Proton Nuclear Magnetic Resonance Study of the Third Component of Complement: Solution Conformation of the Carboxyl-Terminal Segment of C3a Fragment

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ABSTRACT: A proton nuclear magnetic resonance (NMR) study is reported of des-Arg-C3a, which is a 76-residue fragment obtained from the N-terminal portion of the α chain of the third component of human complement. A method of carboxypeptidase digestion/difference spectroscopy [Endo, S., & Arata, Y. (1985) *Biochemistry 24*, 1561–1568] was used for the spectral assignments for Ala-76, Leu-75, Gly-74, His-72, His-67, and Ala-48. On the basis of the NMR results obtained for these residues, we conclude that in aqueous solution (1) the C-terminal segment Leu-73–Ala-76 is free from interactions with the rest of the C3a molecule and (2) the major part of the C-terminal segment takes an ordered conformation. We also suggest that the presence of a *core*, which is formed by segment Tyr-15–Tyr-59 [Huber, R., Scholze, H., Paques, E. P., & Deisenhofer, J. (1980) *Hoppe-Seyler's Z. Physiol. Chem. 361*, 1389–1399], is essential for the C-terminal segment in maintaining the ordered structure in aqueous solution. ^{1}H NMR spectral data were also obtained for the intact C3 from human and porcine sources. The resonances for the C2-H protons of His-67 and His-72, which exist in the C3a part of the human C3 molecule, were assigned. Comparisons of the results obtained with those for des-Arg-C3a demonstrate that (1) upon cleavage of C3a very little change, if any, is induced in microenvironments of His-67 and His-72 and (2) a piece of segment that contains His-72 is exposed to solvent and highly flexible. A possible role of the flexible segment joining C3a and C3b will be discussed briefly.

The third component of complement, C3,[1] plays a crucial role in the cascade of complement for the clearance of foreign materials in the humoral immune response. C3 is a glycoprotein with a M_r of 188 000 consisting of an α chain (M_r 115 000) and a β chain (M_r 75 000) (Tack & Prahl, 1976; Tack et al., 1979). On activating the complement system, C3 convertase (Dias da Silva et al., 1967; Gotze & Muller-Eberhard, 1971) releases C3a anaphylatoxin, which is a 77-residue fragment from the N-terminal portion of the α chain, leaving C3b (Hugli & Muller-Eberhard, 1978). It has been suggested that the proteolytic cleavage leads to a conformational change, resulting in the transacylation of a thiol ester bond that exists in the α chain. C3b thus formed is known to form C5 convertase in conjunction with C3 convertase (Gotze & Muller-Eberhard, 1976; Vogt et al., 1978). The transacylation also results in the formation of a covalent bond with the OH or NH_2 groups of foreign materials (Law et al., 1981; Sim et al., 1981; Thomas et al., 1982; Isenman & Cooper, 1981). It is known that C3b binds with C3b receptors of phagocytic cells (Gigli & Nelson, 1968; Huber et al., 1968; Bianco & Nussenzweig, 1977).

Nucleophilic reagents such as methylamine react with the thiol ester bond (Tack et al., 1980; Harrison et al., 1981; Janatova & Tack, 1981). It has been suggested that the nucleophilic attack induces a conformational change in the C3b part with the C3a part still covalently bonded to C3b. Upon cleavage of the C3a part, structural changes occur very rapidly in the C3b part. By contrast, when C3 is treated with the nucleophilic reagent, conformational change of the C3b part proceeds much more slowly (Isenman et al., 1981).

C3a anaphylatoxin induces contracts of a variety of smooth tissues and enhances vascular permeability (Hugli & Muller-Eberhard, 1978; Lepow et al., 1976). Des-Arg-C3a, which is obtained by enzymatic removal of the C-terminal Arg-77 residue (Muller-Eberhard, 1975), fails to exhibit virtually all the biological activities (Bokisch et al., 1969). On the basis of an CD study of C3a, Hugli et al. (1975) have demonstrated that C3a is structurally unchanged on inactivation by removal of Arg-77. It has been reported that treatment of C3a at 100 °C for 2–3 h, which causes an extensive, irreversible unfolding of the protein, results in a complete loss of the biological activity. It has also been shown that the biological activities are lost almost completely on reduction and alkylation of C3a (Hugli & Muller-Eberhard, 1978). These results indicate that the tertiary structure is an important factor for the expression of the biological activity of C3a.

An X-ray crystallographic study of C3a has been reported by Huber et al. (1980), who demonstrated that (1) segment Tyr-15–Tyr-59 is cross-linked by three disulfide bridges and forms a compact core and (2) the C-terminal segment from Tyr-59 on is extended from the core and takes a helical conformation. Lu et al. (1984) reported that a synthetic segment containing the 21 C-terminal residues of C3a exhibits biological activities similar to natural C3a. They measured CD

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[1] Abbreviations: CD, circular dichroism; CPase, carboxypeptidase; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EACA, ϵ-amino-*n*-caproic acid; NMR, nuclear magnetic resonance; PEG, poly(ethylene glycol); ppm, parts per million; SDS, sodium dodecyl sulfate; STI, soybean trypsin inhibitor; TPCK-trypsin, trypsin pretreated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; C3, third component of complement; C3a, a 77-residue fragment obtained from the α chain of C3; des-Arg-C3a, a 76-residue fragment of C3a in which the C-terminal Arg-77 has been cleaved off by carboxypeptidase N digestion; C3b, a fragment obtained by C3 convertase attack of C3; C3a(Leu-73), a product of CPase digestion of des-Arg-C3a with Leu-73 as the C-terminal residue. Similar notations are used for other products of CPase digestion.

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spectra of this peptide as well as of various synthetic analogues of the C-terminal portion of C3a and concluded that all of these peptides cannot take any ordered structure in aqueous solution. They also showed that an increasing concentration of trifluoroethanol results in the formation of an α-helical structure. It was proposed that the synthetic 21-residue peptide adopts a helical conformation when it is bound to its cellular receptor.

This paper reports a ^{1}H NMR study of human des-Arg-C3a in aqueous solution. It will be shown that des-Arg-C3a gives a number of well-resolved resonances that can be assigned to individual amino acid residues. A method of CPase digestion/difference spectroscopy developed by Endo & Arata (1985) was used for the assignments. On the basis of the NMR results, conformation of the C-terminal segment of des-Arg-C3a in aqueous solution will be discussed. The results obtained were compared with those observed for C3 from human and porcine sources. A technique of *spin diffusion* (Endo & Arata, 1985) was applied to obtain information about resonances originating from amino acid residues that are located in flexible parts of the proteins. We will briefly discuss conformation of a segment that connects C3a and C3b portions of the C3 molecule.

Materials and Methods

The following materials were purchased from the sources indicated: TPCK-trypsin was from Worthington; STI, CPase A, and CPase B were from Sigma; CPase Y was from Oriental Yeast. All other chemicals were reagent-grade and used without further purification.

The isolation of human C3 was carried out as described by Nagasawa & Stroud (1977) with some minor modifications. A 200-mL sample of fresh plasma was diluted with 400 mL of 20 mM sodium phosphate buffer, pH 8.0, containing 20 mM Na_3EDTA, 100 mM EACA, and 10 mM benzamidine hydrochloride. It was fractionated on a QAE-Sephadex A-50 column. The C3-containing fractions were further purified on a heparin–Sepharose column followed by a CM-Sephadex C-50 column. Porcine C3 was isolated in a manner similar to that used for the isolation of human C3. Des-Arg-C3a was prepared essentially according to the method of Fernandez & Hugli (1976).

For qualitative determination, e.g., of the elution position of human and porcine C3 from chromatographic steps, two-dimensional immunodiffusion was performed. In the case of human C3, hemolytic activity assays were carried out as described by Tack & Prahl (1976). The degree of lysis was determined spectrophotometrically at 413 nm following removal of unlysed cells and cellular debris by centrifugation. Purity of protein preparations was checked by carrying out in the Laemmli system SDS–polyacrylamide gel electrophoresis under reducing conditions with slab gels of 7.5% acrylamide (Laemmli, 1970). Spectrophotometric titration of SH content in C3 preparations with DTNB was performed as described by Janatova et al. (1980). The protein concentration of C3 solution was determined by measuring the absorption at 280 nm, with an absorption coefficient $A^{1\%}_{1cm}$ of 9.7 (Tack & Prahl, 1976).

Sample Preparations for ^{1}H NMR Measurements. C3 solutions were concentrated on a Toyo UHP-62 ultrafilter with a UK-50 membrane and further concentrated on an Amicon Microdiaflowsystem 8MC with a PM 30 membrane to 1 mL. Finally, the solution was transferred on to a Toyo microultrafilter, and the buffer was replaced with deuterated phosphate buffers with desired concentrations and pH. pH titration of C3 solutions was made by adding 0.5 M NaOD, starting with D_2O solutions at pH 5.2 or above. Titrations of the free SH group, which is formed by activation of the internal thiol ester bond, show that (1) sample solutions typically contain about 15% of an inactivated form of C3 immediately after preparation for NMR measurements and (2) approximately 10% of additional C3 molecules become inactivated during NMR measurements. Lyophilized samples of des-Arg-C3a were dissolved in D_2O at an NaCl concentration of 200 or 400 mM. Typically, 7 and 0.5 mg of proteins were dissolved in 0.3 mL of D_2O buffers for the NMR measurements of C3 and des-Arg-C3a, respectively. All pH values reported in this paper were uncorrected meter readings of D_2O solutions made with an electrode standardized with H_2O buffers.

^{1}H NMR Measurements. ^{1}H NMR spectra were recorded on a Bruker WM-400 spectrometer operating at 400 MHz in the Fourier-transform mode. All chemical shifts are given in ppm from external DSS (5% in D_2O). Unless otherwise stated, NMR measurements were made at 25 °C.

Results and Discussion

^{1}H NMR Spectra of Des-Arg-C3a. Figure 1 gives an example of spectra of human des-Arg-C3a. The spectrum contains a number of well-resolved peaks in both the aromatic and aliphatic regions. On the basis of chemical shift data along with spin-decoupling patterns, four Ala, two His, two Leu, three Met, three Thr, and two Tyr resonances can be identified in the spectrum.

The amino acid sequences of human and porcine C3a are shown in Figure 2 (Hugli & Muller-Eberhard, 1978). Human C3a contains four Ala, two His, seven Leu, three Met, three Thr, and two Tyr residues. The Ala and Thr CH_3 resonances can be differentiated by the observed magnitudes of vicinal spin-coupling constants (Bundi & Wuthrich, 1979). For the identification of the Met residues, oxidation of des-Arg-C3a by hydrogen peroxide was performed as described by Akasaka et al. (1982). The spectra given in Figure 3 clearly indicate that three peaks designated as Met a, Met b, and Met c actually originate from the Met residues. As Figure 3 shows, two Met residues are quickly oxidized with similar half-lives of about 40 min, whereas the third Met residue is oxidized only very slowly. It should also be noted that, as the oxidation proceeds, the number and intensity of resonances presumably due to oxidized Met residues change in a complicated way.

Spectral Assignments. For the assignments of resonances originating from the C-terminal segment of des-Arg-C3a, a method of CPase digestion/difference spectroscopy, which makes use of in situ proteolytic cleavage of the C-terminal amino acid residues (Endo & Arata, 1985), was employed. Spectra of des-Arg-C3a were observed at different stages of proteolyses by CPase Y. In order to confirm the proposed assignments, the sample solutions were subjected to ultrafiltration, and the composition of the free amino acids in the filtrate was analyzed by ^{1}H NMR. C-Terminal residues newly formed by the proteolyses were also identified by observing the pH dependence of the chemical shifts of the corresponding signals. As expected, the further the proteolyses proceed, the slower the reaction becomes. This increases the heterogeneity of the C-terminal segments of the products obtained by the proteolyses. In order to minimize the effect of heterogeneity on the observed difference spectra, differences were taken before the proteolyses proceed too far. This however inevitably resulted in a decrease in intensity observed for the peaks in the difference spectra (see Figure 4).

(A) Ala-76, Leu-75, Gly-74, and Leu-73. Spectrum b in Figure 4 shows the difference 0 min − 20 min, which was obtained by taking the difference between spectra observed

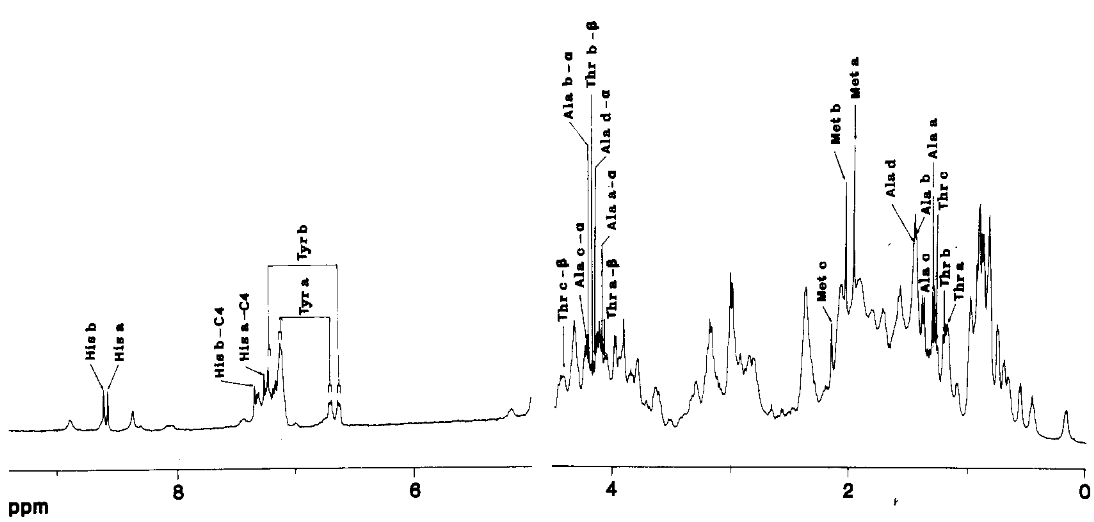

FIGURE 1: The 400-MHz ^1H NMR spectrum of des-Arg-C3a (0.5 mg in 0.3 mL of 0.2 M NaCl/D_2O, pH 4.5). The HDO peak was presaturated in the gated-decoupling mode for 0.5 s at a radiofrequency level of $\gamma H_2/(2\pi) = 70$ Hz. The free induction decay was recorded with 8K data points and a spectral width of ±2500 Hz. A total of 800 transients was acquired, and a line broadening of 0.5 Hz was applied prior to Fourier transformation. Chemical shifts are in ppm from external DSS (5% in D_2O). The probe temperature was 25 °C.

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                1.                                10
Human    C3a    NH2-Ser-Val-Gln-Leu-Thr-Glu-Lys-Arg-Met-Asn-Lys-Val-Gly-Lys-Tyr-
Porcine  C3a    NH2-Ser-Val-Gln-Leu-Met-Glu-Lys-Arg-Met-Asn-Lys-Leu-Gly-Gln-Tyr-

                            20                                30
                -Pro-Lys-Glu-Leu-Arg-Lys-Cys-Cys-Glu-Asp-Gly-Met-Arg-Gln-Asn-
                -Ser-Lys-Glu-Leu-Arg-Arg-Cys-Cys-Glu-His-Gly-Met-Arg-Asn-Asn-

                                                  40
                -Pro-Met-Arg-Phe-Ser-Cys-Gln-Arg-Arg-Thr-Arg-Phe-Ile-Ser-Leu-
                -Pro-Met-Lys-Phe-Ser-Cys-Gln-Arg-Arg-Ala-Gln-Phe-Ile-His-Gln-

                            50                                60
                -Gly-Glu-Ala-Cys-Lys-Lys-Val-Phe-Leu-Asp-Cys-Cys-Asn-Tyr-Ile-
                -Gly-Asn-Ala-Cys-Val-Lys-Ala-Phe-Leu-Asn-Cys-Cys-Glu-Tyr-Ile-

                                                  70
                -Thr-Glu-Leu-Arg-Arg-Gln-His-Ala-Arg-Ala-Ser-His-Leu-Gly-Leu-
                -Ala-Lys-Leu-Arg-Gln-Gln-His-Ser-Arg-Asn-Lys-Pro-Leu-Gly-Leu-

                        77
                -Ala-Arg-COOH
                -Ala-Arg-COOH
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FIGURE 2: A comparison of the amino acid sequences of human C3a and porcine C3a.

at 0 min and 20 min after the addition of CPase Y. Similar notations will be used hereafter. This result clearly indicates that at this stage of proteolysis significant shifts are observed for Leu a, Leu b, Ala a, Ala b, and Gly a resonances. Assignemnts of these resonances were made as follows. The chemical shifts for Leu A and Ala A [new peaks that become observable as the proteolyses proceed will be identified by capital letters] coincide with those of the corresponding free amino acids. No other peaks corresponding to free amino acids are observable. Gly a exhibits a significant shift, giving Gly A. However, Gly A is *not* a singlet, and its chemical shift is quite different from that of the free amino acid glycine at the pH value used. These results indicate that (1) under the present condition of the proteolysis Ala-76 and, with only very little delay, Leu-75 are cleaved off, leaving Gly-74 as the C-terminal residue, and (2) Ala a and Leu b originate from Ala-76 and Leu-75, respectively. It should also be noted in spectrum b that small but significant shifts are observed for Leu b and Ala b.

Spectrum c in Figure 4 gives the difference 40 min − 100 min. This result shows that Gly-74 is cleaved off at this stage, giving a sharp singlet Gly A′ that is due to the free amino acid

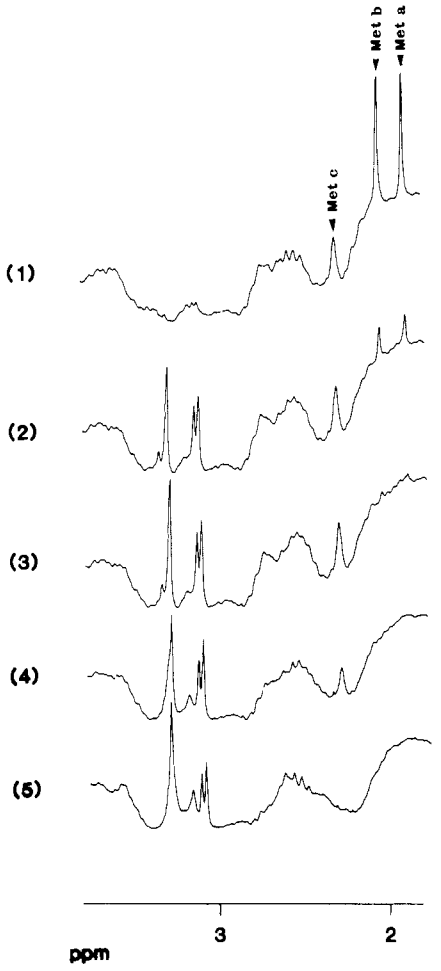

FIGURE 3: Effects of hydrogen peroxide on the ^1H NMR resonances of the SCH_3 protons of the three Met residues in des-Arg-C3a (0.5 mg in 0.3 mL of 0.2 M $NaCl/D_2O$, pH 6.5). Spectrum 1 was observed in the absence of hydrogen peroxide; 3 μL of 30% hydrogen peroxide was added to the sample solution, which was then incubated at 25 °C. Spectra 2–5 were recorded 80 min, 160 min, 7 days, and 20 days after the addition of hydrogen peroxide.

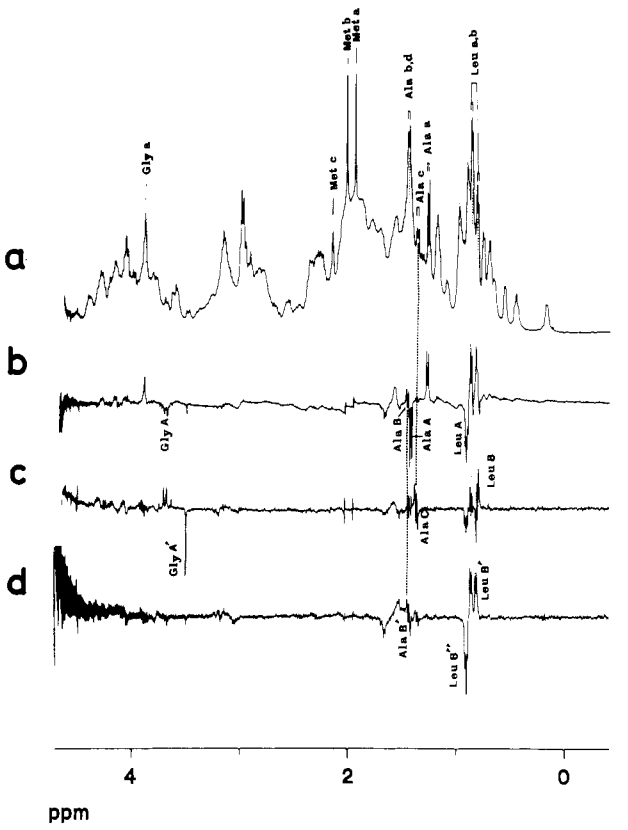

FIGURE 4: Proteolytic cleavage by CPase Y of des-Arg-C3a as followed by difference spectroscopy. A 0.5-mg sample of des-Arg-C3a was dissolved in 0.3 mL of 0.2 M $NaCl/D_2O$, pH 6.5. After measurement of spectrum a, the sample solution was incubated at 25 °C with CPase Y (enzyme/substrate ratio = 1:2000). Spectra 0 min, 20 min, 40 min, 100 min, and 360 min were recorded at 0, 20, 40, 100, and 360 min, respectively. (Sepctrum b) 0 min – 20 min; (spectrum c) 40 min – 100 min; (spectrum d) 100 min – 360 min. Spectral conditions are as described in Figure 1.

glycine. We therefore are able to assign Gly a to Gly-74. At this stage of proteolysis, Leu B, Ala c, and His a also exhibit significant shifts. The chemical shift changes observed in the His resonances will be described in the following section. Spectrum d in Figure 4 is the difference 100 min – 360 min. The chemical shift for Leu B″ coincides with that for the free amino acid leucine. This indicates that Leu b originates from Leu-73. In the experiments described above, distinction of the Leu signals from Val and Ile signals was unambiguously made by confirming the existence of signals for the free amino acid leucine. It was also observed that the free amino acid leucine actually exists in a solution obtained by ultrafiltration of the reaction mixture.

(*B*) *His-72 and His-67.* As Figure 5 shows, two His C2-H proton resonances His a and His b are observed, corresponding to the two His residues His-67 and His-72 of human C3a (see Figure 2). Figure 5a shows how His a and His b resonances change their chemical shifts on proteolytic cleavages of the C-terminal segment. The results are schematically summarized in Figure 5b.

In the present experiment, des-Arg-C3a was first incubated with CPase Y. In Figure 5, spectra 1–3, which were observed upon removal of Gly-74, clearly indicate that His A corresponds to His a of C3a(Leu-73). Upon cleavage of Leu-73, His A again shifts downfield, giving His A′ (spectra 4 and 5). It was confirmed by pH titration experiments that His A′ originates from the C-terminal His residue. His b stays at the same chemical shift up to this point of proteolysis. We therefore assign His A′, His A, and His a to His-72 of C3a-(His-72), C3a(Leu-73), and des-Arg-C3a, respectively. This also indicates that His b is due to His-67. At this stage, an additional amount of CPase Y combined with CPase B was added. This resulted in spectra 6–8, where the C2-H proton resonance that is due to the free amino acid histidine can be identified (His A″). The assignment of the free histidine peak was unambiguously done by adding a small amount of the authentic sample of histidine into the NMR tube. It was also observed that His b gradually lost its intensity with concomitant appearance of His B. It was confirmed that at this stage of proteolysis the free amino acid arginine was released and that two of the four Ala residues were cleaved off. Upon further proteolysis, His B was replaced by another peak, His B′, whose chemical shift is quite similar to that of His A′. His B′ then disappeared, and the intensity of His A″, which is due to the free amino acid histidine, increased instead (data not shown). These results indicate that His B corresponds to His-67 of C3a(Ala-68). pH titration experiments were performed at NaCl concentrations of 0.2 and 0.4 M at 25 °C. Figure 6 shows titration data observed for His-67 and His-72 of des-Arg-C3a in 0.4 M NaCl, 25 °C. A least-squares fitting to sigmoidal curves gave the following pK_a values at 25 °C: 6.1 (μ = 0.2) and 6.2 (μ = 0.4) for His-67; 6.5 (μ = 0.2) and 6.6 (μ = 0.4) for His-72.

(*C*) *Ala-70, Ala-68, and Ala-48.* There are four Ala residues in C3a (see Figure 2). Proteolyses by CPase Y were

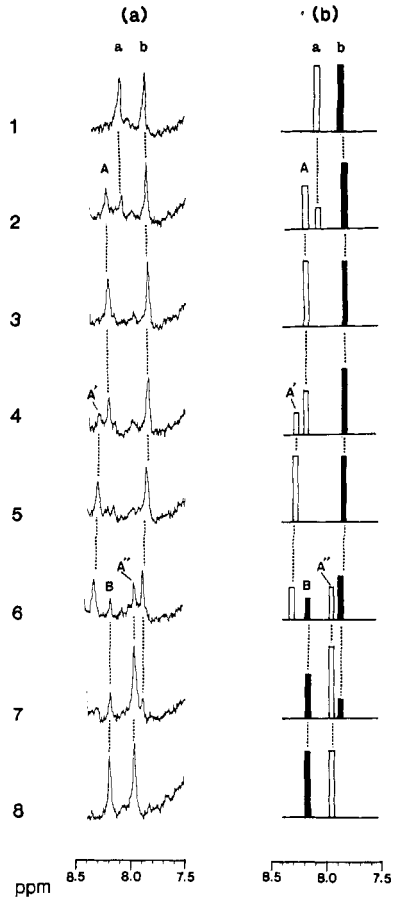

FIGURE 5: Changes in spectra of the C2-H proton of the two His residues of des-Arg-C3a (a). These results are schematically represented in (b). Des-Arg-C3a (0.5 mg in 0.3 mL of 0.2 M NaCl/D_2O, pH 6.5) was incubated at 25 °C with CPase Y (enzyme/substrate ratio = 1:2000). After spectrum 5 was observed, CPase B (enzyme/substrate ratio = 1:4000) with an additional amount of CPase Y (enzyme/substrate ratio = 1:2000) was added. Changes 1–3 and changes 4 and 5 correspond to the cleavages at the C-terminal side of Gly-74 and Leu-73, respectively. Changes 6–8 probably reflect a cleavage at the C-terminal side of Ala-68.

followed as in (B) with difference spectroscopy, and the results are reproduced in Figure 7. Ala a gives pH-dependent chemical shifts (see Figure 8). On cleavage of Ala-76 and Leu-75, Ala a is replaced by Ala A, whose chemical shift coincides with that of the free amino acid alanine (spectrum b). This result indicates that Ala a is due to Ala-76. As Figure 8 shows, Ala b and Ala d overlap with each other above pH 5. Measurements made below pH 5 demonstrated that of the two Ala resonances one is shifted significantly (Ala b), giving Ala B, whereas the other does not show any change in chemical shift (Ala d). No significant change in chemical shift for Ala c is observed either at this stage of proteolysis. When Gly-74 is cleaved off, Ala c exhibits a significant shift, giving Ala C (spectrum c). Even if the proteolysis proceeds further and gives C3a(Ala-68), no change in chemical shift is observed for Ala d. This indicates that Ala d is due to Ala-48. Therefore, Ala b and Ala c may be assigned to Ala-70 and Ala-68, or vice versa, respectively. As shown above, Ala b starts to shift upon cleavage of Leu-75. By contrast, Ala c exhibits a significant shift only when Gly-74 is cleaved off. It is likely that Ala b and Ala c are due to Ala-70 and Ala-68, respectively.

Conformation of the C-Terminal Segment of Des-Arg-C3a in Aqueous Solution. On incubating des-Arg-C3a with CPase Y, Ala-76 and Leu-75 are cleaved off very quickly. This suggests that these two residues are exposed and freely accessible to the enzyme. The difference spectra given in Figure

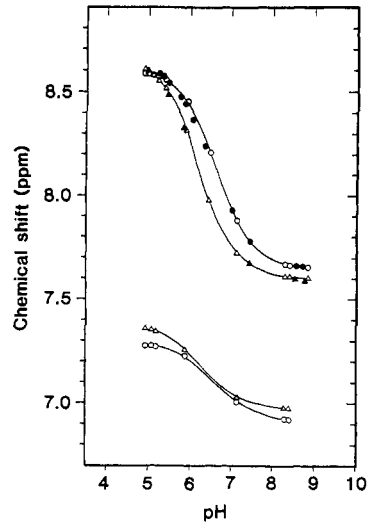

FIGURE 6: pH dependence of the chemical shifts of the C2-H proton of the two His residues observed for des-Arg-C3a and the intact human C3. (△) and (○) correspond to His-67 and His-72 of des-Arg-C3a, respectively. Solid lines were drawn by a least-squares fitting of sigmoidal curves to the data for des-Arg-C3a. Titration data observed with human C3 are represented by (●) and (▲), which correspond to peaks 1 and 2 of Figure 9a, respectively. μ = 0.4, 25 °C.

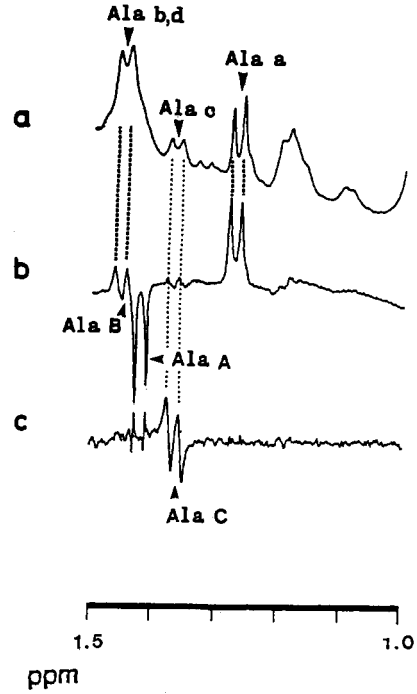

FIGURE 7: Changes in spectra of the three Ala residues in des-Arg-C3a as followed by difference spectroscopy. A total of 0.5 mg of des-Arg-C3a was dissolved in 0.3 mL of 0.2 M NaCl/D_2O, pH 6.5. Enzyme/substrate ratio = 1:2000. Incubation was at 25 °C. Spectrum a was observed before the addition of CPase Y. Spectra b and c are the differences taken before and after the cleavages of Leu-75 and Gly-74, respectively. Ala b and Ala d overlap with each other at the pH value used for the measurements (see text).

4 demonstrate that the loss of Ala-76 and Leu-75 affects the chemical shifts of the Leu-73 and Gly-74 resonances. It should be noted that a significant shift is also observed for Ala b. Cleavage of Gly-74 results in changes in chemical shifts for Ala c as well as those for Leu-73 and His-72. As described above, Ala b and Ala c are assigned to Ala-70 and Ala-68 (or vice versa), respectively. This means that effects of cleavages of Leu-75 and Gly-74 reach Ala residues that are separated by at least four amino acid residues from the cleavage sites. As Figure 8 shows, the Ala c titration data exhibit an inflection

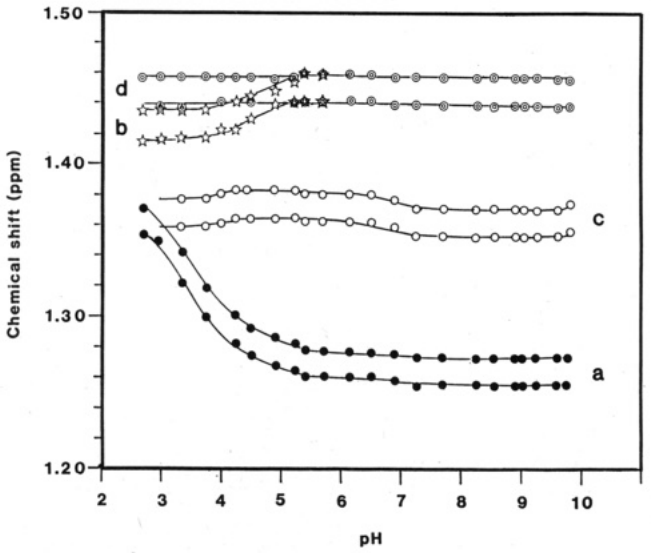

FIGURE 8: pH dependence of the chemical shifts of the CH_3 protons of the four Ala residues for des-Arg-C3a. $\mu = 0.4$, 25 °C. Assignments were as follows: a, Ala-76; b, Ala-70 (or Ala-68); c, Ala-68 (or Ala-70); d, Ala-48. Correspondence of titration curves b and d to Ala b and Ala d, respectively, was established by pH titration experiments below pH 5 with a C3a sample where proteolysis proceeded beyond Ala-68.

at pH ~6.5. This pH value coincides with the pK_a of His-72 (see Figure 6). These results strongly suggest that the chain segment Ala-68-Ala-70 is in close spatial proximity to His-72.

We have previously reported that the combination of CPase digestion and difference spectroscopy can be used to discuss conformation of C-terminal segments (Endo & Arata, 1985). When the C-terminal segment is extended and does not have any ordered structure, significant effects of cleavage by CPase can be detected only for the amino acid residue adjacent to the new C-terminal residue. This was typically demonstrated in the case of the $F(ab')_2$ fragment of the human IgG1 immunoglobulin. By contrast, in the case of the Fab fragment, interactions of the C-terminal residue with other parts of the molecule result in significant shifts for amino acid residues that are remote from the cleavage site. It is quite likely that cleavage of a C-terminal segment forming a helix would be reflected on the chemical shifts of amino acid residues that are separated by three to five residues from the cleavage site. On the basis of these results, we conclude that in aqueous solution the major part of the C-terminal segment of des-Arg-C3a takes an ordered conformation. The present NMR results also show that the Leu-73–Ala-76 segment is free from interactions with the amino acid residues between Ser-1 and His-67. It should be noted that the above conclusion was obtained independent of X-ray crystallography.

An X-ray crystallographic study of C3a demonstrates that (1) segment Tyr-15–Tyr-59 is cross-linked by three disulfide bridges and forms a compact *core* and (2) the C-terminal segment from Tyr-59 on is extended from the core and takes a helical conformation (Huber et al., 1980). The X-ray data also show that the C3a molecule exists as a dimer in the crystal. This suggests a possibility that the dimerization contributes to the stabilization of the helical conformation of the C-terminal segment. However, the present NMR study has demonstrated that the C-terminal segment of des-Arg-C3a takes an ordered conformation in aqueous solution, where it is known that des-Arg-C3a is monomeric (Huber et al., 1980). In marked contrast, Lu et al. (1984) have reported that a synthetic 21-residue C-terminal peptide does not take any ordered conformation in aqueous solution. It was also observed

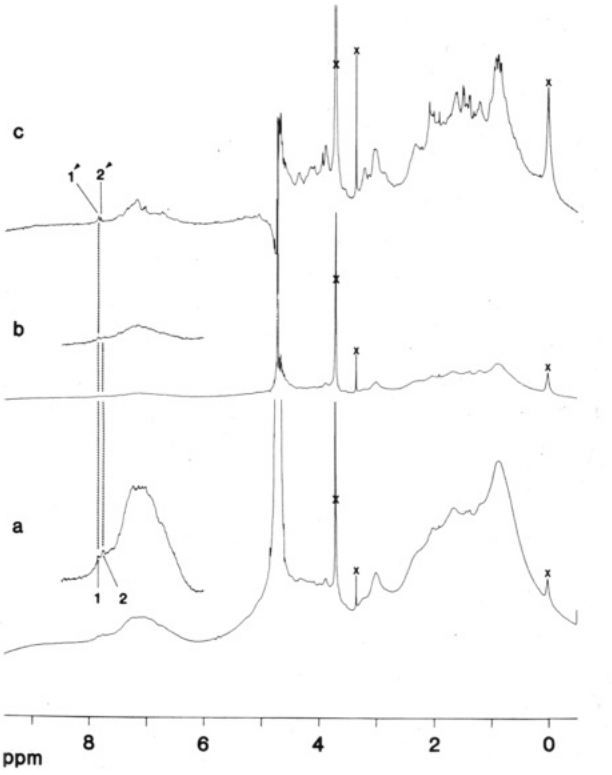

FIGURE 9: The 400-MHz ^1H NMR spectra of the intact human C3 (7 mg in 0.3 mL of 600 mM $NaCl/D_2O$, 20 mM phosphate, pH 7.2). Spectra a and b were observed in the absence and presence of a presaturation pulse, respectively. For presaturation, a radiofrequency pulse was applied for 0.5 s at a level of $\gamma H_2/(2\pi) = 70$ Hz. For each measurement, 3000 transients were accumulated. Spectrum c was observed after the addition of trypsin. Trypsin was added at an enzyme/substrate ratio of 1:100, and the sample solution was incubated at 25 °C for 2 min in an NMR tube. STI was then added to terminate the proteolysis, and 960 transients were acquired. Other spectral conditions are as described in Figure 1. The peaks marked × are due to low molecular weight contaminants.

by CPase digestion/difference spectroscopy that cleavages proceeding beyond His-67 affect significantly the chemical shifts of resonances originating from the rest of the C-terminal as well as those from the core. We therefore suggest that the presence of the core is essential for the C-terminal segment in maintaining the ordered structure in aqueous solution.

His-67 and His-72 Resonances as Probes for the Conformation of a Sequence Joining C3a and C3b Portions in the Intact C3 Molecule. Figure 9a,b shows ^1H NMR spectra of the intact human C3. Spectrum a was observed without presaturating the HDO resonance. The presaturation, which causes saturation of the backbone CH resonances at the same time, results in *spin diffusion* (Endo & Arata, 1985). As spectrum b shows, several resonances are observed on the broad background. The two peaks observed in the aromatic region (peaks 1 and 2) give pH-dependent chemical shifts as shown in Figure 6, where the titration data for the intact C3 are compared with those for des-Arg-C3a. All data presented in Figure 6 were obtained at 25 °C at a NaCl concentration of 400 mM. As Figure 6 shows, the titration data for peaks 1 and 2 coincide with those for His-72 and His-67 of des-Arg-C3a, respectively. For peak 1, a least-squares fitting using a sigmoidal curve gave a pK_a of 6.5 with limiting shifts of 7.66 and 8.60. Peak 2 can be observed above pH 7 or below pH 6; the line widths of this peak become unobservably broad between pH 6 and pH 7.

On incubating C3 with trypsin in 600 mM $NaCl/D_2O$ at pH 7.2 with an enzyme/substrate ratio of 1:100, several sharp

resonances become observable (see Figure 9c). In the aromatic region, peaks 1 and 2 disappear, and new peaks 1′ and 2′ become observable instead. Similar experiments were performed with porcine C3 (data not shown). The intact porcine C3 gave four peaks in the aromatic region. Upon incubation with trypsin, it gives three sharp resonances. As Figure 2 shows, porcine C3 has three His residues (His-25, His-44, and His-67) in the C3a portion. It has been shown that trypsin digestion releases C3a fragment (Bokisch et al., 1969). These results strongly suggest that the peak 1 and peak 2 observed for human C3 are due to His residues in the C3a portion. As Figure 6 shows, the titration data observed for His-72 of des-Arg-C3a coincide with those for peak 1 of intact C3. This demonstrates that peak 1 is due to His-72. The NMR results also show that the His-67 titration data for des-Arg-C3a coincide with those for peak 2, although the amount of data is limited due to the failure of observing the signals between pH 6 and pH 7. It is quite likely that peak 2 is due to His-67.

We have shown that the titration data observed for a His residue that exists in the hinge segment of human IgG1 immunoglobulins coincide with those for simple His-containing peptides (Arata et al., 1980; Endo & Arata, 1985). It has also been demonstrated that spin–diffusion extends only very slowly to the hinge segment. On the basis of these NMR data, we have concluded that the His residue in the hinge region of IgG1 is located in the segment that is both exposed to the solvent and highly flexible (Endo & Arata, 1985). It is of great interest that the His-72 titration data observed for both des-Arg-C3a and intact C3 are very close to those for the hinge His residue. Figure 9a,b shows that, in the intact C3, spin diffusion extends only very slowly to His-72; very little change in intensity is observed for peak 1 (His-72), whereas more than 70% of the intensity is lost in the case of peak 2 (His-67). Comparisons of the results obtained for C3 with those for IgG1 strongly suggest that, in the intact C3 molecule, His-72 is located in a segment that is both exposed to the solvent and flexible. An interesting possibility is that the segment that contains His-72 extends to Arg-77 joining C3a and C3b and renders it as the site of proteolytic attack by C3 convertase.

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REFERENCES

Akasaka, K., Fujii, S., & Hatano, H. (1982) *J. Biochem. (Tokyo) 92*, 591.

Arata, Y., Honzawa, M., & Shimizu, A. (1980) *Biochemistry 19*, 5130.

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

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

Bundi, A., & Wuthrich, K. (1979) *Biopolymers 18*, 285.

Dias da Silva, W., Eisele, J. W., & Lepow, J. H. (1967) *J. Exp. Med. 126*, 1027.

Endo, S., & Arata, Y. (1985) *Biochemistry 24*, 1561.

Fernandez, H. N., & Hugli, T. E. (1976) *J. Immunol. 117*, 1688.

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

Gotze, O., & Muller-Eberhard, H. J. (1971) *J. Exp. Med. 134*, 90.

Gotze, O., & Muller-Eberhard, H. J. (1976) *Adv. Immunol. 24*, 1.

Harrison, R. A., Thomas, M. L., & Tack, B. F. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7388.

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

Huber, R., Scholze, H., Paques, E. P., & Deisenhofer, J. (1980) *Hoppe-Seyler's Z. Physiol. Chem. 361*, 1389.

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

Hugli, T. E., Morgan, W. T., & Muller-Eberhard, H. J. (1975) *J. Biol. Chem. 250*, 1479.

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

Isenman, D. E., Kells, D. I. C., Muller-Eberhard, H. J., & Pangburn, M. K. (1981) *Biochemistry 20*, 4458.

Janatova, J., & Tack, B. F. (1981) *Biochemistry 20*, 2394.

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

Laemmli, V. K. (1970) *Nature (London) 227*, 680.

Law, S. K., Minich, T. M., & Levine, R. P. (1981) *Biochemistry 20*, 7457.

Lepow, J. H., Wilms-Kretschmer, K., Patrick, R. A., & Rosen, F. S. (1976) *Am. J. Pathol. 61*, 13.

Lu, Z.-X., Fok, K.-F., Erickson, B. W., & Hugli, T. E. (1984) *J. Biol. Chem. 259*, 7367.

Muller-Eberhard, H. J. (1975) *Annu. Rev. Biochem. 44*, 697.

Nagasawa, S., & Stroud, R. M. (1977) *Immunochemistry 14*, 749.

Sim, R. B., Tuose, T. M., Paterson, D. S., & Sim, E. (1981) *Biochem. J. 193*, 115.

Tack, B. F., & Prahl, J. W. (1976) *Biochemistry 15*, 4513.

Tack, B. F., Morris, S. C., & Prahl, J. W. (1979) *Biochemistry 18*, 1497.

Tack, B. F., Harrison, R. A., Janatova, J., Thomas, M. L., & Prahl, J. W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7388.

Thomas, M. L., Janatova, J., Gray, W. R., & Tack, B. F. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1054.

Vogt, W., Schmidt, G., von Buttlar, B., & Dieminger, L. (1978) *Immunology 34*, 29.