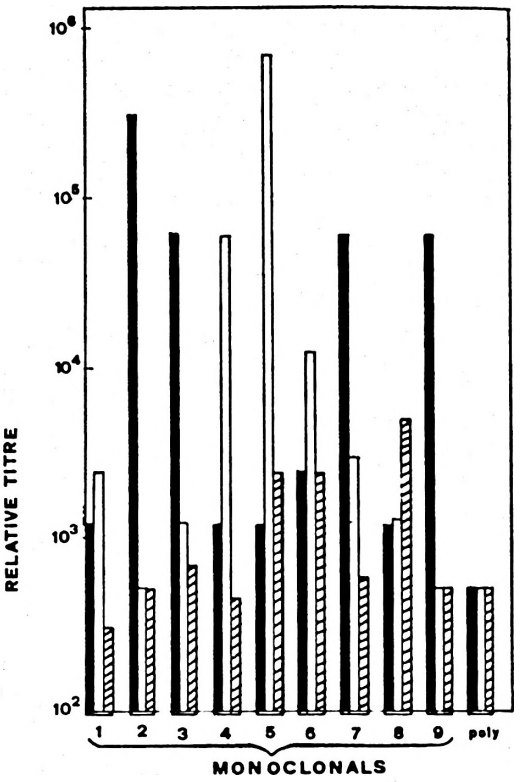

Fig. 1. *Relative titre of ascites anti-CRP in CRP, anti-CRP-PE and denatured-CRP assays.* The monoclonal antibodies were from ascitic fluids induced by hybridomas producing anti-CRP antibodies and diluted in dilution buffer. Ascites from mice injected with parental myeloma did not react in CRP assay. Relative titre was defined as the inverse of the dilution of ascites at which the activity at 1/100 dilution was reduced to 50 %. The plates were coated with CRP (■), PE (□) and denatured-CRP (▨).

denatured-CRP coated plates. The strongest relative titre was for monoclonal 8 and somewhat lower for monoclonals 5, 6, and 7, the rest being 2-3 fold smaller. Overall titres were smaller in this than in the other two assays (fig. 1).

No mouse CRP was detected in the ascitic fluids containing the monoclonal antibodies by anti-CRP-PE assay at dilutions of 1/12,500 to 1/100, so that no interference would be excepted in all

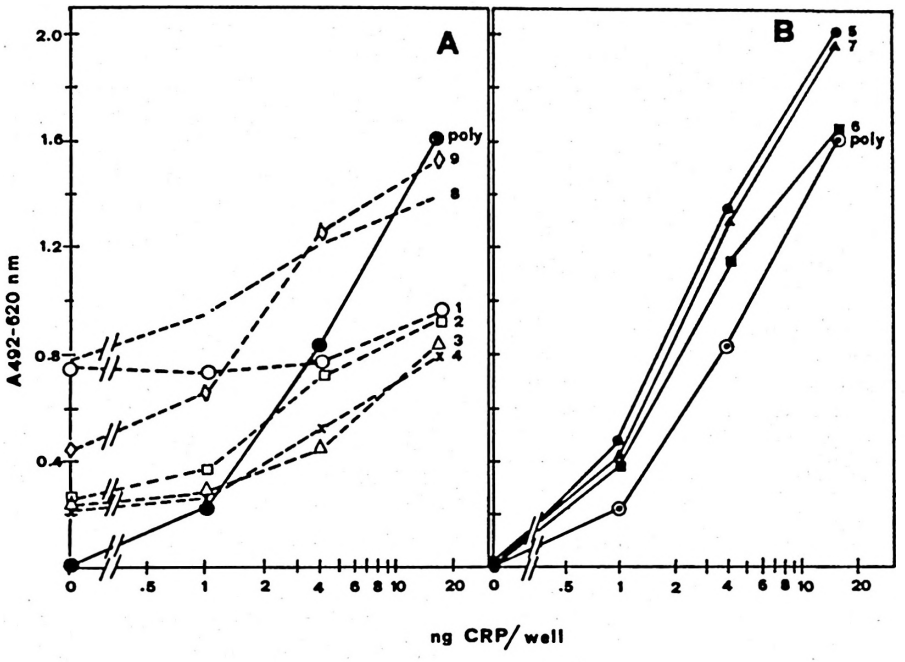

Fig. 2. *Performance of monoclonals in the CRP binding to PE assay at different CRP concentrations.* After incubating with CRP, monoclonals were added at the following dilutions from ascitic fluids: at 1/20, 2 and 3; at 1/100, 4, 9 and polyclonals; at 1/500, 1, 7 and 8; at 1/2,500, 6; and 1/12,500, 5. The different dilutions tried to compensate the titres observed in figure 1. Averages from duplicates are presented.

these assays due to mouse CRP cross-reactions (not shown).

Studies with monoclonal antibodies. — Further experiments focused on the interference of monoclonals in the CRP binding to PE, by studying the inhibition by PE of the binding of monoclonals to solid-phase CRP, the binding of monoclonals to CRP bound to polyclonal anti-CRP in the absence of Ca^{++}, and the inhibition by monoclonals of the binding of CRP to PE.

Solid-phase CRP was saturated with 100 mM PE, the monoclonals were added and monoclonal binding was measured. Monoclonals 1 and 6 had more than 80 % inhibition whereas 2, 3, 7, 8 and 9 were 20-50 % inhibited and 4 and 5 were not inhibited. Mouse polyclonal anti-CRP was 93 % inhibited.

To measure the Ca^{++} dependence of the binding of monoclonals to CRP, wells were coated with 2 μg/well of IgG anti-CRP from rabbit. The assay was performed in the presence of either 2 mM Ca^{++} or 0.5 mM EDTA. The polyclonal anti-CRP to CRP binding was independent of the Ca^{++} concentration as shown by immunodiffusion experiments. In the absence of Ca^{++}, most monoclonals retained 40-90 % of the binding to CRP and monoclonals 5 and 6 retained 100 %. This result was confirmed by immunodiffusion, since the nine monoclonals (ascitic fluids) gave identity or partial identity lines on immunodiffusion when confronted with highly purified CRP (1.1 mg/ml) whether in the presence of either 2 mM Ca^{++} or 0.5 mM EDTA (not shown).

To measure the capacity of the CRP-anti-CRP complexes formed in solution

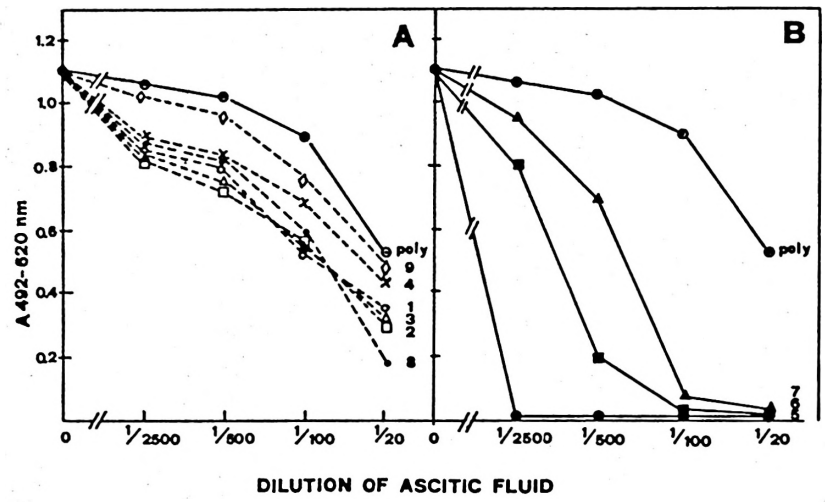

Fig. 3. *Competition between monoclonals anti-CRP and monoclonal 5 for binding to CRP bound to PE.* PE coated plates were allowed to react with 16 ng of CRP/well/100 μl for 1 hour. After plates were washed twice, 100 μl of diluted ascitic fluid was added and incubated for 1 hour. After washing twice, 100 μl of horseradish peroxidase conjugated to monoclonal 5 at 1/1,000 dilution was added and incubated for 30 minutes. Averages from duplicates are given.

to bind to solid-phase PE, 0.5 ml of 160 ng/ml CRP in dilution buffer was incubated for 1 hour at room temperature with 30 μl of each ascitic fluid. Then 100 μl of the mixture was added to the solid-phase PE and the assay continued as indicated in Materials and Methods.

The percentage of binding to PE of the CRP-anti-CRP complexes with respect to CRP alone were lower than 4 % except for monoclonal 8 that was 21 % (not shown). From all these experiments the anti-CRP monoclonal antibodies could be divided into at least two different groups: one group not interfering with the PE binding (principally defined by monoclonal 5) and another group interfering with the PE binding (principally defined by monoclonal 2). To further delineate both groups, competition experiments between monoclonals were designed by labelling monoclonal 5 with horseradish peroxidase. Figure 3 B shows that only monoclonals, 5, 6 and 7 compete with 5 for binding to CRP bound to PE. Polyclonal anti-CRP from mouse and the rest of the monoclonals competed much less efficiently (fig. 3 A).

Antigenicity of the PE binding site of CRP. — Anti-CRP antiserum when passed through the column lost its anti-CRP activity. Anti-CRP was eluted from the column with a yield of 2 mg/ml of antiserum. All the anti-CRP activity was IgG as indicated by reducing polyacrylamide gel electrophoresis (not shown). When this purified anti-CRP IgG was passed again through the same column saturated with PE, only 20 % of the anti-CRP activity was bound to the column (figure 4).

Trypsin digestion. — Figure 5 A shows the Sephadex G-50 protein profile of the partial trypsin digest of CRP after 12 or 48 hours of incubation. After 12 hours, three peaks were obtained: at the void volume (peak I), at about 5,000 daltons

(peak II) and at about 2,000 daltons (peak III). After 48 hours of incubation peak I remained, a new peak at about 17-18,000 daltons appeared, peak II increased and peak III was displaced to the internal volume. Figure 5 B shows the binding to PE of the fractions corresponding to the protein profile of figure 5 A. After 12 hours of trypsin digestion the peak of binding to PE coincided with the void volume but after 48 hours the peak of binding to PE was displaced to the 17-18,000 daltons peak. No significant binding to PE was detected in peak II at 5,000 daltons. The binding to PE profile was similar whether polyclonal or monoclonal 5 anti-CRP antibodies were used in the assay, but no profile was observed with the use of monoclonal 2 (not shown). When analyzed by denaturing gel electrophoresis, peak I contained the same band as unfractionated undigested CRP at 21,000 daltons, a 18,000 daltons band and a very faint band at about 5,000 daltons. Peak II contained the band at about 5,000 daltons. To investigate the monoclonal binding capacity of peak II, undigested CRP and peak II from the 48 h digest were dried onto microtiter wells (0.15 μg of protein per well) and allowed to react for 1 h with 10 μg of either monoclonal 2 or monoclonal 5. After washing, enzyme immunoassay was with rabbit anti-mouse IgG labelled with peroxidase.

The reaction of monoclonal 5 with peak II was 42 percent of the reaction observed with undigested CRP. By contrast, the reaction of monoclonal 2 with peak II was only 5 percent of the reaction observed with undigested CRP.

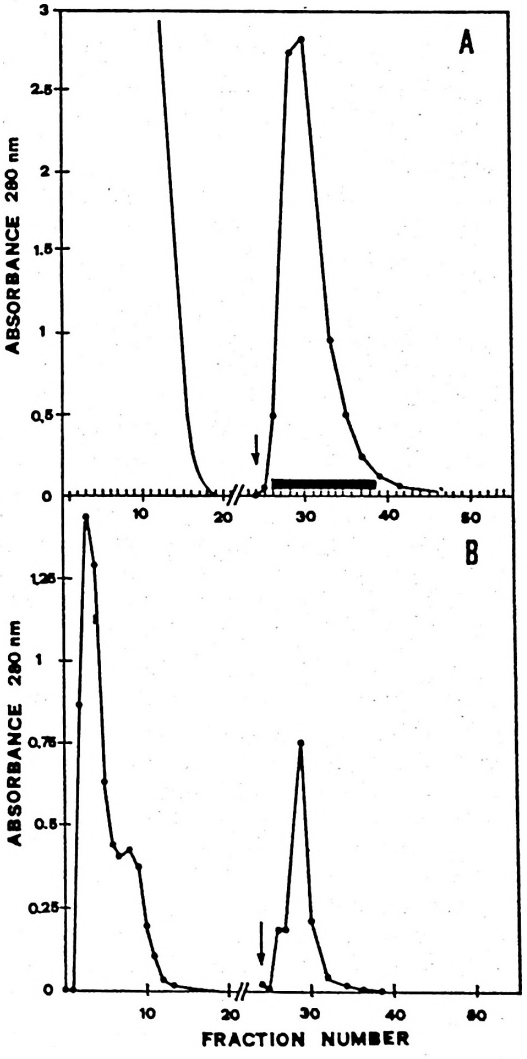

Fig. 4. *Purification of polyclonal anti-CRP by CRP affinity column (A) and rechromatography of purified polyconal anti-CRP by PE-saturated CRP affinity column (B).*

Ten ml of goat anti-human CRP from Biomerieux dialyzed against 20 mM Tris, 100 mM NaCl, 10 mM $CaCl_2$, pH 8 were passed through a CRP-Sepharose column at 3 ml/hour. Bound anti-CRP was eluted with 50 mM diethylenamine pH 11 in 1.5 ml fractions over tubes filled out with 50 μl 3 M Tris pH 5. Twenty mg of anti-CRP were recovered. After washing the column, 15 ml of 0.1 M PE in the buffer was passed through the column and incubated overnight, then the column was again washed with the same buffer and 5 mg of the pooled anti-CRP from the column A dialyzed against the same buffer was passed again through the column. Bound CRP was eluted as above. The arrow indicates the begining of elution. Black bars indicate the fractions pooled.

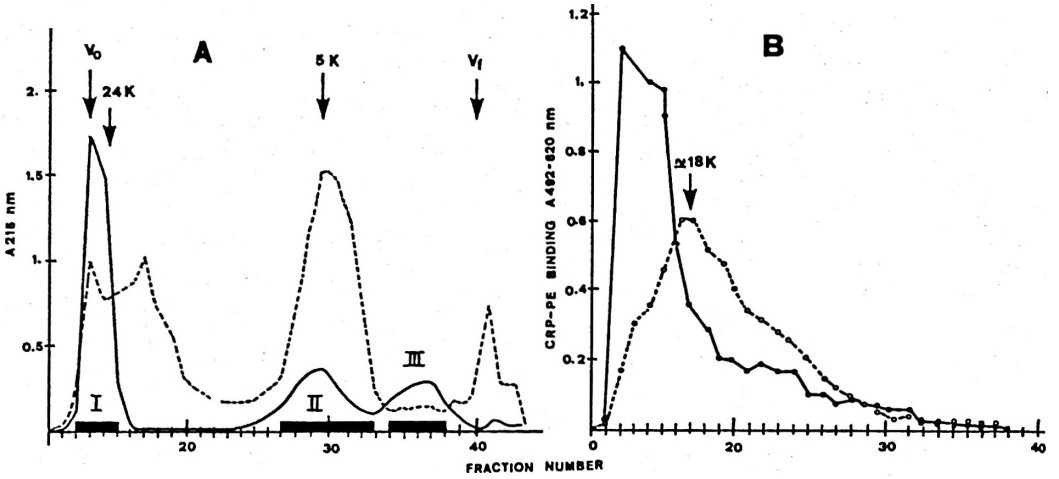

Fig. 5. *Sephadex G-50 profile of partial trypsin CRP digestion (A) and binding to PE of the fractions (B).* One mg of CRP was digested with trypsin (1:2 w/w) by 12 (———) or 48 (- - -) hours at 37° C in 3 ml 0.1 N NH_4HCO_3 pH 8. The digest was separated in a Sephadex G-50 column (50 × 1 cm) in 0.1 N NH_4HCO_3. Fractions of 1.2 ml were collected. Pooled fractions designated I, II and III were lyophilized and redissolved in 1 ml of 0.1 N NH_4HCO_3. CRP binding to PE assay was performed with either polyclonal or monoclonal 5 anti-CRP conjugated to peroxidase.

Mercaptoethanol treatment. — Mercaptoethanol was added at 720 mM final concentration during 30 minutes at room temperature. Afterwards samples were diluted 31-fold in dilution buffer with 46 mM of iodoacetamide and again 31-fold in dilution buffer with 1.5 mM of iodoacetamide and kept overnight at 0° C before assay. After this treatment the samples were assayed for binding to PE. In the absence of Ca^{++}, Mercaptoethanol treatment abolished 99 % of the CRP binding to PE; in the presence of Ca^{++}, however, 60 % of the CRP binding to PE remained. Results were similar when polyclonal or monoclonal 5 conjugates.

Hydrophobicity profile. — The hydrophobicity profile of CRP (8) shows six major hydrophilic sites (fig. 6): 2 (A and F) on the amino and carboxyl terminals and 4 in two groups (B-C and D-E) separated by a short hydrophobic valley. There is a part which is neither hydrophilic nor hydrophobic between the highly hydrophilic regions E and F. The hydrophilic double B-C is located inside the internal bisulphide bridge (fig. 6).

Discussion

One of the CRP fragments obtained by trypsin digestion of CRP (peak II at about 5,000 daltons, fig. 5) was shown to correspond to residues 105-169 in similar trypsin digest (17) but had no binding to PE and was not recognized by monoclonal 2, thus suggesting the absence of the PE binding site outside the internal bisulphide bridge (residues 36 to 78). Accordingly, the use of reducing agents such as mercaptoethanol (but only in the absence of Ca^{++}) abolished the CRP binding to PE. If histidine residues are implicated in the CRP binding of Ca^{++} as suggested, the presence of Ca^{++} could

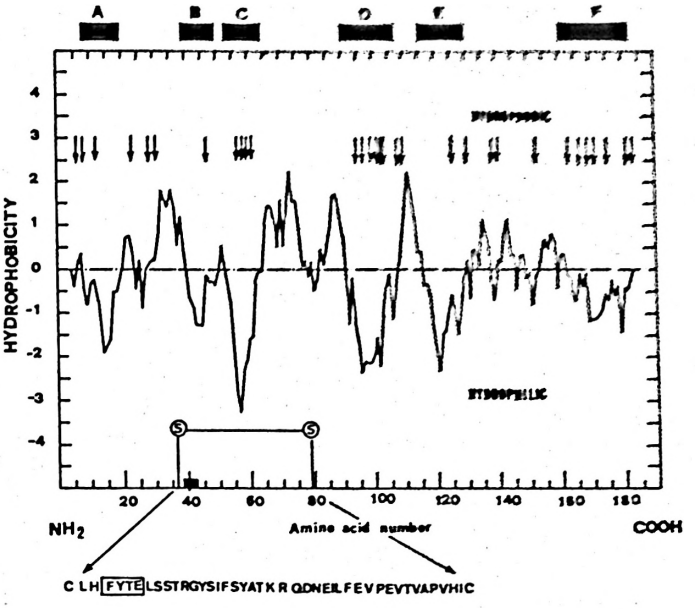

Fig. 6. *Hydrophobicity profile of CRP, position and sequence of internal bisulphide bridge.*
The method of KYTE and DOOLITTLE (8) was used to calculate the hydrophobicity profile. The black box indicates the position of the sequence Phe-Tyr-Thr-Glu, (FYTE). Black upper boxes show the most important hydrophilic regions. The internal sequence of the bisulphide loop is shown enlarged. Arrows indicate free NH_2 corresponding to arginine, lysine, asparagine and glutamine residues. The sequence data is from OLIVEIRA *et al.* (17).

serve to hold the bisulphide loop by the 2 histidines present around the internal bisulphide bridge on the CRP structure: ...Cys^{36} - Leu^{37} - His^{38} - ...His^{76} - $Ileu^{77}$ - Cys^{78}... (fig. 6). The putative phosphorylamine main binding sequence located inside the bisulphide bridge (...Phe^{39}-Tyr^{40}-Thr^{41}-Glu^{42}...) could thus be held by calcium together with other residues probably implicated in the binding of CRP to phosphorylamines (10, 20, 21), even when the bisulphide bridge is disrupted by the mercaptoethanol treatment.

A different relative antigenicity of the 2 regions seems to be supported by, 6-7 monoclonals of nine interfering with the CRP binding to solid-phase PE (fig. 2 and 3), 80 % of the polyclonal anti-CRP was not retained in CRP affinity columns saturated with PE (fig. 4), 90 % inhibition by PE of the mouse polyclonal anti-CRP binding to solid-phase CRP and the fact that mouse polyclonal anti-CRP behaves as most of the monoclonals when competing with 5 (fig. 3).

All these results suggest that most of the CRP molecule participates in the binding to PE including the sequences inside or around the bisulphide bridge.

Ca^{++} induced conformational changes in the CRP molecule have been implicated in this binding by the use of anti-CRP monoclonal antibodies (12). No evidence in favour of monoclonals whose binding to CRP depends on the Ca^{++}-induced conformational change of CRP was obtained in this work, either by enzyme immunoassay or by immunodiffusion. These discrepancies could be explained by the different monoclonals employed in each work or by the use of solution (12) or solid-phase (this work) assays. Immunodiffusion data showed evidence that reaction of purified CRP with the monoclonals did not need the presence of Ca^{++}. In this instance, the immunoprecipitation might be due to the pentameric nature of CRP rather than to the bifunctional monoclonal antibodies.

Acknowledgements

The author wishes to thank Dr. J. Moscoso, Gloria Morago and J. Martínez, for obtaining and processing the hybridomas used in this work and Dr. J. Albar all of them of INVESGEN for obtaining the hydrophilicity profile in the «Departamento de Inmunología de la Fundación Jiménez Díaz» (Madrid), to Angeles Samino for technical assistance, and Dr. J. Fernández-Santarén of «Centro de Biología Molecular» (Madrid) for reviewing the manuscript.

Resumen

Se estudia la interferencia de los anticuerpos policlonales y monoclonales, la digestión con tripsina y el tratamiento con mercaptoetanol, en la reacción de la proteína C reactiva (PCR), con la fosforiletanolamina (PE) ligada a una fase sólida. De nueve anticuerpos monoclonales anti-PCR estudiados, 6-7 interfieren con la reacción PCR-PE, representados por el monoclonal 2; el resto, representado por el monoclonal 5, no interfiere. La digestión de la PCR con tripsina da lugar a fragmentos que reaccionan con PE y el monoclonal 5, pero no con el monoclonal 2. La reacción PCR-PE es inhibida por tratamiento de la CPR con mercaptoetanol. Estos resultados, junto con la estimación de la antigenicidad del sitio de la PCR que reconoce a la PE y con las características del perfil hidrofóbico de la PCR, sugieren que la mayoría de las secuencias hidrofílicas de la PCR contribuyen a la reacción PCR-PE, excepto una región definida por el monoclonal 5. Es probable que dichas secuencias estén dentro o alrededor del puente disulfuro interno existente en cada monómero de la PCR pentamérica.

Palabras clave: Proteína C-reactiva, Anticuerpos monoclonales, Fosforiletanolamina, Región ligadora.

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