INTERPLAY OF J CHAIN AND DISULFIDE BONDING IN ASSEMBLY OF POLYMERIC IgM

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science Graduate Department of Immunology University of Toronto

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Abstract

Normal mouse IgM is synthesized as hexamers in the absence of J chain and as pentamers in its presence. Previous work has suggested that polymer size is also closely related to formation of the inter-\(\mu\) chain disulfide bond mediated by cysteine 414, one of three cysteines involved in inter-\(\mu\) chain bonding. This correlation in turn suggested that formation of C414-C414 might be required for J chain to influence how IgM assembles and that formation of C414-C414 might affect the J chain/IgM stoichiometry. To test such hypotheses we have used cell-lines that either express or do not express J chain to produce IgM in which serine was substituted for C414. In contrast to the case of IgM assembled from normal \(\mu\) chains, IgM-S414 was secreted mostly as pentamers and tetramers but not as hexamers, irrespective of J chain synthesis. These results indicate that the role of J chain as modulator of IgM structure and function requires C414. Moreover, a more detailed analysis of the structure of J-plus and J-minus IgM-S414 revealed that J chain, in fact, influenced the nature of secreted IgM-S414: in the absence of J chain, some IgM-S414 was secreted as dimers and trimers, while in the presence of J chain, some IgM was secreted as non-covalently assembled pentamers. These results imply that disulfide bonding can occur differently from the pattern depicted in conventional models of IgM structure.
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Interplay of J chain and disulfide bonding in the assembly of polymeric IgM
Introduction

Polymeric IgM is an array of μ2L2 subunits that are covalently linked through three inter-μ disulfide bonds: C337 in the Cμ2 domain, C414 in the Cμ3 domain, and C575 in the Cμ4-tail domain. The following discusses general features of IgM structure, assembly and function, with particular emphasis on the role of J chain and disulfide bond arrangements.

The Immunoglobulin domain

The immunoglobulin superfamily consists of adhesion molecules (e.g. CD2, ICAM-1, 2, and 3, LFA-3 and V-CAM-1), co-stimulatory molecules (e.g. CD-28, CTLA-4), cell surface markers (e.g. Thy-1), peptide-presenting molecules MHC class I and II, and cell-surface receptors such as Fc receptors, the TCR and the Immunoglobulins. The Ig domain folds into two layers of anti-parallel β-sheet, enclosing a hydrophobic interior. Generally, one layer has three strands (Y face) while the other has four (X face), and the strands are connected by flexible loops of varying length. A highly conserved internal disulfide bond bridges the two faces thus stabilizing the tertiary structure. This structure can tolerate mutations that neither change the hydrophobicity of the interior nor introduce β-breaking residues in the β-sheets (1). Ig domains are encoded by single exons such that the 3' end of the preceding exon encodes the first position in the codon and the 5' end of the next exon encodes the second position of the codon. Thus, an open reading frame is always maintained after evolutionary random shuffling of exons. Gene duplication and diversification take advantage of these features of the Ig domain, giving rise to many functionally different molecules that normally contain several Ig domains (1).

The prototypical pattern of Ig domain construction, based on analogy to IgG structure, is diagrammed (Fig. 1). The V domains are longer than the C domains by
Figure 1. Ig domain structure. Rectangles denote β-strands. Arrows denote connecting loops in N-terminal to C-terminal direction. An immunoglobulin V domain differs from the C domain by two extra β-strands following 3-1 strand.
approximately 16 amino acid residues. Hence, the V domain has two more β-strands between 3-1 and 4-3 such that the Y face has five strands and the X face has four strands (2-4). The Cµ3 domain, if analogous to Cγ2, resembles an intermediary to the V and C domain structure: it has two faces of 4 strands each (5).

Ig domains in the IgG molecule, interact and form modules consisting of two domains (6). Ig domains in the IgM molecule are thought to behave in a similar manner. That is, the V domain of the light chain interacts with the V domain of the heavy chain, Cµ1 domain interacts with the CL chain, the Cµ2 domain interacts with the Cµ2 domain of its neighboring μ chain, and the Cµ4 domain interacts with another Cµ4 domain. V domains interact though their Y face (five strand face), while the C domain interact through their X face (four strand face). This interaction is made possible in IgG by hydrophobic patches on the solvent-exposed side of the β-strand (2-4, 7). The Cµ3 domain, like the Cε3, Cδ2, Cα2, and Cγ2 domains, lacks these hydrophobic patches and is unable to dimerize with a partner (5).

Polymerization

Several studies indicate that μ2L2 monomers are the basic polymerizing blocks that lead to assembly of secreted IgM. Fractionation of pulsed intracellular immunoglobulin pools showed a component that contained a heavy and a light chain (8, 9). This pool persisted after a 3 hr incubation during which higher order polymers were not detected intracellularly, but IgM polymers were detected extracellularly. The authors took this evidence to argue that polymerization and secretion are quick and nearly spontaneous events that occur after μL formation. The work of Brewer et al (10) supports these early observations. They found that the intracellular pool consisted mostly of μL halfmers and monomers with relatively little intermediates (dimers and trimers) and polymers. The assembly intermediates contained immature N-linked oligosaccharides, and were multiples
of μL halfmers (μL, μ2L2, μ3L3, ..., μ10L10) that were found to be both covalently and non-covalently assembled. Some of these intermediates were also associated with J chain. The kinetics of IgM assembly were uncovered using pulse-chase experiments. After five minutes of pulsing, intracellular halfmers and monomers were labeled. By twenty minutes of chasing, IgM polymers could be detected both intracellularly and extracellularly (10).

Bergman and Kuehl (11) showed that the formation of γL halfmers may even occur during the translation of the heavy and light chain. QAE-Sephadex purification of microsomal nascent polypeptides from MPC 11 (IgG producer) followed by specific immunoprecipitation of light chains, yielded disulfide-linked heavy-light chain halfmers on SDS-PAGE. This co-translational association of heavy and light chain in the lumen of the ER is probably the initiating event for IgM polymerization. However, light chain is not necessary for polymerization. Bornemann et al (12) have examined IgM polymerization in NYCH.μκ and NYCH.μ, a κ loss variant of NYCH.μκ hybridoma cell line. They found that the μ chains polymerize to μ10 polymers which are retained in the ER bound to the molecular chaperone BiP. This would suggest that polymerization of IgM occurs exclusively in the ER.

Consistent with this hypothesis brefeldin A and low temperatures, which block export of proteins from the ER, do not affect IgM polymerization (10). Shachar et al (13), however, argue against the notion of ER polymerization of IgM. Their evidence is three fold: i) galactosylated monomers and polymers were observed. Assuming that these monomers can still polymerize, these results may suggest that monomer to polymer conversion occurs after the monomers reach the trans-Golgi galactosylation compartment; ii) hampering of Golgi function by Tris base also hampered IgM polymerization; and iii) inhibition of protein export from the ER with brefeldin A, and energy poisons also reduced the amount of total pentameric IgM (extracellular and intracellular). The authors conclude that polymerization probably occurs after monomers assembled in the ER are channeled to the trans-Golgi. The contradiction between these two groups may be resolved if assembly
of IgM is in equilibrium with the amount of intracellular IgM. Thus, treatment of cells with brefeldin A would result in lower amounts of total pentameric IgM (13), because cells are not able to secrete the IgM and the intracellular assembly process is saturated. Observations by Shachar et al (13), then, do not necessarily argue against the ability of IgM to be polymerized exclusively within the ER (10).

Quality control mechanisms in IgM assembly

BiP is a 78 KDa protein implicated in proper assembly of the IgM molecule. It was originally discovered by Haas and Wabl (14), when they observed that the μ chain of an Abelson transformed B cell line was intracellularly associated with a chain other than the light chain. This new chain was named heavy chain Binding Protein or BiP. Using immunoperoxidase staining, BiP was localized to within the cisternal spaces of the RER (15). The binding site of BiP to heavy chain was found to be in the CH1 and not in the CH2 or the CH3 (16). BiP could only interact with heavy chains that were not associated with the light chain (17), and binding of light chain to the heavy chain, in fact, replaced BiP (18). Hendershot and Kearney (19) showed that BiP was involved in post-translational processing of the μ chain in lymphoid cell lines representing all stages of B cell development. Cloning of the gene encoding BiP revealed that the gene was consitutively expressed in various murine tissues, suggesting that BiP might have a more general function (20). In support of this prediction, the chaperone-like behavior of BiP has been further extended to include other proteins as well, e.g., prolactin (21), vesicular stomatitis virus G protein (22), TCR α chain (23), mutant Insulin receptor (24).

BiP, in addition to ensuring heavy-light chain pairing, has also been implicated in monitoring polymer assembly. It had long been observed that B cells, despite expressing secreted-μ splice form, do not secrete IgM. In 1988 Sitia et al (25) made the observation that although the L29 μ B cell lymphoma, similar to other B cell lines, synthesized both μm
and μs proteins, μm was transported to the cell surface, whereas μs protein was degraded intracellularly. Co-immunoprecipitation studies showed that the μs protein was associated with BiP, while μm was not. This suggested that the involvement of BiP is not restricted to proper assembly of μL halfmers. Since the only difference between μm and μs is in the tail, efforts were concentrated on finding the motif responsible for retention of μs protein in I.29μ B cells by BiP. The authors found that the mutation of C575 in the Cμ tail to an alanine residue resulted in secretion of the μs protein (26). In addition, whereas μs chain with deleted Cμ1 and a wild type tail still associated with BiP, mutating C575 to alanine abolished this association. As such, BiP interaction with the μ chain occurs at two distinct sites: Cμ1 and Cμ4-tail, possibly through free cysteines.

The role of the μ-tail piece (μtp), however, in retention is not clear. On the one hand, the A575 mutation in the μtp also leads to decreased intracellular transit time and increased efficiency of secretion of the IgM by plasmacytoma hosts. Also, transplantation of μtp onto IgG (26) or onto Cathepsin D (27) results in ER retention of these chimeric molecules. On the other hand IgG-μtp chimeric molecules constructed by other laboratories are efficiently secreted (28-30), and mutation of C575 to a serine does not significantly affect the ratio of secreted IgM to μ mRNA (31). Moreover, there are other mutations in the Cμ3 region that inhibit polymer assembly but do not affect efficient secretion (32).

Despite these controversies, the proposed quality control mechanism is that μ chains that have not completely polymerized by C575-C575 bonds would be retained through their free thiols by BiP. In support of this argument, Alberini et al (33), found that adding reducing agents to the supernatant at concentrations that did not affect cell viability, terminal glycosylation, or retention through the KDEL mechanism, resulted in secretion of IgM assembly intermediates that supposedly have exposed thiols. That is, the intermediates must have been retained through a disulfide interchange reaction. Neither chloroquine nor brefeldin A inhibited degradation of incomplete IgM assembly intermediates suggesting an
Figure 2. Hypothesized mechanism of IgM polymerization. Association of heavy and light chain may occur during translation. BiP ensures that unpaired heavy chains are retained until they interact with a light chain partner through the Cμ1 domain of the heavy chain. Within five minutes, intracellular dimers and monomers are readily detectable. Once seeded with IgM monomers, polymerization and secretion may occur within twenty minutes. Thiol-mediated retention mechanism is postulated to ensure complete polymerization of IgM by ensuring complete oxidation of C575.
ER degradation mechanism (27). In addition, Brewer and Corley have observed that whereas high mannose IgM monomers and assembly intermediates have free thiols, secreted polymers, intermediates and monomers are fully oxidized. Thus, thiol-mediated retention only acts on incompletely oxidized species (34).

**J chain**

J chain is an acidic 15KDa, 137 amino acid protein with eight cysteine residues. It is exclusively found in covalent association with secreted IgA dimers and tetramers and with secreted IgM pentamers (35-38). J chain synthesis is developmentally regulated in B lymphocytes, and expression in murine B cells coincides with synthesis of the secreted μ heavy chain (39). The upstream region of the J chain gene becomes increasingly hypersensitive to nucleases as a B cell progresses toward the plasma cell stage (40). Interleukins that induce growth and differentiation of B cells such as IL-2 and IL-5, also induce J chain production (41-43). Moreover, J chain induction by IL-2 and IL-5 is inhibited by IL-4 (43) which induces class switch from IgM isotype to the IgE and IgG isotypes. C14 and C68 of the J chain form disulfide bonds with C471 in the α chain and C575 in the μ chain, in both cases the penultimate amino acid (36, 44, 45). Although it has been thought that J chain is only involved with IgM and IgA polymers, recently, J chain has also been found in invertebrates that do not have any known immunoglobulins (46).

The data from early studies of J chain stoichiometry indicate 0.7 J chains/IgM polymer (38, 47, 48). The accuracy of this calculation is uncertain. First it is unclear whether J chain was present in each IgM molecule. As noted above, normal IgM is usually a mixture of hexamers and pentamers. Inasmuch as hexamers, and perhaps even some pentamers, lack J chain the natural heterogeneity of IgM might account for the submolar value. It is also possible that some IgM pentamers contain more than one J chain.
Although the J chain is bound to C575 of the \( \mu \) tail piece, the \( \mu \)tp is not sufficient to mediate J chain incorporation. Several groups have transplanted \( \mu \)tp to IgG, resulting in variable degrees of secretion and polymerization of the hybrid antibody (26, 29, 49). However, such secreted polymers do not contain J chain (29). Wiersma et al (30) studied IgM structures that influence J chain incorporation by analyzing IgM with deleted domains, or IgM with IgG domains. It was found that none of \( \text{C} \mu 1 \), \( \text{C} \mu 2 \) or \( \text{C} \mu 3 \) was absolutely necessary for J chain incorporation. The role of \( \text{C} \mu 4 \) could not be uncovered because replacing \( \text{C} \mu 4 \) with \( \text{C} \gamma 3 \) resulted in secretion of monomers. This study also revealed that glycosylation at asparagine 563 is important for J chain incorporation.

J chain is not required for IgM polymerization. Previously, it was thought that the primary function of the J chain was to initiate (48, 50, 51) or terminate (52) polymerization, and early efforts at \textit{in vitro} IgM polymerization yielded mixed results as to whether J chain was (53, 54) or was not (55, 56) required in this process. Recent experiments, however, clearly show the ability of IgM to polymerize in the absence of J chain. Glioma transfectants of mouse \( \mu \) chain and \( \lambda \) light chain produce and secrete IgM polymers (pentamers and/or hexamers) in the absence of J chain (57), and IgM-wt is secreted as a combination of J chain containing pentamers and J chain-deficient hexamers (58-61).

It has been argued that J chain affects the rate of polymerization and secretion of IgM. Thus, Niles at al (61) found that transfection of J chain into four different J chain deficient cell lines lead to more rapid secretion of the IgM. In a similar system, however, transfection of WEHI-231 cells with a J chain-expressing plasmid vector did not alter IgM secretion, although the J chain was incorporated into polymeric IgM (62). It is not clear why these two sets of observation are discrepant. With the availability of J chain knock-out mice (63, 64) these questions will probably be settled.

\textit{Interchain disulfide bonding}
Wild-type IgM (C337, C414, C575 are available for disulfide bonding) is secreted as pentamers and hexamers (>90% combined), monomers (<5%), and halfmers (~1%). Analysis of IgM secreted by cells carrying a μ transgene with various cysteine to serine substitutions has revealed the role of each cysteine in IgM polymer assembly as outlined below.

C575 is important in covalent assembly of polymers from monomers, and to a lesser degree in assembly of monomers from halfmers (31). Thus, IgM-S575 secretes a small fraction (~17%) of J chain-deficient, covalent hexamers, and a predominant fraction of monomers. Compared to IgM-wt, IgM-S575 monomer secretion appeared to be impaired because the ratio of secreted monomers to secreted halfmers was lower than in IgM-wt. Moreover, IgM-S(337,414) with only C575 available for inter chain disulfide bonding is secreted as a combination of polymers, intermediates and monomers comparable to IgM-wt (31, 58).

C414 does not seem to have significant effect on polymer or monomer assembly. Thus, IgM-S414 is secreted as mostly covalent pentamers and tetramers that are associated with J chain. IgM-S414 also contains a significant amount of intermediately sized polymers (dimers and trimers). Moreover, IgM-S(337,575), with only C414 available for disulfide bonding, is mostly halfmeric (31, 65, 66).

C337 is important for covalent assembly of monomers from halfmers, and to a lesser degree in assembly of polymers from monomers. Thus, IgM-S337 is secreted as non-covalently assembled, J chain-containing pentamers most of which dissociate under denaturing conditions to covalent monomers and dimers. The monomer to halfmer ratio of IgM-S337 is also relatively low. Moreover IgM-S(414,575), with only C337 available for disulfide bonding, is mostly monomeric (31, 65).

Thus, C337, and to a lesser extent C575, are important in assembly of μ2L2 monomers, and C575 is important is assembly of μ10L10 or μ12L12 pentamers and
hexamers from μ2L2 monomers. C414 does not seem to have a significant role in polymerization.

Since monomers form before polymers, and since C337 is implicated in monomerization and C575 is implicated in polymerization, it is possible that the C337-C337 is the first inter-μ chain bond to form (67). Similar to γL co-translational association, C337-C337 may form between nascent μ chains. Since C575S mutation affects monomer formation less than the C337S mutation, C575-C575 bonds probably occur much less frequently than C337-C337 bonds at this initial stage. In addition, monomers linked through C575-C575 will most likely be secreted as monomers because μ chains that have associated with the light chain and do not possess a free thiol at C575 are not retained in the ER. Since polymerization occurs rapidly after formation of monomers (8, 10), it is likely that once one C575-C575 forms between two monomers that are linked through C337-C337 bonds, consequent C575-C575 bond formation is facilitated. Alternatively, C337-C337 linked monomers might be transported to a particular compartment within the ER where C575-C575 bond formation is favored. Finally, the quality control mechanism based on oxidation state of C575 (reviewed above) is consistent with the above conclusion that C575 is the most important cysteine for polymerization.

It has been suggested that J chain is required for IgM pentamer assembly, and that absence of J chain directs IgM hexamer assembly (38). IgM-wt hexamers are not associated with J chain (58-60, 68). As mentioned, transfection of J chain-expressing plasmid into J-minus cell lines redirects assembly from hexamers to pentamers (61, 62). Treatment of CH12 inducible B-cell lymphoma with LPS, which does not upregulate endogenous J chain expression but induces IgM secretion, results in secretion of mostly hexameric IgM; however, treatment with IL-5, which induces J chain expression as well as IgM secretion, results in secretion of mostly pentameric IgM (69). Also, IgM-S575 polymers which cannot associate with J chain is mostly hexameric as well (58).
To account for the foregoing observations, our lab proposed that the assembly of pentamers or hexamers is determined by the formation of C414-C414 bonds and the availability of J chain (38, 65). Thus, IgM in which all possible C414-C414 form is a comparatively rigid molecule and requires six subunits to circularize. This observation accounts for the finding that IgM-S575 is a hexamer, as IgM-S575 can be polymerized only by forming all C414-C414. Conversely, IgM in which no C414-C414 can form can be circularized with five or fewer subunits mediated by C575-C575, thus accounting for the structure of IgM-S414. Normal IgM is then thought to represent both these extremes as well as intermediate situation which can occur because not all of the three disulfide bonds must be used in polymerizing IgM-wt. Specifically, a molecule of normal IgM in which only C337-C337 and C575-C575 form could be polymerized as a pentamer or smaller polymer; a molecule in which all C414-C414 form would assemble as a hexamer; and a molecule in which at least one but not all C414-C414 forms would polymerize as a pentamer which includes a J-chain.

Two types of studies have provided information about how the inter-μ chain disulfide bonding is arranged. Early efforts involved partial reduction of the IgM molecule and labeling of the reduced cysteine residues (70-72). Their results suggested that only homopairs (C337-C337, C414-C414, C575-C575) formed (38). These analyses were used to deduce whether these different pairs join μ chain in series or in parallel (Fig. 3) and led to different models for human and mouse IgM, namely that C337-C337 and C575-C575 join μ chains in parallel in human IgM but they are in series in mouse IgM, and C414-C414 is in series with C337-C337 in human IgM but is not formed in mouse IgM. The analysis of cysteine to serine mutants of mouse μ chain, as described above, provided evidence that these simple models required modification. Thus, the finding that IgM-S414 forms a covalently assembled polymer requires that most C337-C337 and C575-C575 join μ chains in series, consistent with Models B and C, but not A (Fig. 3). Similarly, the finding of covalently assembled IgM-S575 polymers requires that C337-C337 is in series with C575-
C575, consistent with models A and B, but not C. Along the same line, the finding of non-covalently assembled IgM-S337, which upon denaturation yields covalent monomers and dimers, requires that C414-C414 is in some cases in series with C575-C575, consistent with models A and C but not B. Therefore, if we restrict the alternatives to models A, B and C, our findings would imply that IgM assembles in at least two ways (both A and B, both B and C, or both A and C).

Another interesting way of assembling IgM is modeled in Figure 1D, which we refer to as having crossed μ chains. In this case each of C414-C414, C337-C337 and C575-C575 is in series with the other two. If IgM is assembled according to such a model, mutant IgM lacking any one of the three cysteines will still be a covalently assembled polymer. In such a model, IgM-S337 non-covalent polymers argue that C414-C414 bonds are not always formed, consistent with early observations by Milstein et al (72). It is also possible that C575-C575 bonding is not orderly (as is assumed in above discussion). Thus, the flexibility of μtp allows any C575 to find a partner with any one of available C575. The μ tail pieces in an IgM polymer are in the middle of the polymeric circle, and as such are sufficiently close to each other to allow non-orderly interactions. This topic is dealt with in greater depth in the Discussion.

**Complement and IgM**

The classical pathway of complement dependent cytolysis is initiated when the latent protease of the first complement component, C1, is activated by a conformational change in C1 which is induced by multivalent binding of C1 to immunoglobulin. IgG and IgM, two immunoglobulin isotypes that can fix complement, have different requirements for C1 activation. In IgG, the C1 binding site is accessible, but free IgG in solution cannot activate complement because the affinity of C1 for IgG is low, and uncomplexed IgG does not sufficiently constrain C1 to impose a conformational change required for C1 activation.
Figure 3. Disulfide bond connectivity. A) C337-C337 is in parallel with C575-C575. Both C337-C337 and C575-C575 are in series with C414-C414. Human IgM is thought to assemble according to A. B) C337-C337 is in series with both C414-C414 and C575-C575. C414-C414 is in parallel with C575-C575. Mouse IgM (with fewer C414-C414 bonds) is thought to assemble according to B. C) C337-C337 is in parallel with C414-C414. Both C337-C337 and C414-C414 are in series with C575-C575. D) C337-C337 is in series both with C414-C414 and C575-C575. C414-C414 is also in series with C575-C575.
Many IgGs bound to a multivalent antigen provides enough avidity and sufficient immobility to bind and distort C1 into activation. In IgM, the C1 binding site is not accessible, and thus, unbound IgM is unable to bind C1. A single molecule of IgM bound to the surface of a cell, however, can activate complement (74, 75). This evidence suggests that interaction of IgM with a multivalent antigen either constructs or exposes a C1 binding site.

Early work using fragments of the IgM suggested that the C1 binding site was located in the Cµ4 domain (76, 77). However, there is increasing evidence that Cµ3 domain is the major if not exclusive site of C1 binding. First, of the Cµ domains, Cµ3 domain is most similar to Cγ2 domain which includes the binding site of IgG (73). Second, cytolytic activity of IgM was inhibited by monoclonal antibodies specific for the Cµ3 domain but not by those directed against other Cµ domains (78). Third, in an effort to identify the structural features of IgM which are necessary for C1 activation, two hybridoma mutants were selected that produced non-cytolytic IgM: in both cases the mutations in the IgM were localized to the Cµ3 domain (79, 80). Recently, using site-directed mutagenesis, Arya et al (81) have described two clusters of charged residues in the Cµ3 domain which when mutated, abrogate the cytolytic activity of the mutant IgM. These mutations, by analogy to IgG structure, are probably located on the Y face of the Cµ3 domain, but are distinct from the C1 binding site regions of the Cγ2 domain (82).

Aside from specific contact residues, the overall disposition of the IgM molecules has also been found to be important in complement activation. Thus, J chain deficient hexamers activate complement 10-20 fold better than J chain-containing pentamers (58, 60, 68). Beale and Fenstein (83) suggested that C414-C414 bonds (all C414-C414 bonds are formed in hexamers, whereas some C414-C414 bonds are formed in pentamers) rigidify the polymer such that the articulation of Fab arms to bind an immobilized antigen is able to expose or construct the C1 binding site. Davis et al (65) and Fazel et al (66) have confirmed
the importance of C414-C414 bonds for complement activation as IgM-S414 activates complement at least 25 fold less efficiently than IgM-wt irrespective of whether IgM-S414 is associated with J chain or not. It is also possible that six monomeric units in a limited space effect a bigger distortion in C1q than five monomeric units would be able to do. That is, the geometry of the IgM polymer may be more important than the rigidity of the IgM polymer. Along the same lines, the six arms of the C1q molecule may fit more tightly onto a hexameric molecule than onto a smaller pentameric molecule. The geometry of a better fit might translate into more efficient activation.
Rationale for the Thesis

Previous studies have shown that C414-C414 disulfide bond formation, unlike C337-C337 and C575-C575 bonds, is not necessary for assembly of covalent IgM polymers (31, 38, 58, 65). Moreover, relatively few C414-C414 bonds are normally formed in mouse pentameric IgM (72). By contrast, most or even all C414-C414 are thought to form in hexameric IgM (38). Hence, absence of J chain is correlated with increased C414-C414 bond formation. Intriguingly, J chain, C337 and C575 seem to pre-date C414 in evolution. While J chain is expressed even in invertebrates (46) and μ chains from all vertebrates studied to date contain C337 and C575, early vertebrates such as cartilaginous and bony fish lack C414 (84, 85). The IgM of these fish is generally pentameric and tetrameric respectively (86). Bony fish tetramers are, to some degree, non-covalently assembled (87-90), suggesting that C414 in mammalian IgM might promote complete covalent assembly. To test whether influence of J chain on polymer type depends on formation of C414-C414 and whether C414 plays a part in covalent assembly of mouse IgM, we analyzed the structure of secreted IgM molecules which bore serine in place of C414 and were produced in J chain-expressing and J chain-deficient hybridoma cells. Our results suggest some modifications to the standard model of IgM structure.
Methods

Cell culture

Vectors and transfected cell-lines have been previously described (58, 65). Tissue culture conditions are as in (79, 92). The CCL-1 fibroblast line was obtained from Dr. F. Tsui (University of Toronto, Toronto, Canada).

Preparation and analysis of IgM

IgM preparation. Labeled IgM was obtained by affinity purification or immunoprecipitation (58) of culture supernatants of cells that had grown overnight at an initial density of 1 x 10^6 cells/ml in 90% methionine-free DMEM and 10% normal DMEM, containing 12% FCS and 100 μCi/ml [35S]-methionine (SJ 204; Amersham, Arlington Heights IL). For immunoprecipitation we used anti-μ antibody (Jackson ImmunoResearch laboratories, West Grove PA) coupled to protein A-agarose.

SDS-PAGE analysis. SDS-PAGE (92) was performed with the following modifications: the upper gel contained 3.0% acrylamide, 0.40% agarose, 0.10% SDS and 0.38 M Tris-HCl, pH 8.8. The lower gel was as described containing 7.5% (non-reducing SDS-PAGE) or 12.5% (reducing SDS-PAGE) acrylamide. Samples were not reduced unless otherwise indicated. To reduce disulfide bonds, the material was boiled for 3 min in 5% (v/v) 2-mercaptoethanol. Samples were visualized by auto-radiography of vacuum-dried gels or by Western blot analysis. Auto-radiographs were quantified by PhosphorImager analysis. Prestained molecular mass markers (Life Technologies, Inc., Grand Island NY) were run with each sample.

Western blot analysis. Samples were transferred to nitrocellulose paper (Schleicher & Schuell, Keene NH), blocked with I-block (Tropix, Bedford MA), probed with either rabbit anti-human J chain with cross-reactivity to mouse J chain (BioGenex, San Ramon CA) followed by alkaline phosphatase-conjugated mouse anti-rabbit IgG (Jackson ImmunoResearch laboratories West Grove PA), alkaline phosphatase-conjugated
goat anti-mouse IgM μ-specific (Jackson) or alkaline phosphatase-conjugated goat anti-mouse kappa (CALTAG, San Francisco CA) antibodies. Blots were washed and J chain, μ or κ bands were visualized by Enhanced Chemiluminescence (ECL) using Nitro-Block II and CSPD substrate (Tropix) as per the manufacturer’s instructions. Luminescence was captured on X-ray film, X-OMAT AR (Kodak, Rochester NY).

**Alkaline urea-polyacrylamide gel electrophoresis (AU-PAGE) analysis.** Samples were reduced in 50mM dithiothreitol (in 100 mM Tris/HCl pH 8.0) and alkylated with 167 mM iodoacetic acid (in 500 mM Tris/HCl pH 8.8). Alkaline urea gel electrophoresis was done according to Reisfeld and Small (93), but urea was not included in the buffer chambers.

**Ultracentrifugation.** Fractionation of IgM was performed by centrifugation in 11-ml linear 5-20% sucrose density gradient as described (58), except that gradients were centrifuged at 23,000 rpm for 16.5 h in an SW41 rotor.

**Northern blot analysis.** 10 μg of total cytoplasmic RNA (94) was denatured with formamide and fractionated by electrophoresis in a 1% agarose gel containing formaldehyde. The blot was probed by a [32]P end-labeled J chain oligonucleotide 5'-GGTACACATGCATTTGTTGT-3' (Vetrogen, London ON), or by an actin DNA fragment that had been [32]P labeled by random priming (NEBlot Kit; New England Biolabs, Beverly MA). Samples were visualized by auto-radiography and quantified by PhosphorImager analysis.

**Hemolysis.** Complement-mediated hemolysis of TNP-coated sheep red blood cells (SRBC) was measured as previously described (79) except hemoglobin release was measured at 405nm.
Results

As noted above, the inter-μ chain disulfide bonds of wild-type mouse IgM-wt are mediated by cysteines C337, C414 and C575. To assess the importance of these disulfide bonds for IgM assembly and function, we have used a gene transfer system based on the Sp6 hybridoma cell line which produces IgM(κ) specific for TNP. We have expressed the normal and variously mutated μ heavy chain genes in the recipient cell lines, IgM10 and X10. These two mutant cell lines were independently derived from Sp6 and lack the μ gene but continue to produce J chain and the TNP-specific κ chain. The mutant products are denoted according to the substituted amino acid. For example, the mutant μ genes, resulting transfectants, and corresponding IgM are denoted as μ-S414, T/μ-S414, and IgM-S414, respectively, for the C414S substitution.

Secretion of IgM-S414 by J chain-deficient and J chain-expressing cell lines. Expression vectors for μ-wt, μ-S414, and μ-S575 were transfected into the κ-producing cell line, X10 (31), and the resulting IgM was examined for J chain. Parallel experiments were conducted with similarly produced IgM10 transfectants which had been generated previously and described in an earlier report from this laboratory (65). IgM was biosynthetically labeled with [35S]-methionine and affinity purified on TNP-Sepharose beads. The eluted material was then fractionated by alkaline-urea polyacrylamide gel electrophoresis (AU-PAGE) and analyzed by auto-radiography. As shown in Figure 4, J chain, the fastest-migrating band, was present in wild-type IgM and absent in IgM-S575, as expected. This analysis also revealed a difference between the earlier (igm10 transfectant) and more recent (X10 transfectant) cell lines in the composition of their IgM-S414: IgM-S414 produced by the original igm10 transfectant, T/μ-S414[0], lacked J chain, but IgM-S414 secreted by the independent X10 transfectants, T/μ-S414[I1-3], contained J chain (data for T/μ-S414[I2,3] not shown). The similar relative intensities of the J, μ and κ bands in wild-type IgM and in IgM-S414[I1], argue that the average J
Figure 4. J chain analysis of wild-type and mutant IgM. IgM secreted by the indicated cell lines was biosynthetically labeled and affinity purified. The IgM was reduced, alkylated and electrophoresed on a 6% alkaline urea gel. J chain is the fastest migrating band as indicated. (Mock) affinity purification of the culture supernatant of the μ− recipient cell line X10 is the negative control. "T IgM-S414" and "O IgM-S414" refer to IgM secreted by T/μ-S414 [I1] and T/μ-S414 [O], respectively.
Figure 5. Analysis of J chain mRNA. 10μg of cytoplasmic RNA was isolated from the indicated cell lines and fractionated by electrophoresis in 1% agarose. The gel was blotted onto nitrocellulose paper and probed with the J chain-specific oligonucleotide and actin gene fragment (see Materials and Methods). CCL-1, a fibroblast cell line was the negative control. "IgM-S414", "IgM-S414" and "IgM-S414" refer to IgM secreted by three independent T/μ-S414 transfecants. "O IgM-S414" refers to IgM secreted by T/μ-S414 [O]. J chain mRNA levels of four independent T/μ-S575 ranged from 80 to 470% of Sp6 hybridoma.
chain content of secreted IgM-S414 and IgM-wt was similar. Therefore, there was no correlation between the lack of inter-μ disulfide bonds at C414 and the incorporation of J chain into polymeric IgM, contrary to the conclusion expressed in the previous report (65).

To understand the basis for the difference between these various μ-S414 transfectants, we compared the J chain mRNA level of the T/μ-S414 [O] and T/μ-S414 [I1-3] transfectants. As shown by Northern blot analysis (Fig. 5), J chain mRNA was evident for all transfectants except T/μ-S414[O], for which J chain mRNA was undetectable. The reason for the lack of normal J chain mRNA in this transfectant is unknown.

Effects of J chain on assembly of IgM-S414. The availability of transfectants which produced IgM-S414 in the presence or absence of J chain allowed us to examine whether J chain alters the assembly or function of IgM-S414. To investigate the effects of J chain on IgM-S414 assembly we analyzed radiolabeled, affinity-purified IgM by SDS-PAGE (Fig. 6). The IgM was then visualized by auto-radiography (Fig. 6A) or by Western blot (Fig. 6B). As indicated in Figure 6, both IgM-wt and IgM-S414 appeared in several discrete polymeric species. For simplicity, we refer to the different IgM-S414 species as S1 to S5, as indicated in Figure 6B. Previous electron-microscopic studies of J-minus IgM-S414 (secreted by T/μ-S414 [O]) indicated the presence of two major polymeric species, pentamers and tetramers (65), and on this basis we suppose that S5 and S4 contained pentamers and tetramers, respectively. The difference in mobility of IgM-wt and IgM-S414 pentamers (Fig. 6A) likely reflects different conformations caused by different disulfide bonding. As indicated by probing with anti-μ antibodies in Figure 6B, both J-plus (secreted by T/μ-S414 [I]) and J-minus IgM-S414 contained species S1-S5, but J chain affected the fraction of IgM appearing as S2 and S3. PhosphorImager analysis of the same preparations indicated that in J-minus IgM-S414 there was nearly three fold more S3 (8-9% compared to 2-3%), and six fold more S2 (7-10% compared to 1-3%) compared to J-plus IgM-S414 (as also seen in Figure 8). By contrast, production of the S5, S4 and S1 species was affected only slightly, if at all, by J chain. Thus, S5, S4 and S1
Figure 6. Analysis of IgM structure and J chain content by SDS-PAGE. IgM was labeled biosynthetically to a specific activity of 3-4x10⁴ cpm/μg and affinity purified. This material was then analyzed by SDS-PAGE and immunoblotting, as follows. (A) The IgM was fractionated using a single phase 3% acrylamide/0.40% agarose SDS-gel. The gel was dried and the IgM was visualized by auto-radiography. (B) The IgM was fractionated using a single phase 3% acrylamide/0.40% agarose SDS-PAGE. The gel was blotted onto nitrocellulose, probed with J chain-specific or μ chain-specific antibodies (Ab μ; Ab J, as indicated) and visualized by enhanced chemiluminescence (ECL). The apparent molecular weight of the S1-S5 species is indicated on the right. IgM-wt pentamers, indicated by (μ2κ2)5J, and hexamers, indicated by (μ2κ2)6, were used as standards to estimate the molecular weight of S5-S1 species.
constituted 45, 25 and 10% respectively of total J-minus IgM-S414, compared to 55, 30 and 12% for J-plus IgM-S414.

As noted above, the AU-PAGE assay system indicated that IgM-S414 incorporated J chain. To determine which of the species, S1-S5, incorporated J chain, we analyzed the material shown in Figure 6B by probing with J chain-specific antibodies. Control experiments showed that the anti-J chain antibody reacted ~5-10 fold more strongly with J-containing IgM than with J-deficient IgM (data not shown), providing a sufficient window for detecting J chain in IgM. These blots showed that J chain is present in the S5 and S4 species but not in the S1 species. J chain was not detected in species S2 and S3, but this negative result might have reflected the low abundance of these species.

To investigate whether IgM-S414 was synthesized as non-covalent structures which would have been disrupted in the SDS-PAGE analysis, we fractionated IgM by sucrose density gradient ultracentrifugation under non-denaturing conditions (Fig. 7) before subjecting the samples to SDS-PAGE. A typical analysis is presented in Figures 7 and 8. Most of the IgM-wt (Fig. 7A) was polymeric (peak fractions 44, 45), but some IgM-wt migrated as a monomer (peak fractions 19, 20). Although the major component of both J-plus (Fig. 7B) and J-minus (Fig. 7C) IgM-S414 sedimented similarly to IgM-wt, i.e., as a full sized polymer, there were several interesting differences between J-plus IgM-S414, J-minus-IgM-414 and IgM-wt. IgM-wt polymers migrated as a broad peak (fractions 48-52; Fig. 7A) which, as previously reported, contains distinct pentameric and hexameric populations migrating at the trailing and the leading edges, respectively (58). By comparison, J-plus IgM-S414 migrated as a narrow, more homogeneous peak (fractions 35-50; Fig. 7B), while J-minus IgM-S414 contained a substantial amount (~20-30%) of material which migrated as smaller, intermediate-sized polymers (fractions 25-35; Fig.7C).
Figure 7. Fractionation of IgM by sucrose density gradient centrifugation. IgM produced by the indicated cell lines was biosynthetically labeled (3-4x10^4 cpm/µg), affinity-purified and centrifuged through a linear, 11 ml 5-20% sucrose gradient. The radioactivity present in 10µl of each fraction of (A) wild-type IgM, (B) J-plus IgM-S414, and (C) J-minus IgM-S414 is shown. The sucrose gradients are representative of at least two independent experiments. The top of the gradient is to the left.
Finally, there was a lower proportion of monomers (fractions 15-25) in IgM-wt than in (J-plus and J-minus) IgM-S414. To confirm that the differences in sedimentation rate did not reflect differences in the size of the μ and κ chains, we analyzed the different species by SDS-PAGE after reduction. These results (not shown) indicated that the μ and κ chains had the same mobility whether they derived from monomeric, intermediately sized, or polymeric IgM-S414, arguing that the differently sedimenting species contained different numbers of similarly sized immunoglobulin chains.

To visualize the covalent structures within the polymeric peak, the fractionated IgM was examined by SDS-PAGE (Fig. 8). Quantification of these species by PhosphorImager indicated that both S5 and S4 of J-plus IgM-S414 (Fig 8A) peaked in fraction 41-44. However, while the S5 species was symmetrically distributed in the SDS-PAGE analysis of the sucrose gradient fractions, S4 had a substantial shoulder on the slower-sedimenting side. Interestingly, the smaller species S1, S2, and S3 peaked in fractions 43-44. This distribution implies that these low molecular weight species must have derived from the polymers. These smaller species were not artifacts of boiling-induced auto-reduction in SDS buffer, as control experiments showed no detectable difference between boiling in the presence or absence of 0.5 M iodoacetic acid, pH 8.6 (data not shown). We conclude that the J-plus IgM-S414 preparation contained two types of pentamers: one type was covalently assembled and migrated as S5, while the other type was partially non-covalent and dissociated in SDS-PAGE to S4 and S1 and to S3 and S2. As outlined in the legend of Figure 8 we estimate that 7-17% of the pentamers and tetramers in fractions 39 to 50 of the polymeric peak was non-covalently assembled. The discordance between the S1 and S4 distributions argues that some S4 derived from material which sedimented more slowly than the major pentameric IgM-S414. That is, S4 derived from two sources, material which sedimented as a pentamer and dissociated into S1 and S4, and material which migrated more slowly and yielded only S4, presumably an IgM-S414 tetramer. Accordingly we refer to this latter material as free tetramers.
Figure 8. Detection of non-covalent IgM-S414. Equal volumes of the indicated fractions from the sucrose density gradients of Figure 7 were immunoprecipitated and fractionated by SDS-PAGE (two phase, discontinuous 3% acrylamide/0.40% agarose -- 7.5% acrylamide gel). The GI "band" is material accumulated at the gel-interface. Position of molecular weight standards are indicated. The species S1-S5 are defined in Figure 3. The amount of non-covalent assembly was calculated as \(((5S1+S2+S3)/(5+S4-(4S1)))\). This represents the percentage of pentameric and pre-existing tetrameric material that dissociated into smaller IgM. The degree of non-covalent assembly is shown in the box below each panel. "nd" denotes that non-covalent assembly could not be detected(<0.5%).
Figure 9. Analysis of J chain content of IgM-S414. The indicated fractions from the gradients presented in Figure 4 were immunoprecipitated, fractionated by SDS-PAGE (discontinuous 3% acrylamide/0.40% agarose -- 7.5% acrylamide gel). The gel was blotted onto nitrocellulose paper and (A) visualized by auto-radiography or (B, C) probed with J chain-specific antibodies and visualized by ECL. (C) is a longer exposure of (B). S5 and S4 species are defined in figure 6. The negative control was a (mock) immunoprecipitation without any added IgM.
In the case of J-minus IgM-S414 (Fig. 8B), we did not detect the S1 species in polymeric fractions, and the S2 and S3 species did not peak in the fractions corresponding to polymeric IgM. This distribution implies that most, if not all, S2 and S3 derived from the smaller (i.e., intermediate) polymers in the sucrose gradient. Therefore, J-plus IgM-S414 contained more non-covalently assembled polymers than did J-minus IgM-S414, and conversely, that J-minus IgM-S414 included more pre-existing intermediate species than did J-plus IgM-S414.

As shown in Figure 9, both S5 and S4 contained J chain. As noted above, the distribution of the S4 and S1-S3 species observed in Figure 8 argues that the S4 species arose both from dissociation of non-covalently assembled S5 and from free tetramers. To ascertain whether S4 derived from free tetramers contained J chain, fractions 35-38, in which most of the S4 is pre-existing, as well as fractions 39, 40 of J-plus IgM-S414 were immunoprecipitated, fractionated by SDS-PAGE, and probed with J chain-specific antibodies. The relative amount of IgM in each band was estimated from the μ-specific blot shown in Figure 9A, while Figures 9B and 9C show the J chain content. This analysis shows that free tetramers contain J chain. Our data, however, does not indicate whether the S4 species derived from non-covalently assembled pentamers contain J chain or not.

Cytolytic activity of IgM-S414. Previous data showed that polymeric (J-minus) IgM-S414 was at least 25-fold less efficient than polymeric IgM-wt in activating complement-mediated hemolysis (65). To assess the cytolytic activity of J-plus IgM-S414, we measured the amount of IgM required to lyse a standard number of TNP-coated SRBC, as determined by hemoglobin release. As shown in Figure 10, lysis by IgM-wt was proportional to added IgM up to a concentration of 40 ng/ml. Little, if any, lysis was seen for either J-plus or J-minus IgM-S414 throughout this concentration range. The cytolytic activity of IgM-wt was not inhibited by the simultaneous addition of up to 40 ng/ml of IgM-S414. These results indicate that J-plus as well as J-minus IgM-S414 was at least 25-fold less hemolytic than wild-type IgM.
Figure 10. Cytolytic activity of wild-type and mutant IgM. IgM produced by the indicated cell lines was affinity purified and eluted. This material was not further fractionated, because IgM-S414 and IgM-wt were predominantly polymeric. DNP-alanine was removed by dialysis, and the IgM was pre-incubated at the indicated concentrations with TNP-coupled erythrocytes. Complement was added, and after a 15 minute incubation the amount of released hemoglobin was measured by the increased absorbance at 405 nm. Complete lysis was defined as released hemoglobin after treatment of SRBC with SDS; percentage of unlysed SRBC was calculated as [(1 - (ODobserved / OD @ 100% lysis)) x 100]. To ascertain whether IgM-S414 might inhibit lysis by IgM-wt, 5 ng/ml IgM-wt supernatant was mixed with varying (0 to 40 ng/ml) concentrations of either J-plus or J-minus IgM-S414 before incubation with the erythrocytes. Released hemoglobin, under these conditions, was not affected by the amount of added IgM-S414 (results not shown).
Discussion

Our analysis of secreted IgM-S414 has revealed several interesting features:

1) Regardless of J chain, IgM-S414 is produced predominantly as (noncytolytic) pentamers and tetrasmers but not as hexamers.
2) The J chain content of IgM-S414 is comparable to that of IgM-wt.
3) J-plus IgM-S414 includes some non-covalently assembled pentamers which are absent in J-minus IgM-S414.
4) J-minus IgM-S414 includes some smaller species (dimers, trimers) which are absent in J-plus IgM-S414.

Several findings taken in the context of the conventional model of IgM (Fig. 11A), in which only adjacent μ chains are disulfide bonded, have suggested that J chain might be needed to circularize the IgM by bridging a gap imposed by formation of C414-C414 bonds. As presented below, our finding of non-covalent pentamers and smaller polymers is difficult to fit with this model. Although the existence of these forms might reflect effects of J chain on secretion kinetics or polymerization, the evidence for such effects is controversial (61, 62). This Discussion will, therefore, focus on how our observations bear on models for the intermolecular disulfide bonding in pentameric IgM.

The hypothesis that J chain might bridge a gap resulting from formation of C414-C414 derives from evidence which relates cytolytic activity and C414-C414 formation and suggests that normal pentameric and hexameric IgM differ in their C414-C414 bonding. Thus, hexameric IgM-S575 which bears six C414-C414 per polymer is 10-20 fold more cytolytic than pentameric IgM-S337, which appears to bear only one C414-C414 per polymer (38, 58, 65). The finding that the cytolytic activity of these mutant pentamers and hexamers was in each case similar to that of wild-type IgM pentamers and hexamers (65) suggested in turn that IgM-wt pentamers bear one C414-C414 bond while IgM-wt hexamers bear more than one, perhaps six, C414-C414 bonds. The role of C414 in cytolytic activity is not established, but it has been suggested that C414-C414 bonds
rigidify the IgM polymer such that binding of antigen can induce a conformational change
and thereby unmask a C1 binding site (83). However, it must be noted that tetrameric bony
fish IgM and pentameric cartilaginous fish IgM, despite a lack of C414, are still able to
activate their respective classical complement pathway (95, 96). It could be that these fish
have evolved a different mechanism to prevent activation of complement proteins by
unbound IgM.

The proposition that pentamers and hexamers differ in the extent of C414-C414
formation suggested a model to explain why J chain is found in pentamers but not in
hexamers (65). The underlying assumptions for this model were that J chain covalently
links the first and the last monomeric unit to complete the polymeric circle (48, 52) (as
depicted in the conventional model) and that circularization is a necessary step in IgM
maturation or secretion. According to this model, formation of C414-C414 closes the gap
between the corresponding subunits, thus increasing the gap between other subunits
(compare Fig. 11B with 11C and 11D). Formation of fewer than four C414-C414 creates a
gap that can be bridged by J chain, but not by the Cμ4-tail domains alone (Fig. 11C).
Formation of four C414-C414 bonds in an uncircularized pentamer results in a final gap
which can be spanned by incorporating one more monomer (Fig. 11D) but not by J chain.
Consequently, J chain is found in pentamers but not hexamers.

Our present data as well as a previous analysis by electron microscopy (65) indicate
that J-minus IgM-S414 is assembled as tetramers as well as pentamers. The presence of
these tetramers argues against this Bridge-the-Gap model, as follows. The inter-subunit
gaps in a J-deficient tetramer (Fig. 11E) should be no larger than the gaps in a pentamer
bearing three C414-C414 bonds (Fig. 11F), as calculated in the legend of Figure 11. If
Cμ4-tail interactions are sufficient to bridge the gaps around a J-deficient tetramer, then
Cμ4-tail interactions should be sufficient, without requiring J chain, to bridge the gaps in
IgM-wt pentamers bearing three or fewer C414-C414 bonds. Therefore, J chain should not
be necessary to circularize pentameric IgM-wt, even when C414-C414 bonds form.
Figure 11. Conventional model of IgM structure. For simplicity, Cμ2-Cμ3-Cμ4-tail domains are drawn to represent an IgM heteromer; inter-μ disulfide bonds are illustrated by either a filled circle (C337-C337 bond connecting Cμ2 domains) or thick lines (C414-C414 or C575-C575 bonds connecting Cμ3 or Cμ4-tail domains respectively). (A) IgM-wt
pentamer as conventionally depicted. All C414-C414 bonds have formed in parallel with C575-C575 and in series with C337-C337 bonds. This model, by convention, does not consider C414-C414 induced gaps (see below) (B-F) Bridge-the-Gap model. Each monomeric unit occupies 60° of the polymeric circle, assuming that a hexamer completely fills the circle and that IgM monomers are inflexible. As illustrated, the angle between adjacent Cμ4-tail domains relates to the size of the gap between monomers. By assumption, there is no gap between monomers that are linked through C414-C414 bonds. (B) IgM pentamer without C414-C414 bonds has five gaps averaging 12° each: (360° - 5 monomers x 60° / monomer) / 5 gaps = 12° / gap. These gaps can be spanned by Cμ4-tail interactions as illustrated. (C) IgM pentamer with three C414-C414 bonds has one gap larger than 30° which can be bridged by J chain but not by Cμ4-tail interactions. (D) IgM pentamer with four C414-C414 bonds has one gap of 60° which can be bridged by an additional IgM monomer but not J chain. (E) IgM-S414 tetramer has four gaps averaging 30° each: (360° - 4 monomers x 60° / monomer) / 4 gaps = 30° / gap. (F) IgM-wt pentamer with three C414-C414 bonds has two gaps of 30° each: (360° - 5 monomers x 60° / monomer) / 2 gaps = 30° / gap, similar in size to gaps in J-minus IgM-S414 tetramer.
Our observation that J-plus non-covalent and J-minus intermediate polymers are secreted, also argues against the conventional model. Detection of non-covalent and small polymers indicates that some μ chains are not joined in series by C337-C337 and C575-C575 bonds. Several observations argue that these polymeric forms of IgM do not reflect failure to form C337-C337 or C575-C575. First, Sitia and colleagues (26, 27, 33, 97) have reported results arguing that free C575 would prevent secretion of IgM. Second, the conventional model in which C414-C414 join μ chains in parallel with C575-C575 predicts that IgM-wt lacking C337-C337 would be non-covalently assembled. However, IgM-wt does not dissociate into smaller units in SDS-PAGE. Therefore the conventional model in which only adjoining μ chains are linked by C575-C575 implies that IgM would either be secreted as a fully covalent polymer or as monomers (31), contrary to our detection of intermediate J-minus and non-covalent J-plus polymers.

In order to conserve formation of all C337-C337 and C575-C575 bonds and yet account for the above observations, we propose a model in which C575-C575 disulfide bonds and J chain can link non-adjoining as well as adjoining μ chains (Fig. 12). According to this model, tetrameric and pentameric IgM-wt polymerize as a smaller circle (smaller radius) than hexameric IgM (Fig. 12A-C), as the subunits flex between domains like scissors-tongs to maintain the inter-domain contacts. In contrast with the Bridge-the-Gap model, J chain interacts with two non-adjoining μ chains (Fig. 12B), and this interaction depends on the diameter of the inner-circle. Thus, if the two μ chains are separated as illustrated for hexameric IgM (Fig. 12C), J chain cannot span the inner-circle. Conversely, incorporation of J chain restricts the size of the inner-circle and thus restricts the number of monomeric units accommodated into the polymer.

This model provides a ready explanation for our findings that J-plus IgM-S414 includes some non-covalently assembled pentamers, and that J-minus IgM-S414 includes some smaller polymers. To account for the presence of non-covalent as well as covalent J-plus IgM-S414 pentamers, we consider first the structure depicted in Figure 12E, in which
J chain joins \( \mu \) chains #2 and #6. The \( \mu \), \( \kappa \) and J chains are covalently bound in one unit \( \mu_{10}k_{10}J \) (species S5). In Figure 12F, however, J chain joins \( \mu \) chains #2 and #5. This structure includes two covalent units (\( \mu_{1-6} \) and \( \mu_{7-10} \)), and on SDS-PAGE is expected to dissociate into \( \mu_{6k6}J \) (species S3) and \( \mu_{4k4} \) (species S2). Figure 12F also suggests an explanation for the smaller polymers of J-minus IgM-S414, as the absence of J chain might permit \( \mu \) chains #9 and #8 to join rather than \( \mu \) chains #10 and #8 with the result that IgM-S414 circular dimers and trimers are pinched off. That is, incorporation of J chain is proposed to hinder some specific \( \mu-\mu \) interactions and thus to direct synthesis of pentameric IgM molecules.
Figure 12. Inner-Circle model. Inter-domain contacts are maintained by flexing IgM monomers that resemble scissors-tongs. By hypothesis, J chain joins two opposing \( \mu \) chains spanning the inner-circle, and the size of the inner-circle in hexamers is too large to be spanned by J chain. (A) IgM-S414 tetramer: the C\( \mu \)4-tail domains are in close proximity, and extensive non-adjoining inter-\( \mu \) interactions occur. (B) IgM-wt pentamer. (C) IgM-wt hexamer: \( \mu \) chains are sufficiently spaced such that only adjoining inter-\( \mu \) interactions take place. (D) IgM monomers from a tetramer, pentamer and a hexamer (left to right) to illustrate the degree of flexing in different sized polymers. (E) Covalent IgM-S414 pentamer in which J chain links \( \mu \) chains \#2 and \#6. (F) Non-covalent IgM-S414 pentamer in which J chain links \( \mu \) chains \#2 and \#5.
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