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Kinetics of Polymerization of a Fluoresceinated Derivative of Complement Protein C9 by the Membrane-Bound Complex of Complement Proteins C5b-8†

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ABSTRACT: The fluorescence self-quenching by energy transfer of FITC-C9, a fluoresceinated derivative of human complement protein C9 [Sims, P. J. (1984) *Biochemistry* (preceding paper in this issue)], has been used to monitor the kinetics of C9 polymerization induced by the membrane-associated complex of complement proteins C5b-8. Time-based measurements of the fluorescence change observed during incubation of FITC-C9 with C5b-8-treated sheep red blood cell ghost membranes at various temperatures revealed that C9 polymerization induced by the C5b-8 proteins exhibits a temperature dependence similar to that previously reported for the complement-mediated hemolysis of these cells, with an Arrhenius activation energy for FITC-C9 polymerization of 13.3 ± 3.2 kcal mol^{-1} (mean ± 2 SD). Similar measurements obtained with C5b-8-treated unilamellar vesicles composed of either egg yolk phosphatidylcholine (egg PC), dipalmitoylphosphatidylcholine (DPPC), or dimyristoylphosphatidylcholine (DMPC) revealed activation energies of between 20 and 25 kcal mol^{-1} for FITC-C9 polymerization by C5b-8 bound to these membranes. Temperature-dependent rates of C9 polymerization were observed to be largely unaffected by the phase state of membrane lipid in the target C5b-8 vesicles. The significance of these observations to the mechanism of C9 activation of membrane insertion is considered.

Cytolytic membrane damage by the serum complement system is initiated upon the association of component C9 with membrane-bound complexes formed of complement proteins C5b, C6, C7, and C8 (Muller-Eberhard, 1975; Esser, 1982; Bhakdi & Tranum-Jensen, 1983).[1] It has recently been demonstrated that the incorporation of C9 into the membrane-bound C5b-8 complex (MC5b-8) results in the copolymerization of multiple molecules of C9 and the exposure of hydrophobic domains within the protein (Podack & Tschopp, 1982; Tschopp et al., 1982; Podack et al., 1982b). Under conditions promoting disulfide exchange, interchain disulfide bonds are formed that can covalently link C9 monomers in the polymerized C9 complex (Ware & Kolb, 1981; Yamamoto & Migita, 1983). By intercalating through bilayer lipid, tubules of polymerized C9 are thought to initiate a change in membrane permeability that leads to the collapse of ionic gradients and consequent cell death (Tschopp et al., 1982).

Considerable insight into the mechanism of membrane damage by these key immunoproteins has been derived from ultrastructural analysis of the membrane-bound C5b-9 complex and from biochemical studies of the association of the proteins either in solution or following extraction from detergent-solubilized membranes [reviewed by Esser (1982), Tschopp et al. (1982), and Bhakdi & Tranum-Jensen (1983)]. Additionally, analysis of the temperature dependence of complement-mediated hemolysis and measurements of the rates of diffusion of various solutes across C5b-9 damaged membranes have provided clues to the molecular events preceding cell lysis [reviewed by Boyle & Borsos (1980) and Esser (1982)]. Nevertheless, a direct kinetic analysis of the terminal molecular event that is presumed to initiate functional membrane damage—the MC5b-8 catalyzed activation and polymerization of C9 monomers—has not previously been possible, due to the inability to directly measure the self-association of

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[1] Complement proteins are named in accordance with recommendations of *Bull W. H. O.* (1968). The prefix "M" (e.g., MC5b-8) is used to indicate that the protein is membrane associated. EAC1–9 refers to an antibody-treated erythrocyte treated with complement proteins C1–C9.

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C9 in situ, during its activation by MC5b-8 and consequent membrane insertion.

In a recent report, we describe how the steady-state fluorescence of a fluoresceinated derivative of C9 (FITC-C9)[2] is self-quenched upon polymerization of the protein, due to intermolecular energy transfer that occurs between closely apposed fluorescein chromophores in the membrane-bound FITC-C9 polymer (Sims, 1984). Using this real-time spectroscopic indicator of C9 self-association, we have directly investigated the kinetics of the MC5b-8-induced activation and polymerization of C9 in situ, during the interaction of these proteins bound to red cell membranes and unilamellar vesicles composed of various phospholipids.

Materials and Methods

Materials. Fluorescein 5-isothiocyanate (FITC; isomer I) was obtained from Molecular Probes. Egg lecithin (egg PC), dimyristoylphosphatidylcholine (DMPC), and dipalmitoylphosphatidylcholine (DPPC) were each obtained in chloroform from Avanti Polar Lipids, Inc. TES was obtained from Calbiochem-Behring. All chemicals were of analytical grade, and all organic solvents were of spectroscopic grade.

Solutions. All solutions were freshly prepared with H_2O obtained by reverse osmosis and ultrafiltration (Millipore Corp.). Except where indicated otherwise, all reagents contained 0.02% (w/v) NaN_3: buffer *I*, 150 mM NaCl–10 mM TES, pH 7.50; *buffer II*, 150 mM NaCl–5 mM sodium phosphate, pH 8.00.

Complement Proteins. Human complement proteins were purified and assayed for immunochemical purity as well as hemolytic and membrane binding activities according to methods previously described (Sims, 1983, 1984).

FITC Labeling of C9 (FITC-C9). Labeling of C9 with fluorescein isothiocyanate (FITC) was performed at pH 8.70 according to methods previously described (Sims, 1984). The input of FITC to C9 monomer was adjusted to 200:1 on a molar basis to achieve final dye/protein ratios of from 1.8 to 2.0. All preparations of FITC-C9 used in these studies chromatographed by polyacrylamide gel electrophoresis as a single homogeneous band of approximately 70,000 M.W., and retained greater than 80% of the hemolytic activity of serum C9 [see Sims (1984)].

Determination of Protein Concentrations and Dye/Protein Ratios. Protein concentrations and dye/protein ratios were determined according to methods previously described (Sims, 1984).

Preparation of Lipid Vesicles. Small unilamellar lipid vesicles were formed of egg PC, DMPC, or DPPC by sonication, according to the methods of Huang (1969). Chloroform was removed from 25 mg of lipid by evaporation under a stream of N_2 and the lipid maintained under <5 torr for 12–16 h. One milliliter of buffer I was added at either 4 (egg PC), 25 (DMPC), or 45 °C (DPPC) and the sample dispersed by vortexing. The samples were sonicated under nitrogen (Heat Systems Ultrasonics Model 375W) to optical clarity and SUV fractions recovered and stored under N_2 at temperatures which were above their respective thermotropic phase transitions, as indicated above. Large unilamellar vesicles of egg PC were prepared by reverse-phase evaporation according to methods previously described (Sims, 1984).

Preparation of Sheep Red Blood Cell Membranes (Ghosts). White ghost membranes of sheep red blood cells (ghosts) were prepared by hypotonic lysis at 2–4 °C (Steck, 1974). One milliliter of packed erythrocytes was hemolyzed in 40 mL of ice-cold 5 mM sodium phosphate, pH 8.0. The membranes were then recovered by centrifugation (10 min, 40000*g*, 2 °C) and washed twice with 40 mL of the hemolyzing solution. The white membranes were suspended in 40 mL of buffer II (prewarmed to 37 °C) and allowed to reseal by a 40-min incubation at 37 °C. The ghosts were then washed twice in ice-cold buffer II and stored as a concentrated suspension (3.5×10^{10}/mL) in this buffer at 2–4 °C. Concentrations of the ghost suspensions were determined by resistive particle counting in filtered saline (Coulter Model ZBI).

Preparation of C5b67 and C5b-8 Membranes. The membrane-bound C5b67 complex (MC5b67) was assembled on lipid vesicles and SRBC ghosts by incubation at 37 °C with purified C5b6 + C7 (Sims, 1983, 1984). In the case of DPPC vesicles, incubation was performed at 42 °C. C5b67 vesicles were prepared by mixing the vesicles with C5b6 followed by the addition with rapid vortexing of C7 at molar equivalence to C5b6. The input of C5b6 + C7 was adjusted to yield a maximal change in FITC-C9 fluorescence (upon addition of excess C8) of 17–21%, which was comparable to that observed for C5b-7 ghosts prepared as described below. Final lipid concentrations of C5b67 vesicle suspensions were 8.3 mg/mL in buffer I. Vesicles adjusted to the same lipid concentrations in the absence of the C5b67 proteins (or vesicles exposed to C5b6 but not C7) served as controls.

C5b67 ghosts were similarly prepared by suspension of the washed ghosts with C5b6 followed by rapid mixing with C7 at molar equivalence (1 mg of C5b67 protein per 5×10^9 ghosts in a total volume of 1.1 mL) and incubation at 37 °C (15 min). Following C5b67 assembly, vesicles and ghosts were used within 6 h.

To form the membrane-bound C5b-8 complex (M5b-8), C8 was added to C5b67 vesicles (10 μg of C8/415 μg of lipid) or C5b67 ghosts (10 μg of C8/1.7×10^8 ghosts) followed by incubation at 37 °C for 30 min. In the case of C5b67 DPPC, incubation was performed at 42 °C. For certain experiments (see below), C5b67 membranes (vesicles or ghosts) were first suspended with FITC-C9 in a stirred cuvette; C5b-9 assembly was then initiated by the addition of C8 (10 μg) to the FITC-C9 + MC5b67 suspensions. C8 was always added as a 1 mg/mL stock in buffer I.

Measurement of FITC-C9 Polymerization by MC5b-8. The polymerization of C9 by membrane C5b-8 was analyzed by measuring the self-quenching of FITC-C9 fluorescence upon binding to C5b-8 membranes, according to methods previously described (Sims, 1984). Typically, C5b67 vesicles (415 μg of lipid) or C5b67 ghosts (1.7×10^8 ghosts) were suspended with 0.7–3 μg of FITC-C9 in a final volume of 2.1 mL of buffer I equilibrated in a stirred 1-cm path-length cuvette. Sample temperature was maintained by thermostatic control at various levels as indicated in the text (see below). Fluorescence (excitation 470 nm; emission 520 nm) was continuously monitored before and after the addition of C8 (0–10 μL of 1 mg/mL stock solution). For certain experiments, C5b-8 membranes (vesicles or ghosts) were first prepared by preincubation of C5b67 membranes with C8 (see above); fluorescence of the FITC-C9 solutions was then continuously measured before and after addition of the C5b-8 membranes (or controls) to the stirred cuvette.

To facilitate comparison of data obtained at various temperatures, data from each experiment were normalized to the

[2] Abbreviations: FITC, fluorescein 5-isothiocyanate; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; SRBC, sheep red blood cells; egg PC, egg yolk phosphatidylcholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine.

steady-state fluorescence intensity that was measured for FITC-C9 (equilibrated at the sample temperature) immediately before FITC-C9 polymerization was initiated by the addition of either C8 (to the MC5b67 + FITC-C9 suspension) or MC5b-8 (to FITC-C9). Correction was made for light scattering due to membranes according to methods previously described (Sims, 1984).

The initial rate (k_0) of the MC5b-8 induced change in FITC-C9 fluorescence was determined by assuming a pseudo-first-order reaction, by linear interpolation of the initial slopes of the fluorescence trace recorded following C8 or C5b-8 membrane additions. Correction was made for sample mixing times (~3 s) and sample dilution (<4%). Sample mixing times were estimated by direct recording of fluorescence upon dye dilution, under conditions identical with those employed for sample additions (above). Additionally, where necessary, correction was made for a spontaneous decrease in FITC-C9 fluorescence due to photobleaching or due to C9 aggregation, which was observed during prolonged incubation of FITC-C9 solutions at temperatures above 40 °C (see Results and Discussion). Calculation of Arrhenius activation energies was performed by using only those data for which the total relative change in FITC-C9 fluorescence—measured 60 min after C8 or MC5b-8 addition—were the same [generally, experiments performed at temperatures between 20 and 40 °C (see Results and Discussion)].

Fluorescence Measurements. Instrumentation employed for fluorescence measurements is described in detail elsewhere (Sims, 1984). Except where indicated otherwise, excitation was at 470 nm (2-nm slits) and emission at 520 nm (8-nm slits). For time-based measurements with membrane suspensions, polarizers were configured vertically (excitation) and horizontally (emission) to minimize the contribution of light scattering. Where necessary, correction was made for light scattering due to vesicle (or ghost) suspensions, by subtraction of the scatter measured for identical suspensions containing unlabeled C9 substituted for FITC-C9 [see Sims (1984)]. To correct for fluctuations in lamp output, the fluorescence emission was always recorded as a ratio to the emission from 3 g/L rhodamine B in ethylene glycol, contained in a front surface cuvette placed in the reference compartment. A Schott RG630 filter was placed before the reference channel photomultiplier tube. Emission spectra were corrected for the wavelength-dependent response of the photomultiplier tubes by using correction factors supplied by the manufacturer. Polarizers were aligned vertically (excitation) and at 54.7° (emission) during excitation and emission scans.

Temperature control of the sample cuvette housing was provided by a circulating bath interfaced to a digital controller (Neslab Models RTE5 and DCR1). Continuous measurement (and feedback control) of the temperature of the sample suspension during fluorescence measurements was provided by a thin-film platinum RTD element (Omega Engineering) immersed into the sample cuvette above the optical path, which was interfaced to the DCR1 bath controller. The platinum RTD was calibrated to ±0.02 °C accuracy with NBS reference thermometers. During scans, sample temperature and fluorescence were simultaneously acquired and stored by computer.

Results and Discussion

Change in FITC-C9 Fluorescence during Incubation with C5b-8-Treated Erythrocyte Ghost Membranes. We have previously shown that the polymerization of FITC-C9—induced either by heating the protein in solution or by its incorporation into the C5b-9 complex bound to lipid vesicles—results in a decrease in the steady-state fluorescence of the chromophore, due to self-quenching energy transfer between closely apposed FITC residues in the polymerized FITC-C9 complex (Sims, 1984). No quenching of fluorescence was observed when FITC-C9 was bound to C5b-8 membranes under conditions restricting the potential for fluorescein to fluorescein energy transfer, confirming the specificity of this probe as an indicator of the aggregation state of the protein in situ (Sims, 1984). In order to apply this spectroscopic indicator to measurements with biological membranes, we first undertook to determine whether similar changes in FITC-C9 fluorescence would be observed upon its incorporation into the polymerized FITC-C9 complex generated during incubation with C5b-8-treated erythrocyte ghost membranes.

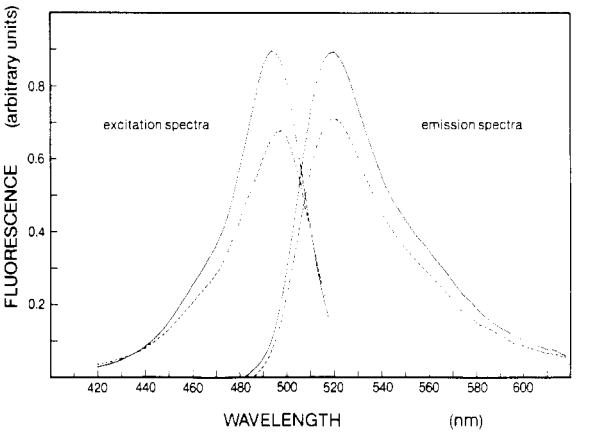

FIGURE 1: Change in FITC-C9 fluorescence due to polymerization by C5b-8 erythrocyte ghost membranes. Corrected visible wavelength excitation and emission spectra of FITC-C9 (dye/protein ratio = 1.9) after incubation (30 min, 30 °C) with either C5b67 ghosts (solid line) or C5b-8 ghosts (dashed line). Both samples contained 0.7 μg of FITC-C9 and 1.7×10^8 ghosts in a total volume of 2.1 mL of buffer I. Samples were equilibrated at 23 °C before spectra were obtained. For the fluorescence excitation spectra, emission was recorded at 540 nm (16-nm slits) and excitation scanned at 2-nm intervals (2-nm slits). For the fluorescence emission spectra, excitation was at 470 nm (8-nm slits), and emission was recorded at 2-nm intervals (2-nm slits). Polarizers were aligned at vertical (excitation) and 54.7° (emission). Spectra are corrected for light scattering due to the ghost membranes and for lamp output and photomultiplier tube response (see Materials and Methods). Excitation peaks are at 494 (C5b67) and 498 nm (C5b-8). Emission peaks are at 520 nm. Spectra shown for FITC-C9 + C5b67 ghosts (corrected for light scattering) are identical with spectra recorded for FITC-C9 before membrane additions (see text).

In Figure 1 are shown the corrected visible wavelength excitation and emission spectra obtained for identical suspensions of C5b-8 ghosts and controls after incubation with FITC-C9 for 60 min at 37 °C. Incubation of FITC-C9 with C5b-8 ghosts generally resulted in a 17–21% decrease in the integrated steady-state emission (for excitation at 470 nm), with a 2–4-nm red shift of the excitation spectrum. No change in FITC-C9 fluorescence was observed upon incubation of the protein either with control ghosts or with C8 in solution (not shown). As had previously been observed with C5b-8-treated lipid vesicles (Sims, 1984), no change in FITC-C9 fluorescence was observed during incubation with C5b-8 ghosts at 0–2 °C (data not shown; see below). Since at these temperatures C9 is known to bind to C5b-8 without activation to its hemolytic conformation (Boyle et al., 1978; Okada et al., 1980), these results confirm that the quenching of FITC-C9 fluorescence which is observed upon its incubation with the C5b-8-treated erythrocyte membranes requires the specific activation of the protein by MC5b-8—inducing FITC-C9 self-association—and is not due to other quenching mechanisms (e.g., quenching of

bound FITC-C9 by aromatic residues on membrane proteins). A detailed analysis of the mechanism of FITC-C9 self-quenching upon polymerization by MC5b-8 has previously been presented, as has a description of both the functional, spectroscopic, and ultrastructural properties of this fluorescent derivative of C9 (Sims, 1984).

Kinetics of FITC-C9 Polymerization by C5b-8 Erythrocyte Ghost Membranes. On the basis of the observed temperature dependence of the lysis of SRBC by antibody and complement, it has previously been concluded that the formation of the cytolytic membrane lesion involves a temperature-sensitive activation step which occurs subsequent to the physical binding of the C5b-9 proteins to the membrane: Binding of C9 to MC5b-8 has been shown to occur rapidly at 0 °C, but no lysis is observed until the cells are warmed (Hadding & Muller-Eberhard, 1969; Boyle et al., 1978; Boyle & Borsos, 1980; Okada et al., 1980). Because the sensitivity of the cell-bound C5b-9 proteins to tryptic inactivation is lost upon warming, it has been inferred that membrane insertion of the cytolytic complement pore occurs during this activation step (Hammer et al., 1977; Boyle et al., 1978). Direct evidence for the insertion of hydrophobic domains of the activated C5b-9 proteins into bilayer lipid has also been provided by ultrastructural analysis (Bhakdi & Tranum-Jensen, 1978, 1979; Bhakdi et al., 1980; Podack et al., 1982a) and photoaffinity labeling (Hu et al., 1979; Ishida et al., 1982; Steckel et al., 1983). On the basis of the measured rates of hemolysis of EACl-9 cells at various temperatures, an activation energy of 13–15 kcal mol^{-1} has been estimated for this terminal cytolytic event (Lauf, 1975; Li & Levine, 1977).

In light of recent evidence that the cytolytic lesion may be initiated by multimeric aggregates of component C9—polymerization of C9 presumably catalyzed during its interaction with the membrane-bound C5b-8 complex (Tschopp et al., 1982)—we undertook to analyze the temperature dependence and activation energy specifically associated with C9 self-aggregation induced by MC5b-8, using the fluorescence self-quenching by energy transfer of FITC-C9 as an indicator of this molecular event.

In Figure 2 are shown typical results obtained when the fluorescence of FITC-C9 was continuously measured during incubation with C5b-8-treated ghosts suspended at various temperatures. FITC-C9 binding and polymerization was initiated either by the addition of C8 to stirred suspensions of C5b67 ghosts + FITC-C9 (previously equilibrated at the temperatures indicated; Figure 2A) or by the addition of C5b-8-treated ghosts (the MC5b-8 complex assembled by prior 37 °C incubation) to a stirred solution of FITC-C9 at the measuring temperature (Figure 2B). In each case, prior equilibration of FITC-C9 at the measuring temperature permitted direct observation of initial sample fluorescence (before FITC-C9 activation) and continuous monitoring of the change in sample fluorescence due to C5b-8-induced aggregation of the protein. Inspection of the figure reveals that at all temperatures the quenching of FITC-C9 fluorescence occurs without a measurable lag phase (resolution limited by ~3-s mixing time in the cuvette; see Materials and Methods) and with an initial rate that is highly dependent upon sample temperature. At temperatures below 4 °C, no measureable change in FITC-C9 fluorescence (relative to controls) was observed. Within the resolution of the sampling methods, similar rates were observed for experiments performed under conditions described for Figure 2A as for Figure 2B (see also Figure 3), suggesting that the assembly and/or activation of C5b-8 was not rate limiting to the observed kinetics of

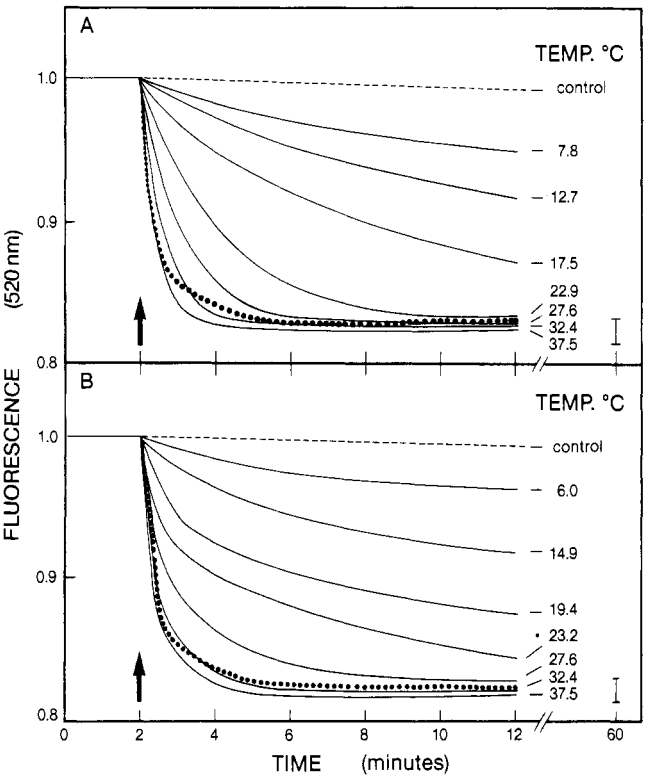

FIGURE 2: Kinetics of FITC-C9 polymerization by C5b-8 erythrocyte ghost membranes. Time-based fluorescence traces obtained during incubation of FITC-C9 with C5b-8-treated ghost membranes. (A) FITC-C9 (0.7 μg) was added to 1.7×10^8 C5b67 ghosts equilibrated in a stirred cuvette at temperatures indicated. After 5-min equilibration in the dark, fluorescence trace was started. At the time indicated by the arrow, 10 μg of C8 (as 1 mg/mL stock) was delivered to the cuvette. Final sample volume was 2.1 mL (see Materials and Methods). Dotted trace (not labeled) was obtained at 42.3 °C (see text). Dashed trace indicates data obtained for control membranes (no C5b67) suspended at 32.4 °C. Other controls are omitted for clarity (see text). Error bar indicates range of fluorescence measured at t = 60 min, for experiments performed at temperatures indicated between 17.5 and 37.5 °C. Shutters were closed at t = 15 min to reduce photobleaching. (B) FITC-C9 (0.7 μg) was equilibrated in a stirred cuvette at temperature indicated. At time indicated by arrow, 1.7×10^8 C5b-8 ghosts were delivered to the cuvette (final suspension volume 2.1 mL). Dotted trace (not labeled) indicates data obtained at 42.3 °C (see text). Dashed trace indicates data obtained when control ghosts + C8 (no C5b67) were added to FITC-C9 equilibrated at 32.4 °C. Other controls are omitted for clarity (see text). Error bar indicates range of fluorescence measured at t = 60 min, for experiments performed at temperatures indicated between 19.4 and 37.5 °C. Shutters were closed at t = 15 min to reduce photobleaching. Fluorescence was continuously monitored with excitation at 470 nm (4-nm slits) and emission at 520 nm (8-nm slits). Polarizers were crossed to minimize effects of sample scattering. Scans measured at various temperatures were normalized and corrected for mixing artifacts observed after C8 (or C5b-8 ghost) additions as described under Materials and Methods. Fluorescence traces obtained by both methods at temperatures below 4 °C superimposed on dashed lines indicated for control membranes (see text).

FITC-C9 fluorescence quenching.

The initial rates of FITC-C9 polymerization, derived from the initial slopes of time-based fluorescence measurements obtained from three separate experiments performed at various temperatures under conditions described for Figure 2, conformed to the Arrhenius equation in the temperature range 15–40 °C, with an apparent activation energy of 13.3 ± 3.2 kcal mol^{-1} (mean ± 2 SD; see Figure 3 and Table I). In this temperature range, the total relative change in FITC-C9 fluorescence (measured after 60 min) was equivalent for all C5b-8 ghost suspensions, suggesting the same equilibrium state of aggregation of the protein (see Figure 2). At low tem-

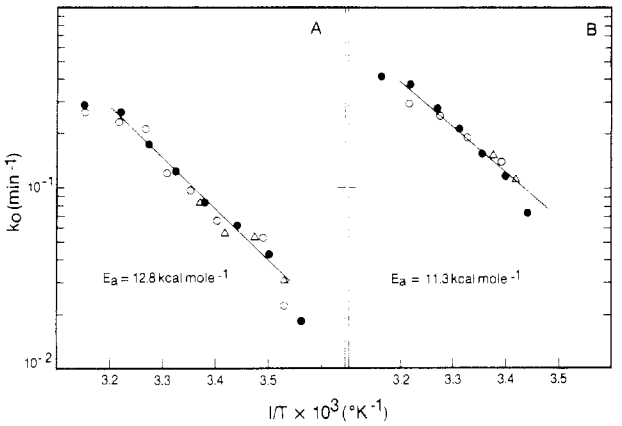

FIGURE 3: Arrhenius plot of FITC-C9 polymerization by C5b-8 erythrocyte ghost membranes. Initial velocities (*ordinate*) were derived from the slopes of time-based fluorescence traces measured as described for Figure 2. *Abscissa* indicates reciprocal absolute temperature (×10^3 K^{-1}). Data were compiled from three separate experiments each performed under conditions described for Figure 2, with FITC-C9 polymerization initiated either by addition of C8 to FITC-C9 + C5b67 ghost suspensions (panel A) or by mixing of preformed C5b-8 ghosts with FITC-C9 (panel B). Symbols indicate data from separate experiments with different preparations of C5b67 ghost membranes. Lines drawn indicate best fit derived by linear regression of data between 15 and 40 °C (see text).

peratures, the initial rate of the fluorescence change slowed considerably, deviating from linearity in the Arrhenius plot (see Figure 3). A similar deviation from linearity at low temperatures has also been described for Arrhenius plots derived from the measured rates of complement-mediated hemolysis (Lauf, 1975; Li & Levine, 1977). Furthermore, at these temperatures (below 15 °C), the total relative change in FITC-C9 fluorescence measured after prolonged incubation (>3 h) with MC5b-8 diminished from that observed at higher temperatures, suggesting a competing process of C9 inactivation or desorption from the membrane at these temperatures. Evidence for C9 desorption from C5b-9-treated erythrocyte membranes has previously been considered on the basis of reaction rates observed for hemolysis (Li & Levine, 1977).

When FITC-C9 was incubated in solution or with control membranes at temperatures above 40 °C, a slow spontaneous decrease in sample fluorescence was often observed (data not shown). This loss in sample fluorescence is likely due to the spontaneous polymerization of the protein which is known to occur at these elevated temperatures (Tschopp et al., 1982; Podack & Tschopp, 1982; Sims, 1984). Furthermore, at temperatures above 40 °C, both the initial rates and equilibrium levels of the C5b-8-induced change in FITC-C9 fluorescence were observed to decline (cf. dotted line, Figure 2), suggesting inactivation of the proteins at these elevated temperatures.

The apparent activation energy we observe for the MC5b-8-induced polymerization of FITC-C9 on SRBC membranes is comparable to previous estimates of 13–15 kcal mol^{-1} required for complement-mediated hemolysis, as derived from the measured rate of hemoglobin release from erythrocytes lysed by antibody and complement (see above). Our results suggest, therefore, either that the polymerization of C9 by the C5b-8 complex is the rate-limiting molecular event associated with formation of the cytolytic lesion or that it is directly coupled to the rate-limiting molecular event.

In a recent report, Podack & Tschopp (1982) describe the kinetics of the spontaneous polymerization of C9 in solution, as indicated by the binding of the polarity-sensitive fluorophore 8-anilinonaphthalene-1-sulfonic acid (ANS). On the basis of

Table I: Arrhenius Activation Energies for FITC-C9 Polymerization [a]

C5b-8 membrane	E_a (kcal mol^{-1})
SRBC ghosts	13.3 ± 3.2
DMPC	24.0 ± 1.9
DPPC	19.8 ± 2.2
egg PC[b]	21.2 ± 1.7
egg PC[c]	21.3 ± 1.6

[a] Activation energies derived from Arrhenius plots of data obtained from experiments performed as described in Figures 2–6. Values shown represent mean ± 2 SD obtained in three separate experiments. [b] C5b-8 assembled on egg PC small unilamellar vesicles. [c] C5b-8 assembled on egg PC large unilamellar vesicles.

reaction rates estimated by ANS binding, they report that the heat-induced polymerization of C9 occurs with an activation energy of 40 kcal mol^{-1}, as compared to 15 kcal mol^{-1} (estimated by light scattering) for the spontaneous polymerization of the protein induced by 1 M guanidine hydrochloride. Our estimate of 13.3 kcal mol^{-1} for the MC5b-8 catalyzed polymerization of FITC-C9 on SRBC ghosts (Table I) is similar to that reported for polymerization induced by denaturation of the protein in guanidine hydrochloride and is consistent with the suggestion made by these authors that MC5b-8 serves to lower the activation energy for spontaneous C9 polymerization by inducing a conformational change within the protein.

FITC-C9 Polymerization by C5b-8 Treated Lipid Vesicles. In several previous studies it has been observed that the release of trapped aqueous markers from cells or liposomes treated with antibody and the complement proteins is influenced by the lipid composition of the target membrane (Hesketh et al., 1972; Abramovitz et al., 1976; Alving, 1977; Ohanian et al., 1978; Shin et al., 1978; Alving et al., 1980). These effects have been variously attributed to the influence of the physical properties of membrane lipid (e.g., viscosity) upon antigenic expression, antibody binding, the efficiency of the complement activation cascades, or the functional state of the C5b-9 pore site. Conclusive evidence that the composition of membrane lipid can directly influence the functional activity of the C5b-9 proteins per se is presented by Shin et al. (1978), who demonstrate that the release of Rb^+ from liposomes treated with the purified C5b-9 proteins varies inversely with either the acyl chain length of constituent phospholipid or the mole fraction of cholesterol. A similar stabilizing effect of cholesterol upon liposomes treated with the C5b-9 proteins was also reported by Hesketh et al. (1972), who proposed that the functional activity of these proteins depends upon the "fluidity" of the target membrane. By contrast, Shin et al. (1978) conclude that the compositional effects of membrane lipid on C5b-9 pore function are *not* directly related to membrane fluidity but to bilayer thickness. Evidence for the influence of membrane thickness on the functional state of the C5b-9 pore has also been considered by Abramovitz et al. (1976).

Because the reaction kinetics we observe for the molecular event of FITC-C9 polymerization by C5b-8 SRBC ghosts are similar to those deduced for the *functional* damage of SRBC membranes induced by these proteins (see above), it was of considerable interest to determine whether this specific reaction step was itself influenced by the composition and/or phase state of lipid in the target membrane. In order to explore this question, the rate of MC5b-8-induced aggregation of FITC-C9 was measured for C5b-8-treated lipid vesicles composed of egg PC, DMPC, or DPPC, during suspension at various temperatures under conditions similar to those described for C5b-8 SRBC membranes. Selection of these lipids permitted analysis of the temperature-dependent rate of FITC-C9 polymerization

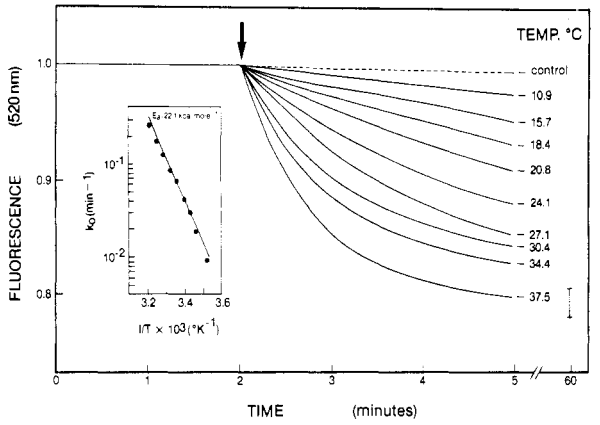

FIGURE 4: Kinetics of FITC-C9 polymerization by C5b-8-treated egg PC vesicles. Time-based fluorescence traces were obtained during incubation of FITC-C9 with C5b-8-treated small unilamellar vesicles composed of egg PC. Experiments shown were performed under conditions described for Figure 2A, except that C5b67 vesicles (415 μg of lipid) substituted for ghost membranes. Arrow indicates time of addition of 10 μg of C8. Similar traces were obtained with C5b-8 egg PC (formed by prior incubation with C8) in experiments performed under conditions described for Figure 2B (not shown). Dashed line indicates data for control vesicles suspended at 30.4 °C; other controls are omitted for clarity. Error bar indicates range of fluorescence measured at t = 60 min, for experiments performed at temperatures indicated between 18.4 and 37.5 °C. Shutters were closed at t = 15 min to reduce photobleaching. Data obtained for C5b-8 vesicles at temperatures below 4 °C superimposed on dashed trace indicated for control membranes (see text). Data are from single experiment. Activation energy derived from Arrhenius plot (insert) is 22.1 kcal mol^{-1}.

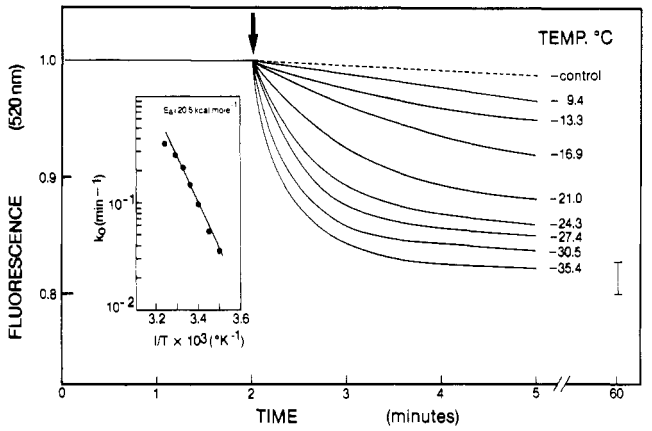

FIGURE 5: Kinetics of FITC-C9 polymerization by C5b-8-treated DPPC vesicles. Data were obtained in single experiment with DPPC vesicles under conditions described for Figure 4. Dashed line indicates data for control vesicles suspended at 30.5 °C; other controls are omitted for clarity. Error bar indicates range of fluorescence measured at t = 60 min, for experiments performed at temperatures indicated between 16.9 and 35.4 °C. Shutters were closed at t = 15 min to reduce photobleaching. Data obtained for C5b-8-treated vesicles at temperatures below 4 °C superimposed on dashed trace indicated for control membranes (see text). Activation energy derived from Arrhenius plot (insert) is 20.5 kcal mol^{-1}.

for C5b-8 membranes maintained at temperatures that were both above and below the thermotropic phase transitions of the constituent lipid. In these experiments, the input of C5b-8 proteins to membrane lipid was adjusted to achieve a total relative change in FITC-C9 fluorescence (at equilibrium) that was comparable to that observed for C5b-8 SRBC ghosts, permitting comparison of reaction rates between these various membranes (see Materials and Methods and Figure 2).

In Figures 4–6 are shown typical results obtained in single experiments with C5b-8-treated vesicles composed of each of

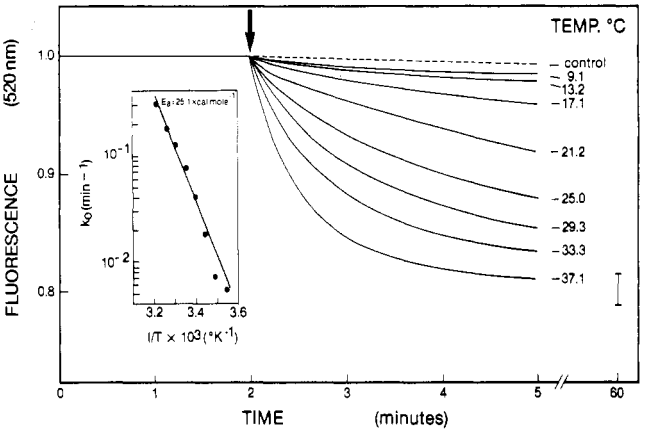

FIGURE 6: Kinetics of FITC-C9 polymerization by C5b-8-treated DMPC vesicles. Data were obtained in a single experiment with DMPC vesicles under conditions described for Figure 4. Dashed line indicates data for control vesicles suspended at 33.3 °C; other controls are omitted for clarity. Error bar indicates range of fluorescence measured at t = 60 min, for experiments performed at temperatures indicated between 21.2 and 37.1 °C. Shutters were closed at t = 15 min to reduce photobleaching. Data obtained for C5b-8-treated vesicles at temperatures below 4 °C superimpose on dashed trace indicated for control membranes (see text). Activation energy derived from Arrhenius plot (insert) is 25.1 kcal mol^{-1}.

these lipids. Inspection of the data of these figures reveals that, despite small variations observed between the lipids examined (see below), reaction rates for MC5b-8-induced self-quenching of FITC-C9 fluorescence were to first approximation comparable (for suspension at corresponding temperatures) irrespective of whether the constituent lipid was predominantly in the gel state (as in the case of DPPC) or in the liquid-crystalline state (as in the case of egg PC). For C5b-8 DMPC vesicles, no obvious discontinuity at the phase transition temperature was observed in Arrhenius plots of data obtained with this lipid (cf. insert Figure 6).

It is of interest to note that the reaction rates observed for FITC-C9 self-quenching by C5b-8 DPPC vesicles (Figure 5) were generally higher (for incubation at corresponding temperatures) than observed for C5b-8 vesicles composed of either egg PC (Figure 4) or DMPC (Figure 6). Since the higher rate observed for C5b-8 DPPC vesicles cannot simply be related to the presence of gel-state lipid in these vesicles (compare rates observed for DMPC at temperatures below 21 °C; Figure 6), one might speculate that these higher rates reflect an increased number of functionally active C5b-8 complexes bound to DPPC (as compared to the other lipids) due either to greater efficiency of C5b67 binding to these membranes (see Materials and Methods) or to enhanced stability of the membrane-bound C5b-8 complexes in DPPC. The relationship of acyl chain length to the functional activity and stability of the membrane-bound C5b-8 proteins is under current investigation.

The equilibrium level of FITC-C9 fluorescence (measured after 60 min) was in general equivalent (relative fluorescence 0.81 ± 0.02, mean ± SD) for all C5b-8 vesicle suspensions, following incubation at temperatures between 20 and 40 °C (see Figure 4–6). For incubation below these temperatures, a significant decrease in the C5b-8-specific change of FITC-C9 fluorescence was observed (see Figures 4–6). Even after prolonged incubation (>3 h), the relative change in FITC-C9 fluorescence measured for C5b-8 vesicles suspended at temperatures below 20 °C generally failed to achieve levels observed for suspension at 20–40 °C (data not shown), suggesting that the equilibrium level of FITC-C9 aggregation is inhibited at temperatures below this range (cf. results for SRBC ghosts,

above). Inhibition of the functional activity of the C5b-9 proteins at these low temperatures has previously been noted (Alving et al., 1980; Boyle & Borsos, 1980). As was observed for SRBC ghosts (see the text above and Figure 2), incubation of FITC-C9 with vesicles at temperatures above 40 °C resulted in a slow spontaneous decrease in fluorescence intensity (and a decrease in the C5b-8-specific response, suggesting aggregation and inactivation of the protein at these elevated temperatures. Accordingly, for these experiments, only data in the temperature range 20–40 °C were analyzed to derive Arrhenius activation energies.

Arrhenius activation energies estimated for the polymerization of FITC-C9 by C5b-8 bound to lipid vesicles were in the range 20–25 kcal mol^{-1}, significantly higher than that determined for C5b-8 bound to SRBC ghost membranes (Table I). The activation energy estimated for C5b-8 DMPC vesicles was found to be slightly higher than for vesicles composed of either DPPC or egg PC. No significant differences were obtained when the Arrhenius activation energies for FITC-C9 polymerization by C5b-8-treated small unilamellar vesicles were compared to C5b-8-treated large unilamellar vesicles composed of the same lipid (egg PC; Table I). These data suggest that the lower activation energy observed in the case of C5b-8-treated ghost membranes is unlikely to be related to either bulk viscosity or radius of curvature of the membranes.

On the basis of the results obtained with C5b-8 vesicles, it would appear that the activation of C9 by MC5b-8 is largely unaffected by the phase state of constituent membrane lipid. This is somewhat surprising in light of the fact that the mobility of membrane constituents decreases by nearly 3 orders of magnitude in the gel state of the matrix lipid (Rubenstein et al., 1979; Peters, 1982). These results may indicate, therefore, that the polymerization of C9 occurs *prior* to membrane insertion (rather than by diffusional aggregation of membrane-inserted C9 monomers). In this context it should be noted, however, that the membrane insertion of C9—as detected by labeling with membrane-restricted photoaffinity probes—has been reported to occur within 5 s after addition to C5b-8 vesicles at 37 °C (Hu et al., 1981). Furthermore, on the basis of estimates of the diffusion coefficient in the gel state of DMPC ($\sim 10^{-10}$ cm^2 s^{-1}) and DPPC ($\sim 5 \times 10^{-12}$ cm^2 s^{-1}) in the absence of cholesterol [estimated from data of Rubenstein et al. (1979)], it is conceivable that aggregation by lateral diffusion of membrane-inserted C9 monomers in the gel state of these vesicles (membrane areas of 10^{-10}–10^{-9} cm^2) occurs on a time scale faster than we are able to resolve in the present experiments. In this context, it is also necessary to consider the likelihood that C9 insertion occurs in a nonrandom fashion, activation and insertion topologically restricted to the site of each membrane-bound C5b-8 complex.

It is of considerable interest that the activation energies we obtain for FITC-C9 polymerization by C5b-8 SRBC ghost membranes are significantly lower than those determined for any of the C5b-8 lipid vesicles (Table I). These results suggest that a constituent of the SRBC membrane exerts a catalytic effet upon the C9 polymerization reaction. In light of accumulating evidence that sulfhydryl reagents such as glutathione can serve to cross-link C9 [C9–C9 interchain disulfide linkages promoted by disulfide exchange; see Ware & Kolb (1981) and Yamamoto & Migita (1983)], one might speculate that the lower activation energies observed for SRBC ghost membranes (as compared to lipid vesicles) may reflect the influence of free sulfhydryls on membrane proteins in promoting disulfide exchange and C9 self-association. The possible role of membrane sulfhydryls in promoting C9 self-association is under current investigation.

Acknowledgments

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Registry No. Complement C9, 80295-59-6; C5b-8, 82903-91-1.

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Covalent Binding Efficiency of the Third and Fourth Complement Proteins in Relation to pH, Nucleophilicity, and Availability of Hydroxyl Groups†

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ABSTRACT: The binding of [^{3}H]glycerol and [^{3}H]putrescine to C3 was studied in a fluid-phase system using trypsin as the C3 convertase. The binding of glycerol showed little variation in the pH range between 6.0 and 10.0. The binding of putrescine (pK_a = 9.0) is rather ineffective below pH 7.5 but becomes more efficient as the pH of the reaction mixture increases. These results agree with the contention that the final step of the binding reaction is the transfer of the acyl group of the exposed thio ester of C3 to a nucleophile since the nucleophilicity of hydroxyl groups is rather independent of pH whereas only the unprotonated form of amino groups is nucleophilic. The inefficient reaction of amino groups with the exposed thio ester of C3 is also supported by the study of the inhibitory activity of serine and its two derivatives, *N*-acetylserine and *O*-methylserine, to the binding of [^{3}H]glycerol to C3. *N*-Acetylserine showed an inhibitory activity equivalent to that of serine, whereas O-methylated serine showed only minimal activity. It can be concluded, therefore, that serine reacts with the thio ester of C3 by its hydroxyl group but not by its α-amino group. The ability of the alcohol group of various alkanes to inhibit the binding of [^{3}H]glycerol to C3 was also studied. The primary alcohols inhibit the binding reaction with an efficiency that is similar to glycerol, and there are no significant differences in the binding efficiencies of methanol, ethanol, 1-propanol, and 1-butanol. Hence, the length of the alkyl chain does not appear to affect the reactivity of the hydroxyl group with the exposed thio ester. On the other hand, secondary and tertiary alcohols are less effective in their inhibition of the binding reaction, indicating that the thio ester, though exposed, is not freely accessible to bulkier molecules. The binding of [^{3}H]glycerol to C4 shows a pH dependence that is similar to C3. However, the binding efficiency of [^{3}H]putrescine to C4 is significantly higher in the pH range 5.0–10.0. These results suggest that there are some differences between the binding sites of C3 and C4, though the differences are not defined at present. The elevated activity of the exposed thio ester of C4 to amino groups may be of physiological significance, since the binding of C4b to IgG of immune complexes by amide bonds may be important in the efficient activation of the classical pathway of complement.

The covalent binding reaction of the third complement protein C3[1] is an integral part of its functional activity (Law et al., 1980b). It allows the protein to bind to all cell surfaces capable of activating the classical and/or the alternative pathways of complement [for review, see Law (1983b)], and it is currently understood as an acyl transfer reaction (Law et al., 1979; Sim et al., 1981) in which an internal thio ester (Janatova et al., 1980; Pangburn & Müller-Eberhard, 1980; Tack et al., 1980; Law et al., 1980b) between a cysteinyl residue and a glutamyl residue in C3 (Tack et al., 1980) becomes available for reaction upon the proteolytic activation of C3 to C3a and C3b* (C3b* is defined as the active state of the C3b molecule). The thio ester can react with water or other molecules that contain either amino or hydroxyl groups in the medium. If, however, C3 is activated sufficiently close to a cell surface, C3b* can react with surface-bound hydroxyl or amino groups to covalently link C3b to the cell surfaces via ester or amide bonds (Müller-Eberhard et al., 1966; Law & Levine, 1977; Law et al., 1979).

We have established a fluid-phase system to study the covalent bonding reaction of C3b* (Law et al., 1981). Radioactive small molecules (S) are incorporated specifically at the binding site of C3b with trypsin as the C3 convertase (Hostetter et al., 1982). The fluid-phase system has the advantage of being comprised solely of defined molecular components in a medium where kinetic parameters are defined. Uncertainties arising from the heterogeneity of cell-surface molecules and the molecular diffusional properties at cell surfaces are eliminated. Using this system, we have shown that the basic scheme of the binding reaction is

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; SBTI, soybean trypsin inhibitor; $NaDodSO_4$, sodium dodecyl sulfate; PPO, 2,5-diphenyloxazole; Me_2POPOP, 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene; C3 and C4, third and fourth complement proteins; C3b* and C4b*, the active states of C3 and C4 induced by proteases; C3* and C4*, the active states of C3 and C4 induced by nonproteolytic reagents; S, substrate molecule in the binding reaction; I, inhibitor of the binding reaction; BE, binding efficiency; IgG, immunoglobulin G. The nomenclature of the complement proteins is that recommended by the World Health Organization (1968).

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