Conformational Changes Accompanying Proteolytic Cleavage of Human Complement Protein C3b by the Regulatory Enzyme Factor I and Its Cofactor H

SPECTROSCOPIC AND ENZYMEOLOGICAL STUDIES*

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The conformational basis for the loss of C3b functional site expression following its conversion to iC3b by the regulatory proteins factor H and factor I has been examined by a number of spectroscopic techniques including the fluorescence of the extrinsic probe 8-anilino-1-naphthalenesulfonate, circular dichroism, and UV absorption difference spectroscopy. These techniques all detected significant conformational alterations accompanying the proteolytic cleavage event. To a substantial extent, the observed spectral changes tended to reverse those seen in the original conversion of native C3 to its functionally active C3b form. Similar changes were also seen when the thiostere-cleaved, but peptide chain-intact, form of C3 was acted upon by the regulatory proteins. The most dramatic effect was seen in the 8-anilino-1-naphthalenesulfonate fluorescence system where the fluorescence enhancement observed upon conversion of C3 to C3b, which presumably reflects an increase in surface hydrophobicity, is lost upon further cleavage of the molecule to iC3b. This marked signal change forms the basis of a simple spectrophotofluorometric assay with which to study the enzymatic properties of factor I for its true substrate: the molecular weight 68,000, respectively, both of which are disulfide-bonded to the M = 75,000 β chain. A subsequent factor I-mediated cleavage removes a 3000-dalton piece from α'-46 and thus the final iC3b fragment consists of 3 disulfide-linked chains: α'-68, α'-43, and β (13). Formation of iC3b correlates with an irreversible loss in both factor B and C5 binding activity (6, 11, 14). The ability to bind factor H is also greatly diminished (14), thereby enabling it to act in a catalytic fashion (13). While the iC3b molecule can no longer bind to receptors of the CR1 class, it does become a ligand for the CR2 and CR3 classes of complement receptors (4). Finally, the ability to bind properdin appears to be unaltered in iC3b (14).

In a previous study, we showed that proteolytic activation of C3 resulted in discrete conformational changes which could be detected by a number of spectroscopic methods (15). Subsequently, we also demonstrated that the same conformational end state could be achieved by nucleophilic scission of the thioester linkage, which is involved in the surface attachment mechanism of C3b, in the absence of any peptide bond cleavage (16). A kinetic analysis of the acquisition of C3b-like functional activity in the nucleophile-modified protein showed that the appearance of these properties largely paralleled the rate of the spectral changes, but was distinctly slower than

Conversion of precursor C3 into its functionally active fragments, C3a and C3b, is the key point of amplification and effector function initiation in the complement system and, as such, the biological properties of these cleavage products must be subject to stringent regulation (for recent reviews on C3 structure and function see Refs. 1–3). The major cleavage fragment, C3b, exhibits a number of stable functional activities which allow the molecule to function as an opsonin in the cytoytic complement reactions. Thus, C3b exhibits a stable binding site for a class of leucocyte receptors known as CR1 receptors (4) and can enter into specific protein-protein interactions with the complement components factor B, properdin, C5, factor H, and factor I† (3). The binding sites for factor B and properdin are respectively involved in the formation and stabilization of the alternative pathway C3 convertase enzyme and the binding of component C5 to C3b is a prerequisite for its cleavage by the C5 convertase enzymes of either pathway (5, 6). Regulation of the aforementioned potentiating enzymes of the complement cascade is achieved through the concerted action of the C3b inactivator enzyme, factor I, and its cofactor H (7–12). One consequence of factor H binding to C3b is that a high affinity site for factor I is created (8). Thus factor H can be thought of as a modulator of the substrate, C3b, for the cleaving enzyme factor I. The mode of cleavage of C3b by factor I involves a scission in the COOH-terminal half of the α chain producing two chains with molecular weights 68,000 (α'-68) and 46,000 (α'-46), respectively, both of which are disulfide-bonded to the M = 75,000 β chain. A subsequent factor I-mediated cleavage removes a 3000-dalton piece from α'-46 and thus the final iC3b fragment consists of 3 disulfide-linked chains: α'-68, α'-43, and β (13).

* The terminology applied to the alternative pathway complement components conforms to the recommendations made by the World Health Organization Committee on Complement Nomenclature (1981) Bull. WHO 59, 489. Factor H was formerly referred to as βH; factor I was formerly referred to as C3bINA or C3b-inactivator; and iC3b, the inactive form of C3b produced by the concerted action of factors H and I, was formerly referred to as C3bi. Other abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate; C3(H2O), thioester bond-hydrolyzed form of peptide chain-intact C3; iC3(H2O), iC3b equivalent of C3(H2O); Pipes, piperezine-N,N'-bis(2-ethanesulfonic acid); NaDodSO4, sodium dodecyl sulfate; n, binding site stoichiometry in units of moles of ligand per mol of protein.

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the rate of the thioester cleavage event (16). Taken together, these findings strongly suggest that the observed conformational changes which accompany either proteolytic activation or direct thioester cleavage were the structural correlations to the acquisition of the various stable functional activities of C3b. As most of the protein ligand interaction sites are lost following conversion of the molecule to iC3b by factors H and I, it was the objective of the present study to investigate the conformational basis for the loss of these functional properties. Formation of iC3b was indeed found to correlate with spectrosopically demonstrable conformational rearrangements. One of the spectral changes (ANS fluorescence) was sufficiently large to form the basis of a continuous monitoring assay for factor I activity and was used to characterize the enzymological properties of this crucial regulatory enzyme of the complement system. A preliminary report of this work has appeared in abstract form (17).

MATERIALS AND METHODS

Chemicals and Reagents—Bovine trypsin, soybean trypsin inhibitor, 5,5'-dithiobis-(2-nitrobenzoic acid), and Pipes were purchased from Sigma. The magnesium salt of 8-aminol-1-naphthalenesulfonate was obtained from Eastman. Fresh frozen human plasma was generously provided by Toronto Western Hospital Blood Bank and was the source material for all complement components.

Purified Complement Components—Factor B (18) and factor I (19) were prepared by methods described previously. Factor H was isolated from a pH 5.6 guinea pig precipitate by gel filtration on Bio-Gel A-1.5m followed by salt gradient elution from DEAE-cellulose (20). C3b was prepared as described by Tack and Prahl (21) and included as a final purification step a QAE-Sephadex A-50 column which separated the hemolytically active and inactive forms of the molecule (22). The protein was stored in a 0.1 M sodium phosphate buffer, pH 7.0, at -70 °C. When thawed, this protein typically revealed an ---SH content on the order of 0.10-0.15 mol/mol as assessed by sulphydryl titration with DTNB (23, 24). C3b was generated from the native protein by trypsic digestion (1%, w/w) for 2 min at room temperature, at which time an equal weight of soybean trypsin inhibitor was added (25). In most cases, no attempt was made to remove either the C3a activation peptide or the inactivated enzyme. A hemolytically inactive and thioester bond-hydrolyzed form of C3b was produced spontaneously during an accidental freeze thaw and is designated C3(H2O).

Spectral Measurements—Using the data acquisition and processing procedures described previously (27), CD, fluorescence, and ultraviolet difference absorption measurements were made by Beckman (28) spectrophotometer, an Aminco SPF 600 spectrophotometer, and a Cary 219 spectrophotometer. Except where indicated otherwise, spectral measurements were made at 25 °C in phosphate-buffered saline (10 mM sodium phosphate, 0.15 M NaCl, pH 7.4).

UV absorption difference spectral changes in C3b or C3b(H2O) produced by catalytic amounts of factors H and I were obtained using a matched pair of rectangular tandem cells. One side of each cell contained 0.9 ml of C3b or C3b(H2O) (1.5 mg/ml) to which had been added a 3% weight equivalent of factor H while the other side contained an exactly equal volume of a dilute solution of factor I (0.02 mg/ml). A premixing baseline was obtained and then the conversion of C3b to iC3b (or C3b(H2O) to iC3b(H2O)) was initiated in the sample cell by repeated inversion. Digestion was allowed to go to completion, as judged by the cessation of any further spectral change, and the difference spectrum was then recorded.

After all spectral measurements, the extent of peptide bond cleavage was assessed by NaDodSO4-polyacrylamide gel electrophoresis.

Protein concentrations were determined spectrophotometrically at 280 nm using E of 0.66 for C3b (21), 12.5 for factor B, 13.0 for tryptase, and 10.8 for factor I. The latter three values were determined from UV absorbance measurements and a concentration determination using a Hilger and Watts Rayleigh interference refractometer (28, 29).

ANS Titration—Additions of ANS to a fluorescence cuvette containing 1.7 ml of protein solution were made from a 50 μl Hamilton syringe controlled by a repeating dispenser (Hamilton) which fed directly into the cuvette via a line passing through its Teflon stopper. In situ mixing was accomplished by means of a motor-driven glass stirrer which was also suspended from the stopper of the cuvette. Excitation was at 386 ± 2.5 nm and protein-dependent ANS fluorescence was monitored at 473 ± 2.5 nm. All fluorescence titration measurements were performed at 25 °C. The observed fluorescence intensity values were first corrected for a small background fluorescence or scattering value and for the dilution effect of the added ligand. These values were then corrected for the inner filter effect of the added ligand using the following relationship (30): Fraction of bound ANS = Fobs/Fmax where Fobs is the corrected fluorescence value of ANS in the presence of protein and Fmax would be the fluorescence intensity of a molar solution of ligand if it were all bound to protein. It is assumed that fluorescence intensity is a linear function of the concentration of bound ligand. Thus, in order to obtain a concentration titration, one titrates ANS into a solution of protein that is of sufficiently high concentration to yield a nearly all of the added ligand in the initial part of the binding curve. In this region, the binding curve is linear and the slope is equal to the molar fluorescence change for bound ANS (i.e. Fmax) at the particular fluorometer settings employed in the experiment. Binding data obtained in this way were then analyzed according to the method of Fairbanks et al. (32) or in 5-15% gradient slab gels using the system of Laemmli (33). Gels were run under reducing conditions. Densitometric scanning of the slab gels was performed on a CanaClo Model G scanning densitometer at 540 nm. RESULTS AND DISCUSSION

ANS Fluorescence as a Probe of the Functional State of C3—Of the various probes used in assessing the conformational alterations accompanying conversion of native C3 to C3b, the largest signal change was observed in the ANS fluorescence monitoring system (15). While the large ANS fluorescence enhancement suggested that there was a considerable increase in surface hydrophobicity in the activated form of C3, it remained to be demonstrated whether this ANS binding was actually reporting on any of the stable protein interaction sites in the C3b molecule. Fig. 1 shows that in the presence of sufficient amounts of factor B (and magnesium ions) to saturate about 70% of the C3b binding sites, there is a 55% decrease in the fluorescence intensity at 473 nm. Factor B by itself shows only minimal ANS fluorescence enhancement. Fig. 1 further demonstrates that the C3b-dependent ANS fluorescence signal is sensitive to the presence of factor H, as well, however, in this case, near saturating amounts of the ligand resulted in a fluorescence decrease which is less than half that observed in the case of C3b-B interaction. Like

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The fractional saturation reported in the text for the fluid phase C3b-B interaction is based on an association constant of 1.4 × 10^4 M^-1 at 25 °C in Pipes-buffered saline containing 5 mM MgCl2. At the time of submission, a reliable value for the association constant governing the C3b-H interaction in the fluid phase was not available. Nevertheless, it is clear that the conditions employed in Fig. 1, curve 2, are near saturating, since increasing the factor H concentration by 50% does not significantly alter the net fluorescence change observed.
The spectra have been corrected for a small contribution due to buffer B, C3b, and the fluorescence to a level below that of the original native protein. The presence of iC3b and iC3(H2O) is explained by the presence of residual uncleaved sites for these two protein ligands of C3b. Since these functional binding sites are lost upon conversion to iC3b, the next series of experiments was designed to investigate the effects of iC3b formation on the binding and fluorescence properties of this hydrophobic probe. In order to minimize the signal change resulting from the addition of factor H alone, the amount of cofactor added was limited to 5% (w/w) of the C3b present (curve 2, Fig. 2A). The addition of factor I (2.5% w/w) to the cuvette resulted in a dramatic decrease in ANS fluorescence to a level below that of the original native protein (curve 4) and the wavelength of maximum fluorescence was red-shifted from 473 to 487 nm. As can be seen from the gel electrophoresis analysis shown in the inset to Fig. 2A, under the digestion conditions used, the cleavage was largely complete after 30 min. Treatment of the native protein with factors H and I as above yielded a fluorescence spectrum which was very similar to that of iC3b (compare curves 4 and 5, Fig. 2A). In this case, however, very little α-43 chain can be detected on the gel, indicating that the bulk of the protein was in the noncleavable native state. Fig. 2B shows that when all of the peptide bond intact C3 is in the form of C3(H2O), treatment with factors H and I to form iC3(H2O) results in a very similar transition to that observed in forming iC3b. The gel analysis (inset, Fig. 2B) shows that at least part of the difference in the absolute magnitude of the ANS fluorescence spectra of iC3b and iC3(H2O) is explained by the presence of residual uncleaved α chain at the time that the spectrum of iC3(H2O) was recorded. It has been shown previously that C3(H2O) is cleaved significantly more slowly by factors H and I than is C3b (16, 34).

Quantitative Binding Analysis of ANS to C3, C3b, C3(H2O), and iC3b—The marked decrease in ANS fluorescence in going from C3b to iC3b could have any of the following origins. (a) The conformation of the protein is altered in such a way that the residues which form the ANS binding sites are no longer contiguous in three-dimensional space and thus the affinity for ANS is greatly decreased. (b) The site for ANS binding is intact, but residues brought near it as a result of the conformational change result in a quenching of the fluorescence emitted from bound ANS molecules. (c) There are multiple binding sites for ANS and the conformational change differentially affects their respective affinities. By carrying out a quantitative analysis of ANS binding, it is possible to distinguish among these alternatives and at the same time gain some insight into the way in which the C3 molecule regulates the expression of its stable functional properties.

An example of an ANS titration experiment with C3b is shown in Fig. 3A. Also shown is the calibration curve used to obtain the molar fluorescence change for bound ANS at 473 nm (Fmax) and the titration curve of ANS into buffer in the absence of protein. Transformation of these data into the form of a Scatchard plot is shown in Fig. 3B. The Scatchard curve indicates a relatively homogeneous binding of ANS to two sites on C3b (n = 2.14 mol/mol) with an association constant of 2.4 × 10^4 M⁻¹. Also shown in Fig. 3B are the data for the thioester-cleaved form of intact C3 (C3(H2O)) which displays ANS binding characteristics virtually identical with those of C3b (Kₐ = 2.4 × 10⁴ M⁻¹, n = 2.06 mol/mol). In titrating iC3b and native C3 with ANS, the assumption has been made that the F_max obtained using high concentrations of C3b was also valid for these two proteins. An additional complicating factor in the case of the native protein was that if the usual quality of C3 was used, the curve was dominated by the C3(H2O) species, which although present at a level of only 10%, has two relatively high affinity sites for ANS. In order to circumvent this problem, C3 was pretreated with catalytic amounts of factors H and I, so that in fact it consisted of about 10% iC3(H2O) and 90% native C3. The Scatchard analysis depicted in Fig. 3C reveals that in iC3b one of the high affinity sites present in C3b is completely lost and that the remaining titratable site displays an approximately 5-fold weaker association constant (Kₐ = 4.5 × 10³ M⁻¹, n = 0.88 mol/mol). It was only possible to obtain data through about 30% of the binding curve because the ANS concentrations required to achieve even this degree of saturation were so high that the inner filter correction approached 60% of the observed fluorescence. The limited data that it was possible to obtain for the native protein suggested that at least some of the molecules displayed one ANS site of binding affinity identical with that observed in the iC3b species (Kₐ = 4.5 × 10³ M⁻¹, n = 0.58 mol/mol). If the assumption made about F_max for the native molecule is incorrect, it would have no effect on the measured association constant, but could account for the nonintegral stoichiometry that was observed. Thus, conversion of native C3 to C3b results in the formation of two relatively high affinity ANS binding sites, while further cleavage to iC3b completely eliminates one of these sites and results in a 5-fold decrease in the affinity of the remaining one.

Assessment of the Conformational Changes Accompanying iC3b Formation by Circular Dichroism and UV Difference Spectroscopy—When a sample of C3b is treated with catalytic amounts of factors H and I, a negative shift is observed over most of the near-UV CD spectral region (Fig. 4). Above 275 nm, the spectrum of iC3b becomes very similar in its shape...
and magnitude to that of the original native molecule, suggesting that some of the tryptophan and tyrosine reporter chromophores might be reverting to the asymmetric environments which they initially experienced in the native protein. Below 275 nm, however, there was a greater deviation between iC3b and the native spectrum, indicating that there remained a rearrangement, which, except for its kinetics, does not seem to be affected by the presence of the covalently attached C3a portion of the molecule. In both cases, blue-shifted spectra are observed and the magnitude of the molar difference absorptivity in the 290 nm region (−1550 \text{ M}^{-1}\text{ cm}^{-1}) indicates the net exposure to the solvent of one tryptophan residue (35). While one cannot ascribe this change with certainty to a specific tryptophan residue, it is nevertheless noteworthy that a reporter tryptophan chromophore experienced the opposite change in the polarity of its microenvironment in the original conversion of native C3 to C3b.

The cleavage of C3(H2O) by factor I to produce the iC3b equivalent of this molecule (iC3(H2O)) is accompanied by the same spectral shifts seen in the conversion of C3b, although the absolute magnitude of the spectral change seen in this case was usually less than that observed in going from C3b to iC3b (data not shown). Once again, at least part of this difference could be accounted for by the presence of residual α chain which was seen on the gel electrophoretic analysis run at the end of the experiment.

If the far-UV region, there is some evidence for a small change in backbone conformation when iC3b is produced from C3b. Whereas C3b displays a single asymmetrical minimum at 213 nm, iC3b shows well defined double minima at 208 and 213 nm and the strength of the signal is decreased approximately 13%. Identical changes were also seen in the conversion of C3(H2O) to iC3(H2O) (data not shown).

The UV absorption difference spectra produced by conversion of C3b and C3(H2O) to iC3b and iC3(H2O), respectively, are shown in Fig. 5. The near identity of these spectra clearly demonstrates that the factor I-produced cleavage in either of these proteins results in a very similar conformational rearrangement, which, except for its kinetics, does not seem to be affected by the presence of the covalently attached C3a portion of the molecule. In both cases, blue-shifted spectra are observed and the magnitude of the molar difference absorptivity in the 290 nm region (−1550 \text{ M}^{-1}\text{ cm}^{-1}) indicates the net exposure to the solvent of one tryptophan residue (35). While one cannot ascribe this change with certainty to a specific tryptophan residue, it is nevertheless noteworthy that a reporter tryptophan chromophore experienced the opposite change in the polarity of its microenvironment in the original conversion of native C3 to C3b.

The minimum at 285 nm contains contributions from both phenolic and indole ring transitions. Making the assumption that the interior of a protein resembles 120% ethylene glycol (35) and that the 1L and 1S absorption bands of tryptophan are both giving rise to difference spectral bands of the same sign (36). One can use the model compound data of Herskovits and Sorensen (37) to calculate that there is a net tyrosine exposure of one residue in going from C3b to iC3b (or C3(H2O) to iC3(H2O)).

Continuous Monitoring Spectrofluorometric Assay of Factor I Enzymatic Activity—The large ANS fluorescence change accompanying C3b conversion to iC3b forms the basis of a simple spectrofluorometric assay for factor I activity. A typical assay tracing is shown in Fig. 6. The tracing to the left of zero time represents the fluorescence at 473 nm of a mixture that is 1.6 \mu M in C3b, 0.16 \mu M in factor H, and 20 \mu M in ANS. After
Conformational Properties of iC3b

**Fig. 3.** Fluorescence titration of C3, C3b, C3(H2O), and iC3b with ANS. Fluorescence emission was monitored at 473 nm (386 nm excitation). A, experimental data set showing the titration of C3b at a concentration of 6.08 μM. The data obtained at high C3b concentration (58.7 μM) were used to determine \( F_{\text{max}} \). The fluorescence change produced by the addition of ANS into a buffer blank is also shown. All fluorescence values have been corrected for dilution and inner filter effects as detailed under “Materials and Methods.” B, Scatchard transformation of the C3b data from A and of ANS binding data obtained for C3(H2O) at a protein concentration of 4.57 μM. C, Scatchard transformation of ANS binding data obtained for C3b (20.6 μM) which has been converted to iC3b by pretreatment with catalytic amounts of factors H and I (1.5% w/w H, 0.2% w/w I, 4 h, 22 °C) and of native C3 (28.3 μM) also treated with factors H and I as above.

The establishment of this initial fluorescence level, the solution was made 0.016 μM in factor I (zero time). The addition of the enzyme resulted in an immediate change in slope without any discernible lag phase. It can also be seen that the assay remains linear for over 80% of the total signal change, indicative of the fact that the true substrate of factor I is the C3b-cofactor H complex and, in an assay where factor H is limiting, the substrate will be regenerated for a considerable portion of the reaction. The inset to Fig. 6 shows the NaDodSO4-polyacrylamide gel electrophoretic analysis of a time course experiment performed under conditions identical with those used in the fluorometric assay. This gel was scanned densitometrically and the areas of the peaks corresponding to \( \alpha' \), \( \alpha'\alpha-68 \), and \( \alpha'-46+\alpha'-43 \) were expressed as a fraction of total C3b peak areas. These peaks were then normalized so that the maximal theoretical change in the peaks could be plotted on the same scale as the fluorescence change. Within the limits of precision attainable from the densitometric measuring procedure, there is a good correlation between the cleavage rate monitored at fixed time points in the gel analysis and the continuous spectrofluorometric assay (Fig. 6).

Using C3b, factor H, and ANS concentrations similar to those used in Fig. 6, it was found that the initial rate of the spectral change shows a linear dependence on the amount of enzyme added. The assay was also completely insensitive to the ANS concentrations used over the range of 10-80 μM.

The spectrofluorometric enzyme assay was used to estimate the Michaelis-Menten constants governing the action of factor I for its complex substrate (i.e. C3b-H). In order to maintain maximal signal with which to monitor the kinetics of cleavage,
the total C3b concentration was kept constant at 1.6 μM and the concentration of true substrate was varied by adjusting the amount of factor H added. Fig. 7 shows the experimental curve of the initial rate at 25 °C as a function of factor H concentration. In order to extract the interaction constant between C3b and factor H. Kazatchkine et al. (38) have reported a value of 10^7 M^-1 for the interaction between factor H and surface-bound C3b at 30 °C. Others (14, 39) have reported higher values, but noted that the binding curves showed indication of receptor heterogeneity. A value for this interaction constant in the fluid phase is as yet not available. The inset to Fig. 7 shows a Lineweaver-Burk plot of the kinetic data in which a K_m value of 10^6 M^-1 has been assumed in calculating the concentrations of the C3b-H complex. The observed K_m and turnover number of this data set are 2.5 × 10^6 M and 2.7 min^-1, respectively. Some experiment to experiment variation in these values was seen. For example, using different preparations of all three proteins, the observed K_m and turnover number were 4-fold and 1.5-fold higher, respectively. If the association constant governing the C3b-H interaction in the fluid phase is in fact greater than that which we have assumed, it will have a negligible effect on the calculated value of K_m since, even using the 10^6 value in the calculation, under the experimental conditions employed greater than 90% of the factor H added would be complexed to C3b. On the other hand, if the fluid phase association constant is on the order of 10^6 M^-1, the calculated value of K_m will be decreased by about 35%. In any event, factor I must be considered an example of an enzyme showing very tight binding of its substrate, but also displaying a low rate of cleavage. Although the range of turnover numbers observed for factor I is at the low end of those observed in other serine proteases for peptide substrates (40), in normal physiological states one would not expect this to be a limiting factor since the enzyme is present in plasma at a molar concentration that is approximately 6% that of the total C3 concentration (3) and the low K_m would allow it to bind even minimal concentrations of C3b or C3(H2O) that may be present in the circulation.

General Discussion—The present study has clearly demonstrated that the factor I-mediated conversion of C3b to iC3b is accompanied by spectroscopically demonstrable conformational changes in the molecule. Very similar transitions were also seen when the C3b-like C3(H2O) form of intact C3 was converted to iC3b. For the most part, the observed spectral changes tended to be of the opposite sense to those originally seen in the conversion of the native protein to C3b. The most dramatic illustration of this effect was seen in the ANS fluorescence experiments where iC3b showed a reversion.
in apparent surface hydrophobicity to the low level displayed by the native protein. As most of the stable ligand interaction sites in C3b are lost upon conversion to iC3b, it is tempting to speculate that their loss is mediated by a reinternalization of stretches of protein chain that had become surface-exposed and contiguous during the original activation process. Further experiments using higher resolution techniques will be required to establish the validity of this hypothesis.

Harrison and Lachmann (13) have shown that the production of iC3b from C3b takes place via two sequential cleavages; the first of these splitting the $M_1 = 114,000$ $\alpha'$ chain into $\alpha'46$ and $\alpha'46$ and the second removing a 3000-dalton peptide from $\alpha'46$. While it cannot be determined from the spectral data obtained at equilibrium whether only the first or both cleavages are required for the induction of the conformational changes leading to the loss of the various protein ligand interaction sites in C3b, the lack of a discernible lag phase in the spectrofluorometric assay of factor I activity would argue that at least the rate-determining step of the conformational transition is mediated by the first cleavage.

The differential sensitivity of the ANS binding sites in C3b to the presence of factor B and factor H may have a number of origins. Clearly, there may be a difference in the extent to which the two protein interaction sites overlap the ANS binding sites, with the overlap being greater for factor B. Alternatively, the effect on ANS binding may not be due to a strict competition, but may be allosterically mediated in either or both cases. The allosteric mode of ANS binding modulation is more likely to apply in the case of the C3b-factor H interaction than to the C3b-factor B interaction. In the former, it is the conformation of the ANS-binding C3b species that is altered as evidence by the enhanced binding of factor I (8), while in the latter it is the non-ANS-binding factor B molecule which presumably undergoes a conformational change and thereby becomes susceptible to cleavage by factor D (41).

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