CALCIUM BINDING PROPERTIES OF THE C1 SUBCOMPONENTS C1q, C1r AND C1s

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1. Introduction

The first component of complement, C1, is a macromolecular complex dependent for its integrity on the presence of Ca^{2+} [1]. However there is little information on the number of calcium atoms involved in maintaining the structure of C1 or the affinity for calcium of the various subcomponents. Moreover it appears that this cation plays a direct inhibitory role in the activation of proenzyme C1r and C1s in the fluid phase [2–5]. Given the major structural changes which occur in the proenzyme C1r and C1s during their activation, it might be expected that some changes in the calcium binding characteristics of these proteins would be observed which could provide informations on the mechanism of activation of C1.

The isolated subcomponents C1r and C1s bind calcium in both the proenzymic and activated forms. In the case of C1s this leads to a dimerization of the molecule [6–8] whereas C1r exists as a dimer in the absence of calcium. The affinity between C1r and calcium is stronger for the activated form of the protease, which confirms the occurence of structural changes in C1r during activation. It has been shown recently that this subcomponent undergoes additional proteolytic cleavages following its activation, which have permitted a tentative location for the calcium binding site [5] on $C\bar{1}r$ to be made.

Abbreviations: EDTA, ethylenediamine tetraacetic acid; K_d, dissociation constant; app. K_m, apparent Michaelis constant; SDS, sodium dodecyl sulphate

Nomenclature: The components of complement are those recommended by the World Health Organization (Bull. WHO 39 (1968) 935–936).

It is not clear from the literature whether C1q has itself any affinity for calcium although from the fact that calcium is necessary for its association with the other subcomponents, we might expect it to bind calcium. To establish whether this is the case, the binding of calcium to purified C1q was also estimated.

Finally the association of the tetrameric complex $C1r_2$–$C1s_2$ with calcium was examined with a view to elucidating the role of calcium in its cohesion. In [5] evidence was presented for contact between the polypeptide chains of the $C\bar{1}r$ and $C\bar{1}s$ subunits in their self association as dimer and in their mutual interactions in the tetrameric $C\bar{1}r_2$–$C\bar{1}s_2$ complex. These data are discussed in the light of the preceding findings.

2. Materials and methods

2.1. *Chemicals*

$^{45}CaCl_2$, spec. act. 0.43 Ci/mol, was obtained from the Commissariat à l'Energie Atomique (Saclay). $TbCl_3$ was from Alfa Products (Danvers, CO). All other chemicals were analytical grade.

2.2. *Complement components*

The methods cited were used for the purification of subcomponents C1q, $C\bar{1}r$ and $C\bar{1}s$ [9] and the proenzymic forms of the two latter [5]. The proteins were prepared as follows for calcium binding estimation: 1 vol. each solution of protein was dialysed exhaustively, for 3 periods of 12 h each time, against 1000 vol. 145 mM NaCl, 5 mM triethanolamine–HCl (pH 7.4) to remove any trace of EDTA. Proteolytic cleavage of $C\bar{1}r$ was obtained by incubating purified

C1r for 5 h at 37°C in 2 mM EDTA, 145 mM NaCl, 5 mM triethanolamine–HCl (pH 7.4) [10]. Subsequently EDTA was removed as above.

Purified $C\bar{1}r$ (C1r), $C\bar{1}s$ (C1s) and C1q were estimated from their A_{280} using, respectively, $E^{1\%}_{1\,cm}$ = 11.5 [11], 9.5 [11] and 6.8 [12]. Molecular weights were taken as 85 000 for $C\bar{1}r$ (C1r) [5], 85 000 for $C\bar{1}s$ (C1s) [8] and 410 000 for C1q [12].

2.3. *Calcium binding*

Calcium binding was measured with a flow dialysis system after [13] and the dialysis cell was constructed in the laboratory by the principles in [14]. Its main features are: diffusion area, 0.5 cm^2; lower chamber vol., 0.2 ml; upper chamber vol., 2 ml. Visking tube membrane (Union Carbide) was used for the dialysis membrane between the two chambers. In each chamber, magnetic stirring was maintained throughout the experiment.

The dialysis buffer, 145 mM NaCl, 5 mM triethanolamine–HCl (pH 7.4) was perfused at a rate of 6 ml/min and the effluent collected automatically in 2 ml fractions. ^{45}Calcium radioactivity was measured on 1 ml aliquots of each fraction mixed with 10 ml scintillation fluid [15] with a SL 3000 Counter (Intertechnique). All calcium binding experiments were performed at 25°C.

Calcium binding is given by the difference between a first profile rate measured in the absence of protein and a diffusion rate measured with proteins in the cell. The estimate is based on the binding of ^{45}Ca to the protein and its subsequent displacement by increasing ^{40}Ca concentration. Since the dialysis leads to negligible loss of ligand from the cell, the same solution of protein can be used for a series of equilibria with stepwise additions of ^{40}Ca. Thus, from a single protein sample, it is possible to collect enough data for a complete binding curve and the calculation of the binding parameters. This is also particularly useful when dealing with unstable proteins since a constant diffusion rate is reached within 1–1.5 min which permits a complete binding assay to be achieved in 45 min. Results presented are in each case the average from at least 3 such experiments and the limits given are the range of values obtained.

Corrections were introduced in the data to take into account the loss of radioactivity in the cell due to dialysis and the dilution of the protein solution due to ^{40}Ca additions. The total loss of radioactivity was always $\leqslant$3%.

3. Results

3.1. *Binding of calcium to $C\bar{1}s$*

A typical ^{45}Ca diffusion rate profile is illustrated in fig.1. In the absence of protein, a maximum diffusion rate appears after the first addition of ^{45}Ca; then succesive additions of ^{40}Ca and the dialysis of ^{45}Ca are responsible for an overall continuous decrease of the diffusion rate. In detail, at each ^{40}Ca addition, the diffusion rate increases transiently then stabilizes below the initial level; this transient increase due to ^{45}Ca bound to the dialysis membrane and release by ^{40}Ca proved to be negligible in regard of the total calcium in the dialysis compartment.

In the presence of protein, ^{45}Ca binding leads to an initial lower diffusion rate which increases upon sequential ^{40}Ca additions to reach values comparable to those obtained in the absence of protein after a large addition of ^{40}Ca. Scatchard plots of the calcium binding (fig.1 insert) are linear, indicating homogeneity of the site. By extrapolation, 12 nmol/mg protein is calculated, corresponding to a binding of 2 Ca/dimeric $C\bar{1}s$ with K_d 40 μM.

3.2. *Effect of pH and protein concentration on the binding of calcium to $C\bar{1}s$*

A series of binding assays with $C\bar{1}s$ at 0.5–1.6 mg/ml all lead to the same 2 mol cation/mol dimeric $C\bar{1}s$.

Varying over pH 5–9, is without effect on the number of binding sites (fig.2 insert) whereas the K_d, which appears unchanged at pH 6–9, increases rapidly at pH 6–5 (fig.2). This could arise from the protonation of residues close to the calcium binding site creating an environment less favorable to the access by Ca^{2+} without diminishing the actual number of sites.

3.3. *Specificity of the binding of calcium to $C\bar{1}s$*

The specificity of calcium binding is assessed by experiments based on the competition between ^{45}Ca and other ions: $^{40}Ca^{2+}$, Ba^{2+}, Sr^{2+}, Tb^{3+}, Mn^{2+} and Mg^{2+}. The ability of these ions to bring about ^{45}Ca displacement from $C\bar{1}s$ is reported in fig.3. The displacement is optimal with $^{40}Ca^{2+}$ while Tb^{3+}, Ba^{2+} and Sr^{2+} compete with comparable affinities; in contrast Mg^{2+} and Mn^{2+} appear poor competitors.

When the competitivity is plotted as a function of crystal ionic radii of the different ions (fig.4), it clearly appears that ions smaller than calcium are ineffective in displacing ^{45}Ca whereas the bigger ions are nearly as effective as ^{40}Ca.

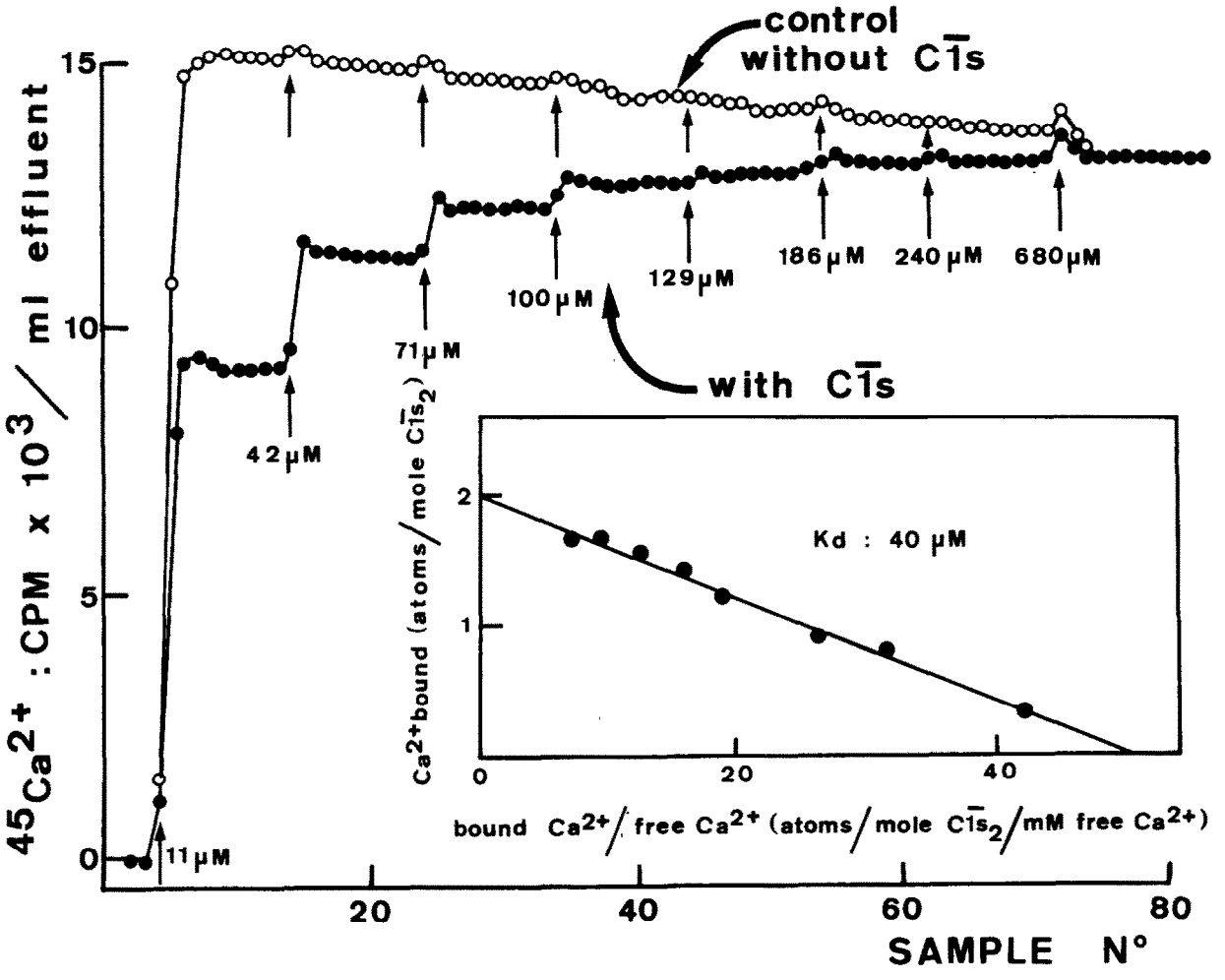

Fig.1. Binding of calcium to C1̄s. The buffer used for protein solution and elution was 145 mM NaCl, 5 mM triethanolamine–HCl (pH 7.4). Collection of samples is started before the injection of 10 μl $^{45}Ca^{2+}$ in the dialysis compartment (11 μM final conc.). Ten samples were collected before each successive $^{40}Ca^{2+}$ addition (10 μl and 40 μl for the final addition). Flowrate 6 ml/min; temp. 25°C; (○) no protein; (●) C1̄s, 1.3 mg/ml.

3.4. *Binding of calcium to C1̄ subcomponents*

Table 1 illustrates the binding of Ca^{2+} to the 3 C1̄ subcomponents. Whereas C1̄s, as shown above, binds 2 Ca/mol dimer (fig.1), C1̄r fixes between 2–3 Ca^{2+}/dimer with K_d 33 μM. It is noteworthy that the precipitation of C1̄r brought about by the presence of Ca^{2+} does not affect the Scatchard plot (fig.5). A mixture of C1̄r and C1̄s in equimolar ratio fixes 5 Ca^{2+} which is equivalent to the number of atoms of Ca^{2+} fixed by the 2 subcomponents when isolated. The K_d in this case is 32 μM, of the same order as the individual K_d values. Thus the tetrameric $C1̄r_2$–$C1̄s_2$ complex does not appear to perturb significantly the sites observed on isolated C1̄r and C1̄s.

C1q also is found to bind 3 Ca^{2+}/mol with K_d ~76 μM.

3.5. *Binding of calcium to proenzymic C1r and C1s*

Table 1 shows that proenzymic C1s binds 2 Ca^{2+}/dimer, a value comparable to the binding by C1̄s, with K_d 27 μM. In the case of proenzymic C1r, only 1 Ca^{2+} is bound/dimer protein (compared to 2.6 for

the activated dimeric $C\bar{1}r$). With equimolar amounts of C1r and C1s, the formation of the tetrameric $C1r_2$–$C1s_2$ complex is accompanied by the binding of 4 Ca^{2+}/tetramer. This value, higher than the sum of the individual binding by C1r and C1s, can reflect conformational changes on C1r and C1s brought about during their incorporation in the $C1r_2$–$C1s_2$ tetramer and resulting in an additional Ca^{2+} binding site.

3.6. *Binding of Ca^{2+} to $C\bar{1}r$ after proteolytic cleavage*

Activated $C\bar{1}r$ incubated for several hours at 37°C undergoes a proteolytic cleavage [10,16,17]. As shown by SDS–polyacrylamide gel electrophoresis, two major fragments are sequentially released by proteol-

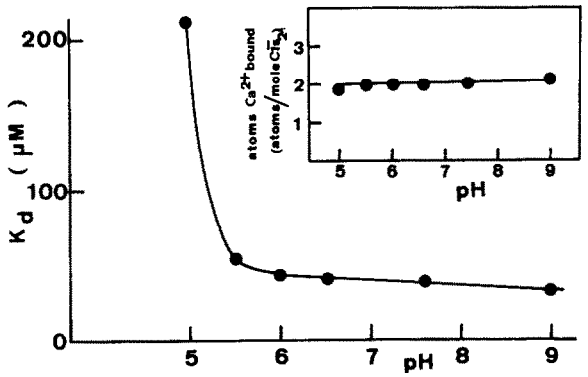

Fig.2. Effect of pH on the binding of calcium to $C\bar{1}s$. $C\bar{1}s$ (1.3 mg/ml) in 145 mM NaCl, 5 mM triethanolamine–HCl (pH 7.4) is adjusted to the desired pH by addition of 1 M sodium acetate (pH 4.0) or 1 M Tris–HCl (pH 9.0) before calcium binding. The dialysis buffer was also ajdusted in the same way. Other conditions as in fig.1. Corrections are applied for dilution due to pH adjustment.

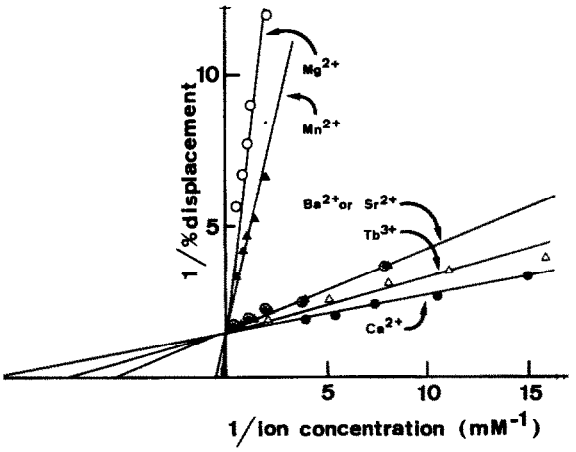

Fig.3. Competition between $^{45}Ca^{2+}$ and divalent or trivalent cations. Basic experimental conditions as in fig.1. For each ion tested, after the initial addition of 10 μl $^{45}Ca^{2+}$, 6 successive additions of the ion under test are made, followed by a final addition of $^{40}Ca^{2+}$ to 680 μM final conc. 0% displacement corresponds to calcium bound after the initial addition of $^{45}Ca^{2+}$ and 100% to calcium bound after the last addition of $^{40}Ca^{2+}$. Double reciprocal plot of 1/% displacement vs. 1/[ion]. All the cations tested are chloride salts.

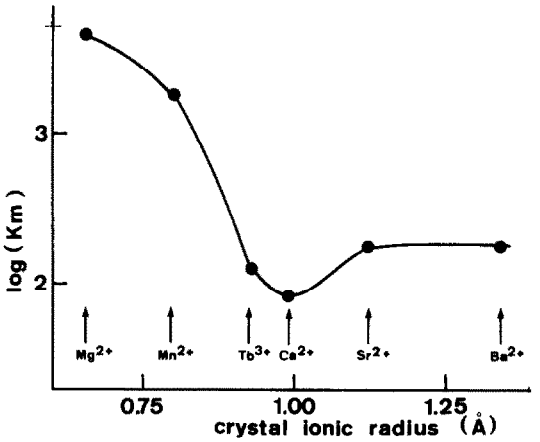

Fig.4. Apparent K_m values for $^{45}Ca^{2+}$ displacement and crystal ionic radii. The respective app. K_m values for $^{45}Ca^{2+}$ displacement are calculated from the double reciprocal plot given in fig.3 for $^{40}Ca^{2+}$, Tb^{3+}, Sr^{2+}, Ba^{2+}, Mn^{2+} and Mg^{2+}. Crystal ionic radii are taken from [20].

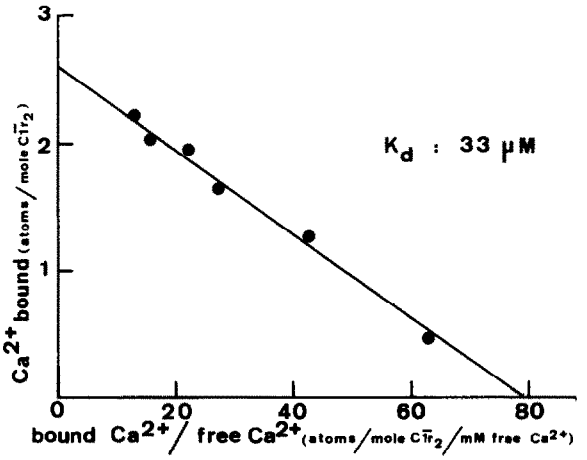

Fig.5. A typical Scatchard plot of Ca^{2+} binding to $C\bar{1}r$. Calcium binding is calculated after correction for volume variation and dialysis of calcium in the dialysis compartment, as in section 2.

Table 1
Calcium binding to $C\bar{1}$ or C1 subcomponents

Subcomponents	No. expt	Proteins (mg/ml)	Ca^{2+} bound/ mol protein	K_d (μM)
$C\bar{1}s_2$	8	1.3	2.0 (±0.1)	40 (±2)
$C\bar{1}r_2$	5	0.6	2.6 (±0.4)	33 (±3)
$C\bar{1}r_2$–$C\bar{1}s_2$	3	0.9	5.0 (±0.4)	32 (±2)
$C1s_2$	3	1.2	2.0 (±0.2)	27 (±2)
$C1r_2$	3	0.7	1.0 (±0.2)	17 (±1)
$C1r_2$–$C1s_2$	3	0.9	4.0 (±0.4)	15 (±1)
C1q	4	3.2	3.0 (±0.2)	76 (±10)

Conditions were as in section 2

ysis of the the A chain (M_r): fragment α (35 000) and fragment β (7000–11 000) (fig.6); this autolytic limited cleavage is completed in 5 h [10].

The residual molecule retains a dimeric structure and still activates C1s but appears unable to form tetrameric complexes when mixed with $C\bar{1}s$ in the presence of calcium.

In keeping with this last observation, we find that this residual $C\bar{1}r$ molecule does not bind calcium in the conditions used for the binding to native $C\bar{1}r$.

4. Discussion

Calcium binding, initially described as a global property of C1, is now shown to be a fundamental property of each of the subcomponents and plays a role in their self-association as dimers as well as a role in their mutual association in the C1 molecule. A role for calcium in C1q renaturation was shown in [21] but no direct evidence for an effect of calcium on C1q, based on CD and hydrodynamic parameters estimation was provided in [22]. For the moment, one cannot say what role calcium plays in the interactions between C1q and the other subcomponents other than that C1q binds calcium with an affinity a little less than that of the other subcomponents and with a valency which is consistent with the threefold basic structure of the molecule.

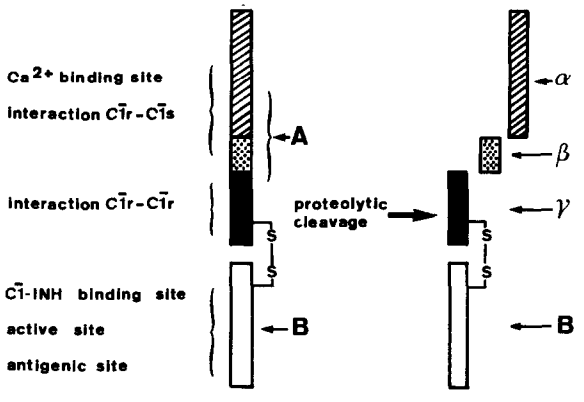

Fig.6. Tentative scheme of $C\bar{1}r$ proteolysis and of biological reactivity location. This simplified scheme does not rule out a different position of the interchain disulfide bond on the A chain.

Proenzymic or activated C1s binds calcium with equivalent valency and with comparable affinities. This binding results in a dimerization of the 4.5 S monomer into a 6.0 S molecule [10]. From peripheral iodination studies of monomers and dimers of C1s, it appears that the dimerization involves mainly the A chains of each monomer [10]; thus it is reasonable to postulate that the two calcium binding sites are located in the A chain of the $C\bar{1}s$ dimer.

In contrast to C1s there is a big difference in the calcium binding characteristics of C1r and $C\bar{1}r$. The proenzymic form binds 1 Ca^{2+}/molecule dimer (at a comparable affinity to that of $C1s_2$) while the activated form appears to bind 2.6 Ca^{2+}/mol dimer. As C1r behaves as a 7 S dimer either in the presence or in the absence of calcium, Ca^{2+} may contribute only to an increase of the intermonomer interaction. The lower valency of the proenzyme form for Ca^{2+} may be related to the failure to extract all the calcium from the $C1r_2$ (buried calcium tightly bound at the monomers interface); there is also the possibility that calcium is involved in the formation of aggregates which are known to occur in Clr and especially $C\bar{1}r$ although this occurrence did not alter the slope of

the Scatchard plots. However in the absence of such a cause the result points to a major molecular rearrangement following activation of C1r. This is reflected by separate results showing that $C1r_2$ is dissociated into monomers by incubation of pH 5.0 whereas $C\bar{1}r_2$ dissociates only at pH 4.0 [5].

As in the case of C1s, peripheral iodination indicates that the two C1r subunits interact through their A chain which are likely therefore to contain the calcium binding site.

Further evidence for this localization is provided by studies with proteolysed $C\bar{1}r$. The residual molecule which contains the intact B chain lacks a portion of the A chain and no longer binds calcium. The fact that this residual molecule still forms dimers indicates that any role played by calcium in this interaction can only be a supplementary one. In fact, calcium may affect directly or indirectly any one or all of these interactions.

The binding of calcium to mixtures of C1r and C1s is accompanied by the formation of tetrameric $C1r_2$–$C1s_2$ complexes by both the proenzymic and activated forms. The total number of atoms bound is of the same order for the activated form of the tetramer while it exceeds by one atom the sum of the individual valencies in the case of the proenzymic form. Calcium modifies the C1r–C1s interaction which was demonstrated to occur in the absence of this cation [10] with the formation of an intermediate trimeric 7.7 S complex. Whether calcium atoms bridge between the subunits cannot be stated at present, they may well function by sustaining the conformation in each subunit which favours the cohesion of the tetramer complex. The role of calcium in the activation of isolated $C1r_2$ has been reported [5,18], calcium appears to inhibit C1r autoactivation. In a proposed autocatalytic mechanism for C1r in which the pro-site of one monomer activates the pro-site of the other, there is need for rotational mobility of the subunits to bring the reacting group of first the C1r and subsequently the C1s subunits into apposition. A role which calcium might play in this mechanism would be to prevent such rotational freedom, thereby preventing the autoactivation. The reinforcement of the intersubunit bonds in the C1r dimer and in the $C1r_2$–$C1s_2$ tetramer by calcium is consistent with this hypothesis. This same mechanism can be invoked to account for the inhibitory activity of calcium in the activation of C1s by $C\bar{1}r$ [2,4–19] and in the proteolysis of $C\bar{1}r$ [5,7]. The specific requirement for calcium in this regard, as shown in [2] is borne out here in which ions of smaller crystal radii (e.g., Mg^{2+}) were found to bind inefficiently.

Acknowledgements

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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] Naff, G. B. and Ratnoff, O. D. (1968) J. Exp. Med. 128, 571–593.
[3] Gigli, I., Porter, R. R. and Sim, R. B. (1976) Biochem. J. 157, 541–548.
[4] Ziccardi, R. J. and Cooper, N. R. (1976) J. Immunol. 116, 496–503.
[5] Arlaud, G. J., Villiers, C. L., Chesne, S. and Colomb, M. G. (1980) submitted.
[6] Valet, G. and Cooper, N. R. (1974) J. Immunol. 112, 1667–1673.
[7] Assimeh, S. N., Bing, D. H. and Painter, R. H. (1974) J. Immunol. 113, 225–234.
[8] Arlaud, G. J., Reboul, A., Meyer, C. M. and Colomb, M. G. (1977) Biochim. Biophys. Acta 485, 215–226.
[9] Arlaud, G. J., Sim, R. B., Duplaa, A.-M. and Colomb, M. G. (1979) Mol. Immunol. 16, 445–450.
[10] Arlaud, G. J., Chesne, S., Villiers, C. L. and Colomb, M. G. (1980) submitted.
[11] Sim, R. B., Porter, R. R., Reid, K. B. M. and Gigli, I. (1977) Biochem. J. 163, 219–227.
[12] Reid, K. B. M., Lowe, D. M. and Porter, R. R. (1972) Biochem. J. 130, 749–763.
[13] Colowick, S. P. and Womack, F. C. (1969) J. Biol. Chem. 244, 774–777.
[14] Remy, R. and Buc, H. (1970) FEBS Lett. 9, 152–156.
[15] Patterson, M. S. and Greene, R. C. (1965) Anal. Chem. 37, 854–857.
[16] Assimeh, S. N., Chapuis, R. M. and Isliker, H. (1978) Immunochemistry 15, 13–17.
[17] Okamura, K. and Fujii, S. (1978) Biochim. Biophys. Acta 534, 258–266.
[18] Ziccardi, R. J. and Cooper, N. R. (1976) J. Immunol. 116, 504–509.
[19] Arlaud, G. J., Reboul, A. and Colomb, M. G. (1977) Biochim. Biophys. Acta 485, 227–235.
[20] Plane, R. A. and Sienko, M. S. (1963) in: Physical Inorganic Chemistry, W. A. Benjamin, New-York.
[21] Heusser, C. H., Boesman, M., Knobel, H. R., Jacot-Guillarmod, H. and Isliker, H. (1975) Immunochemistry 12, 213–219.
[22] Liberti, P. A. and Paul, S. M. (1978) Biochemistry 17, 1952–1958.