CARBOXYTERMINAL RESIDUES OF THE Fc FRAGMENT FROM HUMAN Ig M*

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Previous studies from this laboratory (Mihaesco and Seligmann, 1966) have shown that a large Fcμ fragment can be obtained by papain proteolysis of human IgM globulins if the enzyme-activating thiol concentration in the reaction mixture is kept low. The large molecular size of this Fcμ fragment remained unchanged in dissociating solvents but readily dropped after reduction with 0.1 M 2-mercaptoethanol in saline. From these and other immunochemical data it was infered that the Fcμ fragment is constituted by several disulfide-linked polypeptide segments belonging to all the monomeric subunits, each segment probably representing the carboxyterminal of one μ polypeptide chain (Seligmann and Mihaesco, 1967 ; Mihaesco and Seligmann, 1968).

In order to ascertain such a molecular location of the Fcμ fragment, a direct comparison between their COOH-terminal aminoacids and those present in the parent μ chains seemed necessary. In the present communication, the patterns of the aminoacids released by carboxypeptidase-A (C.P.-A) treatment of the μ chains obtained from the two previously studied Waldenström IgM were compared to those of the reduced and alkylated

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Fcμ fragments (R.A.-Fcμ) of the same IgM. The same COOH-terminal residues were found.

METHODS

The reduction of the IgM globulins was performed in 0.2 M Tris-HCl buffer pH 8.6 with dithiothreitol at a final concentration of 3×10^{-3} M for 30 minutes at 25°C. In these conditions all (and only) inter-chain disulfide bonds are reduced (Miller and Metzger, 1965). Alkylation was performed with 10 per cent molar excess of 1-^{14}C-iodoacetamide (New England Nuclear) of 0.33 C/Mol specific activity during 15 minutes at 25°C in 0.2 M Tris-HCl buffer pH 8.0. This was followed by extensive dialysis in the cold against distilled water and then in 1 M acetic acid. The μ chains were separated from light chains by gel filtration on Sephadex G 100 columns in 1 M acetic acid. They were devoid of any L chain-contamination detectable by Ouchterlony double diffusion tests. The reduction and alkylation of the Fcμ fragments, obtained as previously described (Mihaesco and Seligmann, 1968), was performed in the same way. The protein concentrations were obtained by O.D. spectrophotometric measurements in 0.25 M acetic acid using an absorption coefficient $E^{1\%,\ 1\ cm}_{280}$ of 13.5. The ^{14}C radioactivity was counted in a Beckman C.P.M. liquid scintillation system at a 0.2 % counting error. Hyamine 10 X, dimethyl-P.O.P.O.P. and naphtalene in dioxane was used as scintillator liquid (Bray, 1960 ; Brown and Badman, 1961).

The labeled μ chains and R.A.-Fcμ fragments were digested with DFP*-treated C.P.-A. (Worthington Biochemicals) at an enzyme-to-protein ratio of 1/50 (molar). The enzyme concentration was 0.2 mg/ml in 0.1 M ammonium bicarbonate. The hydrolytic activity at 25°C was stopped after 10 minutes, 60 minutes, 240 minutes and 1,200 minutes by adding 1.0 M acetic acid (final pH = 2.0) to the reaction mixture. This material was spotted on Whatman 3 MM paper and submitted to : 1) Descending partition chromatography with n-butanol-acetic acid-water (4/1/5) V/V and 2) High voltage electrophoresis using a 1.0 M acetic acid-0.75 M

* DFP : Diisopropylfluorophosphate.

formic acid buffer (pH : 1.9). A voltage of 2 Kv was applied for 1 H at 18°C. After drying, the aminoacids released by C.P.-A were revealed with ninhydrin.

RESULTS AND DISCUSSION

The two aminoacids released by C.P.-A from both μ chains after 10 and 60 minutes had Rf values and electrophoretic mobilities identical to those of carboxamido-methyl-cysteine (C.A.M.C.) and tyrosine. In addition, an appreciable amount of material remained at the point of application. After 240 minutes glycine, alanine, leucine, threonine and valine were detected in addition to C.A.M.C. and tyrosine. The same pattern was found after 1,200 minutes.

The C.P.-A. digest of both R.A.-Fcμ fragments showed at 10 minutes C.A.M.C., tyrosine and an additional spot identified as leucine. At later stages of digestion the patterns of the released aminoacids were identical to those found for μ chains at the corresponding stages. Attempts to identify the first aminoacid residue released by C.P.-A from μ chains and R.A.-Fcμ, using either a low enzyme to protein ratio or shorter periods of incubation, were unfruitful.

Autoradiographic plates taken from the developed and stained paper chromatograms demonstrated that the only radioactive spots were those corresponding to the C.A.M.C. and to the application area. No ninhydrin-positive and/or radioactive material was released from freshly prepared material. From measurements of the ^{14}C-radioactivity, it was found that 4.4. ^{14}C-C.A.M.C. residues were present per each μ chain and that the R.A.-Fcμ fragments contained 1.9 residues assuming that the molecular weights were 72,000 (Miller and Metzger, 1966) and 32,000 (Onoue, Kishimoto and Yamamura, 1968) for μ chains and R.A.-Fcμ respectively. These values are in agreement with previous data on the number of disulfide bonds in IgM (Miller and Metzger, 1965).

It was found that the release of C.A.M.C. by C.P.-A was slow but progressive and did approach the theoretic maximum only in the μ chains of IgM(S) after 240 minutes digestion (96.3 %). In IgM(D) μ chains the

yield was 49.3 % and for both R.A.-Fcμ fragments it was much lower than expected. These low yields are probably due to a high adsorption of C.A.M.C. on the non-migrating material since higher yields were obtained by high voltage electrophoresis.

These results support the previously proposed location of the Fcμ fragments at the COOH-terminus of the μ chains. The COOH-terminus is probably analogous for both IgM globulins but the simultaneous release of C.A.M.C. and tyrosine precluded any choice between the two possible COOH-terminal sequences : -Tyr-Cyst or -Cyst-Tyr. While Doolittle and al. (1966) favoured the first alternative, Abel and Grey (1967) found the following COOH-sequence : Ala-Gly-Thr-Cys-Tyr. All these residues were found in both R.A.-Fcμ fragments and μ chains of our two IgM globulins. The early presence of leucine as an additional residue in R.A.-Fcμ digests might be attributed to the higher susceptibility of this fragment to C.P.A. hydrolysis as compared to that of the whole μ chains. Alternatively one might suspect that in some of the IgM molecules, the papain used to prepare the Fcμ fragments could have hydrolysed several peptide bonds at the COOH-terminal of the μ chains, as described for γ chains (Turner and Bennich, 1968). Thus, the Fcμ fragment represents the "junction region" of the μ chains. It is reasonable to assume that one of the two -S-S inter-μ-chain bonds found in the R.A.-Fcμ fragments is intra-subunit whereas the other is inter-subunit. However it is not possible from the present data to know which one of these -S-S- bonds is located at the C-terminus.

Recent electron microscopic data offered convincing evidence in favour of a circular structure of the IgM molecule (Svehag, Chesebro and Bloth, 1967 ; Chesebro, Bloth and Svehag, 1968). Preliminary electron-microscopic studies (Svehag and Chesebro, 1968) have shown that unreduced Fcμ fragments of IgM(S) and IgM(D) possess a ring-shaped structure. This finding is in good agreement with the present chemical results.

REFERENCES

Abel, C. and Grey, H. (1967). Science, 156, 1609.
Bray, G. (1960). Anal. Biochem., 1, 279.

Brown, W.D. and Badman, H.G. (1961). Biochem. J., 78, 571.
Chesebro, B., Bloth, B. and Svehag, S.E. (1968). J.Exp.Med., 127, 399.
Doolittle, F.R., Singer, S.J. and Metzger, H.(1966). Science, 154, 1561.
Mihaesco, C. and Seligmann, M.(1966). C.R.Acad.Sci.Paris, 262, 2661.
Mihaesco, C. and Seligmann, M.(1968). J. Exp. Med., 127, 431.
Miller, F. and Metzger, H. (1965). J. Biol. Chem., 240, 4740.
Miller, F. and Metzger, H. (1966). J. Biol. Chem., 241, 1732.
Onoue, K., Kishimoto, T. and Yamamura, Y. (1968). J.Immunol., 100, 238.
Seligmann, M. and Mihaesco, C. (1967). in : Nobel Symposium 3. Gamma Globulins. Structure and Control of Biosynthesis, Almqvist and Wiksell Ed., Stockholm, 169.
Svehag, S.E., Chesebro, B. and Bloth, B. (1967). in : Nobel Symposium 3. Gamma Globulins. Structure and Control of Biosynthesis, Almqvist and Wiksell Ed., Stockholm, 269.
Svehag, S.E. and Bloth, B. (Unpublished results).
Turner, M.W. and Bennich, H. (1968). Biochem. J., 107, 171.