Non-enzymic activation of the covalent binding reaction of the complement protein C3

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The covalent binding of [^3H]glycerol to C3 by the transfer of the acyl group of the internal thioester of C3 to the hydroxy group of glycerol can be activated either proteolytically by trypsin or by various chaotropes and denaturants. The activation of binding by trypsin or KBr showed similar dependence on the concentration of glycerol, indicating a similar activation mechanism. It is therefore concluded that the conformational change of the protein is the critical step in the binding reaction, and that the conversion of C3 into C3b under physiological conditions is only a means to induce the conformational change. Guanidinium chloride induces the binding of glycerol to C3 at concentrations of about 1 M. On increasing the concentration of guanidinium chloride the extent of binding declines and is accompanied by an increase in the autolytic cleavage reaction [Sim & Sim (1981) *Biochem. J.* **193**, 129–141]. The autolytic cleavage reaction is therefore not independently activated with respect to the binding reaction. Its occurrence, however, is structurally restricted under physiological or limited denaturing conditions and is permissible only when C3 is brought to a higher denaturation state.

The covalent binding reaction of C3/C3b (Law & Levine, 1977) has been described as an acyl-transfer reaction (Law *et al.*, 1979, 1980; Sim *et al.*, 1981; Hostetter *et al.*, 1982). A thioester (Janatova *et al.*, 1980; Tack *et al.*, 1980; Pangburn & Müller-Eberhard, 1980), which is normally hidden in the native C3 molecule, becomes exposed upon the cleavage of C3 to C3b. The exposed thioester is free to react with hydroxy or amino groups of surrounding molecules to form ester- or amide-linked complexes (Law *et al.*, 1979, 1981; Hostetter *et al.*, 1982). Alternatively, the exposed thioester is hydrolysed. Thus, in an aqueous medium, the range over which C3 can mediate the binding reaction is spatially limited by diffusion. C3b can only bind to cell surfaces on which it is activated (Müller-Eberhard *et al.*, 1966; Götze & Müller-Eberhard, 1970). Bystander cells, in general, are outside the diffusion range of the activated molecule. C3b-mediated activities, such as assembly of the terminal complement components (Mayer *et al.*, 1981), and

complement-mediated phagocytosis (Griffin, 1977), are therefore restricted to cells that can activate the classical or alternative pathways of complement. Normal host cells, which do not activate the complement pathways, are free from the binding of C3b. [For reviews on the activation of the complement pathways, see Porter & Reid (1979) and Müller-Eberhard & Schreiber (1980)].

C3 has a molecular weight of about 187 000, with two disulphide-linked polypeptide chains: C3 α , of mol.wt. 110 000, and C3 β , of mol.wt. 75 000 (Nilsson *et al.*, 1975; Bokisch *et al.*, 1975). The activation of C3 involves the cleavage of a 77-amino-acid fragment (C3a) from the *N*-terminus of the α -chain (Hugli, 1975). The thioester is between the cysteinyl residue and the second glutamyl residue of a -Gly-Cys-Gly-Glu-Glu- stretch in the C3 α -polypeptide (Tack *et al.*, 1980).

With a fluid-phase system, the binding of radioactive small molecules (S) to C3/C3b in the presence of trypsin was studied as a function of the concentration of S (Law *et al.*, 1981). The result is consistent with the reaction scheme below:

Abbreviations used: BE, binding efficiency; PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene; SDS, sodium dodecyl sulphate; the nomenclature of the complement proteins is that recommended by the World Health Organisation (1968, 1981); C3*, C3b*, the active states of C3 and C3b; C3i, inactivated C3.

where C3b* represents the activated state of C3b with an exposed but intact thioester, and C3b-S the covalently linked complex between C3b and S.

There are two other features of C3 related to its possession of an internal thioester. Circular-dichroism (c.d.) measurements in the near-u.v. have revealed that the conformational state of native C3 and C3b are different, and that the difference is attributed to the breakage of the thioester in the conversion of C3 into C3b (Isenman *et al.*, 1981). The other feature is the autolytic cleavage reaction of C3 under denaturing conditions (Janatova *et al.*, 1980; Sim & Sim, 1981). The α -polypeptide of C3 undergoes a peptide-bond cleavage into two fragments of sizes 74 and 46 kDa when native C3 is denatured in SDS or guanidinium chloride, or at high temperature.

The relationship between the binding reaction, the autolytic cleavage reaction and the conformational change of C3 is not clear, though they all proceed in association with the breakage of the thioester. In the present paper the binding reaction is further studied by the binding of glycerol to C3 activated either by proteolysis or in the presence of chaotropic reagents. The results suggest that limited conformational change of the protein is an essential step in activating the binding reaction, and that autolytic cleavage occurs upon extensive denaturation of the protein.

Materials and methods

Chemicals and reagents

Outdated human plasma was obtained from the National Blood Transfusion Service, John Radcliffe Hospital, Oxford, U.K. Glycerol [AR (analytical reagent)], KBr [SLR (standard laboratory reagent)], KSCN (SLR), urea (AR), naphthalene, PPO, and dimethyl POPOP were purchased from Fisons Ltd, Loughborough, Leics., U.K. SDS (specially pure), and iodoacetamide were from BDH Ltd., Poole, Dorset, U.K. Guanidinium chloride (ultrapure) was from Bethesda Research Laboratories, Rockville, MD, U.S.A. [^3H]Glycerol (200 mCi/mmol) in sterile aqueous solution, and [^{14}C]methylamine hydrochloride (44 mCi/mmol) were products of New England Nuclear Corp., Boston, MA, U.S.A. Iodo-[^{14}C]acetamide (53 mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. NCS tissue solubilizer was from Amersham Corp., Arlington Heights, IL, U.S.A.

Complement proteins

C3 was purified from plasma as described by Hammer *et al.* (1981). C3, at 5 mg/ml, was stored in 25 mM-potassium phosphate/2 mM-EDTA/100 mM-NaCl/0.01% NaN_3 , pH 7.5, at 4°C. The activity of C3, as determined by its [^{14}C]methylamine-binding

capacity (Law *et al.*, 1980), was found to be 85–90% immediately after purification, and gradually decreased to about 40% in 4 months at 4°C.

[^3H]Glycerol binding and inactivation of C3

[^3H]Glycerol was mixed with glycerol to the desired specific radioactivity (25–100 mCi/mmol) and the water was removed in a Speed Vac concentrator (Savant Instruments, Hicksville, NY, U.S.A.). The [^3H]glycerol was dissolved by the addition of a predetermined amount of 25 mM-sodium phosphate/100 mM-NaCl, pH 7.5. Iodo-[^{14}C]acetamide was mixed with iodoacetamide to a desired specific radioactivity (10–25 mCi/mmol).

[^3H]Glycerol, iodo-[^{14}C]acetamide, C3, and buffer were added to a reaction tube. The reagent to inactivate C3 was added last. The composition of the final mixture varied between experiments: [^3H]glycerol ranged from 7 to 25 mM, C3 from 10 to 25 μM (2–5 mg/ml), and iodo-[^{14}C]acetamide from 20 to 30-fold molar excess over C3. The final volume was between 100 and 120 μl . The incubation was carried out at 37°C for 1 h. A 10–15-fold volume excess, again dependent on the experiment of 0.25 M-Tris/137 mM-glycerol, pH 8.3, was added. The mixture was left at room temperature for an extra 1 h. A 100 μl portion of 25 mM-L-cysteine was added and allowed to react with excess iodoacetamide. The sample was dialysed against 25 mM-sodium phosphate/100 mM-NaCl, pH 7.5, at 4°C.

In experiments where trypsin was used to activate the binding reaction, trypsin [0.5% (w/w) with respect to C3], was added at the beginning of the incubation period. A 2-fold (w/w) excess of soybean trypsin inhibitor over trypsin was added after 5 min. The mixture was left at 37°C for the rest of the 1 h incubation.

The final concentration of glycerol in the reaction mixture was determined by counting a small portion (1 μl) for radioactivity.

The [^3H]glycerol cannot be effectively removed by dialysis without the addition of SDS (Law *et al.*, 1981). However, in the present experiments SDS was not compatible with most of the reagents used. Hence, the samples were analysed by SDS/polyacrylamide-gel electrophoresis and the covalent binding of [^3H]glycerol to C3 was determined by counting the radioactivity in association with various C3 bands. The removal of iodo-[^{14}C]acetamide was complete upon dialysis.

The non-specific binding of [^3H]glycerol to C3 varies according to the reagents used. For this reason, the binding of [^3H]glycerol to the C3 β -chain was used as an indicator of the amount of non-specific binding. C3 incubated with [^3H]glycerol in buffer was included in the experiment as a control. The ratio of the amount of radioactivity

recovered from all six gel slices to that from C3 β (slice no. 5) was obtained (e.g., see Fig. 1, sample h). The ratio had a numerical value between 2.3 and 2.5. Non-specific binding under each set of incubation conditions was taken as this ratio multiplied by the amount of radioactivity from the β -chain.

Protein determination

The concentration of protein in samples was determined by the method of Lowry *et al.* (1951), bovine serum albumin being used as the standard.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was by the method of Laemmli (1970) with gels containing 7% (w/v) acrylamide. Gels were stained and destained in Coomassie Blue as described by Gigli *et al.* (1976). Stained gels were scanned at 540 nm with a Vitatron densitometer.

C.d. measurements

C.d. measurements were made with a Roussel Jouan Dichrograph, Mark II. The kinetics of the change in c.d. of C3 in 2 M-KBr, 37°C, was at 264.5 nm (Isenman *et al.*, 1981).

Scintillation counting

Scintillation counting was carried out in a LKB 1211 Minibeta liquid-scintillation counter. Liquid samples (50–200 μ l) were counted in 3.5 ml of 1,4-dioxan/naphthalene (20 g/litre)/PPO (5 g/litre). Gel slices were first solubilized in 1.5 ml of NCS tissue solubilizer (9:1, v/v, with respect to water) at 56°C for 2 h before the addition of 15 ml of toluene/PPO (5 g/litre)/dimethyl-POPOP (0.3 g/litre).

For counting the radioactivity of double-labelled ($^3\text{H}/^{14}\text{C}$) samples, the ^3H window was set between 008 and 090 and that for ^{14}C between 090 and 165. The counting efficiencies of ^3H were 33% for both scintillants, and the cross-over to the ^{14}C window was negligible (<1%). The counting efficiencies of ^{14}C were 62 and 75%, with cross-over to the ^3H window (counts in ^3H channel/counts in ^{14}C channel) of 62 and 32% for the dioxan and toluene scintillants respectively.

Results

Binding of [^3H]glycerol to C3 by non-enzymic activation

C3 (10 μM) was incubated with [^3H]glycerol (100 mCi/mmol, 15 mM) in the presence of different reagents at pH 7.5, 37°C for 1 h. A 25-fold molar excess of iodo[^{14}C]acetamide (11.3 mCi/mmol) over C3 was also included in the reaction mixture to label the free thiol group on inactivated C3 as an indicator for the extent of inactivation in each sample. After

the removal of radioactive small molecules by dialysis, the samples were analysed by SDS/polyacrylamide-gel electrophoresis. The results are shown in Fig. 1(a). The α - and β -bands of C3 and C3b are identified in all samples. Bands of high molecular weight both at the top of the gel and at about 140 000 mol.wt. are also detected in C3

Fig. 1. Binding of [^3H]glycerol to C3 in different reagents

C3 (10 μM) was incubated with [^3H]glycerol (15 mM) in the presence of: a, 2 M-KBr; b, 0.5 M-KSCN; c, 1 M-guanidinium chloride; d, 3 M-urea; e, 0.015% SDS; f, 0.5% trypsin (w/w) with respect to C3; g, 0.1 M-methylamine; and h, buffer. (a) The polypeptide patterns on SDS/polyacrylamide-gel electrophoresis after incubation. Brackets (I) nos. 1–6 indicate the slices cut out for radioactivity measurements. (b) Histograms of the radioactivity counts (in c.p.m./ μg of C3). The slice numbers correspond to the brackets marked in (a).

samples treated with either 0.5 M-KSCN, 1 M-guanidinium chloride, 3 M-urea, or 0.015% SDS (lanes b-e). Appearance of the high-molecular weight bands was not obvious in C3 samples incubated in KBr trypsin, methylamine or buffer (lanes, a, f, g and h). In 0.015% SDS at least some of the C3 underwent autolytic cleavage, yielding the characteristic polypeptides of mol.wts. 74000 and 46000 (lane e) (Sim & Sim, 1981). Autolytic cleavage activity of C3 may also be present in 1 M guanidinium chloride. The effect, however, is less pronounced (lane c; also see below).

The ^3H radioactivity in association with each band was measured. The results are shown in Fig. 1(b). Significant [^3H]glycerol binding to the α -chain of C3 in comparison with the binding to the β -chain was demonstrated for the incubation of C3 in KBr, KSCN, guanidinium chloride, or urea, as well as when trypsin was used to activate the binding reaction (Fig. 1b; lanes a, b, c, d and f). It is not obvious from the results presented in Fig. 1(b), lane e that binding took place in 0.015% SDS (see Table 1 and the Discussion section). No significant binding was observed when C3 was incubated with [^3H]glycerol in the presence of 100 mM-methylamine (Fig. 1b, lane g). Non-specific binding under each set of incubation conditions was estimated as the amount of radioactivity bound to the β -chain multiplied by 2.3. Specific binding under each set of incubation conditions is listed in Table 1. It should be emphasized that the S.E.M. shown only represents the statistical error in the measurements. Systematic

variation, such as the possibility of differential non-specific binding of [^3H]glycerol to the α - and β -chains under different conditions, is not taken into account.

The absence of [^3H]glycerol binding to C3 in 100 mM-methylamine could be due to the high concentration of methylamine, which can also react with the exposed thioester during the binding reaction. C3 was incubated with [^3H]glycerol (100 mCi/mmol, 7 mM) in the presence of 10 mM-methylamine or 1 M-guanidinium chloride or both. The $t_{1/2}$ of C3 inactivation in 10 mM-methylamine is about 30 min (Law *et al.*, 1980), and that in 1 M-guanidinium chloride is about 1.5 min (see below). No binding was observed when C3 was incubated with [^3H]glycerol in 10 mM-methylamine. However, the presence of 10 mM-methylamine only showed insignificant inhibition of the binding of [^3H]glycerol to C3 in 1 M-guanidinium chloride. The results (Table 2) indicates that methylamine inactivates C3 without activating the binding reaction.

Binding of [^3H]glycerol to C3 in KBr: dependence on glycerol concentration

To test whether the binding of glycerol to C3 as induced by KBr and other chaotropes and denaturants is similar to the binding during the proteolytic conversion of C3 into C3b, the binding of [^3H]glycerol (25 mCi/mmol) to C3 in 2 M-KBr was studied as a function of glycerol concentration. The reaction was carried out at pH 7.5 and allowed to proceed for 1 h at 37°C. The reaction was stopped by the addition of 0.4 ml of 25 mM-sodium phosphate/100 mM-NaCl/137 mM-glycerol, pH 7.5. The activity of C3 in the experiment was determined by its methylamine-binding capacity (Law *et al.*, 1980) and was found to be about 60%.

The samples were run on a SDS/polyacrylamide slab gel. The radioactivity in association with the C3 α -chains was measured for each sample. The background level of binding was estimated from control samples where C3i (C3 pre-inactivated in

Table 1. [^3H]Glycerol binding and inactivation of C3. C3 was incubated with [^3H]glycerol in the presence of different reagents at 37°C, pH 7.5, for 1 h. Specific binding is expressed in c.p.m./ μg of C3 and is not corrected for the C3 activity (68%) or the percentage inactivation. The S.E.M. shown indicates the confidence in establishing positive or negative binding. Percentage inactivation was measured by the percentage appearance of the maximal detectable level of the thiol group (0.68 mol/mol of C3).

Inactivating reagent	Concn.	Inactivation (%)	Specific binding (c.p.m./ μg of C3)
KBr	2 M	99	43.1 $\pm$ 3.4
KSCN	0.5 M	73	34.9 $\pm$ 3.6
Guanidinium chloride	1 M	97	32.2 $\pm$ 3.0
Urea	3 M	106	41.2 $\pm$ 3.7
SDS	0.015%	71	9.0 $\pm$ 3.2
Trypsin	0.5%*	100	26.9 $\pm$ 3.4
Methylamine†	0.1 M	94	1.5 $\pm$ 4.3

* W/w with respect to C3.

† The inactivation of C3 in methylamine was carried out at pH 8.0 instead of 7.5.

Table 2. Inactivation of C3 in methylamine. C3 was incubated with [^3H]glycerol (7 mM) in 10 mM-methylamine and/or 1 M-guanidinium chloride at pH 8.0, 37°C, for 1 h. The C3 activity is 50% (see also the legend to Table 1).

Reagent	Inactivation (%)	Specific binding (c.p.m./ μg of C3)
Methylamine (10 mM)	79	-0.7 $\pm$ 4.5
Methylamine (10 mM) and guanidinium chloride (1 M)	99	28.1 $\pm$ 3.3
Guanidinium chloride (1 M)	99	30.0 $\pm$ 3.2

Fig. 2. Binding of $[^3\text{H}]$ glycerol to C3 as a function of glycerol concentration

C3 was incubated in 2M-KBr (O) or trypsin (●) in different concentrations of $[^3\text{H}]$ glycerol at pH 7.5, 37°C, for 1 h. The double-reciprocal plot of binding efficiency (BE) against glycerol concentration is shown.

2M-KBr, 37°C, 1 h) was used in place of C3. The specific binding of $[^3\text{H}]$ glycerol to C3 induced by KBr was obtained by subtracting the background binding at each concentration of glycerol. The binding efficiency (BE), defined as the molar ratio of covalently linked C3–glycerol complexes to total C3, at each concentration of glycerol, was calculated, correcting for the C3 activity of 60% (Law *et al.*, 1981). Fig. 2 shows the double-reciprocal plot of the binding efficiency against the concentration of glycerol. A straight line intersecting the y-axis at about 1 is obtained.

An experiment using trypsin [0.5% (w/w) with respect to C3] to activate binding was done in parallel. Preformed C3b was used for the determination of background binding. The incubation was at 37°C for 5 min when soya-bean trypsin inhibitor at 2-fold weight excess over trypsin was added. The result is also shown in Fig. 2.

Kinetics of inactivation and $[^3\text{H}]$ glycerol binding of C3 in 2M-KBr

C3 (13 μM) was incubated with $[^3\text{H}]$ glycerol (100 mCi/mmol, 8 mM) in 2M-KBr at 37°C. A 25-fold molar excess of iodo $[^{14}\text{C}]$ acetamide (23 mCi/mmol) over C3 was included in the reaction mixture to label the free thiol group generated during the course of incubation. At specific times, 125 μl portions were removed from the reaction mixture and immediately added to 1.5 ml of 0.25M-Tris/137 mM-glycerol, pH 8.3, at room temperature. The samples were left at room temperature for a further 1 h to allow the labelling of the thiol groups to go to completion. The ^3H and ^{14}C radioactivity in association with the α - and β -chains of C3 were measured as described above.

The aim of the experiment was to determine if there is a difference between the kinetics of the glycerol binding and the inactivation of C3, as measured by the appearance of the free thiol group. It was first established that the incubation of C3 in KBr at concentrations of 0.2M or less, pH 8.3, at 37°C or room temperature for 2 h, did not lead to the exposure of the free thiol (results not shown). Hence we may assume that the inactivation reaction was stopped upon dilution of the reaction mixture in a 12-fold volume excess of Tris buffer, and that subsequent incubation did not lead to additional inactivation of C3. The binding of $[^3\text{H}]$ glycerol to C3 was also assumed to stop when the reaction mixture was added to 0.25M-Tris with 137 mM-glycerol (200-fold molar excess).

The non-specific binding of iodo $[^{14}\text{C}]$ acetamide to C3 was negligible. Non-specific binding of $[^3\text{H}]$ -glycerol was taken to be 2.5 multiplied by the amount of radioactivity bound to the β -chain.

The data on $[^3\text{H}]$ glycerol binding and the appearance of the free thiol group were normalized by expressing them as a percentage of the maximum values, which were obtained after 1 h of incubation. The normalized data points are shown in Fig. 3. The degree of uncertainty in the data of the experiment was indicated by the error bars shown for the two points at 0 and 100%, where replicate samples were obtained. Within the accuracy of the experimental system, no difference between the kinetics of inactivation and that of $[^3\text{H}]$ glycerol binding can be confidently established. The t_1 for both reactions was between 3 and 4 min.

Similar results were obtained when the reaction was carried out at a glycerol concentration of 22 mM, or when guanidinium chloride (1M), in 11 mM- or 23 mM-glycerol, was used as the inactivation reagent. t_1 for C3 inactivation in 1M-guanidinium chloride was between 1 and 2 min (results not shown).

C.d. measurements at 264.5 nm showed a positive change of about 25 degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ when

Fig. 3. Kinetics of inactivation and $[^3\text{H}]$ glycerol binding of C3 in 2M-KBr

C3 ($13\mu\text{M}$) was incubated with $[^3\text{H}]$ glycerol (8mM) in 2M-KBr. Inactivation of C3 (●) was determined as the percentage appearance of maximal detectable level of the thiol group, measured by labelling with iodo $[^{14}\text{C}]$ acetamide. $[^3\text{H}]$ Glycerol binding (○) is expressed as a percentage of maximal binding after 1 h of incubation.

C3 is converted into C3b. This value is in agreement with the results by Isenman *et al.* (1981). Inactivated C3, in 2M-KBr, showed similar $[\theta]_M$ as C3b. The kinetics of the change of c.d. of C3 in 2M-KBr was monitored at 264.5 nm. t_4 was about 5 min. The results are summarized in Table 3.

Inactivation of C3 in guanidinium chloride

The denaturation of C3 by heat, SDS or guanidinium chloride leads to the breakage of a specific peptide bond at the thioester site (Janatova *et al.*, 1980; Sim & Sim, 1981; Thomas *et al.*, 1982). This phenomenon has been termed 'autolytic cleavage' and its occurrence is specific for native C3 with an intact thioester. To determine the relationship between the autolytic-cleavage reaction and the binding reaction, C3 was incubated with $[^3\text{H}]$ -glycerol at different concentrations of guanidinium chloride. The samples were analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 4a). The behaviour of C3 in guanidinium chloride can be described in three phases. No apparent modification of C3, as assessed by SDS/polyacrylamide-gel electrophoresis, was observed when C3 was incubated in guanidinium chloride of less than 0.5M. At guanidinium chloride concentrations of 1–1.5M, high-molecular-weight complexes appear, indicating the occurrence of the binding reaction (see above). The intensity of the high-molecular-weight bands declines in C3 samples incubated in guanidinium chloride at 2M and above. At the same time, bands corresponding to the 74 000- and 46 000-mol.wt. polypeptides, characteristic of the autolytic cleavage of C3, become evident.

Specific binding of $[^3\text{H}]$ glycerol to C3 was estimated as described above, with the background non-specific binding taken to be the radioactivity associated with the β -chain multiplied by a factor of

Table 3. Inactivation and inactivation-induced conformational change of C3

C3 was incubated in 2M-KBr, pH 7.5, 37°C. The change in c.d. of C3 was monitored at 264.5 nm. The t_4 values for inactivation and binding were from a separate experiment (Fig. 3). Corresponding data from the inactivation of C3 by trypsin or methylamine (Isenman *et al.*, 1981) are included for comparison.

Incubation conditions	$\Delta[\theta]_M$ at 264.5 nm C3→C3i/C3b (in degrees · cm ² · dmol ⁻¹)	t_4 (min)		
		Conformational change (c.d. at 264.5 nm)	Inactivation	Binding
KBr, 2M, pH 7.5, 37°C	+25	4–6 min	3–4 min*	3–4 min†
Trypsin, 1% (w/w with respect to C3), room temperature	+20	<2 min‡	<2 min‡	—
Methylamine, 50 mM, pH 8.0, 23.5°C	+26	520 min	27 min§	—

* Measured by the appearance of the free thiol group (Fig. 3).

† Measured by the incorporation (binding) of $[^3\text{H}]$ glycerol to C3 (Fig. 3).

‡ Not explicitly determined, it was estimated that the rate of change in c.d. was limited by the proteolytic conversion of C3 into C3b (Isenman *et al.*, 1981).

§ Measured by the loss of haemolytic activity (Isenman *et al.*, 1981).

Fig. 4. [^3H]Glycerol binding and autolytic cleavage of C3 in different concentrations of guanidinium chloride. C3 was incubated with [^3H]glycerol in the presence of: a, 0 M; b, 0.25 M; c, 0.5 M; d, 1 M; e, 1.5 M; f, 2 M; and g, 2.5 M-guanidinium chloride, for 1 h at 37°C. (a) The Coomassie Blue-stained gel. (b) Specific [^3H]glycerol binding (●) and autolytic cleavage (○) of C3 in different concentrations of guanidinium chloride. Autolytic cleavage was quantified by scanning the gel in (a) with a Vitatron densitometer at 540 nm. Percentage autolytic cleavage is defined as $100 \times (\text{sum of area under peaks 74 000 and 46 000 mol.wt.}) / (\text{sum of area under C3}\alpha \text{ and peaks at 74 000 and 46 000 mol.wt.})$.

2.5. Autolytic cleavage was quantified by scanning the Coomassie Blue-stained gel (Fig. 4a). The percentage of autolytic cleavage was estimated by the ratio of the sum of the area under the peaks 74 000 and 46 000 mol.wt. to the sum of the area under the C3 peak and those under the peak 74 000 and 46 000 mol.wt. The results are shown in Fig. 4(b). Attempts to use the labelling of the free thiol group, which should only be found in the C3 α -chain and the band at 46 000 mol.wt. (Thomas *et al.*, 1982), as a means to quantify autolytic cleavage failed because of significant disulphide exchange at high (>2 M) guanidinium chloride concentrations.

Samples of C3, after incubation in different concentrations of guanidinium chloride for 1 h, were adjusted to a concentration of 3 M-guanidinium chloride and incubated for a further 1 h. Autolytic cleavage was observed for samples whose first incubation was in 0.5 M-guanidinium chloride or less, indicating that the absence of autolytic cleavage (Fig. 4a) was due to the stability of native C3 in the first incubation. No autolytic cleavage, however, was observed for samples originally in 1–1.5 M-guanidinium chloride, indicating that C3 has been inactivated in the first 1 h (results not shown).

Similar results were obtained by using SDS instead of guanidinium chloride. However, the results are less reproducible with respect to the range of SDS concentrations over which binding and/or autolytic cleavage occur. The [^3H]glycerol incorporation to C3 in the presence of SDS was never established to a higher confidence than in the results presented in Fig. 1 and Table 1.

Discussion

The initial event in the covalent binding of C3b to cell surfaces (Law & Levine, 1977) is the proteolytic conversion of C3 into C3b* by a surface-bound C3-convertase, where C3b* is defined as the active state of the molecule. The conversion exposes the thioester in C3b*, allowing either the transfer of the acyl group from the thioester to a surface-bound hydroxy or amino group, yielding a surface-bound C3b molecule, or the hydrolysis of the thioester to yield fluid-phase C3b. The description of the 'exposure' of the thioester presupposes a conformational change in the molecule induced by the conversion. This was supported by experiments showing the difference between C3 and C3b in their 8-anilino-naphthalene-1-sulphonate-binding properties, and their c.d. in the near u.v. (Isenman *et al.*, 1981). A question remains as to whether the enzymic conversion of C3 directly activates the covalent binding reaction and the conformational change is incidental, or the enzymic conversion of C3 is simply a means to induce the conformational

change of the molecule, which is the effective activation step in the binding reaction.

Various chaotropes such as KBr, KSCN (Dalmasso & Müller-Eberhard, 1966), denaturants such as urea and guanidinium chloride, and ionic detergents such as SDS (Janatova *et al.*, 1980), are known to inactivate C3. The inactivation is invariably associated with the appearance of the thiol group (Janatova *et al.*, 1980), implying the cleavage of the thioester bond. In some cases it was demonstrated that the inactivated C3 attains certain C3b-like properties (Pangburn *et al.*, 1981). For example, inactivated C3 is cleaved by factor I in the presence of factor H, a property of C3b but not that of native C3 (Pangburn *et al.*, 1977; Crossley & Porter, 1980). If conformational change is indeed the activation step in the binding reaction, it should be possible to induce the C3 binding reaction to proceed with some of these reagents. This has been studied in a fluid-phase system where [^3H]glycerol was incubated with C3 in the presence of various chaotropes and denaturants.

Binding of [^3H]glycerol to C3 was demonstrated in 2M-KBr, 0.5M-KSCN, 1M-guanidinium chloride, 3M-urea, and, at a lower level of confidence, in 0.015% SDS. Moreover, the binding in KBr as a function of glycerol concentration was shown to follow a straight line in a double-reciprocal plot (Fig. 2), indicating the relationship:

$$(\text{BE})^{-1} = 1 + m[\text{glycerol}]^{-1}$$

where m is the slope of the line. The result is similar to that demonstrated for the activation of the binding reaction with trypsin (Law *et al.*, 1981; see also Fig. 2). Hence the binding reaction of C3 as induced by chaotropes can be described in a similar scheme:

where C3i denotes inactivated C3 with a hydrolysed thioester, and C3* the activated state of C3 in chaotropes. The similarity between the binding reaction activated by KBr and by trypsin indicates that the conversion of C3 into C3b is not an absolute prerequisite for binding. The conversion may serve only as a means to alter the conformation of C3, which is in effect the activation mechanism. The description of the 'exposure' of the thioester in the activation process is possibly correct. This is further supported by the observation that the change in c.d. at 264.5 nm of C3 in 2M-KBr has a similar t_1 to that of inactivation and binding (Table 3).

The appearance of bands of high-molecular-weight when C3 is inactivated under different conditions (Fig. 1a) can be ascribed to the formation of covalently linked C3 multimers. The exact nature

of these bands, whether they are of α - α , α - β , or any other combinations, was not further analysed.

The inactivation of C3 by methylamine is by a different mechanism. Whereas all other reagents used in the experiments presented here inactivate C3 by altering its conformation, leading to the exposure of the thioester, methylamine inactivates C3 by the direct attack on the thioester of native C3. The attaining of a final C3b-like conformation follows much slower kinetics in comparison with the loss of haemolytic activity (Table 3) (Isenman *et al.*, 1981). Following this argument, the inactivation of C3 by methylamine should not induce the binding of glycerol to C3, as is experimentally demonstrated (Fig. 1, Tables 1 and 2).

When C3 is incubated at elevated temperature ($>65^\circ\text{C}$), or in the presence of high concentrations of guanidinium chloride or SDS, it undergoes the autolytic-cleavage reaction (Janatova *et al.*, 1980; Sim & Sim, 1981). It was proposed that the cleavage arise from the nucleophilic attack of the thioester by the peptide nitrogen atom of the glutamyl residue to form an internal pyroglutamic acid residue, followed by the hydrolysis of the peptide bond at the pyroglutamic residue (Howard, 1981). As a result, the C3 α -chain is split into two fragments, a 46 kDa N-terminal fragment and a 74 kDa C-terminal fragment (Thomas *et al.*, 1982). Although it has been shown that under certain conditions the autolytic-cleavage reaction can be driven to completion (Sim & Sim, 1981), the state of the molecules that do not undergo autolytic cleavage in incomplete reaction is unclear. Fig. 4 shows that those molecules that do not go through an autolytic-cleavage reaction probably undergo a binding reaction, since glycerol incorporation to the intact α -chain of C3 was observed in samples where partial autolytic cleavage was detected.

The incubation of C3 in 1M-guanidinium chloride is sufficient to bring about the binding reaction but not the autolytic-cleavage reaction, which is only detected at higher concentrations of the denaturant. It is apparent that an extensive denaturation of the protein is required for the autolytic-cleavage reaction to take place, indicating that, under physiological or limited denaturation conditions, the peptide nitrogen atom was restricted from attacking the thioester to form the internal pyroglutamic acid residue. The restriction is likely to be conformational, since more denaturing conditions, such as higher temperature or higher concentrations of denaturants, remove the constraint, and the attack of the thioester by nucleophiles (binding) and that by the peptide nitrogen atom (autolytic cleavage) become competing reactions (Fig. 4b).

The binding reaction of C3 as an acyl transfer mediated by an internal thioester can only be explained by an activated thioester. Whereas the t_1

for the hydrolysis of thioesters at neutral pH in aqueous medium is in the order of 10 min (Fedor & Bruice, 1965), the half-life of C3b* has been estimated to be in the order of milliseconds (Bokisch *et al.*, 1975) or even as low as 60 μ s (Sim *et al.*, 1981). The existence of other amino acid residues that participate in the binding reaction in a catalytic capacity was considered (Law *et al.*, 1980; Davies & Sim, 1981; Thomas *et al.*, 1982). It is shown here that the conformational change of C3 plays an important part in activating the binding reaction, and it is likely that this conclusion is applicable to the binding activity of other thioester-containing proteins such as C4 (Campbell *et al.*, 1981; Harrison *et al.*, 1981) and α_2 -macroglobulin (Howard, 1981).

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