Margolis, R. L., & Wilson, L. (1980) *Cell (Cambridge, Mass.) 18*, 673–678.
Murphy, D. B., Johnson, K. A., & Borisy, G. G. (1977) *J. Mol. Biol. 117*, 33–52.
Olmsted, J. B., & Borisy, G. G. (1973) *Biochemistry 12*, 4282–4289.
Penningroth, S. M., Cleveland, D. W., & Kirschner, M. W. (1976) in *Cell Motility* (Goldman, R., Pollard, T., & Rosenbaum, J., Eds.) p 1233–1257, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Scheele, R. B., & Borisy, G. G. (1979) in *Microtubules* (Roberts, K., & Hyams, J. S., Eds.) pp 175–254, Academic Press, New York.
Sheir-Neiss, G., Lai, M. H., & Morris, N. R. (1978) *Cell (Cambridge, Mass.) 15*, 639–647.
Shelanski, M. L., Gaskin, F., & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 765–768.
Sloboda, R. D., Rudolph, S. A., Rosenbaum, J. L., & Greengard, P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 177–181.
Snyder, J. A., & McIntosh, J. R. (1976) *Annu. Rev. Biochem. 45*, 699–720.
Timasheff, S. N., & Grisham, L. M. (1980) *Annu. Rev. Biochem. 49*, 565–592.
Vallee, R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3206–3210.
Veech, R. L., Lawson, W. R., Cornell, N. W., & Krebs, H. A. (1979) *J. Biol. Chem. 254*, 6538–6547.
Webb, B. C., & Wilson, L. (1980) *Biochemistry 19*, 1993–2001.
Weingarten, M. D., Lockwood, A. H., Hwo, S.-Y., & Kirschner, M. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1858–1862.
White, H. D., Coughlin, B. A., & Purich, D. L. (1980) *J. Biol. Chem. 255*, 486–491.
Wilson, L., & Bryan, J. (1974) *Adv. Cell Mol. Biol. 3*, 21–72.
Wilson, L., & Margolis, R. L. (1978) in *ICN–UCLA Conference on Cell Reproduction* (Prescott, D., Dirksen, E., & Fox, C. F., Eds.) pp 241–258, Academic Press, New York.
Wilson, L., Anderson, K., & Chin, D. (1976) in *Cell Motility* (Goldman, R., Pollard, T., & Rosenbaum, J., Eds.) pp 1051–1064, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Nucleophilic Modification of Human Complement Protein C3: Correlation of Conformational Changes with Acquisition of C3b-like Functional Properties†

David E. Isenman,* David I. C. Kells, Neil R. Cooper, Hans J. Müller-Eberhard, and Michael K. Pangburn

ABSTRACT: Inactivation of C3 by enzymatic cleavage, nucleophilic addition, or slow freezing and thawing resulted in the acquisition of similar end-state conformations as judged by near-UV circular dichroism. Although inactivation by the two nonenzymatic processes involves no peptide bond scission, the inactivated C3 resembled C3b in that it possessed a free sulfhydryl group not present in the native protein and an increased surface hydrophobicity as evidenced by enhanced binding of the fluorophore 8-anilino-1-naphthalenesulfonate (ANS). The C3b-like functional properties of modified C3 [Pangburn, M. K., & Müller-Eberhard, H. J. (1980) *J. Exp. Med. 152*, 1102–1114] may thus be understood in terms of the similarity of its conformation to that of C3b. The rate of the conformational change following proteolytic cleavage was fast and appeared to be limited by the rate of the enzymatic reaction. In contrast, the rate of conformational change following addition of methylamine was slow and rate limited by the conformational rearrangement itself, not by the chemical modification. A kinetic analysis of the changes in circular dichroism and ANS fluorescence enhancement suggested that the nucleophilic addition was spectroscopically undetectable and was followed by a minimally biphasic, spectroscopically demonstrable conformational rearrangement. The appearance of C3b-like functional activity in nucleophile-modified C3 largely parallels the time course of the spectroscopically detectable conformational change but is distinctly slower than the rate at which hemolytic activity is lost. While fully transconformed methylamine-inactivated C3 can bind factor B and is susceptible to cleavage by C3b inactivator and its cofactor β1H, this cleavage occurs at a substantially slower rate than the equivalent process in C3b. The implications of these findings in terms of the mechanism through which the alternative pathway of complement is initiated are discussed.

C3 is a highly versatile serum protein which plays a pivotal role in host defense and the inflammatory functions of complement. As such, C3 is the precursor of several physiologically occurring fragments which have different biological activities (Müller-Eberhard & Schreiber, 1980). The protein has a molecular weight of 187 500 (Tack & Prahl, 1976) and is composed of two polypeptide chains, the molecular weights of which are approximately 115 000 (α chain) and 75 000 (β chain). Upon complement activation, the enzyme C3 convertase cleaves peptide bond 77 of the α chain of C3, thereby producing the fragments C3a (M_r 9000) and C3b (M_r 178 500) (Hugli & Müller-Eberhard, 1978). C3a constitutes one of the three anaphylatoxins of the complement system, and C3b fulfills various functions in the cytolytic and opsonic com-

† From the Department of Biochemistry, University of Toronto, Toronto, Canada M5S 1A8 (D.E.I. and D.I.C.K.), and the Department of Molecular Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037 (N.R.C., H.J.M.-E., and M.K.P.). *Received December 18, 1980.* Supported by Grant MA-7081 of the Medical Research Council of Canada and by Grants AI 07007, CA 14692, 1 S07 RR 05514, and HL 16411 from the U.S. Public Health Service. D.E.I. is the recipient of a Medical Research Council of Canada Scholarship. H.J.M.-E. is the Cecil H. and Ida M. Green Investigator in Medical Research, Research Institute of Scripps Clinic. This is Publication No. 2320 from the Research Institute of Scripps Clinic.

0006-2960/81/0420-4458$01.25/0

plement reactions. In its nascent state, C3b exhibits a metastable binding site (Müller-Eberhard et al., 1966; Pangburn & Müller-Eberhard, 1980) through which it may form an ester (Law et al., 1979) or possibly an amide bond with surface constituents of biological particles (Campbell et al., 1980). Through this mechanism, C3b becomes firmly attached to targets of complement attack. Bound C3b exerts opsonic activity because it is capable of binding to specific complement receptors on the surface of phagocytic cells (Gigli & Nelson, 1968; Huber et al., 1968; Bianco & Nussenzweig, 1977). It is an essential ingredient of the cytolytic pathway because it constitutes a subunit of the C3 and C5 activating enzymes of complement (Götze & Müller-Eberhard, 1976; Vogt et al., 1978). C3b participates in the formation and the control of these crucial enzymes by entering into specific protein–protein interactions with several complement constituents (Müller-Eberhard & Schreiber, 1980), including factor B, properdin, C5, β1H, and C3b inactivator. Since none of the aforementioned properties of C3b are expressed by native C3, it was postulated that the enzymatic conversion of C3 to C3b is accompanied by discrete conformational changes as a structural correlate to the acquisition of these new activities. Such conformational changes have indeed been established by spectroscopic methodology (Isenman & Cooper, 1981).

Recently, a novel form of C3 has been described which, although intact with regard to its peptide structure, is hemolytically inactive and displays C3b-like functional properties (Pangburn & Müller-Eberhard, 1980). Because this form can bind and modulate factor B and thus generate a fluid-phase C3 convertase, it has been proposed to be the form of C3 that is directly responsible for the initiation of the alternative complement pathway. C3 with C3b-like functional properties was produced by treatment of native C3 with methylamine or with chaotropic agents or by slowly freezing and thawing the protein. Whereas the first treatment was shown to result in incorporation of 1 mol of methylamine per mol of protein (Pangburn & Müller-Eberhard, 1980; Tack et al., 1980a,b; Howard, 1980), the latter two treatments are believed to cause the reaction of 1 mol of H_2O with 1 mol of protein. Like C3b, methylamine-modified C3b bound 1 mol of iodoacetamide per mol of protein, while native C3 failed to react with this compound (Pangburn & Müller-Eberhard, 1980; Janatova et al., 1980a). Because the methylamine-substituted glutamyl residue and the carbamido-methylated cysteinyl residue were both found in a peptide derived from the α chain, separated only by two amino acid residues (Tack et al., 1980b), it was proposed that unmodified native C3 contains an internal thioester bond (Pangburn, 1980; Janatova et al., 1980a; Tack et al., 1980a,b; Pangburn & Müller-Eberhard, 1980). Accordingly, C3 modified by treatment with methylamine or chaotropic agents is thought to differ from native C3 in that it is devoid of the putative thioester bond.

It is the purpose of this paper to show that when native C3 was modified to assume the functional characteristics of C3b, it also assumed the conformational characteristics of C3b as evidenced by spectroscopic methods, although no peptide bonds were broken in the process. Compared to the rate of chemical modification, the rate of conformational rearrangement of C3 was slow and similar to the rate of acquisition of C3b-like functional properties. This finding suggests that the expression of C3b-like activities by modified C3 is dependent on the acquisition of a C3b-like conformation.

Materials and Methods

Materials. The following materials were purchased from the sources indicated: bovine trypsin and lactoperoxidase were obtained from Worthington (Freehold, NJ); soybean trypsin inhibitor, methylamine, and iodoacetamide were from Sigma (St. Louis, MO); *n*-butyl phthalate was from Fisher Scientific (Fairlawn, NJ); the magnesium salt of 8-anilino-1-naphthalenesulfonate was from Eastman (Rochester, NY); sodium [^{125}I]iodide and iodo[^{14}C]acetamide were from Amersham (Oakville, Ontario). Fresh frozen human plasma was generously provided by the Canadian Red Cross and the Toronto Western Hospital blood bank.

Purified Components. Factor B (Götze & Müller-Eberhard, 1971), β1H and C3bINA[1] (Pangburn et al., 1977), and C5 (Tack & Prahl, 1976) were prepared as described previously. C3 was prepared essentially according to the method of Tack & Prahl (1976) with the following modifications. The resins DEAE-Sephacel (Pharmacia) and Bio-Gel A-1.5M (Bio-Rad) were substituted for DEAE-cellulose and Sepharose 6B, respectively. In addition, as a final purification step, the posthydroxylapatite C3 pool was applied to a column of QAE-A50 Sephadex (20 mM Tris-HCl and 70 mM NaCl, pH 7.8) (Pharmacia) and eluted with a salt gradient ranging from 9 to 25 mS (Janatova et al., 1980b). C3b was generated from intact C3 by tryptic digestion (1% w/w) for 2 min at room temperature at which time an equal weight of soybean trypsin inhibitor was added (Bokisch et al., 1969). Protein iodination was accomplished by using the lactoperoxidase procedure of Marchalonis (1969).

Buffers. The following veronal–NaCl buffers were used (Rapp & Borsos, 1963): VB, veronal-buffered saline, pH 7.3, containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (μ = 0.15); GVB, VB containing 0.1% gelatin; GVBE, GVB made 10 mM in EDTA; GVB-Mg^{2+}, GVB in which the Mg^{2+} concentration has been increased to 5 mM. Phosphate-buffered saline (PBS) consisted of 10 mM sodium phosphate and 0.15 M NaCl, pH 7.4. Tris-buffered saline (TBS) consisted of 10 mM Tris-HCl and 0.15 M NaCl, pH 7.4.

C3 Hemolytic Activity Assays. The preparation of $EAC4^{oxy}2$ cells and their use together with purified C5 and KSCN/hydrazine-treated guinea pig serum (as a source of C6–9) for the effective molecule titration of C3 have been described previously (Cooper & Müller-Eberhard, 1970). The time course of C3 hemolytic inactivation at various temperatures by methylamine (50 mM, pH 8.0) was followed by measuring the residual C3 hemolytic activity of time-point aliquots which had been diluted 200-fold into ice-cold GVBE. Results were calculated on the basis of the "one-hit" hypothesis and are expressed as a percentage of the C3 activity present at time zero.

Preparation of Methylamine-Modified C3. Methylamine-modified C3, C3(CH_3NH_2), was prepared by incubating native C3 with 50 mM methylamine (in TBS, pH 8.0) for 1 h at 37 °C. Excess reagent was removed by dialysis against TBS, pH 7.4.

Radioalkylation of Free Sulfhydryl Groups. Samples of 300 μL of C3 or its derivatives (1–2 mg/mL) in 50 mM Tris-HCl and 0.15 M NaCl, pH 8.2, buffer were made 0.19 mM in iodo[^{14}C]acetamide (17 000 cpm/nmol) and incubated at room temperature for 1 h. Unreacted iodoacetamide was removed by exhaustive dialysis against PBS. The number of free sulfhydryl groups present per molecule of C3 in each sample was determined from liquid scintillation counting data

[1] Abbreviations used: C3bINA, C3b inactivator; C3(CH_3NH_2), C3 modified by incorporation of methylamine; iC3b, C3b inactivated by cleavage with C3bINA; $NaDodSO_4$, sodium dodecyl sulfate; ANS, 8-anilino-1-naphthalenesulfonate; CD, circular dichroism; DTT, dithiothreitol; *Z*, average number of hemolytically effective molecules per cell.

using the known specific activity of the iodo[^{14}C]acetamide and the protein concentration determined by the method of Lowry et al. (1951). The concentration of C3 used to construct the standard curve was determined spectrophotometrically at 280 nm using 9.7 as the value for $E^{1\%}_{280nm}$ (Tack & Prahl, 1976). Small corrections were made for nonspecific binding of the radiolabel by running a parallel series of samples which were pretreated for 1 h at room temperature with excess (1 mM) nonradioactive iodoacetamide.

Factor B Binding Assay. The ^{125}I–factor B binding assay was a modification of the assay described by Kazatchkine et al. (1979). EAC4^{oxy}23b cells prepared by the addition of 100 μg of C3 per 10^9 EAC4^{oxy}2 cells were used as the source of solid-phase C3b. In a standard assay, 10^7 EAC4^{oxy}23b cells were incubated with various concentrations of ^{125}I-labeled B in a total volume of 210 μL of GVB-Mg^{2+} for 15 min at room temperature. The incubation mixture (180 μL) was layered on top of 180 μL of *n*-butyl phthalate in a 400-μL polypropylene microfuge tube. With a Beckman B microfuge, the cells were pelleted through the oil phase within 15 s. The bottoms of the tubes were cut off, and the radioactivity in the pellet was determined. As a correction for nonspecific binding and/or trapping, we subtracted the radioactivity pelleted in tubes containing ^{125}I-labeled B and 10^7 EAC4^{oxy}2 cells. Inhibition of ^{125}I-labeled B binding by fluid-phase C3b or methylamine-treated C3 was performed at a ^{125}I-labeled B concentration sufficient to give approximately 50% saturation of sites. This concentration of radiolabeled factor B was preincubated for a minimum of 30 s with the inhibitor protein in a total volume of 190 μL of GVB-Mg^{2+}. EAC4^{oxy}23b (20 μL, containing 10^7 cells) were then added, and the tubes were incubated for a further 15 min at room temperature at which time the specifically cell-bound radioactivity was determined as described above. Results for methylamine-treated C3 are expressed as a percentage of the maximal inhibition achieved with C3b according to the following relationship:

$$\text{percent factor B binding activity} = \frac{T - M}{T - I} \times 100$$

where T is the specifically cell-bound radioactivity in the absence of fluid-phase inhibitor protein and M and I represent the specifically cell-bound radioactivities when time-course aliquots of methylamine-treated C3 (M) or C3b (I) are present in the fluid phase.

NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. NaDodSO$_4$–polyacrylamide gel electrophoresis was performed in 5.6% gels according to the method of Fairbanks et al. (1971). The stained gels were scanned at 560 nm in a Unicam SP1800 spectrophotometer equipped with a densitometer accessory. The areas under the peaks were determined by planimetry, and the area of each peak was expressed as a percentage of the total peak areas.

Cleavage of C3 or C3(CH_3NH_2) with β1H and C3bINA. The cleavage reaction was performed in TBS at 37 °C for 10 min and terminated by addition of NaDodSO$_4$ sample buffer containing DTT and heating at 100 °C for 2 min. The extent of cleavage was then analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis.

Circular Dichroism Spectra. CD measurements were performed with a Jasco J-41A spectropolarimeter (Japan Spectroscopic Co.) equipped with a built-in data processor. Unless otherwise indicated, spectra were signal averaged 8 times and were corrected for base-line nonlinearities by subtraction of an equally signal-averaged solvent blank. Results are expressed in terms of mean residue ellipticity (Adler et al., 1973) using a mean residue weight of 110.

Table I: Correlation of C3 Hemolytic Activity and the Presence of a Free Sulfhydryl Group

protein	mol of SH radioalkylated/mol of protein	hemolytic activity (Z/μg)
native C3 (prepn 1)	0.14	2.3
native C3 (prepn 2)	0.20	2.0
C3b (prepn 1)[a]	1.30	0
C3b (prepn 2)[a]	0.98	0
C3(CH_3NH_2)	0.85	0
C3(CH_3NH_2) + limited trypsinization	0.90	0
freezer-inactivated C3[b]	1.02	0
freezer-inactivated C3[b] + limited trypsinization	1.09	0

[a] C3b was prepared from native C3 by limited trypsin cleavage. [b] Freezer-inactivated C3 refers to C3 which had lost hemolytic activity during an accidental freezer thawing.

ANS Fluorescence Measurements. ANS fluorescence emission spectra were recorded in an Aminco SPF500 spectrofluorometer (American Instrument Co., Silver Springs, MD) in the ratio mode. Excitation was at 386 nm, and emission spectra were recorded over the range of 400–625 nm using 5-nm band-pass excitation and emission slits. Unless indicated otherwise, the cell compartment was thermostated to 25 °C.

Kinetic CD and Fluorescence Measurements. For kinetic experiments in which the spectral change at a single wavelength was continuously monitored, the analogue signal of either instrument was digitized and recorded on magnetic tape by means of a time-base data logger built by the Medical Computing Facility of the University of Toronto. The data were analyzed by iteratively fitting the curves to a theoretical model described by the differential rate equations presented in the appendix. Minimization was done with a nonlinear least-squares program based on the Marquardt algorithm (Marquardt, 1963; Meeter, 1965).

Results

Correlation of Loss of C3 Hemolytic Activity and the Appearance of a Free Sulfhydryl Group. It has recently been reported that C3 activation by enzymatic cleavage, or inactivation by nucleophilic agents, led to the appearance of a free sulfhydryl group (Janatova et al., 1980a,b; Pangburn & Müller-Eberhard, 1980). Table I compares native C3, methylamine-treated C3, and enzymatically generated C3b with respect to their hemolytic activity and the degree of alkylation by iodo[^{14}C]acetamide. Hemolytically active native C3 had less than 0.2 mol of free sulfhydryl per mol of protein. Exposure of native C3 to methylamine, or to trypsin, abolished hemolytic activity and raised SH exposure to approximately 1 mol/mol of protein. A preparation of C3 inactivated by an accidental freezer thaw is also seen to contain 1 mol of free sulfhydryl per mol of protein. Neither C3(CH_3NH_2) nor freezer-inactivated C3 showed significant increases in sulfhydryl exposure after these proteins were subjected to limited trypsinization.

Circular Dichroism. In a previous study (Isenman & Cooper, 1981), it was demonstrated that enzymatic activation of C3 leads to alterations in its tertiary structure as evidenced by pronounced positive shifts in several of the near-ultraviolet CD spectral bands. As shown in Figure 1, the near-UV CD spectrum of C3 inactivated by the freezer thaw and showing an exposed sulfhydryl group demonstrated a marked similarity to that of C3 which had been converted to C3b by limited trypsinization. Trypsinization of this C3 led to only small

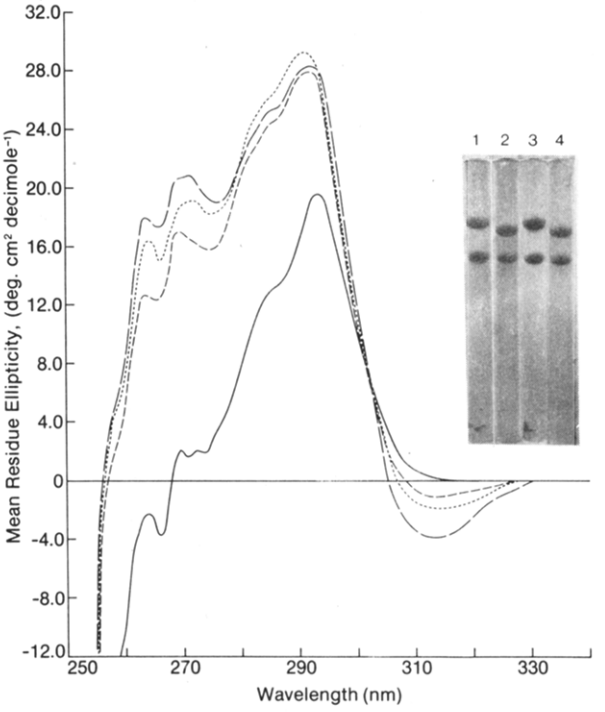

FIGURE 1: Near-ultraviolet circular dichroism spectra of hemolytically active C3 (—), C3 digested with trypsin (1% w/w) for 2 min to form C3b (– – –), "freezer-inactivated" C3 (- - -), and freezer-inactivated C3 digested with trypsin as above (···). The inset depicts the $NaDodSO_4$–polyacrylamide gel electrophoresis analysis, performed under reducing conditions, of the proteins whose spectra were recorded: (1) native C3, (2) trypsinized C3, (3) freezer-inactivated C3, (4) trypsinized, freezer-inactivated C3.

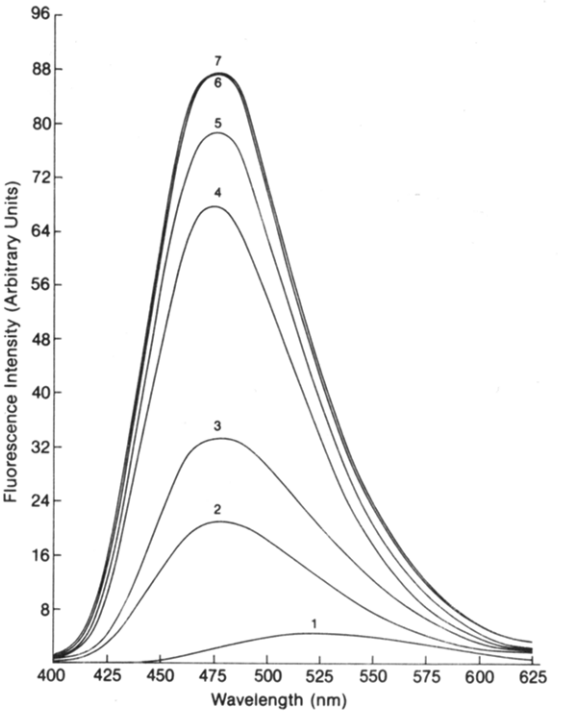

FIGURE 2: Emission spectra of 10^{-5} M ANS in the presence of TBS buffer alone (1), native C3 (2), native C3 left at room temperature for 48 h prior to the spectral measurement (3), freezer-inactivated C3 (4), native C3 incubated with methylamine (50 mM, pH 8.0) for 48 h at room temperature (5), native C3 trypsinized (1% w/w) for 2 min (6), and freezer-inactivated C3 trypsinized (1% w/w) for 2 min (7). The protein concentration in all cases was 1.7×10^{-6} M. Excitation was at 386 nm, and 5-nm band-pass excitation and emission slits were used. The spectra have been corrected for a small contribution due to buffer scattering.

changes in the spectrum. The inset of Figure 1 shows the $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of the proteins whose spectra were recorded. It is evident that like hemolytically active C3, the inactivated C3 molecule had an intact α chain which was converted to an α' chain after trypsin treatment. In the far-UV region, all the spectra were indistinguishable and were characterized by a broad negative band centered at 213 nm (–6200 deg·cm²/dmol, data not shown). Thus, as was previously shown for enzymatic conversion of C3 to C3b (Isenman & Cooper, 1981), inactivation of C3 without peptide bond cleavage but with exposure of a sulfhydryl group led to subtle changes in tertiary structure without significant alteration of the secondary structure.

ANS Fluorescence. The emission spectrum resulting from the binding of the fluorophore 8-anilino-1-naphthalenesulfonate (ANS) to C3 displays a very pronounced enhancement following enzymatic conversion of C3 to C3b (Isenman & Cooper, 1981). As can be seen in Figure 2, under standard conditions of 10^{-5} M ANS and 1.7×10^{-6} M C3 in TBS, pH 7.4, this enhancement is approximately 4.2-fold at 473 nm (compare spectra 2 and 6 of Figure 2). Under the same conditions, the fluorescence of ANS in the presence of C3 treated with methylamine for 48 h at room temperature approached that of enzymatically activated C3 (spectrum 5, Figure 2). A control C3 sample sham incubated for 48 h at room temperature also displays a small increase in ANS fluorescence (spectrum 3, Figure 2) relative to that of the unincubated protein. The C3 preparation inactivated during the freezer thaw exhibited a 3.3-fold enhancement of ANS fluorescence (spectrum 4, Figure 2). Limited trypsinization of this protein gave rise to an immediate further increase in ANS fluorescence to the level of the spectrum of C3b (compare spectra 6 and 7, Figure 2). Thus, the increase in surface hydrophobicity, as reflected by an increased tendency to bind ANS, has occurred in the methylamine-treated and the "freezer-inactivated" C3 without peptide bond cleavage. ANS fluorescence enhancement resulting from methylamine (50 mM) inactivation of C3 was very slow, and only about 30% of the anticipated change had occurred after 2 h at 25 °C. The addition of trypsin (1% w/w) to the inactivation mixture at this time resulted in a rapid increase in ANS fluorescence to the level expected for the fully transconformed molecule.

Table II: Kinetic Constants for the rate of C3 Hemolytic Inactivation by 50 mM Methylamine at pH 8.0[a]

temp (°C)	pseudo-first-order rate constant (s^{-1})	pseudo-first-order $t_{1/2}$[b] (min)	calcd second-order rate constant[c,d] (M^{-1} s^{-1})
37	2.11×10^{-3}	5.45	4.22×10^{-2}
30	8.57×10^{-4}	13.4	1.71×10^{-2}
23.5	4.3×10^{-4}	26.7	8.61×10^{-3}

[a] The reaction buffer was 0.01 M Tris-HCl and 0.15 M NaCl, pH 8.0, at 23.5 °C. No attempt was made to compensate for the 0.2 pH unit drop which this buffer displays at 37 °C. [b] $t_{1/2}$ is defined as $0.69/k$ for first-order or pseudo-first-order reactions. [c] Calculated by dividing the pseudo-first-order rate by the molar concentration of methylamine. [d] The second-order rate constants obtained at the various temperatures when plotted in the form of an Arrhenius plot [i.e., $\ln k_{12}$ vs. $1/T$ (K)] yield a straight line from which an activation energy of 21.4 kcal/mol is calculated.

Comparison of the Rate of Inactivation of C3 Hemolytic Activity and the Rate of the Spectral Change following the Addition of Methylamine. Determination of the time course of inactivation of C3 by methylamine was performed under pseudo-first-order conditions (1.7×10^{-6} M C3 and 50 mM methylamine, pH 8.0) at several temperatures. The results of these experiments are shown in the form of first-order

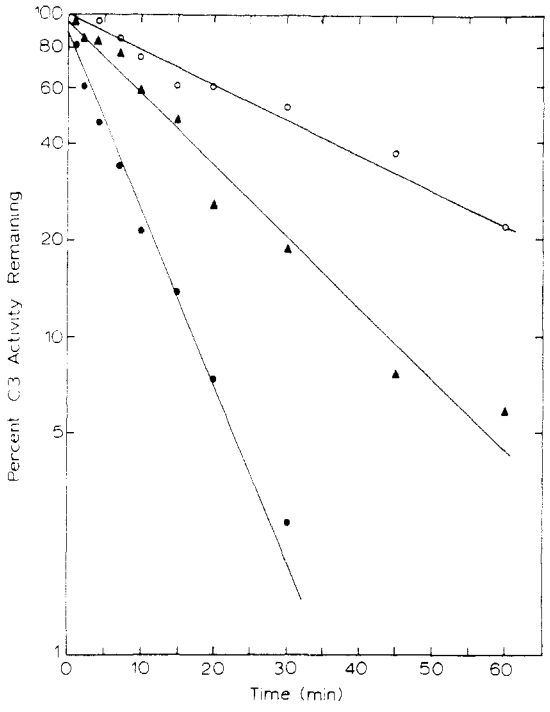

FIGURE 3: Pseudo-first-order kinetic plots of loss of C3 hemolytic activity by 50 mM methylamine (in TBS, pH 8.0) at 37 (●), 30 (▲), and 23.5 °C (○). Control incubation in TBS showed no inactivation during the time frame of the experiment. The initial protein concentration in the incubation mixture was 1.7×10^{-6} M.

kinetic plots in Figure 3. From the slopes of these graphs, the kinetic constants shown in Table II were determined. Thus, at 23.5 °C, the half-time of inactivation was 27 min, and at 37 °C, it was 5.5 min. The inclusion of ANS in the incubation mixture did not affect the rate of inactivation. Figure 4 depicts the time course of the near-UV CD change following methylamine addition. As was the case for enzymatic conversion of C3 to C3b, inactivation with methylamine resulted in the spectral bands below 305 nm becoming more positive and in the appearance of the negative band centered at 312 nm. Assuming that spectrum 6 (i.e., at 3030 min) represented an equilibrium value, we plotted the kinetics of the signal change at 264.5 nm in the form of a first-order plot (see inset, Figure 4). The large deviation of the point at zero time from the extrapolated value at time zero of the slow phase suggests that a minimally biphasic mechanism is needed to account for the kinetics of the conformational transition. The slow phase, which accounted for 73% of the spectral change, had a half-time of 520 min ($k_{slow} = 2.21 \times 10^{-5}$ s^{-1}) as 23.5 °C. Thus, the major portion of the spectroscopically observed conformational change occurred at a rate that was almost 20-fold slower than the rate of inactivation by methylamine at the same temperature (Figure 3). Similar treatment of the data using the signal change at other wavelengths (272.5, 284, and 293 nm) gave comparable results, although because of the smaller signal to noise ratio, the scatter of the points was larger.

Continuous Monitoring of the CD and ANS Fluorescence Signal Changes. In order to obtain sufficient data to perform a detailed kinetic analysis of the conformational changes following nucleophilic inactivation of C3, the CD signal at a fixed wavelength was continuously monitored following the addition of 50 mM methylamine. The results of such an experiment performed at 37 °C and monitored at 264.5 nm are shown in Figure 5. Attempts to analyze the data phenomenologically in terms of a sum of two exponents (Vuk-Pavlović et al., 1978; Isenman et al., 1979) were partially successful at the longer time periods but failed to yield a

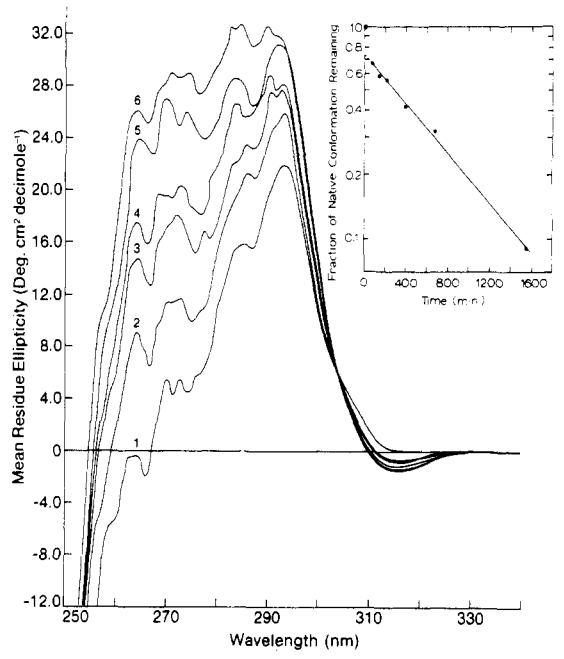

FIGURE 4: Near-UV CD time-course scans of C3 treated with 50 mM methylamine at 23.5 °C, pH 8.0, for (1) 0, (2) 67, (3) 396, (4) 669, (5) 1548, and (6) 3030 min. The time taken to accumulate four scans was 14 min, and the times indicated represent the total elapsed time following methylamine addition when two of the four scans had been completed. The inset depicts the signal change at 264.5 nm in the form of a first-order kinetic plot. It was assumed that the mean residue ellipticity at this wavelength after 3030 min (spectrum 6) represented an equilibrium value and that therefore the difference in ellipticity between spectra 1–5 and spectrum 6 was proportional to the concentration of C3 in the native conformation.

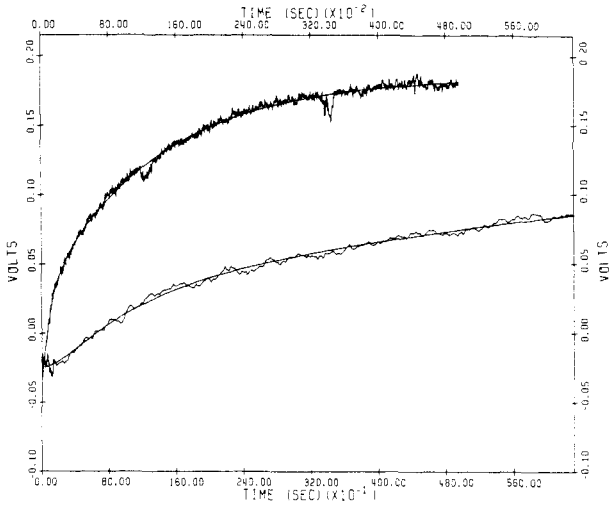

FIGURE 5: Continuous monitoring of the CD spectral change at 264.5 nm following the addition of 50 mM methylamine at 37 °C and pH 8.0. The lower curve (and lower time scale) represents an expansion of the first 10% of the recorded data. The upper curve (and upper time scale) shows the complete data set. The units of the ordinate are derived from the digitization process where 1 V is equal to 8.06 mdeg of ellipticity. The noisy curve depicts the raw data, while the smooth curve is calculated from the fit parameters derived from a numerical treatment of the data in terms of mechanistic Scheme I (see text). The experiment was done at a protein concentration of 4.15×10^{-6} M in a 1-cm cell using a full-scale expansion of 5 mdeg. The pen period was 16 s for the first 2 min of the reaction and 64 s thereafter.

satisfactory fit during the first 5 min of the reaction where there was a definite indication of a lag phase. The appearance of a similar lag phase is also apparent from the kinetics of the methylamine-induced ANS fluorescence change at 37 °C (Figure 6). It was clear that in neither experiment would a

Table III: Rate Constants Derived from a Mechanistic Analysis of the Spectral Change in C3 Inactivated with Methylamine[a]

signal monitor	k_0 (s^{-1})	k_1 (s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})	k_4 (s^{-1})	ΔS_1	ΔS_2
CD 264.5 nm	4.5×10^{-3}	7.9×10^{-4}	2.9×10^{-4}	10.4×10^{-5}	7.0×10^{-8}	0.28	0.72
SD[b]	0.5×10^{-3}	0.8×10^{-4}	0.8×10^{-4}	1.2×10^{-5}	3.4×10^{-8}	0.03	0.0014
ANS fluorescence	4.5×10^{-3}	10.4×10^{-4}	2.2×10^{-4}	10.9×10^{-5}	6.1×10^{-9}	0.24	0.76
SD[b]	0.4×10^{-3}	0.8×10^{-4}	0.6×10^{-4}	0.7×10^{-5}	2.2×10^{-9}	0.0016	0.0024

[a] Inactivation conditions were 50 mM methylamine, 37 °C, pH 8.0. [b] SD, the standard deviation, refers to the error in the kinetic parameters estimated by the fit procedure for a given data set.

Scheme I

$$A \xrightarrow[\text{nucleophile}]{k_0} B \underset{k_2}{\overset{\Delta S_1,\ k_1}{\rightleftharpoons}} C \underset{k_4}{\overset{\Delta S_2,\ k_3}{\rightleftharpoons}} D$$

simple sum of exponentials analysis succeed in fitting the data. Therefore, an attempt was made to fit both the CD and the ANS fluorescence data according to the mechanism shown in Scheme I, where A represents the native protein, B represents the nucleophile-modified but otherwise conformationally unchanged molecule, C represents an intermediate conformational state, and D represents the final conformational state. The model further assumes that the nucleophilic attack is a pseudo-first-order irreversible process which by itself does not give rise to a spectral change, while the conformational transitions are equilibrium processes, each of which gives rise to a certain fraction (ΔS_1, ΔS_2) of the signal change observed. The rate constants k_0 through k_4 as well as ΔS_1, ΔS_2, and the value of the signal at time zero were treated as free parameters in an iterative fit of the data to theoretical curves derived from the differential rate equations which describe the above mechanism (see Appendix for further details).

It can be seen in Figures 5 and 6 that the mechanism is largely successful in fitting the experimental curves. The kinetic parameters shown in Table III indicate that the first conformational transition (B to C) is a reversible process, while the transition to the final state is irreversible and thus provides the driving force for the overall reaction. The slow conformational process accounts for 72% of the total CD signal change and 76% of the total ANS fluorescence change, in agreement with the value obtained from the semilogarithmic plot of the CD data shown in Figure 4. The value for the half-time of the nucleophilic attack derived from the mechanistic analysis is 2.5 min, which is about twice as fast as the measured rate of loss of hemolytic activity (Figure 3). The similarity between the kinetic constants derived by using the two different spectral probes suggests that both phenomena are manifestations of the same, or at least very similar, conformational transitions in the C3 molecule. The experimental curves were also fitted to two simplified versions of Scheme I. In accordance with the negligibly small value determined for k_4 when the full version was employed, the C to D transition was assumed to be an irreversible step. In this case, similarly good fits of the data were obtained without substantially altering the values of the remaining seven parameters. In contrast, a shortened mechanism which had only one spectroscopically observable conformational change (i.e., the C to D transition was eliminated) failed to successfully fit the experimental data.

Kinetics of the Acquisition of Functional C3b-like Activity following Nucleophilic Inactivation. In view of the evidence that the rate of transition to a C3b-like conformation was considerably slower than the rate of nucleophilic attack, experiments were performed to determine whether the functional properties of methylamine-modified C3 appeared concurrent to the cleavage of the putative thioester or whether expression

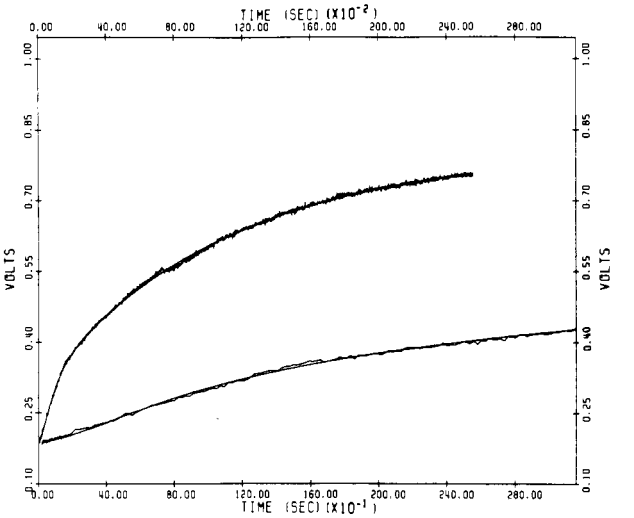

FIGURE 6: Continuous monitoring of the ANS fluorescence signal change following the addition of 50 mM methylamine at 37.5 °C and pH 8.0. As before, the lower curve and lower time scale represent an expansion of the first 10% of the reaction while the upper curve and time scale depict the complete data set. The smooth curve is calculated from the fit parameters derived from a numerical treatment of the data in terms of mechanistic Scheme I (see text). The protein concentration was 1.7×10^{-6} M, the ANS concentration was 10^{-5} M, excitation was 386 nm (1-nm band-pass), and emission was monitored at 473 nm (10-nm band-pass). These slit settings were employed in order to minimize the effects of photobleaching while still maintaining a good signal to noise ratio.

of C3b functions awaited completion, or partial completion, of the spectroscopically detected conformational changes.

The two C3b functional activities which were kinetically assessed are the ability of the methylamine-treated molecule to bind factor B (in the presence of Mg^{2+}) and to be cleaved by a mixture of β1H and C3bINA.

(*i*) *Factor B Binding by C3(CH_3NH_2).* The time course of appearance of factor B binding activity following the addition of 50 mM methylamine to C3 at 37 °C is shown in Figure 7. The assay conditions used (see Materials and Methods and legend to Figure 7) result in a 40% background activity for the unmodified protein. It can, however, be seen that approximately 25 min are required following the addition of methylamine for 50% of the nucleophile-induced increase in factor B binding activity to occur. By contrast, 96% of the hemolytic activity had been lost at that time ($t_{1/2}$ = 5.45 min). It is clear that the rate of appearance of the binding site for factor B is far slower than the rate of hemolytic inactivation and thus more closely resembles the rate of the conformational transition observed spectroscopically. The observed rate must be considered an estimate because of several difficulties inherent in this assay. For example, the hyberbolic shape of the assay's C3b concentration dependence will tend to yield an overestimation of the rate at which factor B binding activity is acquired. The assay is also prejudiced toward shorter half-times because the incubation period introduced a significant delay during which both the nucleophilic attack and the conformational transition could continue. These latter

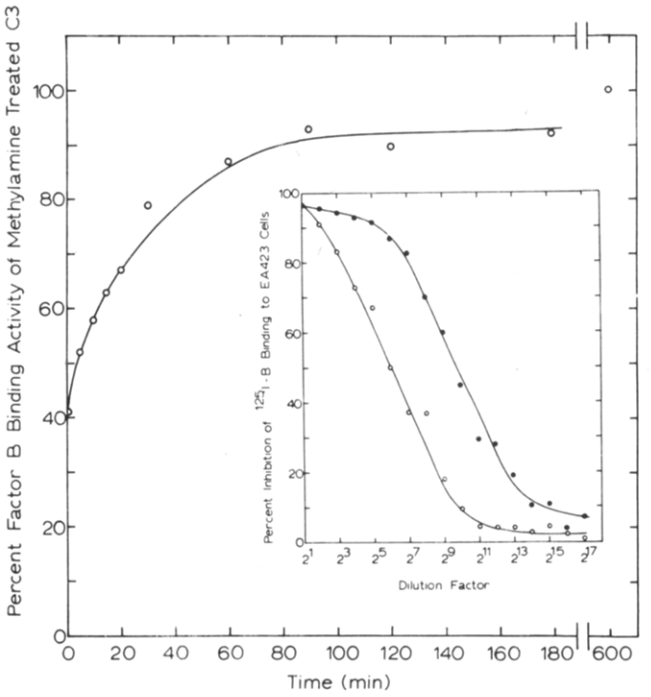

FIGURE 7: Time-course appearance of fluid-phase factor B binding activity by C3 treated with 50 mM methylamine at 37 °C. The times indicated represent the time of sampling of the incubation mixture. The final concentration of C3(CH_3NH_2) or C3b used in the assay system (after 10-fold dilution from the incubation mixture) was 1.02×10^{-7} M. This concentration of protein was chosen since it gave the best sensitivity; that is, C3b at this concentration inhibited 80% of ^{125}I-labeled B uptake onto cell-bound C3b while the unmodified protein at the same concentration inhibited only 36% of ^{125}I-labeled B uptake onto the cells. The experiment in which this was determined is shown in the inset. The initial concentration of C3b (●) or native C3 (○) (i.e., before any dilution) was 10.17×10^{-6} M. See Materials and Methods for further details of the assay system and data manipulations.

effects were minimized by dilution and by lowering the temperature during the binding assay to 23 °C. Although native C3 should theoretically be without activity in this assay, at sufficiently high concentrations it was inhibitory. As can be seen in Figure 7 (inset), the concentration of native C3 required for 50% inhibition of ^{125}I-labeled B uptake onto the C3b-bearing cells is 13-fold higher than the equivalent point in the dose–response curve of C3b. Inhibition by the native protein was almost certainly due to the spontaneous thioester cleavage of 10–15% of the protein by the solvent. Indeed, between 0.1 and 0.2 mol of free SH per mol of native C3 has been consistently observed (Isenman et al., 1980; Pangburn & Müller-Eberhard, 1980; Janatova et al., 1980a).

(*ii*) *Cleavage of C3(CH_3NH_2) by β1H and C3bINA.* Following nucleophilic inactivation with methylamine, C3 has been shown to become susceptible to cleavage by C3bINA and β1H (Pangburn & Müller-Eberhard, 1980). The time course of the appearance of this property was examined. Aliquots taken at various times were incubated for 10 min at 37 °C with sufficient C3bINA and β1H to completely cleave an equivalent amount of C3b. The extent of cleavage was monitored by $NaDodSO_4$–polyacrylamide gel electrophoresis of the reduced protein. As can be seen in gel 15 of Figure 8 (upper portion), C3b thus treated yields the known three-chain pattern of α′-67, α′-40, and β (Pangburn et al., 1977). The equivalent cleavage of nucleophile-inactivated C3 produces a larger α-chain fragment of M_r 76 000 (α-76) due to the presence of the C3a domain. The almost identical molecular weights of α-76 and the β chain preclude their separation on $NaDodSO_4$–polyacrylamide gel electrophoresis. The data

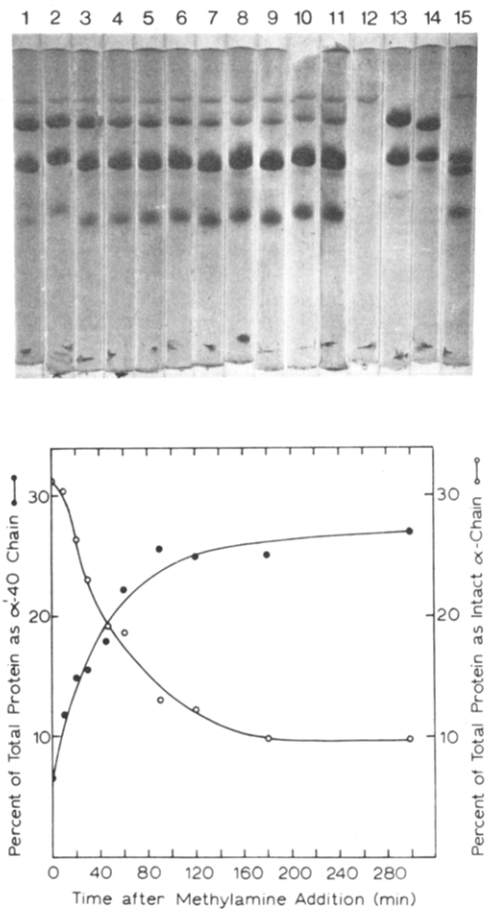

FIGURE 8: $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of the time-course appearance of susceptibility to cleavage of C3bINA and β1H of C3 treated with methylamine (50 mM, pH 8.0, 37 °C). C3 (1.43×10^{-6} M) sampled at various times after methylamine addition was pulsed for 10 min at 37 °C with β1H (1.57×10^{-7} M) and C3bINA (1.53×10^{-7} M) prior to boiling in $NaDodSO_4$ sample buffer containing reducing agent: (1) 0, (2) 10, (3) 20, (4) 30, (5) 45, (6) 60, (7) 90, (8) 120, (9) 180, (10) 300, and (11) 600 min; (12) β1H and C3INA mixture; (13) untreated native C3; (14) untreated C3b; (15) C3b pulsed for 10 min with β1H and C3bINA as above. The lower portion of the figure depicts the quantitation of α and α′-40 chains determined from densitometric scans of gels 1–10.

shown in the lower part of Figure 8 indicate that the 50% completion point occurs 35–40 min after the addition of methylamine. The assay introduced a 10-min delay which tended to give an overestimation of the rate of conformational change. Even assuming that the half-time of 35 min is not an underestimate, it is obvious that the acquisition of a conformation susceptible to C3bINA cleavage is a relatively slow process.

The rate constants derived from fitting the conformational transitions monitored by the ANS fluorescence signal change to the mechanism described by Scheme I have been used to generate theoretical progress curves for species A through D (Figure 9). Also shown in Figure 9 are the curves depicting the appearance of factor B binding activity and susceptibility to cleavage by a C3bINA–β1H mixture. These latter curves were derived from the experimental data shown in Figures 7 and 8 after subtraction of the background values of the respective assay systems and normalizing all values to the same molar concentrations. Although one must bear in mind that, for reasons mentioned previously, the kinetic–functional assays are at best semiquantitative, it can nevertheless be seen that neither functional activity correlates with the appearance of species B (i.e., nucleophilic incorporation per se). Factor B binding activity does, however, correlate quite well with the appearance of species C, while susceptibility of the molecule

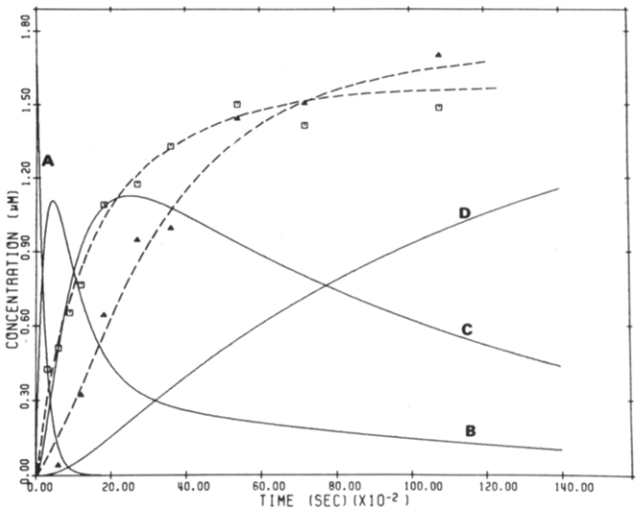

FIGURE 9: Theoretical progress curves of species A through D of mechanistic Scheme I using the kinetic constants derived from a fit of the ANS fluorescence data (Figure 6 and Table III). The appearance of factor B binding activity (□) and susceptibility to cleavage by a C3bINA–β1H mixture (▲) are superimposed. These points were derived from the experimental data shown in Figures 7 and 8 after subtraction of the background values of the respective assay systems. The effective concentrations of the activity profiles have been normalized to the same protein concentration used in the ANS fluorescence experiment (1.7×10^{-6} M). The dashed lines joining these points are provided as a visual aid and do not imply a mechanistic interpretation.

to cleavage by the C3bINA–β1H mixture is somewhat slower than the appearance of C, although clearly not slow enough to correlate with the appearance of species D.

Comparison of the Cleavage Rate of C3(CH_3NH_2) and C3b by C3bINA and β1H. The inability of a significant portion of C3(CH_3NH_2) to be cleaved by C3bINA within 10 min even after the conformational rearrangement was complete suggested that the rate of cleavage of C3(CH_3NH_2) was slower than the comparable rate of cleavage of C3b. To test this hypothesis, we compared the rate of cleavage of C3b and fully transconformed C3(CH_3NH_2), incubated 12 h at 37 °C (Figure 10). While conversion of C3b to iC3b was essentially complete within 10 min, conversion of C3(CH_3NH_2) required 1 h.

Discussion

The multiplicity of functions expressed by activated C3 (C3b) and the key role which this component plays in both pathways of complement activation make this molecule a unique entity in which to study protein structure–function relationships. A number of physicochemical changes are associated with conversion of C3 to C3b (Isenman & Cooper, 1981), including alterations in the environment of certain aromatic residues, a decreased susceptibility of disulfides to reduction, and an increase in surface hydrophobicity. It could be argued that the observed differences reflect cleavage-induced allosteric changes in conformation or exposure of residues initially sequestered from solvent by the C3a domain. The demonstration in the present study that the hydrolysis of a putative internal thioester results in very similar physicochemical changes strongly argues in favor of the allosteric mechanism.

The kinetics of the conformational changes induced by the two distinct modes of C3 inactivation are revealing. Whereas proteolytic conversion of C3 gives rise to a very rapid conformational change, nucleophilic cleavage of the thioester bond in the absence of peptide bond cleavage gives rise to a much

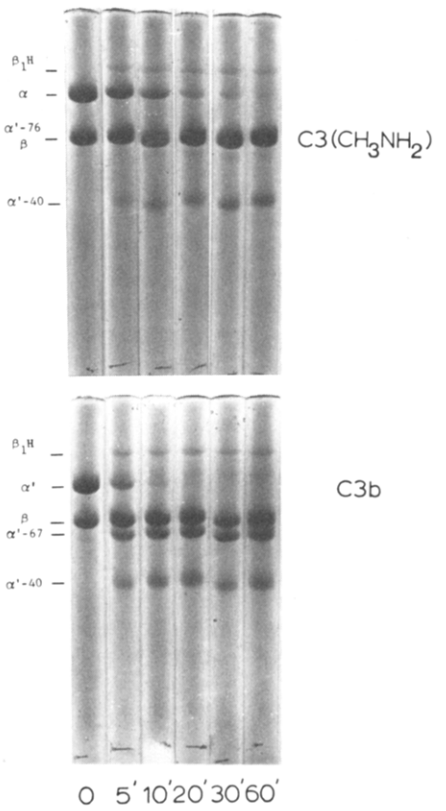

FIGURE 10: Comparison of the cleavage rate by C3bINA and β1H of C3(CH_3NH_2) and C3 which had been converted to C3b by trypsinization. C3(CH_3NH_2) was incubated at 37 °C for 12 h prior to the experiment to allow the nucleophile-induced conformational change to go to completion. The concentrations of C3(CH_3NH_2)/C3b, β1H, and C3bINA were 1.5×10^{-6} M, 1.41×10^{-7} M, and 4.6×10^{-8} M, respectively. The incubation was carried out at 37 °C.

slower conformational transition. If nucleophilic conversion is allowed to go to completion without completion of the accompanying conformational change, cleavage of the α chain by trypsin will rapidly increase the change to the value characteristic of C3b. These observations suggest the following mechanism for the conformational rearrangement which results in expression of the functional properties of C3b: Proetolytic cleavage of the C3 α chain by one of the C3 convertases and removal of C3a facilitate a rapid rearrangement. This exposes previously concealed portions of the molecule, including the internal thioester, to the solvent. Either it is transesterified to a nucleophile-bearing surface (Law et al., 1979), leading to binding, or the competing reaction with water results in the loss of the metastable binding site. The scission of the internal thioester bond of C3 by nucleophilic addition probably favors the release of conformational strain in the same region of the molecule. In this case, however, the still attached C3a polypeptide appears to impose constraints on the resulting conformational change, slowing down the rate of change.

The slow thawing process which resulted in inactivation of C3 and which has been reported by several groups (Von Zabern et al., 1980; Janatova et al., 1980b) was presumably mediated by water hydrolysis of the thioester bond. Only in the presence of chaotropic agents, which may perturb the three-dimensional folding of C3 and thus make the putative thioester more accessible to the solvent, does water hydrolysis of this bond occur at a significant rate in solution.[2] It may

[2] M. K. Pangburn, R. D. Schreiber, and H. J. Müller-Eberhard, unpublished experiments.

be that similar perturbations of the tertiary structure of C3, which lead to a greater solvent accessibility of the internal thioester, were imposed by changes in the ice lattice structure as the temperature slowly rose from −70 °C to near the freezing point.

In view of the functional properties of thioester-hydrolyzed, but otherwise intact, C3, a role for this protein in the formation of the initial C3 convertase of the alternative pathway has recently been proposed (Pangburn & Müller-Eberhard, 1980). Since spontaneous hydrolysis of native C3 is continuously occurring, albeit at a very slow rate, the fact that this altered form of C3 is susceptible to the action of β1H and C3bINA would prevent an accumulation of this form of the enzyme. On the other hand, sufficient C3b must be generated from C3 to assure deposition of C3b and formation of C3 convertase on an activating surface which restricts control of the enzyme by β1H and C3bINA. A number of observations in the present study tend to suggest a mechanism through which this delicate balance is achieved. For example, while fully transconformed C3(CH_3NH_2) appears to be comparable to C3b in its ability to bind factor B and thereby initiate the positive feedback of the alternative pathway, the rate of inactivation of this form of C3 by C3bINA and β1H is significantly slower than the rate of inactivation of C3b. In this way, the lifetime of the initial fluid-phase C3 convertase would be increased, thereby enhancing the likelihood of initial C3b deposition on surfaces. If the appearance of C3b-like functional properties requires completion of the spectrally observed conformational transition, then there is no apparent physiological advantage brought about by the relatively slow kinetics of this process. If, however, factor B binding can be expressed by a conformational intermediate which precedes the formation of a species susceptible to the concerted action of the regulatory proteins β1H and C3bINA, then a mechanism would be provided for the continuous low-level fluid-phase activation of the alternative pathway. Our data suggest that the acquisition of factor B binding activity precedes the susceptibility of C3(CH_3NH_2) to cleavage by C3bINA and β1H by 10–15 min at 37 °C. A definitive conclusion on this point, however, awaits the development of more sensitive monitoring systems.

Acknowledgments

The superb technical assistance of Laura D. Simpson is gratefully acknowledged.

Appendix

Differential rate equations were written to describe the mechanism shown in Scheme I. The finite difference approximations to the equations are shown.

$$\Delta A(t_{i+1}) = -k_0 A(t_i)\Delta t \quad (1)$$

$$\Delta B(t_{i+1}) = [k_0 A(t_i) - k_1 B(t_i) + k_2 C(t_i)]\Delta t \quad (2)$$

$$\Delta C(t_{i+1}) = [k_1 B(t_i) - (k_2 + k_3) C(t_i) + k_4 D(t_i)]\Delta t \quad (3)$$

$$\Delta D(t_{i+1}) = [k_3 C(t_i) - k_4 D(t_i)]\Delta t \quad (4)$$

$\Delta A(t_{i+1})$ approximates the differential change in the molar concentration of A in Δt seconds at time t_{i+1}. Similar definitions apply to ΔB, ΔC, and ΔD. Due to the long reaction times, it was computationally useful to use two values for Δt, 1.5 s for times less than 8000 and 7.5 s for longer times. With complex mechanisms such as those examined here, it is imperative for the success of the finite difference methods that Δt be small enough to approximate the true differential, dt. Values 0.5 and 0.1 times the Δt's shown yielded no significant change in the parameters fitted but greatly increased the computer time charges for the calculations. The concentration of each species X at each time point, t_i, was calculated with

$$X(t_i) = X(t_{i-1}) + \Delta X_i \quad (5)$$

It was assumed that there was no spectroscopic signal change associated with the nucleophilic addition to A. The molar signal change observed in converting B to C was V_1 and for converting C to D was V_2. $V(t_i)$, the computed spectroscopic signal at time t_i, is given by

$$V(t_i) = V_0 + \sum_{j=1}^{i} V_1[\Delta C(t_j)] + V_2[\Delta D(t_j)] \quad (6)$$

where V_0 is the signal in volts at the start of the experiment.

The signal changes for each of the two equilibrium processes were expressed as fractions of the total molar signal change for the overall process: $\Delta S_1 = V_1/(V_1 + V_2)$ and $\Delta S_2 = V_2/(V_1 + V_2)$.

References

Adler, A. J., Greenfield, N. J., & Fasman, G. D. (1973) *Methods Enzymol. 27*, 675–735.

Bianco, C., & Nussenzweig, V. (1977) *Contemp. Top. Mol. Immunol. 6*, 145–175.

Bokisch, V. A., Müller-Eberhard, H. J., & Cochrane, C. G. (1969) *J. Exp. Med. 129*, 1109–1130.

Campbell, R. D., Dodds, A. W., & Porter, R. R. (1980) *Biochem. J. 89*, 67–80.

Cooper, N. R., & Müller-Eberhard, H. J. (1970) *J. Exp. Med. 132*, 775–793.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Gigli, I., & Nelson, R. A., Jr. (1968) *Exp. Cell Res. 51*, 45–67.

Götze, O., & Müller-Eberhard, H. J. (1971) *J. Exp. Med. 134*, 90s–108s.

Götze, O., & Müller-Eberhard, H. J. (1976) *Adv. Immunol. 24*, 1–35.

Howard, J. B. (1980) *J. Biol. Chem. 255*, 7082–7084.

Huber, H., Polley, M. J., Linscott, W. D., Fudenberg, H. H., & Müller-Eberhard, H. J. (1968) *Science (Washington, D.C.) 162*, 1281–1283.

Hugli, T. E., & Müller-Eberhard, H. J. (1978) *Adv. Immunol. 26*, 1–53.

Isenman, D. E., & Cooper, N. R. (1981) *Mol. Immunol. 18*, 331–339.

Isenman, D. E., Lancet, D., & Pecht, I. (1979) *Biochemistry 18*, 3327–3336.

Isenman, D. E., Sundsmo, J. S., & Cooper, N. R. (1980) *J. Immunol. 125*, 1798–1805.

Janatova, J., Lorenz, P. E., Schechter, A. N., Prahl, J. W., & Tack, B. F. (1980a) *Biochemistry 19*, 4471–4478.

Janatova, J., Tack, B. F., & Prahl, J. W. (1980b) *Biochemistry 19*, 4479–4485.

Kazatchkine, M. D., Fearon, D. T., & Austen, K. F. (1979) *J. Immunol. 122*, 75–81.

Law, S. K., Lichtenberg, N. A., & Levine, R. P. (1979) *J. Immumol. 123*, 1388–1394.

Lowry, O. H., Rosebrough, N. J., Farr, L., & Randal, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Marchalonis, J. J. (1969) *Biochem. J. 113*, 299–305.

Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11*, 431–441.

Meeter, D. A. (1965) University of Wisconsin Computing Centre, I.D. Code C0017-11/S0017-00.

Müller-Eberhard, H. J., & Schreiber, R. D. (1980) *Adv. Immunol. 29*, 1–53.

Müller-Eberhard, H. J., Dalmasso, A. P., & Calcott, M. A. (1966) *J. Exp. Med. 123*, 33–54.
Pangburn, M. K. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1755.
Pangburn, M. K., & Müller-Eberhard, H. J. (1980) *J. Exp. Med. 152*, 1102–1114.
Pangburn, M. K., Schreiber, R. D., & Müller-Eberhard, H. J. (1977) *J. Exp. Med. 146*, 257–270.
Rapp, H. J., & Borsos, T. (1963) *J. Immunol. 91*, 826–832.
Tack, B. F., & Prahl, J. W. (1976) *Biochemistry 15*, 4513–4521.
Tack, B. F., Harrison, R. A., Janata, J., & Prahl, J. W. (1980a) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 700.
Tack, B. F., Harrison, R. A., Janatova, J., Thomas, M. L., & Prahl, J. W. (1980b) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5764–5768.
Vogt, W., Schmidt, G., von Buttlar, B., & Dieminger, L. (1978) *Immunology 34*, 29–40.
Von Zabern, I., Nolte, R., & Vogt, W. (1980) *J. Immunol. 124*, 1543.
Vuk-Pavlović, S., Blatt, Y., Glaudemans, C. P. J., Lancet, D., & Pecht, I. (1978) *Biophys. J. 24*, 161–174.

Isolation of Immunoglobulin Messenger Ribonucleic Acid from Human Lymphoblastoid Cell Lines†

Harald V. Molgaard, Lawrence Weir, John Kenten, Frans Cramer, Carol K. Klukas, Hannah Gould,* and John R. Birch

ABSTRACT: Eight human lymphoblastoid cell lines were screened for levels of synthesis and secretion of immunoglobulins G and M (IgG and IgM). These cell lines produced between 2 and 20% of immunoglobulin, as a proportion of total protein synthesis, and secreted the nascent immunoglobulins with characteristic half-times varying between 4 and 40 h. The secreted immunoglobulins exhibited equimolar ratios of heavy (H) and light (L) chains, consistent with the formula for complete immunoglobulins (H_2L_2); IgG was secreted in the monomer (7S) form, whereas IgM was secreted in varying proportions of the monomeric (8S) and pentameric (19S) forms. One cell line (RPMI 1788) produced a 2-fold molar excess of light over heavy chains, but did not secrete the excess light chains. The conditions of tissue culture were varied to optimize the production of immunoglobulins. Using RPMI 1788, which produced IgM(λ), it was found that substituting swine for calf serum and harvesting cells in the late-exponential phase of growth stimulated total protein synthesis, preferential immunoglobulin synthesis, the rate of immunoglobulin secretion, and the assembly of pentameric IgM. Messenger ribonucleic acid (mRNA) was isolated from two of the cell lines, one (RPMI 1788) producing μ and λ chains and the other (Bristol 7) producing γ and κ chains. The messenger RNA was fractionated by sucrose-gradient centrifugation and translated in a rabbit reticulocyte lysate, and the translation product was characterized by polyacrylamide gel electrophoresis before and after immunoprecipitation with specific antisera. RPMI 1788 contains a minor μ-chain-encoding mRNA, which sediments at 19 S and codes for a 67 400-dalton polypeptide, in addition to the major μ-chain-encoding mRNA, which sediments at 18 S and codes for a 65 000-dalton polypeptide in vitro. The RPMI 1788 λ-chain mRNA sediments at 13 S and codes for a polypeptide 31 000 daltons, longer than the mature λ chain synthesized in vivo. Bristol 7 yielded just two messenger activities, one at 16 S coding for a γ chain of 51 000 daltons, and the other at 12–13 S coding for a κ chain of 28 650 daltons. Sucrose-gradient fractionation resulted in a 10-fold purification of the immunoglobulin messenger RNA in the isolated fractions relative to that of the total. Immunoglobulin heavy or light chains comprised about 1% of the translation product of the gradient fractions. The proportion of immunoglobulin synthesized in the cells in culture (ca. 10% of total protein synthesis) was 2 orders of magnitude greater than that observed on translation of the isolated messenger RNA in vitro (ca. 0.1% for the unfractionated RNA). Further work is required to establish the basis for this large discrepancy.

The cloned genes for specific human immunoglobulins would be useful tools in molecular immunogenetics and might also provide, through bacterial expression, a useful source of proteins for immunotherapy. The technology for the stimulation, selection, and immortalization of human lymphocytes producing immunoglobulins of defined specificity, and for cloning the cells producing homogeneous idiotypes, is now available [see, e.g., Steinitz et al. (1979a,b)]. We show here that the isolation of messenger ribonucleic acid (mRNA) coding for immunoglobulin chains in vitro, suitable for enzymatic synthesis of the genes and cloning in bacteria, is possible, despite the fact that the levels of messenger RNA seem to be considerably lower than in mouse myelomas, the usual source of immunoglobulin mRNA, or in hybridomas made by fusing human lymphocytes with mouse myelomas (Dolby et al., 1980).

† From the Department of Biophysics, King's College London, London WC2B 5RL, England (H.V.M., L.W., J.K., F.C., C.K.K., and H.G.), and Searle Research Laboratories, High Wycombe, United Kingdom (J.R.B.). *Received October 6, 1980.* This work was supported by Medical Research Council Programme Grant G969/509/B, Science Research Council Project Grant Gr/B09681, CASE Awards N1268 and B2259, and U.S. Public Health Service Grant AI-05877.

Materials and Methods

Cell Lines. RPMI 1788, BRI 7, BRI 8, BEC 11, and MICH were supplied by Searle Research Laboratories. SMI 4, TAY 3, and DAUDI were provided by Dr. C. M. Steel. The

0006-2960/81/0420-4467$01.25/0