EVIDENCE FOR C-TERMINAL INTRA-SUBUNIT DISULPHIDE BRIDGES BETWEEN IMMUNOGLOBULIN-M HEAVY CHAINS

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1. Introduction

In a previous paper [1] it was reported that treatment of a human monoclonal 19 S immunoglobulin-M (IgM) with 5 mM dithiothreitol (DTT) (partial reduction) followed by alkylation with iodo 2-^{14}C-acetic acid gave 100% conversion to 7 S subunits that had all their inter-chain disulphide bridges broken. Tryptic digestion of the heavy (μ) polypeptide chain from these subunits gave five labelled peptides (μ1, μ2, μ3, μ4 and μ5). Reduction of the 19 S IgM with 0.125 mM DTT (selective reduction) followed by alkylation with iodo 2-^{14}C-acetic acid gave only 50% conversion to 7 S subunits that had most of their inter-chain bridges still intact. These subunits were isolated and further reduced with 5 mM DTT then alkylated with unlabelled iodoacetic acid. Tryptic digestion of the heavy chain from these subunits gave only three labelled peptides (μ2, μ3 and μ5). Peptides μ1 and μ4 were unlabelled showing that they must have formed intact inter-chain bridges in the 7 S subunits and therefore were not involved in the disulphide bridge that links subunits to one another.

By means of enzymic fragmentation and peptide analysis it was subsequently shown [2] that peptide μ1 was involved in the light-heavy chain disulphide bridge, peptide μ2 formed an intra-subunit bridge in the F(ab$'$)$_2$ region and that peptides μ3, μ4 and μ5 lay in the Fc region or C-terminal half of the heavy chain (fig. 1). It was also shown [2] that peptides μ3 and μ5 most probably arose from the same part of the heavy chain and differed only in the nature of attached carbohydrate. However, the relative positions of μ3/5 (involved in the inter-subunit bridge) and μ4 (involved in an intra-subunit bridge) in the Fc region remained to be determined.

Other workers [3, 4] have reported that the heavy chain of IgM has an inter-chain disulphide bridge next to a C-terminal tyrosine residue and it was suggested that this bridge might form the linkage between subunits. Aminoacid analysis of the labelled peptides μ1, μ2, μ3, μ4 and μ5 showed [2] that only μ4 contained tyrosine and therefore was likely to be C-terminal. However, since this tryptic peptide also contained a residue of lysine it could not conclusively be regarded as C-terminal. If peptide μ4 was C-terminal it would mean that the disulphide bridge at the C-terminus of the heavy chain is an intra-subunit bridge and not the inter-subunit bridge. Confirmation of this is described in the present paper.

2. Methods

The preparation of heavy chain from partially reduced IgM and selectively reduced IgM, as well as complete reduction and tryptic digestion of the chain, was carried out as previously described [1]. Details of paper electrophoresis and aminoacid analysis of peptides have already been given [2]. Cyanogen bromide fragmentation of completely reduced chain was carried out as described by Press, Piggot and Porter [5]. Radioactivty of peptides was measured on paper cut-outs of uniform size by means of a flow counter. The amount and purity of each peptide was determined by eluting the cut-outs with constant boiling HCl followed by hydrolysis and aminoacid analysis of the eluant. Blank cut-outs

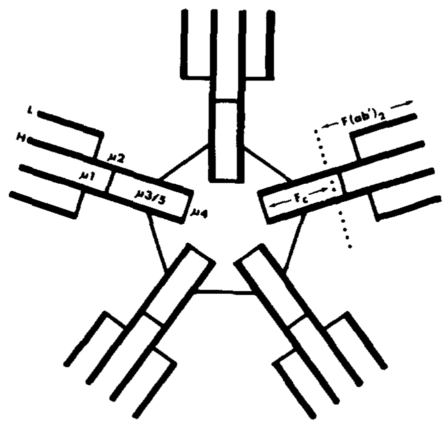

Fig. 1. Diagrammatical representation of a molecule of IgM made up of 5 4-chain subunits. H, heavy chain, L, light chain. Thick lines indicate polypeptide chains and thin lines represent inter-chain disulphide bridges. $\mu1$, $\mu2$, $\mu3/5$ and $\mu4$ indicate the approximate positions of peptides associated with the inter-chain bridges. The dotted line indicates the position where a sub-unit can be enzymically cleaved into $F(ab')_2$ and Fc fragments.

were measured and analysed in an identical manner.

3. Results and discussion

Cyanogen bromide fragmentation of heavy chain from the partially reduced IgM gave a labelled peptide having the composition CMCys$_1$, Asp$_1$, Thr$_2$, Ser$_1$, Gly$_1$, Ala$_1$, Tyr$_1$ which is identical with that of the C-terminal peptide reported by Wikler, Kohler, Shinoda and Putnam [6]. However, the same peptide obtained from the heavy chain of selectively reduced IgM was unlabelled (6.3 nmole gave 1 cpm) even though peptides $\mu3$ and $\mu5$ from this chain were appreciably labelled (4.5 nmole gave 189 cpm and 4.2 nmole gave 195 cpm respectively). Thus the disulphide bridge at the C-terminus must have remained intact during the formation of subunits by selective reduction and must therefore be an intra-subunit bridge.

Further confirmation was obtained by studying the tryptic peptide $\mu4$. Preliminary evidence that this peptide is C-terminal was obtained when treatment of the labelled peptide with carboxypeptidase-A [7] liberated an appreciable amount of tyrosine and a small amount of labelled CM-cysteine. However, significant amounts of leucine and valine were also released, probably due to other proteolytic activity in the enzyme preparation. Further evidence was obtained by treating $\mu4$ with chymotrypsin [8] which gave a labelled peptide having the composition CMCys$_1$, Asp$_2$, Thr$_2$, Ser$_2$, Gly$_1$, Ala$_1$, Val$_2$, Met$_1$, Leu$_1$, Tyr$_1$. This is consistent with the known last nine residues of heavy chain [6] plus five additional ones. Final proof that $\mu4$ is C-terminal was obtained as follows. The labelled peptide was converted to a carbamoylmethylmethionine form [9] and then cleaved by conversion of carbamoylmethylmethionine to homoserine [9]. A labelled peptide was obtained that had the composition CMCys$_1$, Asp$_1$, Thr$_2$, Ser$_1$, Gly$_1$, Ala$_1$, Tyr$_1$ which was identical with that of the C-terminal cyanogen bromide fragment. Since the disulphide bridge associated with peptide $\mu4$ remains intact in the subunits prepared by selective reduction of IgM [1] the disulphide bridge at the C-terminus of the heavy chain must be an intra-subunit bridge.

The results presented here together with those previously reported [1, 2] are represented in fig. 1. This structure for IgM is supported by results from other laboratories [10–12].

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