TENTATIVE AMINO ACID SEQUENCE OF THE CONSTANT REGION OF NORMAL PIG IMMUNOGLOBULIN λ-CHAINS

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1. Introduction

Polypeptide chains prepared from normal immunoglobulins represent microheterogeneous mixtures the components of which differ by amino acid sequence in certain (so-called variable) regions. Elucidation of primary structure of variable as well as constant regions of microheterogeneous chains is a difficult task requiring, particularly, suitable fragmentation procedures. Peptides originating from constant regions could hardly be isolated from the jumble of peptides originated in variable regions unless the parent chains are fragmented into sufficiently large pieces. Perhaps due to this problem, amino acid sequences of constant regions of immunoglobulin light chains known so far were derived, with a few exceptions, from homogeneous myeloma proteins or homogeneous elicited antibodies. Complete data exist on human and murine λ-chain constant regions; fragmentary data are available on pig, chicken and rabbit λ-chain constant regions [1].

We have found that λ-chains prepared from normal pig immunoglobulin G afford, under suitable conditions of tryptic digestion, several large fragments, most of them containing some unsplit lysyl bonds. Such fragments are excellent candidates for automatic Edman degradation since ε-amino groups of lysine side chains can be converted into strongly polar 4-sulfophenyl-thiocarbamyl groups, thereby making the fragments resistant to extractive losses which occur during degradation [2]. In this way, 50 amino acid residues have been sequenced in a fragment which is 56 residues long. Production of large fragments by partial tryptic hydrolysis can be recommended as a general procedure for automatic sequencing of proteins.

2. Materials and methods

Preparation of pig λ-chains and its resolution into λ(15), λ(16) and λ(17) subpopulations was described in detail previously [3]. Diphenylcarbamoyl chloride-treated trypsin was purchased from Calbiochem. Carboxypeptidase C (Röhm), ethylene imine (Koch-Light), and dithiothreitol (Koch-Light) were also commercial products. The reagents used for automatic Edman degradation were purchased from Beckman. Those used for manual Edman degradation were pure commercial products repurified as recommended by Edman [4]. 4-Sulfophenyl isothiocyanate was a gift from Dr J. C. Jaton, Basel Institute of Immunology.

Details of partial tryptic hydrolysis was fully described in a previous publication [5]. In short, λ(15) chains were digested at an enzyme: substrate ratio 1:400 for 1 h, then additional 1.5 h at the enzyme : substrate ratio 1 : 200. The digest was freeze-dried. Fractionation of large peptides was effected by gel filtration on a column of Sephadex G-25 equilibrated with 0.2% ammonium carbonate [5] and by ion-exchange chromatography on SE-Sephadex C-25 equilibrated with 5 mM potassium formate buffer pH 3.0, 8 M in urea. Reduction and aminoethylation was performed as described [6,7]. The method of manual Edman degradation was that of Blombäck et al. [8] with minor modifications. Automatic Edman degradation proceeded on the

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Beckman Sequencer 890 C. For degradation of the peptide TAE-2 (1 μmol), the standard Beckman program 'Fast Peptide' (dimethylallylamine buffer, benzene washing, no ethylacetate washing and single cleavage) was used. With the peptide TAE-1, the first cycle of degradation was performed manually using 5 mg of solid 4-sulfophenyl isothiocyanate (Braunitzer reagent I) per 1 μmol of peptide. During the second (i.e. first automatic) cycle of degradation, prolonged ethylacetate washing and vacuum drying steps were employed [9]. For the first 20 cycles, the automatic degradation proceeded according to a modified Beckman program 'Fast Protein' (1 M quadrol buffer, benzene and ethylacetate washings, double cleavage, all the vacuum drying steps moderately prolonged). Afterwards, the same modified program, but with a single cleavage, was used until the end of degradation. Phenylthiohydantoin derivatives of amino acids were identified by thin-layer chromatography on silica gel-precoated aluminium plates 'Silufol' (Kavalier, Czechoslovakia) using ethylacetate-acetic acid [4] and chloroform-acetone [10] as solvent systems. Detection was done with ammonia and iodine vapours [10]. Selected phenylthiohydantoin derivatives were identified by gas chromatography according to Pisano and Bronzert [11].

To obtain small tryptic peptides, the fragments were digested with trypsin in 0.2% ammonium carbonate for 3 h at an enzyme : substrate ratio 1 : 100. Small peptides were fractionated by paper chromatography and high voltage paper electrophoresis [12]. Digestion with carboxypeptidase C was performed according to a described procedure [13]. Amino acid analyses were performed according to Spackman et al. [14]. Tryptophane was determined spectrophotometrically [15].

3. Results

Partial tryptic digest of the λ(15) chains was resolved into three peaks by gel filtration on Sephadex G-25 [5]. Peptide material from the breakthrough peak was subjected to ion-exchange chromatography on SE-Sephadex at pH 3.0 (fig.1A). After aminoethylation, the material from the peak V was rechromatographed on SE-Sephadex (fig.1B). Two large fragments TAE-1

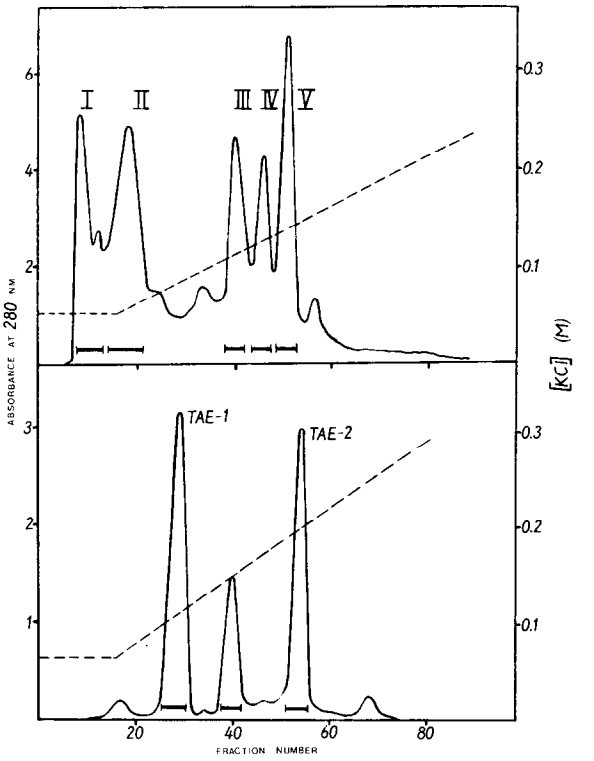

Fig.1. Isolation of the fragments TAE-1 and TAE-2. Upper part: ion-exchange chromatography of a peptide fraction from partial tryptic digest of the λ(15) chains on SE-Sephadex C-25 column (2.3 × 80 cm) in a formate buffer pH 3.0 with urea (5 mM potassium formate, 0.05 M potassium chloride, 8 M urea, pH adjusted with formic acid). After application of the sample (1.9 g in 25 ml) the column was eluted by a linear gradient of potassium chloride in the buffer. The mixer contained 750 ml of the buffer and the reservoir 750 ml of the buffer containing 0.25 M potassium chloride. Peptides from pooled fractions were freed of urea by the passage through the column of Sephadex G-25 equilibrated with 0.2% formic acid and freeze-dried. Lower part: ion-exchange chromatography of aminoethylated fraction V on SE-Sephadex C-25 column (1.3 × 32 cm) in formate buffer pH 3.0 with urea (5 mM potassium formate, 0.06 M potassium chloride, 8 M urea, pH adjusted with formic acid). After application of the sample (70 mg in 5 ml) the column was eluted by a linear gradient of potassium chloride in the buffer. The mixer contained 140 ml of the buffer, and the reservoir 140 ml of the buffer containing 0.30 M potassium chloride. Peptides from pooled fractions were freed from urea by the passage through the column of Sephadex G-25 equilibrated with 0.2% formic acid and freeze-dried. Solid line: absorbance at 280 nm. Broken line: concentration of KCl in the effluent.

Table 1
Amino acid composition of the fragments TAE-1, TAE-2 and their subfragments TAE-1-T-1, TAE-2-T-1. Numbers represent molar ratios. The number of amino acid residues in the determined sequence (cf. fig.2) is given under 'found'. The values for serine, threonine and tyrosine were extrapolated to zero time of hydrolysis, those for valine and isoleucine were taken from 70-h hydrolysates. AE-Cys stands for S-(2-aminoethyl)-cysteine.

	TAE-1	found	TAE-2	found	TAE-1-T-1	TAE-2-T-1
AE-Cys	0.9	1	1.0	1	–	–
Lys	4.0	4	2.9	3	2.1	1.1
His	–		1.1	1	–	1.0
Asp	3.1	3	2.9	3	–	–
Thr	9.7	10	2.9	3	5.3	2.0
Ser	4.3	4	7.8	8	1.1	–
Glu	4.0	4	4.0	4	1.9	2.9
Pro	5.1	5	–		0.8	–
Gly	5.0	5	2.0	2	2.9	1.1
Ala	4.9	5	4.1	4	1.0	–
Val	5.9	6	1.8	2	1.8	1.8
Ile	1.0	1	0.8	1	–	0.8
Leu	4.3	4	2.1	2	–	–
Tyr	1.0	1	1.9	2	–	–
Phe	2.1	2	1.0	1	–	–
Trp	0.8	1	0.8	1	–	–
Total	56.1	56	37.1	38	16.9	10.7

and TAE-2 were isolated in pure form (table 1). The fragment TAE-1 was modified with 4-sulfophenyl isothiocyanate and subjected to automatic Edman degradation. 50 Amino acid residues were degraded; 47 of them were positively identified. The average repetitive yield of the degradation was 96%. Tryptic hydrolysis of the fragment TAE-1 afforded a heptadecapeptide TAE-1-T-1 (table 1), 13 *N*-proximal amino acids of which were identified by manual Edman degradation. The complete amino acid sequence of the fragment TAE-1 was accomplished by carboxypeptidase C digestion of the peptide TAE-1-T-1 (cf. fig.2).

The fragment TAE-2 being subjected to automatic Edman degradation, its 29 *N*-terminal amino acid residues were identified providing an overlap to the sequence around a half-cystine residue determined previously [12] (cf. fig.2). The average repetitive yield of the degradation was 92%. Tryptic hydrolysis of the fragment TAE-2 afforded an undeca-peptide TAE-2-T-1 (table 1). Its C-terminal residue, upon carboxypeptidase C digestion, was found to be a lysine. Since the values of valine and isoleucine in 20-h amino acid analyses of the peptide TAE-2-T-1 are substantially lower than those in 70-h analyses, these two residues must participate in the same peptide bond; this identifies a valine residue as the nearest neighbour of the isoleucine residue, thus completing the C-terminal sequence Ile-Val-Glu-Lys. The penultimate glutamyl residue is present in free acid form as documented by the electrophoretic mobility of the peptide TAE-2-T-1 at pH 5.6.

4. Discussion

In our earlier work [12,16], amino acid sequence around disulfide bridges of the pig λ(15) chains was established (cf. fig.2). Based on this knowledge, the fragments TAE-1 and TAE-2 are identified as representing large sections of the constant region of the λ-chains. Amino acid sequence in both these fragments displays pronounced homology to the primary structure of the constant regions of human [17] and murine [18] type Bence Jones proteins. By this homology the fragments can be aligned, together with

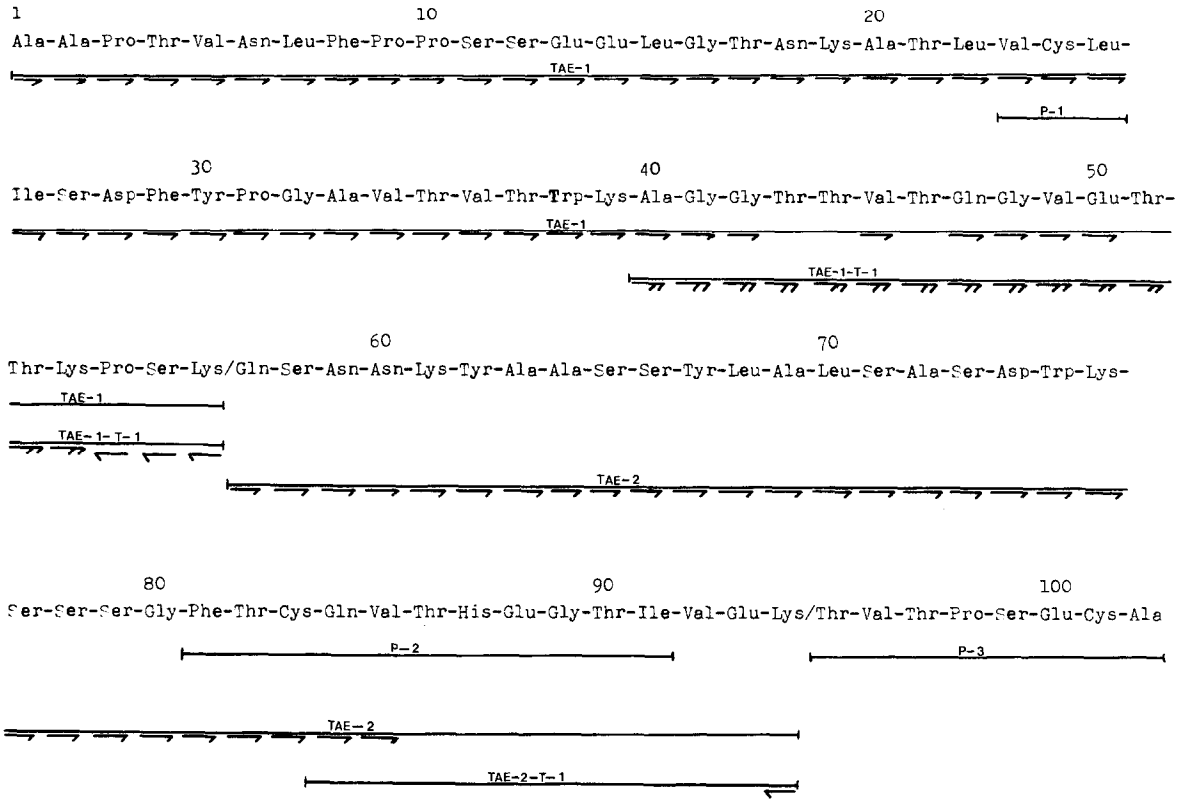

Fig.2. Determination of the amino acid sequence of the constant region of pig immunoglobulin λ-chains. The arrows designate individual peptides isolated from various tryptic digests (see the text). ——→ Sequence determined by automatic Edman degradation. —→→ Sequence determined by manual Edman degradation. ←—— Sequence determined by carboxypeptidase C digestion. Arrows designated P1, P2, and P3 indicate amino acid sequence known previously [12,15]. Alignment of fragments TAE-1, TAE-2 and P3 was accomplished according to homology with primary structures of constant regions of human [17] and murine [18] Bence Jones proteins.

a C-terminal nonapeptide of the pig λ-chains [16], in a contiguous sequence accounting for the primary structure of the whole constant region of the pig λ-chains.

The work described in this paper showed, in agreement with previous experience [19–22], that with a suitable fragmentation strategy sequencing of constant sections of microheterogeneous polypeptide chains is quite feasible. This opens the possibility of studying immunoglobulins from species in which monoclonal proteins have not been found. To this purpose, automatic sequencing of large fragments seems to be one of the most effective methods.

The fragments TAE-1 and TAE-2 studied in this paper originated from the λ(15) chains, i.e. a subpopulation comprising about one third of the whole of λ-chains [3]. A possibility of rare amino acid replacements existing in pig λ-chain constant region (such as isotypic or allotypic replacements in human and rabbit light chains) could not therefore be excluded.

The percentage of identical amino acid residues between pig and human sequences amounts to 75%, that between pig and murine sequences is 67%. Positions in which interspecies amino acid replacements occur are not distributed randomly along the constant region. Detailed analysis of the pattern of this distribution requires special mathematical treatment and will be published in a separate paper.

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