A Blocked N-Terminal Residue in the Light Chain of Rabbit Immunoglobulin G

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The light chain of rabbit immunoglobulin G was shown to contain 15–20% blocked *N*-terminal residue. The blocked residue is pyrrolid-2-one-5-carboxylic acid, and most of the chains that contain this residue have the *N*-terminal sequence pyrrolid-2-one-5-carbonyl-valine.

Rabbit IgG* is composed of four polypeptide chains, two light and two heavy chains (Cohen & Porter, 1964). Nevertheless, only about 1.4 moles of free α -amino *N*-terminal residue (1 mole of alanine and 0.4 mole of other amino acids such as aspartic acid, threonine and serine) are found/mole of rabbit IgG (Porter, 1950; McFadden & Smith, 1955; Cebra, Givol, Silman & Katchalski, 1961). Further investigation (Fleischman, Pain & Porter, 1962) has revealed that all of the free *N*-terminal amino acids reside in the light chains, whereas the heavy chains contain a blocked *N*-terminal residue, which is PCA (Wilkinson, Press & Porter, 1966). Determination of free *N*-terminal residues in isolated light chains has demonstrated that each mole contains 0.45 mole of alanine and 0.08 mole of aspartic acid (calculated on the basis of the data of Fleischman *et al.* 1962); more recently Doolittle (1966), using the phenyl isothiocyanate method, also found substantially less than 1 mole of free *N*-terminal residue/mole of light chain.

It would therefore be expected that an appreciable fraction of rabbit IgG light chains would contain a blocked *N*-terminal residue. In human immunoglobulin light chains, it has already been demonstrated that most of the λ -type chains have PCA as *N*-terminal residue (Hood, Gray & Dreyer, 1966). We now report the presence of an *N*-terminal PCA residue in 15–20% of the light chains of rabbit IgG, most of it (about 75%) being recovered as the dipeptide PCA-valine.

MATERIALS AND METHODS

Rabbit IgG and light chain. Rabbit IgG was prepared from a pooled rabbit serum by precipitation at 40% saturation of $(\text{NH}_4)_2\text{SO}_4$ at 4°, followed by chromatography on DEAE-cellulose in 17.5 mM-sodium phosphate buffer,

pH 6.3 (Levy & Sober, 1960), and concentration by vacuum dialysis. The light chain was prepared as described by Fleischman *et al.* (1962). It was completely reduced in 8 M-urea–0.5 M- β -mercaptoethanol–0.5 M-tris-HCl buffer, pH 8.2, at 37° for 3 hr., and alkylated by the addition of ethyleneimine (twice the amount of the β -mercaptoethanol) and incubation for 1 hr. at room temperature while the pH was maintained at pH 8.2–8.5 by the addition of 3 N-HCl. The solution was dialysed exhaustively against water and freeze-dried. An $E_{1\text{cm}}^{1\%}$ value 11.8 at 280 m μ was used to estimate the amount of light chain (Crompton & Wilkinson, 1963), and its molecular weight was taken as 22000.

Pronase. This was obtained from Calbiochem, Los Angeles, Calif., U.S.A.

Amino acids and peptides. PCA was purchased from Mann Research Laboratories (New York, N.Y., U.S.A.) and *N*-acetylglutamine, glutamic acid γ -benzyl ester and valine benzyl ester hydrochloride were obtained from Yeda (Rehovoth, Israel). *N*-Carboxy-DL-alanine anhydride was prepared by the method of Sela & Berger (1955). *N*-Acetylglutamic acid was prepared as described by Greenstein & Winitz (1961). PCA-valine was prepared by a method similar to that used by Press, Piggot & Porter (1966), except that valine benzyl ester hydrochloride was used as starting material instead of valine *tert*.-butyl ester. The benzyl ester group was removed from the dipeptide derivative by hydrogenation in acetic acid, in the presence of palladium on charcoal, to give free PCA-valine.

Preparation of *N*-acetylglutamyl-valine. *N*-Acetyl- γ -benzylglutamic acid was prepared by a method similar to that of Greenstein & Winitz (1961), in which acetic anhydride was added to a solution of glutamic acid γ -benzyl ester in water at pH 7–8. The pH was maintained by the addition of NaOH. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. On addition of light petroleum, crystallization occurred. The material was recrystallized from methanol–water and from ethyl acetate–light petroleum and had m.p. 127–129° (Found: C, 60.0; H, 6.2; N, 4.9. Calc. for $\text{C}_{14}\text{H}_{17}\text{NO}_5$: C, 60.2; H, 6.1; N, 5.0%).

N-Acetyl- γ -benzylglutamic acid (597 mg.; 2.5 m-moles) and *N*-hydroxysuccinimide (288 mg.; 2.5 m-moles) were dissolved in about 10 ml. of anhydrous 1,4-dioxan. Dicyclohexylcarbodi-imide (516 mg.; 2.5 m-moles) in 10 ml. of 1,4-dioxan was added at 2° and the mixture was stirred for

* Abbreviations: IgG, immunoglobulin G; PCA, pyrrolid-2-one-5-carboxylic acid; [Glu, blocked glutamic acid.

6 hr. Acetic acid (0.1 ml.) was added to destroy excess of dicyclohexylcarbodi-imide and the precipitate of dicyclohexylurea was filtered off. After concentration of the filtrate *in vacuo* no crystalline *N*-hydroxysuccinimide ester of *N*-acetyl- γ -benzylglutamic acid could be obtained. Therefore the oily substance was redissolved in 10 ml. of dichloromethane and a solution of valine benzyl ester hydrochloride (610 mg.; 2.5 m-moles) in 20 ml. of dichloromethane was added, followed by triethylamine (0.237 ml.; 2.5 m-moles). The mixture was stirred overnight at room temperature. After dilution with more dichloromethane the solution was extracted three times with 0.5N-HCl, washed with water, extracted with 0.3M-NaHCO₃ and washed again with water. After drying over Na₂SO₄ and concentration, light petroleum was added to induce crystallization. The substance was recrystallized from ethyl acetate-light petroleum and from benzene-light petroleum. It had m.p. 99–100° and the yield was 440 mg. (38%) (Found: C, 66.8; H, 6.8; N, 6.2. Calc. for *N*-acetyl- γ -benzylglutamyl-valine benzyl ester, C₂₆H₃₂N₂O₆: C, 66.7; H, 6.9; N, 6.0%).

The *N*-acetyl- γ -benzylglutamyl-valine benzyl ester (400 mg.) was dissolved in acetic acid and hydrogenated with palladium on charcoal as catalyst. No crystalline compound could be obtained after evaporation of the acetic acid *in vacuo*. A sample of this material was subjected to acid hydrolysis with 6N-HCl and amino acid analysis showed the composition to be: Glu, 1.00; Val, 1.03. Chromatography and electrophoresis under various conditions showed one component only (Table 3).

Amino acid analyses. These were performed with a Beckman model 120B amino acid analyser equipped with an EEL model 106/20 high-sensitivity unit. *C*-Terminal residue was determined by hydrazinolysis as described by Press *et al.* (1966).

RESULTS

Experiment 1. Fully reduced aminoethylated light chain (191 mg.) was digested with Pronase at pH 8.2 at 37° with an enzyme/protein ratio of 1:25 (w/w) in the cell of the pH-stat. Digestion was complete after 2–3 hr., and the alkali uptake (425 μ moles) corresponded to cleavage of 34% of the peptide bonds, assuming pK 7.9 for the α -amino groups. The digest was applied to a column (28 cm. $\times$ 1.2 cm.) of Dowex 50 (X2; H⁺ form; 50–100 mesh) and eluted with water at 4° (Narita & Ishii, 1962). The elution of peptides was followed by measuring the extinction of the eluate at 215 m μ . Only one peak (between 12 and 50 ml. of eluate) was obtained. Part of the eluate was hydrolysed and analysed for the recovery of amino acids (Table 1), and the rest was subjected to preparative paper chromatography in pyridine–3-methylbutan-1-ol–water (7:7:6, by vol.) (Baglioni, 1961) on Whatman 3MM paper. The peptides (Fig. 1) that were ninhydrin-negative were located on a guide strip by the hypochlorite–starch–iodide technique (Pan & Dutcher, 1956) and were eluted from the paper with water. Amino acid analysis of the three fractions from Fig. 1 showed that fraction 1

Table 1. Yields of amino acids in the water eluate from a Dowex 50 column of a Pronase digest of light chain of rabbit IgG

In Expt. 2 the Pronase digest was treated with 1-fluoro-2,4-dinitrobenzene and the Dowex 50 eluate was passed through talc and Sephadex columns. Experimental details are given in the text.

Fig. 1. Separation of blocked *N*-terminal peptides from a Dowex 50 water eluate by paper chromatography in pyridine–3-methylbutan-1-ol–water (7:7:6, by vol.). Expt. 1: the Pronase digest was passed through Dowex 50 without further treatment. Expt. 2: the Pronase digest was treated with 1-fluoro-2,4-dinitrobenzene. Expt. 3: the Pronase digest was treated with *N*-carboxy-DL-alanine anhydride. Experimental details are given in the text.

contained only the dipeptide [Glu-Val, in a yield accounting for 66% of the glutamic acid and 95–100% of the valine present in the original eluate. Hydrazinolysis of this dipeptide gave only valine (80% yield), which therefore must be the *C*-terminal residue of the dipeptide. Fraction 2 contained glutamic acid, accounting for another 17% of the total amount of glutamic acid in the eluate, and also small amounts (about 25% of that of glutamic acid) of threonine, serine, glycine and alanine. High-voltage electrophoresis at pH 3.5 of

Table 2. Yields of [Glu and [Glu-Val as isolated by paper chromatography or electrophoresis from Pronase digests of rabbit IgG light chain

The Pronase digest of Expt. 2 was treated with 1-fluoro-2,4-dinitrobenzene; the values are corrected for the loss during extraction. The Pronase digest of Expt. 3 was treated with *N*-carboxy-DL-alanine anhydride, and the isolation of [Glu-Val and [Glu was performed by high-voltage paper electrophoresis at pH 3.5. Experimental details are given in the text.

	Yields of [Glu and [Glu-Val (mole/mole of light chain)		
	Expt. 1	Expt. 2	Expt. 3
[Glu	0.04	0.035	0.04
[Glu-Val	0.16	0.09	0.11

Table 3. Electrophoretic and chromatographic characterization of blocked N-terminal glutamic acid derivatives

R_{PCA} , Rate of migration relative to that of PCA on paper chromatography in pyridine-3-methylbutan-1-ol-water (7:7:6, by vol.).

	Relative electrophoretic mobility		R_{PCA}
	pH 3.5	pH 6.5	
PCA	(1.00)	(1.00)	(1.00)
<i>N</i> -Acetyl-Glu	0.67	1.23	0.71
<i>N</i> -Acetyl-Gln	0.62	0.80	0.94
PCA-Val	0.53	0.70	1.60
<i>N</i> -Acetyl-Glu-Val	0.42	0.92	0.88
[Glu	1.00	1.00	1.00
[Glu-Val	0.53	0.70	1.60

fraction 2 showed that most of its glutamic acid appeared as free [Glu with a mobility corresponding exactly to that of PCA. As a routine, the eluate from Dowex 50 was divided into two halves; one was subjected to electrophoresis to isolate [Glu, and the other was chromatographed to isolate [Glu-Val. The yields of [Glu and [Glu-Val are shown in Table 2. Fraction 3 contained most of the other amino acids that were present in a small yield in the eluate (Table 1). This fraction was not investigated further, since fractions 1 and 2 together accounted for about 80% of the glutamic acid and 100% of the valine, which were the major components in the eluate from Dowex 50. The electrophoretic and chromatographic mobilities of the [Glu-Val and [Glu isolated from fractions 1 and 2 (Table 3) suggested that they were PCA-Val and PCA respectively. It was, however, essential to ascertain that [Glu-Val and [Glu were not

derived from glutamine, at an internal position of the light chains, which might have cyclized on the Dowex 50 column; this was demonstrated by either blocking or modifying at neutral or alkaline pH all the α -amino groups produced by the Pronase digestion.

Experiment 2. In this experiment the Pronase digest was treated with 1-fluoro-2,4-dinitrobenzene by the method of Wilkinson *et al.* (1966) and Press *et al.* (1966), to block the new α -amino groups. After dinitrophenylation the reaction mixture (about 20 ml.) was adjusted to pH 2 by the addition of formic acid and was extracted twice with ether (40 ml. each) and four times with ethyl acetate (40 ml. each) to remove the DNP-peptides. When the same extraction procedure was applied to synthetic PCA-Val and to PCA (2 μ moles in 20 ml.), 38% and 15% respectively were extracted into the organic phase, and these values were used as correction factors in the experiment with light chain. After the extraction with ethyl acetate the solution was evaporated to dryness, and the residue was dissolved in water and passed through a Dowex 50 column (28 cm. $\times$ 1.2 cm.). The water eluate was evaporated to dryness, dissolved in *N*-acetic acid and passed through a column (11 cm. $\times$ 0.5 cm.) of talc in *N*-acetic acid (Wilkinson *et al.* 1966) and then through a Sephadex G-10 column (125 cm. $\times$ 1.2 cm.) in 0.02 *M*-ammonia. The material eluted at the position corresponding to that of a sample of synthetic PCA-Val was eluted separately and collected, and a portion was analysed, after hydrolysis, for the recovery of amino acids (Table 1); this material still contained traces of DNP-peptides. Electrophoresis or paper chromatography (Fig. 1) of this material gave the same fractions as in the experiment without blocking with 1-fluoro-2,4-dinitrobenzene, and the yields of [Glu and [Glu-Val (Table 2) were also similar.

Experiment 3. In this experiment the new N-terminal residues were modified by reaction with *N*-carboxy-DL-alanine anhydride (Katchalski & Sela, 1958). After Pronase digestion of light chain (160 mg.) the pH was adjusted to 7.0, the reaction mixture (12 ml.) was cooled to 0° and *N*-carboxy-DL-alanine anhydride (250 mg. in 5 ml. of anhydrous 1,4-dioxan) was added. The pH was kept at 7.0 by the addition of 2*N*-alkali in the cell of the pH-stat and the reaction was continued for 2 hr. Under these conditions the free α -amino groups, including any *N*-terminal glutamine, would serve as initiators for the polymerization of the anhydride, and all the peptides would have *N*-terminal alanine residues and would not pass through Dowex 50. The reaction mixture was concentrated *in vacuo* to remove 1,4-dioxan and was passed through a Dowex 50 column (28 cm. $\times$ 1.2 cm.). The water eluate was concentrated and passed through a

Sephadex G-10 column (125 cm. $\times$ 1.2 cm.) in 0.02 M ammonia. All the material was eluted in a position corresponding to that of a sample of synthetic PCA-Val. Upon chromatography of this material under the conditions described in Experiment 1, two additional fractions containing alanine were detected, one running slightly faster than fraction 1 ([Glu-Val] and the other overlapping fraction 2 ([Glu]). The position of these fractions is shown in Fig. 1, and the amount of alanine present in each fraction is given with respect to the amount of glutamic acid. A better separation was achieved by high-voltage electrophoresis at pH 3.5, where the fractions of [Glu and [Glu-Val were isolated in similar yields to those in previous experiments (Table 2). Here, too, additional fractions containing only alanine were observed: one migrating between [Glu and [Glu-Val (mobility 0.75 of that of PCA) and the other migrating more slowly than [Glu-Val (mobility 0.45 of that of PCA). The same alanine-containing fractions were also obtained in a control experiment, when *N*-carboxy-DL-alanine anhydride was treated alone in water under the same conditions. They probably arose from *N*-terminal-blocked derivatives of alanine known to be formed in minute amounts under the conditions of polymerization (Sela & Berger, 1955), and probably bore no relation to the *N*-terminal-blocked peptides of the light chain. As judged from the recovery of the [Glu and [Glu-Val (Table 2), this method of modifying the *N*-terminal residues, formed by Pronase digestion, to *N*-terminal alanine seems more advantageous than that of blocking with 1-fluoro-2,4-dinitrobenzene.

Nature of the *N*-terminal blocking groups. PCA is known to be more labile to alkali treatment than the *N*-acetyl blocking group (Dekker, Stone & Fruton, 1949). The dipeptide [Glu-Val was therefore tested for the alkali-lability of the *N*-terminal blocking group by the method of Wilkinson *et al.* (1966). After incubation of [Glu-Val, *N*-acetylglutamic acid and *N*-acetylglutamine in *N*-sodium hydroxide at room temperature for 24 hr., the degrees of hydrolysis of the *N*-terminal bonds were found to be 50%, 8% and 16% respectively. This and the identity of [Glu-Val and synthetic PCA-Val in electrophoresis and chromatography (Table 3) provide strong evidence that the *N*-terminal residue is PCA. The free [Glu of fraction 2 must also exist as PCA, according to its mobility in different systems (Table 3).

DISCUSSION

The experiments reported here establish that some of the light chains of rabbit IgG contain PCA as a blocked *N*-terminal residue, most of it being recovered as PCA-Val and a small portion as free

PCA. The free PCA is not derived from PCA-Val, since further digestion of PCA-Val with Pronase did not release any free valine, and hence rabbit IgG light chains that have *N*-terminal PCA appear to vary in their residue next to the PCA. The low yield of *N*-terminal PCA (0.15–0.20 mole/mole of light chain) is to be expected from the work of Fleischman *et al.* (1962), which shows that most of the *N*-terminal residues of the rabbit IgG light chains exist in the free α -amino form. However, in view of the correlation between *N*-terminal PCA and antigenic types of light chains from other species, this yield seems significant.

If PCA could be considered as the typical *N*-terminal residue for the λ -type human light chains (Hood *et al.* 1966; Wikler, Titani, Shinoda & Putnam, 1967), it is suggested, by analogy, that rabbit IgG light chains also could be divided into two types of polypeptide chains, although no antigenic distinction between them has yet been reported. Human λ -type light chains represent about 30% of the total light chain population (Mannik & Kunkel, 1963); however, not all of the λ -type light chains have *N*-terminal PCA (Wikler *et al.* 1967). The yield of *N*-terminal PCA reported here is therefore not far from that expected by comparison with human light chains. Doolittle & Astrin (1967) have deduced, from a comparison of the *C*-terminal peptide of rabbit light chains with that of human light chains, that the bulk of rabbit light chains are of the κ -type. This, together with the finding reported here, implying the presence of λ -type chains, would bring rabbit IgG light chains in line with the general occurrence of two types of light chains in several species.

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REFERENCES

- Baglioni, C. (1961). *Biochim. biophys. Acta*, **48**, 392.
- Cebra, J. J., Givol, D., Silman, H. I. & Katchalski, E. (1961). *J. biol. Chem.* **236**, 1720.
- Cohen, S. & Porter, R. R. (1964). *Advanc. Immunol.* **4**, 287.
- Crompton, M. J. & Wilkinson, J. M. (1963). *Biochem. J.* **88**, 228.
- Dekker, C. A., Stone, D. & Fruton, J. S. (1949). *J. biol. Chem.* **181**, 719.
- Doolittle, R. F. (1966). *Proc. nat. Acad. Sci., Wash.*, **55**, 1195.
- Doolittle, R. F. & Astrin, K. H. (1967). *Science*, **156**, 1755.
- Fleischman, J. B., Pain, R. H. & Porter, R. R. (1962). *Arch. Biochem. Biophys. Suppl.* **1**, p. 174.
- Greenstein, J. P. & Winitz, M. (1961). *Chemistry of the Amino Acids*, p. 1948. New York: John Wiley and Sons Inc.
- Hood, L., Gray, W. R. & Dreyer, W. J. (1966). *J. molec. Biol.* **22**, 179.

- Katchalski, E. & Sela, M. (1958). *Advanc. Protein Chem.* **13**, 243.
- Levy, H. B. & Sober, H. A. (1960). *Proc. Soc. exp. Biol., N.Y.*, **103**, 250.
- McFadden, M. L. & Smith, E. L. (1955). *J. biol. Chem.* **214**, 185.
- Mannik, M. & Kunkel, H. G. (1963). *J. exp. Med.* **117**, 213.
- Narita, K. & Ishii, J. (1962). *J. Biochem., Tokyo*, **52**, 367.
- Pan, S. C. & Dutcher, J. D. (1956). *Analyt. Chem.* **28**, 836.
- Porter, R. R. (1950). *Biochem. J.* **46**, 473.
- Press, E. M., Piggot, P. J. & Porter, R. R. (1966). *Biochem. J.* **99**, 356.
- Sela, M. & Berger, A. (1955). *J. Amer. chem. Soc.* **77**, 1893.
- Wikler, M., Titani, K., Shinoda, T. & Putnam, F. W. (1967). *J. biol. Chem.* **242**, 1668.
- Wilkinson, J. M., Press, E. M. & Porter, R. R. (1966). *Biochem. J.* **100**, 303.