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Soluble C3 proconvertase and convertase of the classical pathway of human complement

Conditions of stabilization *in vitro*

Marie-Bernadette VILLIERS, Nicole M. THIELENS and Maurice G. COLOMB
Equipe de Recherche 'Immunochimie-Système Complémentaire' du D.R.F.-G. et de l'U.S.M.-G, Associée au C.N.R.S. (Unitée Associée no. 556) et à l'I.N.S.E.R.M. (Unité no. 238), Laboratoire de Biologie Moléculaire et Cellulaire, Centre d'Études Nucléaires de Grenoble, 85X, 38041 Grenoble Cedex, France

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Soluble classical-pathway C3 convertase and proconvertase were prepared from purified C4b–C2ox complex in the presence of Ni^{2+}; the two complexes, stable for at least 15h at 4°C, were isolated by sucrose-density-gradient ultracentrifugation. The C3 convertase alone was able to cleave C3, and its decay was accelerated in the presence of C4-binding protein. The individual roles of Ni^{2+} and I_2 treatment of C2 in the stabilization of the complexes seemed to be different and additive. $^{63}Ni^{2+}$ binding coupled to h.p.l.c. analysis showed that $^{63}Ni^{2+}$ bound only to the C2ox proteolytic fragment a (1 mol/mol) with a K_d of 26 μM. Competition studies between Ni^{2+} and Mg^{2+} indicated that only half of the Ni^{2+} bound to the C3 convertase was removed by Mg^{2+}, whereas, in the same conditions, Ni^{2+} bound to C2ox proteolytic fragment a was not displaced, suggesting the presence of two sets of sites on the convertase. EDTA prevented the formation of both C3 convertase and proconvertase; EDTA had no effect on the preformed C3 convertase, whereas it dissociated the preformed proconvertase.

Activation of the classical pathway of complement proceeds, from an initiation step at the level of C1, through a sequential assembling of two convertases, the C3 convertase and the C5 convertase, resulting in a limited proteolytic cleavage of C3 and C5 into fragments C3a and C3b and fragments C5a and C5b respectively (Reid, 1983). The C3 convertase C4b–C2a is assembled, in the presence of Mg^{2+}, from the corresponding fragments of C4 and C2 produced by a dual limited proteolysis of these two proteins by activated $C\bar{1}$ (Ziccardi, 1981; Thielens *et al.*, 1984).

In several recent papers analysing the formation of human C3 convertase on insoluble acceptors, the emphasis has been put on C4: the proteolytic cleavage of C4 by $C\bar{1}$ leads to the opening of an intrachain thioester bond, accounting for a covalent binding of C4b to nucleophilic receptors (Law *et al.*, 1980; Reboul *et al.*, 1980; Campbell *et al.*, 1980, 1981; Sim *et al.*, 1981; Reboul *et al.*, 1981; Isenman & Kells, 1982). The second substrate of $C\bar{1}$, C2, is cleaved after its binding to C4b, in the presence of Mg^{2+} (Vogt *et al.*, 1982); the resulting C4b–C2a complex is able to proteolyse C3 into C3a and C3b peptides, through a serine proteinase activity located in the C2a moiety of the complex. The C3 convertase appears as a highly unstable association with a half-life of the order of 10min at 37°C (Kerr, 1980); different protein factors are involved in its control: decaying factors, such as the C4-binding protein (Gigli *et al.*, 1979; Haydey *et al.*, 1980) or the CR1 receptor (Iida & Nussenzweig, 1981), and stabilizing factors, such as the classical-pathway nephritic factor (Daha & Van Es, 1980; Gigli *et al.*, 1981). Other conditions have also been shown to stabilize the C3 convertase *in vitro*. Thus Polley & Müller-Eberhard (1967) showed that treatment of C2 by low concentration of I_2 allowed the formation of a stable C4b–C2oxa complex; this effect of I_2 remains unclear, but appears to proceed through a reaction of I_2 with a free thiol group in C2, with the subsequent formation of an intra-

Abbreviations used: the nomenclature of complement components is that recommended by the World Health Organisation (1968); activated components are indicated by a bar; C2ox is I_2-treated C2; C2oxa and C2oxb are the corresponding proteolytic fragments.

molecular covalent bond (Parkes *et al.*, 1983). More recently also, Fishelson & Müller-Eberhard (1982) demonstrated that a stabilized C4b–C2a convertase is obtained *in vitro* on EAC$\overline{1}$ intermediates when Ni^{2+} is substituted for Mg^{2+} in the incubation medium.

Our initial purpose was to assemble *in vitro* a soluble C3 convertase suitable for physicochemical studies (electron microscopy, small-angle neutron scattering). The present paper provides evidence showing that a stable C3 proconvertase and a stable C3 convertase can be prepared *in vitro* from their individual purified subcomponents. The binding of Mg^{2+} and Ni^{2+}, which appears critical for the stability of these complexes, has been investigated in detail on the bimolecular complexes and on the individual proteins; the role of these ions is discussed in detail.

Materials

Human citrated plasma was obtained from the Centre de Transfusion Sanguine (Grenoble, France). Serum was prepared as described by Arlaud *et al.* (1977).

Yeast alcohol dehydrogenase, horse spleen apoferritin and ox liver catalase were obtained from Calbiochem.

Antiserum to human C3 was purchased from Behring. $^{63}NiCl_2$ (specific radioactivity 0.7Ci/mmol.) was from Amersham International.

Purified C$\overline{1}$s, C2, C4 and C4bp were prepared from human serum as described by Arlaud *et al.* (1979), Thielens *et al.* (1982), Reboul *et al.* (1979) and Villiers *et al.* (1981) respectively.

Isolated proteins were estimated from their absorbance at 280nm, by using $A^{1\%}_{1cm} = 9.5$ for C$\overline{1}$s (Sim *et al.*, 1977), 10.0 for C2 (Thielens *et al.*, 1982) and C4 (Reboul *et al.*, 1979), and 9.3 for C4bp (Villiers *et al.*, 1981).

M_r values were taken as 85000 for C$\overline{1}$s (Sim *et al.*, 1977), 100000 for C2 and I_2-treated C2, 68000 for C2a and 29000 for C2b (Thielens *et al.*, 1982) and 660000 for the major form of C4bp (Villiers *et al.*, 1981).

Methods

I_2 treatment of C2

I_2-treated C2 (C2ox) was prepared by reaction of C2 with I_2 as described by Parkes *et al.* (1983). Residual free I_2 was eliminated from the protein solution (350μl) by four successive dialyses at 4°C against 1 litre of 150mM-NaCl/5mM-Veronal buffer pH7.2. Protein was then used within 3 days.

Cleavage of C2 (or C2ox) by C$\overline{1}$s

C2 or C2ox (1mg/ml) was incubated with C$\overline{1}$s (0.2mg/ml) in 150mM-NaCl/5mM-Veronal buffer, pH7.2, for 20min at 37°C, with a C2/C$\overline{1}$s molar ratio of 80:1.

Preparation of C4b

C4b was obtained by incubation of purified C4 (0.2mg/ml) with C$\overline{1}$s (0.7mg/ml) for 15min at 37°C, at a C4/C$\overline{1}$s molar ratio of 20:1. The incubation medium was then concentrated by ultrafiltration on an Amicon PM10 ultrafilter. When experiments required C4b free of any trace of C$\overline{1}$s, purified C4 (1.5mg/ml) was incubated in the presence of Sepharose 4B-bound C$\overline{1}$s (3.3mg of C$\overline{1}$s/ml of Sepharose) prepared as described by Reboul *et al.* (1981) for 15min at 37°C; a C4/C$\overline{1}$s molar ratio of 1:1 was used. At the end of the incubation, the Sepharose–C$\overline{1}$s was removed by centrifugation in a disposable 1ml syringe fitted with a porous polyethylene disc (at 100*g* for 2min).

Formation of a soluble C3 convertase

Except when otherwise mentioned, classical C3 convertase was obtained by a two-step incubation. First, 40μg of C4b was incubated with 21μg of C2ox and 132μM-$NiCl_2$ in 125μl of 150mM-NaCl/5mM-Veronal buffer, pH7.2, for 5min at 20°C. Then, after addition of 4μg of C$\overline{1}$s in 40μl of the same buffer, a second incubation followed for 10min at 20°C (final $NiCl_2$ concentration was 0.1mM).

Classical C3 convertase functional assay

Fractions (50μl) were incubated with 50μl of human serum for 5min at 37°C to test its ability to cleave C3. Then the samples were submitted to crossed immunoelectrophoresis as described by Laurell *et al.* (1976) in the presence of antiserum to human C3 in the second dimension.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Reduced proteins were prepared as described by Arlaud *et al.* (1979). Polyacrylamide (5%, w/v) gels were prepared by the method of Fairbanks *et al.* (1971). Protein staining with Coomassie Blue and destaining was as described by Weber & Osborn (1969).

Polyacrylamide-gel electrophoresis under non-denaturing conditions

Polyacrylamide (8%, w/v) gels and samples were prepared by the method of Davis (1964). Protein staining and destaining was as above.

Sucrose-density-gradient ultracentrifugation

Ultracentrifugation was performed at 4°C in 5–20% (w/v) sucrose density gradient in 150mM-NaCl/5mM-Veronal buffer, pH7.2, containing 0.1mM-$NiCl_2$ and/or 1mM-$MgCl_2$ or 5mM-EDTA.

The rotor was operated for 15h at 176000*g* ($r_{av.}$ 9cm) as described by Martin & Ames (1961). Horse spleen apoferritin (17.6S), ox liver catalase (11.4S) and yeast alcohol dehydrogenase (7.6S) were used as standards to determine $s_{20,w}$. After centrifugation, fractions (120μl) were collected from the top of the tubes and proteins were estimated in each fraction at 595nm after Coomassie Brilliant Blue staining as described by Bradford (1976). Samples containing $^{63}Ni^{2+}$ were filtered, before ultracentrifugation, through 1ml of Sephadex G-50 (fine grade) equilibrated in 150mM-NaCl/5mM-Veronal buffer, pH7.2, as described by Penefsky (1977), to eliminate excess free radioisotope. Centrifugation and protein estimation were as described above. After A_{595} measurements, 1ml portions of each sample were withdrawn and added to 10ml of scintillation fluid prepared as described by Patterson & Greene (1965), and the radioactivity was measured on a Kontron SL 3000 β-radiation counter.

$^{63}Ni^{2+}$-binding assays

Binding of $^{63}Ni^{2+}$ to isolated proteins or C3 convertase was measured by equilibrium dialysis in a Dianorm apparatus equipped with two micro-cells (250μl) and Visking tube membrane (Union Carbide) (M_r exclusion 10000). Dialysis was carried out at 4°C for 16h under continuous rotation. For isolated proteins, one cell compartment was filled with 1mg of protein/ml in 160μl of 150mM-NaCl/5mM-Veronal buffer, pH7.2, and the other compartment was filled with the same buffer containing $^{63}Ni^{2+}$ at different concentrations. Binding assays of $^{63}Ni^{2+}$, in the presence of Mg^{2+}, to the soluble C3 convertase, prepared as described above, were slightly different: the convertase prepared in the presence of 28μM-$^{63}Ni^{2+}$ was incubated with Mg^{2+} at different concentrations for 5min at 20°C and injected into one compartment of the dialysis cell, and the second compartment received buffer containing the same ions. (The low concentration of Ni^{2+} was chosen in order to avoid a non-specific binding to the protein.) After equilibrium dialysis, the content of each compartment was collected and analysed: 20μl portions were used for protein determination by using the method described by Bradford (1976), with purified protein as standard in the case of isolated protein; radioactivity was measured on duplicate 20μl portions each mixed with 1ml of the dialysis buffer and 10ml of scintillation fluid. Radioactivity in the compartment without protein corresponded to free $^{63}Ni^{2+}$ and in the other compartment it corresponded to bound plus free $^{63}Ni^{2+}$. In the case of isolated proteins, the number of sites and the K_d values were calculated from the curvilinear Scatchard (1949) plot.

Separation of I_2-treated C2a and C2b

The separation of C2oxa and C2oxb was achieved at room temperature by using h.p.l.c. A 100μl fraction was loaded on a 7.5mm × 600mm column of TSK G3000 SW (LKB Produkter) equilibrated in 150mM-NaCl/5mM-Veronal buffer, pH7.2, and eluted with the same buffer at a flow rate of 1.1ml/min, with the use of a Waters Associates System equipment (model 6000A). Proteins were detected from their absorbance at 280nm, and 700μl fractions were collected and submitted to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Results

Stabilization of a soluble C3 convertase

When C3 convertase was prepared from C4b and C2 in the presence of Mg^{2+}, only two peaks were observed on sucrose-density-gradient ultracentrifugation, corresponding to C4b (7.7S) and C2a (4.7S). When C3 convertase was generated from C4b and C2ox in the presence of Ni^{2+}, two additional faster-sedimenting peaks, one at 9.4S, the second at the bottom of the tube, were observed (Fig. 1). C3 convertase activity was detected in the 9.4S peak, indicating that this peak corresponds to a stabilized C3 convertase that remains functional after 15h at 4°C (Fig. 2*a*). A contaminating C3 convertase activity was also found at the level of the 7.7S peak, this probably being due to the proximity of the 9.4S peak. C3 convertase activity was also observed at the bottom of the gradient, in possible relation with a dimerization of C4b, as reported previously (Reboul *et al.*, 1979), which would probably thus form a higher-M_r convertase. Formation of the convertase with C4 cleaved at low concentration led to a decrease of this fast-sedimenting peak, which supports this view. Addition of 160μg of C4-binding protein to the preformed convertase led to the abolition of the 9.4S peak, with proteins sedimenting at 4.7S and at the bottom of the gradient.

In order to evaluate the individual role of C2ox and Ni^{2+} in the C3 convertase stabilization, the complex was prepared from either C2ox in the presence of Mg^{2+} or C2 in the presence of Ni^{2+}. In both cases a peak of complex was observed, but in a lesser amount than in the previous experiment, whereas the amount of residual free C4b and C2a (or C2oxa) increased; in addition, when C3 convertase was prepared from C2 in the presence of Ni^{2+}, a shift of the *s* value of the peak to 8.9S was observed (Table 1*a*). Thus oxidation of C2 and substitution of Ni^{2+} for Mg^{2+} are individually less efficient than their association for the stabilization of the convertase.

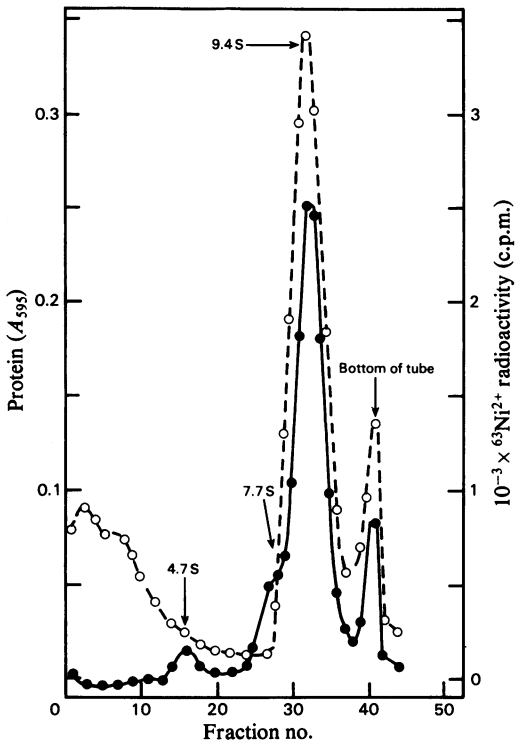

Fig. 1. *Formation of a stabilized soluble C3 convertase: incorporation of* $^{63}Ni^{2+}$

C4b (40 μg) was incubated with C2ox (21 μg) in 125 μl of 150 mM-NaCl/5 mM-Veronal buffer, pH 7.2, containing 132 μM-$NiCl_2$ for 5 min at 20°C. A second incubation after addition of $\overline{C1}$s (4 μg) in 40 μl of the same buffer containing $NiCl_2$ followed for 10 min at 20°C. The reaction mixture was filtered through a Sephadex G-50 (fine grade) column and subjected to 5–20%-sucrose-density-gradient ultracentrifugation at 176000*g* ($r_{av.}$ 9 cm) for 15 h at 4°C. The fractions were analysed for proteins by Coomassie Brilliant Blue staining (●) and for radioactivity (○).

Stabilization of a soluble C3 proconvertase

C4bC2ox complexes were observed from the incubation of C4b and C2ox in the absence of any $\overline{C1}$s. Peaks were observed at the same *s* value as those obtained in the presence of $\overline{C1}$s, but only a very low C3 convertase activity was detected in this peak, in conditions where otherwise a very large proteolytic activity was observed for an active C3 convertase (Fig. 2*b*). Analysis of the complexes by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicated that C2 had not been cleaved. These results indicate the presence of a C4bC2ox procomplex, also stable for 15 h at 4°C. In the same conditions, the amount of residual free C2ox was less after proconvertase formation than after convertase formation, suggesting that the procomplex was more stable than the complex (Table 1). The individual contributions of Ni^{2+} and C2ox to the stabilization of the proconvertase were investigated as for the convertase: substitution of Ni^{2+} by Mg^{2+} led to a small decrease of the 9.4S peak. This peak was shifted to 8.8S and 8.1S respectively when Ni^{2+} or Mg^{2+} was incubated with C2 and C4b; a parallel increase in the amount of residual free proteins was observed (Table 1*b*).

Binding of $^{63}Ni^{2+}$ *to complexes and individual proteins*

The analysis of the stabilized C3 convertase and proconvertase prepared in the presence of $^{63}Ni^{2+}$ showed in both cases a major radioactive peak at 9.4S, reflecting a binding of Ni^{2+} to the stabilized C3 convertase (Fig. 1) and proconvertase.

Binding assays were carried out on individual proteins. No significant $^{63}Ni^{2+}$ binding was observed to C4 or C4 incubated in the presence of $\overline{C1}$s. Similarly, for C2 treated as C4, no significant $^{63}Ni^{2+}$ binding was observed. When binding assays were carried out with C2ox, the $^{63}Ni^{2+}$ bind-

Table 1. *Stabilization of the C3 convertase and proconvertase*

Samples (125 μl) containing C4b (40 μg), C2 or C2ox (21 μg) and $MgCl_2$ (660 μM) or $NiCl_2$ (132 μM) in 150 mM-NaCl/5 mM-Veronal buffer pH 7.2, were incubated for 5 min at 20°C. After a second incubation (10 min at 20°C) in the presence (*a*) or in the absence (*b*) of $\overline{C1}$s (4 μg in 40 μl of the same buffer), samples were analysed by sucrose-density-gradient ultracentrifugation in the presence of either $MgCl_2$ (500 μM) or $NiCl_2$ (100 μM). Proteins were estimated by Coomassie Brilliant Blue Staining. Results are expressed as percentages of residual free C2a (or C2) compared with the control (C4b omitted).

(*a*) Convertase

	Control	C2–Mg^{2+}	C2–Ni^{2+}	C2ox–Mg^{2+}	C2ox–Ni^{2+}
Residual free C2a or C2oxa (%)	100	100	70	53	9
Sedimentation coefficient (S)	No convertase	No convertase	8.9	9.4	9.4

(*b*) Proconvertase

	Control	C2–Mg^{2+}	C2–Ni^{2+}	C2ox–Mg^{2+}	C2ox–Ni^{2+}
Residual free C2 or C2ox (%)	100	68	47	8	4
Sedimentation coefficient (S)	No proconvertase	8.1	8.9	9.4	9.4

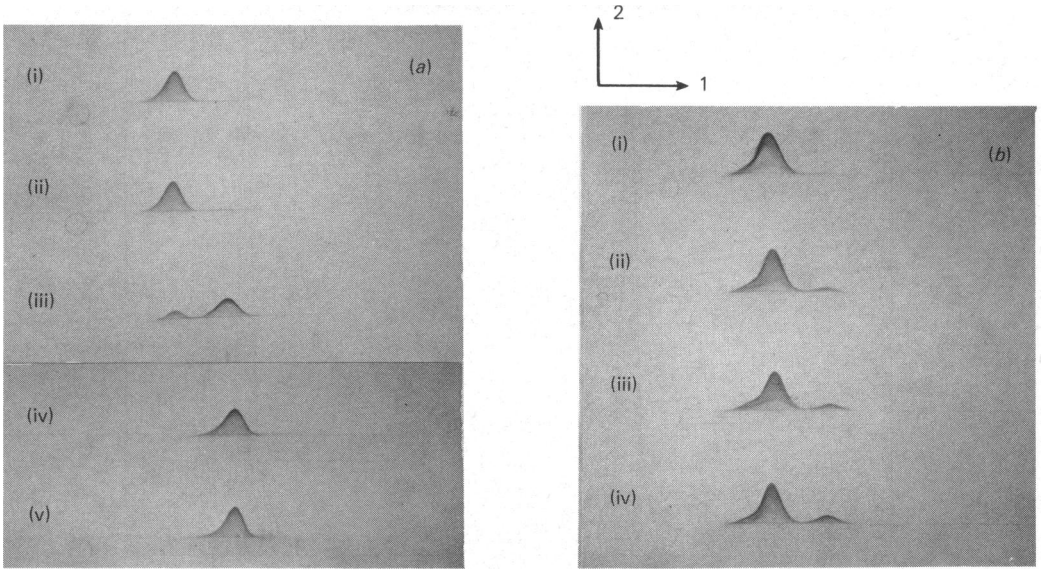

Fig. 2. *Analysis by cross-immunoelectrophoresis of the cleavage of C3 by C3 convertase or C3 proconvertase after sucrose-density-gradient ultracentrifugation*

(*a*) C3 convertase. Human serum (50 μl) was mixed with fractions (50 μl) corresponding to the different peaks observed on sucrose-density-gradient ultracentrifugation in 150 mM-NaCl/5 mM-Veronal buffer, pH 7.2, containing 0.1 mM-$NiCl_2$ and incubated for 5 min at 37°C as indicated: (i) control (serum alone); (ii) 4.7S peak; (iii) 7.7S peak; (iv) 9.4S peak; (v) bottom of the tube. (*b*) C3 proconvertase. Human serum (50 μl) was mixed with fractions (50 μl) corresponding to the 9.4S peak observed in the same conditions as described above, and incubated at 37°C as indicated: (i) control (serum alone), 15 min; (ii) 5 min; (iii) 10 min; (iv) 15 min. Crossed immunoelectrophoresis in 1% agarose with EDTA/Veronal buffer, pH 8.8 was carried out as described by Laurell *et al.* (1976) in the presence of antiserum to human C3 in the second dimension. 1, First migration; 2, second migration.

ing was very unstable and its quantification was difficult because of poor reproducibility; however, it was always less than 1 $^{63}Ni^{2+}$ ion/molecule of C2ox. The binding of $^{63}Ni^{2+}$ to C2oxa+C2oxb was stable; calculation from a Scatchard plot indicated a binding of 0.8 $^{63}Ni^{2+}$ ion/molecule of cleaved C2ox, with a K_d value of 26 μM (Fig. 3). In order to localize the Ni^{2+}-binding site in C2ox fragments, C2oxa+C2oxb was incubated with 28 μm-$^{63}Ni^{2+}$ for 10 min at 20°C and fragments were separated by h.p.l.c. Two peaks were obtained, corresponding to C2oxa and C2oxb; the radioactivity was located in the first peak (Fig. 4*a*). This result was confirmed by polyacrylamide-gel electrophoresis in non-denaturing conditions. Two samples containing C2ox cleaved by $C\bar{1}s$ were submitted to electrophoresis: one gel was stained with Coomassie Blue, the other was immersed in a $^{63}Ni^{2+}$ solution (28 μM) for 1 h, then quickly rinsed in water, and radioactivity was measured as described by Villiers *et al.* (1982). Two protein fractions were separated in these conditions: a major one, more cathodic, representing 65% of the total, labelled by $^{63}Ni^{2+}$ and corresponding to C2oxa, and a minor one, more anodic, which did not bind $^{63}Ni^{2+}$ and corresponding to C2oxb. The effect

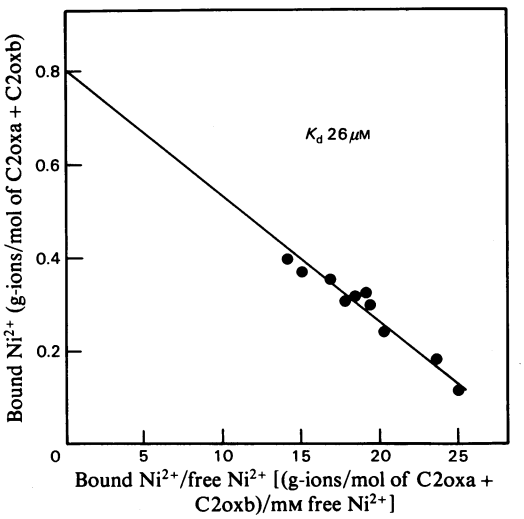

Fig. 3. *Scatchard plot of $^{63}Ni^{2+}$ binding to C2oxa+C2oxb*

C2oxa+C2oxb (160 μg; 1 mg/ml) in 150 mM-NaCl/5 mM-Veronal buffer, pH 7.2, were dialysed to equilibrium (16 h at 4°C) against 160 μl of the same buffer containing $^{63}Ni^{2+}$ at different concentrations (7 to 35 μM). Proteins were estimated by Coomassie Brilliant Blue staining.

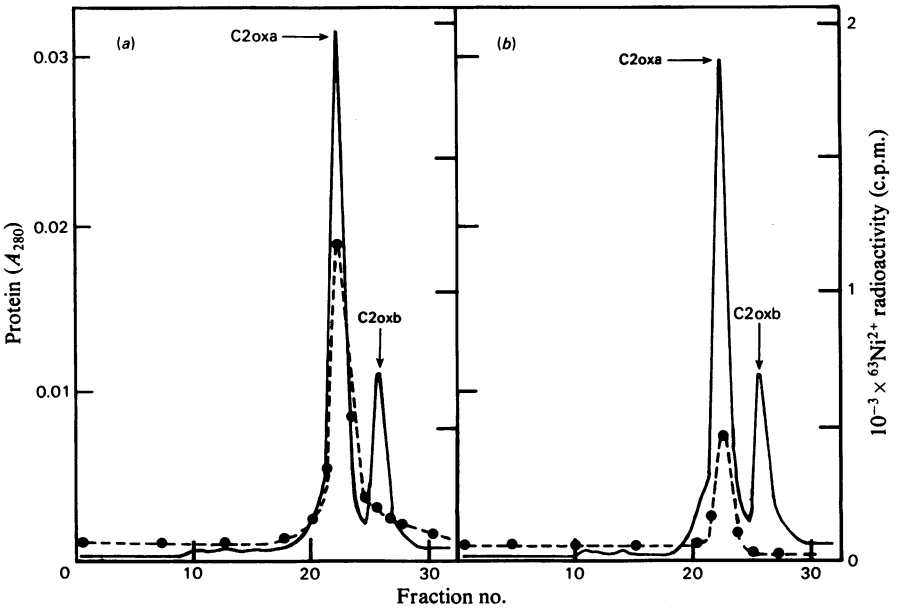

Fig. 4. *Distribution of $^{63}Ni^{2+}$ binding in C2oxa and C2oxb analysed by h.p.l.c.*
C2oxa + C2oxb (1 mg/ml) in 150 mM-NaCl/5 mM-Veronal buffer, pH 7.2, were first incubated with 28 μM-$^{63}Ni^{2+}$ for 10 min at 20°C in the absence (*a*) or in the presence (*b*) of 5.3 mM-EDTA. Samples (100 μl) were loaded on a 7.5 mm × 600 mm h.p.l.c. column of TSK G3000 SW equilibrated in the above-mentioned buffer. Proteins were eluted at a flow rate of 1.1 ml/min with the same buffer and detected from their absorbance at 280 nm (——); fractions were collected (700 μl) and analysed for radioactivity (●).

Table 2. *Role of Mg^{2+} and Ni^{2+} in the stabilization of the C3 convertase*
Samples (115 μl) containing C4b (40 μg) and C2ox (21 μg) in 150 mM-NaCl/5 mM-Veronal buffer, pH 7.2, were incubated for 5 min at 20°C (first incubation). Then, 40 μl of $\overline{C1s}$ (4 μg) in the same buffer was added and mixtures were further incubated for 10 min at 20°C (second incubation). Ions (0.1 mM-$NiCl_2$ or 0.5 mM-$MgCl_2$, final concentrations) were present during the first or the second incubation, as indicated in the Table. Samples were analysed by sucrose-density-gradient ultracentrifugation in the presence of either 0.1 mM-$NiCl_2$ or 0.5 mM-$MgCl_2$ or both. Proteins were estimated by Coomassie Brilliant Blue staining. Results are expressed as percentage of residual free C2oxa compared with the control (C4b omitted).

	Convertase					Control
First incubation	Ni^{2+}	Mg^{2+}	$Ni^{2+}+Mg^{2+}$	Mg^{2+}	–	Ni^{2+}
Second incubation	–	–	–	Ni^{2+}	Ni^{2+}	–
Residual free C2oxa (%)	12	50	12	19	25	100

of EDTA on the binding of $^{63}Ni^{2+}$ was studied, by again using h.p.l.c. C2oxa and C2oxb were incubated first in the presence of 28 μM-$^{63}Ni^{2+}$ for 10 min at 20°C, then in 5 mM-EDTA for another 10 min at 20°C before separation by h.p.l.c. C2oxa contained only 44% of the radioactivity compared with that measured in the same fraction in the absence of EDTA (Fig. 4*b*). This result suggests a partial removal by EDTA of $^{63}Ni^{2+}$ bound to C2oxa.

Role of ions on the C3 convertase and proconvertase stabilization

An optimal stabilization of the C3 convertase was observed when C4b and C2ox were incubated in the presence of 0.1 mM-Ni^{2+} before addition of $\overline{C1s}$; simultaneous addition of 1 mM-Mg^{2+} did not modify the results. When Mg^{2+} alone was present, C3 convertase was formed in smaller amount than when Ni^{2+} was subsequently added at the same time as $\overline{C1s}$. In all cases Mg^{2+} was less efficient than Ni^{2+} in the stabilization of the C3 convertase, even when used in a 10-fold molar excess over Ni^{2+} (Table 2).

Displacement of bound $^{63}Ni^{2+}$ by Mg^{2+} was observed. A plateau corresponding to a maximal displacement of 52% of bound $^{63}Ni^{2+}$ was reached from a Mg^{2+}/Ni^{2+} molar ratio of 10:1 (Fig. 5); in

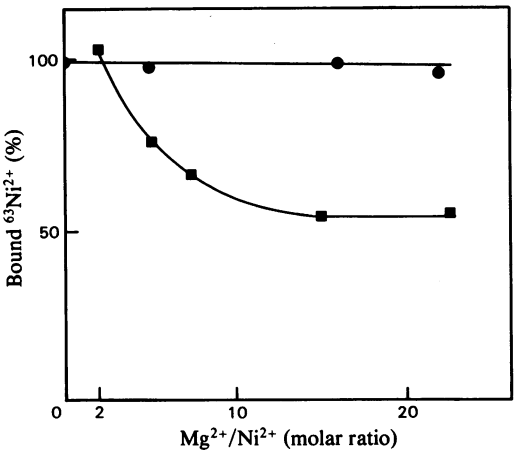

Fig. 5. *Displacement by Mg^{2+} of Ni^{2+} bound to C3 convertase or to cleaved C2ox*

C3 convertase (■): 145 μl of C4b (40 μg) and C2ox (21 μg) in 150 mM-NaCl/5 mM-Veronal buffer, pH 7.2, were incubated in the presence of 32 μM-$^{63}Ni^{2+}$ for 5 min at 20°C. Then, 4 μg of $C\bar{1}s$ in 10 μl of 150 mM-NaCl/5 mM-Veronal buffer, pH 7.2, was added and samples were further incubated for 5 min at 20°C. Cleaved C2ox (●): 145 μl of cleaved C2ox (120 μg) in 150 mM-NaCl/5 mM-Veronal buffer, pH 7.2, was incubated in the presence of $^{63}Ni^{2+}$ for 10 min at 20°C. In both cases, a further incubation followed for 5 min at 20°C after addition of 0 to 560 μM-Mg^{2+} in 10 μl of the same buffer as above. Each sample (160 μl) was dialysed to equilibrium (16 h at 4°C) in micro-cells against 160 μl of buffer containing $^{63}Ni^{2+}$ and Mg^{2+} at the concentrations used for the corresponding protein sample. Proteins were estimated by Coomassie Brilliant Blue staining. Results are expressed as percentages of a control without $MgCl_2$.

contrast, no displacement of bound $^{63}Ni^{2+}$ on C2oxa by Mg^{2+} was observed (Fig. 5). These results seem to reflect the presence of two kinds of binding sites for Ni^{2+} in the C4bC2oxa complex.

The 9.4S peak was not detected when the convertase or the proconvertase was prepared in the presence of 5 mM-EDTA. The incubation of the preformed C3 convertase in the presence of 5 mM-EDTA for 5 min at 20°C was without effect on the convertase, whereas the incubation of the preformed C3 proconvertase in the same conditions led to a large decrease of the 9.4S peak (90%).

Discussion

A soluble classical C3 convertase prepared from purified C4b, C2ox and $C\bar{1}s$ in the presence of Ni^{2+} is stable during at least 15 h at 4°C. This convertase, with a sedimentation coefficient of 9.4S, is able to cleave C3 and is destabilized by C4-binding protein, as expected. These observations confirm other results on the C3 convertase stabilization, either on the cell-bound complex (Fishelson & Müller-Eberhard, 1982) or on the soluble convertase (Kerr, 1980). The C3 convertase obtained in the above conditions is stable enough for physicochemical studies, such as electron microscopy or small-angle neutron scattering for instance.

Similarly, a soluble proconvertase was prepared from C2ox and C4b without any trace of $C\bar{1}s$; C4b–C2 interaction has been previously mentioned in experiments in which gel filtration of affinity chromatography was used (Kerr, 1980; Nagasawa & Stroud, 1977; Thielens *et al.*, 1982). The isolation of a C4bC2ox complex in the presence of Ni^{2+} demonstrates the reality of this C4b–C2 interaction. The sedimentation characteristics of the proconvertase are very similar to those of the convertase, suggesting the presence of C2b in the convertase [a C4b–C2b interaction was mentioned by Nagasawa & Stroud (1977) and Kerr (1980)]. The isolated proconvertase is without appreciable enzymic activity. Of the two parameters influencing the C3 convertase and proconvertase stability, oxidation of C2 seems to be the most effective, with an additive effect when Ni^{2+} and C2ox are used simultaneously. The same conclusion is drawn from a shift of the sedimenting peaks of the complexes (Table 1), which reflects a different affinity between the two constitutive proteins of the convertase depending on ions and C2 treatment.

The proconvertase formation is corroborated by other observations. The amount of C3 convertase is enhanced when C4b and C2ox are allowed to interact in the presence of Ni^{2+} before the C2ox cleavage by $C\bar{1}s$. It was also shown that the kinetics of C2 cleavage by $C\bar{1}s$ is increased when C4b is present (Thielens *et al.*, 1984).

Whereas $^{28}Mg^{2+}$ has a very short half-life, $^{63}Ni^{2+}$ is a very stable isotope, which allows an evaluation of the role of Ni^{2+} in the convertase formation and, indirectly, the role of Mg^{2+} by using Ni^{2+}/Mg^{2+} competition. Direct demonstration of Ni^{2+} incorporation into the classical C3 convertase is in good correlation with observations on the alternative-pathway C3 convertase, but the limited solubility of the C4b–C2oxa complex formed in the presence of Ni^{2+} prevents more precise determinations. However, quantitative determination of $^{63}Ni^{2+}$ binding is possible on individual proteins or their fragments: among C2, C2ox, C2a+C2b, C2oxa+C2oxb, C4 and C4a+C4b, only C2oxa+C2oxb bind $^{63}Ni^{2+}$ in a reproducible manner (1 ion/molecule of cleaved C2ox). The separation of C2oxa from C2oxb allows us to localize the Ni^{2+} binding on C2oxa, which might

suggest a particular role of Ni^{2+} on the catalytic activity of this fragment in the convertase. This binding is similar to the binding observed for Bb, but with a higher affinity (K_d 26 μM compared with 120 μM) (Fishelson *et al.*, 1983). This difference in affinity can be related to treatment of C2 by I_2, as binding of Ni^{2+} is not observed with native C2.

The observation that Ni^{2+} increases the amount of C3 convertase even when it is added after pre-incubation of C4b and C2ox with Mg^{2+} indicates either a greater affinity for Ni^{2+} than for Mg^{2+} or a specific binding site for Ni^{2+}. As a matter of fact, the study of the Ni^{2+}/Mg^{2+} competition indicates that only half of the Ni^{2+} bound to the convertase is removed by Mg^{2+}, whereas, in the same conditions, no displacement of Ni^{2+} bound to C2oxa occurs. These results suggest the presence of two kinds of Ni^{2+} binding sites.

EDTA prevents the formation of the C3 convertase and proconvertase. When it is added after the formation of these complexes, the proconvertase is dissociated, as expected, whereas the convertase is not affected. It should be noted that a stabilized alternative-pathway C3 convertase has also been isolated by sucrose-density-gradient ultracentrifugation in the presence of EDTA by Fishelson & Müller-Eberhard (1983). This inaccessibility to EDTA contrasts with the decrease of 50% of Ni^{2+} bound to C2oxa in the presence of EDTA. It seems plausible that, besides the Ni^{2+}-binding site on C2a involved in the cohesion with C4b, C2a contains a second Ni^{2+}-binding site; the eventual role of this site in the enzymic specificity of the complex or in its control by regulatory factors, for instance, remains to be defined. Similar arguments have been developed by Fishelson & Müller-Eberhard (1983) concerning the stabilizing effect of Gd^{2+} on the alternative-pathway C3 convertase.

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