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Intermolecular disulfide bonding in IgM: effects of replacing cysteine residues in the μ heavy chain

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The conventional model of polymeric IgM depicts a unique structure in which the μ heavy chains and J chain are joined by well defined disulfide bonds involving cysteine residues at positions 337, 414 and 575 of the μ chain. To test this model, we have used site directed mutagenesis to produce IgM in which these cysteines have been replaced by serine. In each case the single mutants were able to assemble polymeric IgM, which was analyzed for its size, morphology, J chain content and activity in complement dependent cytolysis. Whereas normal polymeric IgM is composed predominantly of pentameric and hexameric molecules, the mutant IgM-Ser414 is covalently assembled as pentamers and smaller forms; IgM-Ser575 is assembled as covalent hexamers. IgM-Ser337 appears to include the same pentameric and hexameric forms as normal IgM except that, unlike normal polymeric IgM, most pentameric/hexameric IgM-Ser337 is not covalently assembled. J chain is present in polymeric IgM-Ser337 but absent in polymeric IgM-Ser414 and IgM-Ser575. IgM-Ser414 is defective in activating the classical pathway of complement dependent cytolysis. Our observations are consistent with models in which the covalent linkages between μ chains are mediated by disulfide bonded Cys337-Cys337, Cys414-Cys414 and Cys575-Cys575 but indicate that the arrangement of these Cys-Cys pairs in series and in parallel varies among and within IgM molecules. Our results suggest that (i) formation of Cys414-Cys414 rigidifies the IgM; (ii) this rigidity is necessary for activation of complement component C1; (iii) assembly of hexameric IgM is associated with formation of all possible Cys414-Cys414; (iv) pentameric IgM lacking J chain can be assembled if no Cys414-Cys414 forms; and (v) pentameric IgM containing J chain is assembled if some, but not all, Cys414-Cys414 form.

Key words: disulfide bonds/J chain/polymeric IgM/site-directed mutagenesis

Introduction

Immunoglobulins (Ig) possess two distinct types of polypeptide chain, light (L) and heavy (H) chains, held together by both covalent and non-covalent bonds to form the so called immunoglobulin H_2L_2 monomer. The polymeric immunoglobulins, IgA and IgM, assemble multiples of the H_2L_2 monomer subunit. IgA is produced as dimers, and to a lesser extent trimers and tetramers, in addition to monomers (Della Corte and Parkhouse, 1973). IgM is assembled predominantly into pentamers, although hexamers and monomers are also detected (Parkhouse *et al.*, 1970; Dodler, 1971; Eskeland and Christensen, 1975; Cattaneo and Neuberger, 1987; Davis *et al.*, 1988). In addition, polymeric Ig contains a third polypeptide, J chain, which is covalently linked at a ratio of one J chain per polymer (Halpern and Koshland, 1973; Chapuis and Koshland, 1974).

The traditional model of IgM depicts five monomer subunits joined by disulfide bonds in a circular array, and derives from a combination of mol. wt analyses and electron microscopic observations. Studies in which IgM was partially reduced to monomers to label the resulting cysteine residues indicate that in the human μ heavy chain there are four cysteines available for intermolecular disulfide bonding, at positions 136 (Cμ1 domain), 337 (Cμ2 domain), 414 (Cμ3 domain) and 575 (tail) (Figure 1a; Beale and Feinstein, 1969). By analogy with IgG, Cys136 is assumed to form a disulfide bond with the light chain. Figure 1b shows one particular model for inter-μ chain disulfide bonding, where μ chains are joined by the pairs Cys337-Cys337, Cys414-Cys414 and Cys575-Cys575. As depicted in this model, Cys337-Cys337 is said to join μ chains in parallel with Cys575-Cys575, and these two pairs are said to be in series with Cys414-Cys414. This model was originally suggested on the basis of work which identified the cysteine residues released after partial reduction of polymeric human IgM (Beale and Feinstein, 1969; Milstein *et al.*, 1975). In their study of mouse IgM, Milstein *et al.* (1975) did not detect the use of Cys414 in inter-μ chain bonding, suggesting that Cys337-Cys337 and Cys575-Cys575 might be arrayed in series (Figure 1c). Analysis of disulfide linked peptides has suggested that J chain binds to Cys575 (Mestecky and Schrohenloher, 1974). The finding that J chain containing dimers (μ_4L_4) could be recovered without reducing the cysteines of J chain has suggested that J chain bridges two monomers (Chapuis and Koshland, 1974). The reasons for supposing that J chain might be bound by Cys414 as well as Cys575 are considered in the discussion, as are the distinctions between models (b)-(e) in Figure 1.

In order to test the importance of the various cysteine residues in IgM assembly and function, we have specifically mutated the mouse μ heavy chain gene to substitute serine residues for the various cysteines. We report here our observations on the structure and cytolytic activity of the IgM assembled from these mutant μ chains.

Results

Production of mutant IgM

We have assumed that Cys136 binds only to the light chain and constructed mutants in which one of the other three

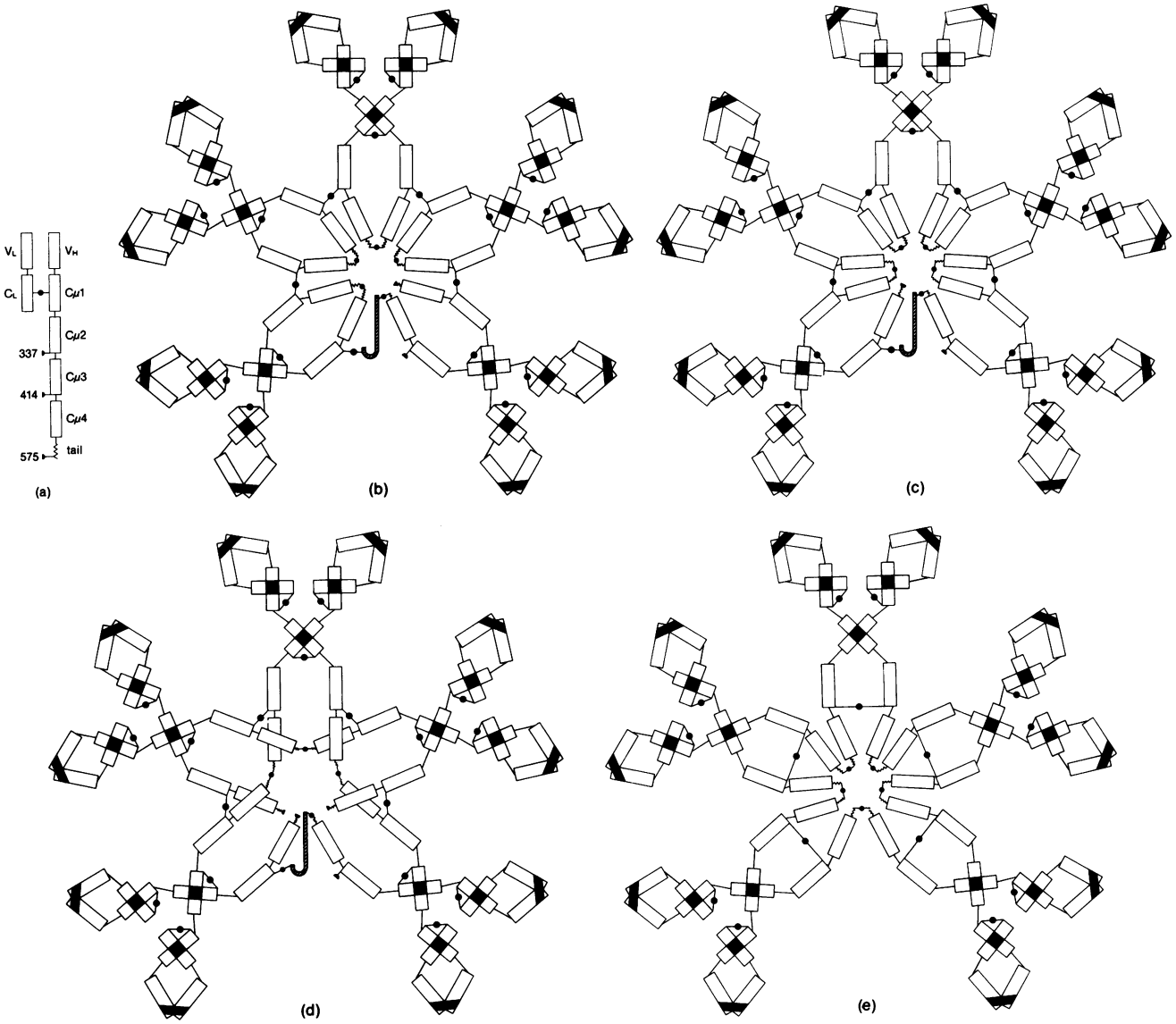

Fig. 1. Models of IgM structure. The domains of the variable (V) and constant (C) regions of the L and H chains are represented as open rectangles. The carboxy-terminal 19 residue tail is represented by the jagged line. Disulfide bonds are represented as —●—, unpaired cysteine residue as —◀. The black quadralaterals indicate domains which are held together by non-covalent interactions. J chain is shown here as bonded to Cys414 as well as to Cys575 (see Discussion). (**a**) Location of the cysteine residues which are available for inter-molecular disulfide bonding. (**b**) The homo pair Cys337–Cys337 is in parallel with Cys575–Cys575, and both are in series with Cys414–Cys414. (**c,d**) The homo pair Cys337–Cys337 is in series with Cys414–Cys414 and Cys575–Cys575. In (**c**) Cys414–Cys414 and Cys575–Cys575 join μ chains in parallel. In (**d**) Cys414–Cys414 and Cys575–Cys575 join μ chains in series. (**e**) Covalently assembled polymeric IgM-Ser337, where Cys414–Cys414 is in series with Cys575–Cys575.

cysteine residues was altered to serine. The naming convention for the mutants is that the altered amino acid is designated. Thus, we constructed the mutant vectors pμ-Ser337, pμ-Ser414 and pμ-Ser575, as well as the double mutant pμ-Ser414Ser575, as described in Materials and methods. The gene transfer system used here is derived from the hybridoma, Sp6, which produces mouse IgM(κ) which binds the haptens dinitrophenyl (DNP) and trinitrophenyl (TNP) with equal affinity. The TNP specific μ gene is inserted into the transfer vector pSV2neo and is assayed by transfer to the Sp6 derived mutant cell line, igm10, which lacks the μ gene of Sp6 but still produces the TNP specific κ chain. The wild type and mutant μ genes were transferred to igm10 and those G418 resistant transformants producing the highest amounts of IgM were subcloned and analyzed further. To control for the possibility that an extraneous mutation had occurred during vector construction or propogation, two independent pμ-Ser414 and pμ-Ser414Ser575 plasmids were transferred into igm10.

Effects of mutations on IgM assembly

IgM was biosynthetically labeled, affinity purified and sedimented through a sucrose gradient under non-denaturing conditions. The sedimentation profiles of mutant and wild-type IgM are shown in Figure 2(a–c). As described previously (Davis *et al.*, 1988), IgM-wt preparations include 3–10% monomers (peak fraction ~15) and 70–90% polymers; the polymers are heterogeneous, such that the major species are pentamers (peak fraction ~38) and hexamers (peak fraction ~42). The amount of hexamer varies substantially in different preparations, as can be

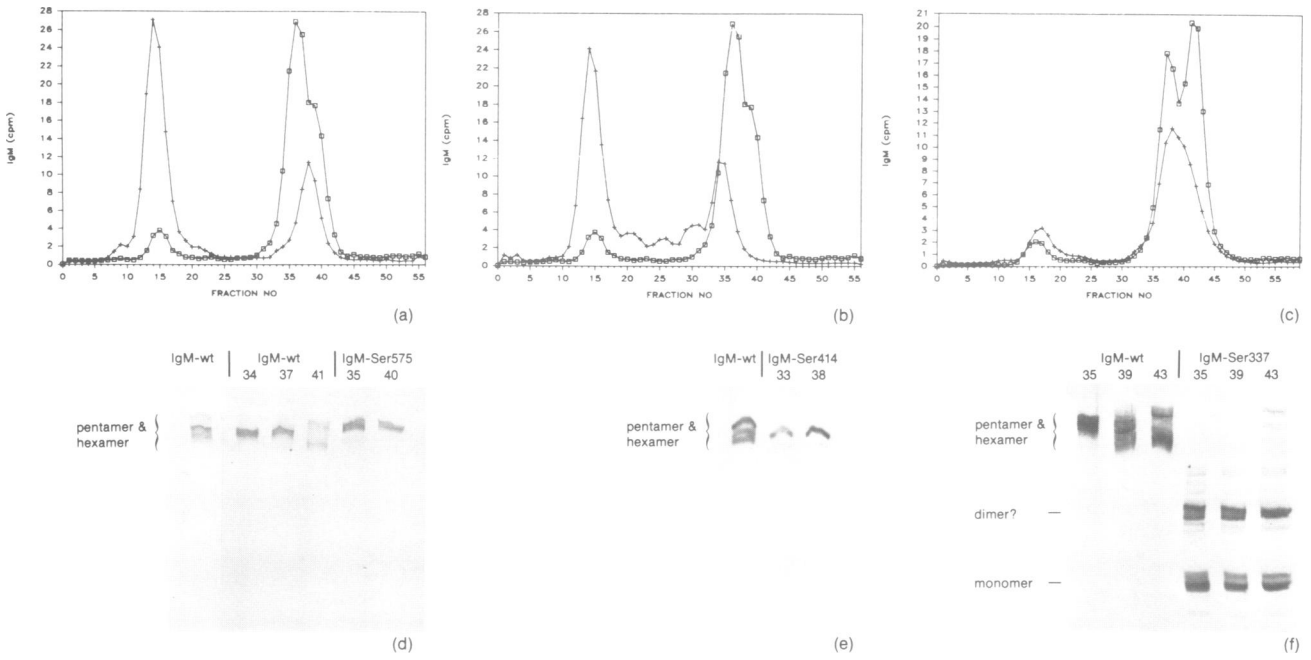

Fig. 2. Comparison of mutant and wild-type IgM. (**a**–**c**) Sucrose gradient fractionation. Wild-type (□——□) and mutant (+——+). IgM was biosynthetically labeled, affinity purified and sedimented on parallel sucrose gradients. (**a**) IgM-wt and IgM-Ser575, (**b**) IgM-wt and IgM-Ser414, (**c**) IgM-wt and IgM-Ser337. The top of the gradient is to the left. (**d**–**f**) The indicated fractions of mutant and wild type IgM were analyzed by SDS–PAGE without reduction of disulfide bonds. The left lanes in (d) and (e) contain unfractionated, i.e. uncentrifuged, IgM-wt.

appreciated by comparing panels (a) and (c). The double mutant, IgM-Ser414Ser575, sediments in a single peak which co-migrates with monomeric IgM-wt (data not shown). Sucrose gradient sedimentation of the IgM mutants in which a single cysteine was altered show that they all assemble polymers, although with different efficiencies. The amount of polymer varies from one experiment to another, but in general IgM-Ser575 and IgM-Ser414 assemble 20–40% of the secreted immunoglobulin into polymers of similar size to IgM-wt, whereas 65–75% of secreted IgM-Ser337 is polymeric. Most of the other IgM is monomeric, with the exception of IgM-Ser414, which assembles species intermediate between monomer and wild-type polymer. The sedimentation rate of the polymeric peak is different for the different mutants. The IgM-Ser575 polymer peak co-sediments with the faster IgM-wt species (Figure 2a). A small shoulder preceding the main polymer peak (5–10% of the polymer) is also present. The fastest IgM-Ser414 polymer co-sediments with the slower IgM-wt species (Figure 2b). IgM-Ser337 yields a broad band of polymeric IgM in a profile similar to IgM-wt preparations (Figure 2c).

We examined the polymeric IgM molecules on SDS–PAGE to determine if these polymers were held together by covalent bonds (Figure 2d–f). Under non-reducing conditions, no free light chains nor light chain dimers were evident, confirming that the light chains are covalently bound to the μ chain and that Cys136 of the μ chain is unavailable for inter-μ chain bonds. As we have previously reported, IgM-wt polymers migrate as three bands on SDS–PAGE: the slower sedimenting IgM-wt corresponds to the band of intermediate mobility on SDS–PAGE and was identified by electron microscopy as pentameric IgM. The more rapidly sedimenting IgM which contains hexamers gives two bands on SDS–PAGE, one which migrates slower than the pentamer and one which migrates more rapidly (Davis *et al.*, 1988).

The IgM-Ser575 and IgM-Ser414 polymers are both covalently assembled. IgM-Ser575 from the main polymer peak of the sucrose gradient co-migrates on SDS–PAGE with the slow band from IgM-wt (Figure 2d), and has been shown to be predominantly hexameric IgM (Davis *et al.*, 1988). A minor component of faster mobility is also seen in fraction 35 from IgM-Ser575. The IgM-Ser414 polymer co-migrates on SDS–PAGE with the intermediate pentameric IgM-wt band (Figure 2e). In contrast, the IgM-Ser337 polymers give multiple species on SDS–PAGE (Figure 2f). One of the predominant bands migrates as a monomer, whilst the slower major band might be a dimer; less intense bands migrating between monomer and pentamer/hexamer are also seen. Thus, many of the IgM-Ser337 pentamers/hexamers purified on the sucrose gradient are held together by non-covalent interactions. Fractions from the sucrose gradients shown in Figure 2 were also analyzed after reduction of disulfide bonds. After reduction the mutant and wild-type polymeric IgM yielded heavy and light chain bands of the same mobility on SDS–PAGE (data not shown), implying that the heterogeneity seen on the sucrose gradients was not due to contaminating non-immunoglobulin (radiolabeled) structures.

Analysis of the μ heavy chains of IgM-Ser337 argues that the monomeric IgM-Ser337 does not derive from non-covalently assembled polymeric IgM-Ser337 which degraded in the course of purification. That is, we have previously shown that the μ chains of monomeric IgM are glycosylated differently from the μ chains of the polymer, and as a consequence migrate more slowly in SDS–PAGE (Davis *et al.*, 1989). The same difference is seen between the μ chains of monomeric and polymeric IgM-Ser337 (data not shown), arguing that the monomeric IgM-Ser337 was secreted in the monomer form.

The implications of these findings for the pattern of disulfide bonding are considered further in the discussion.

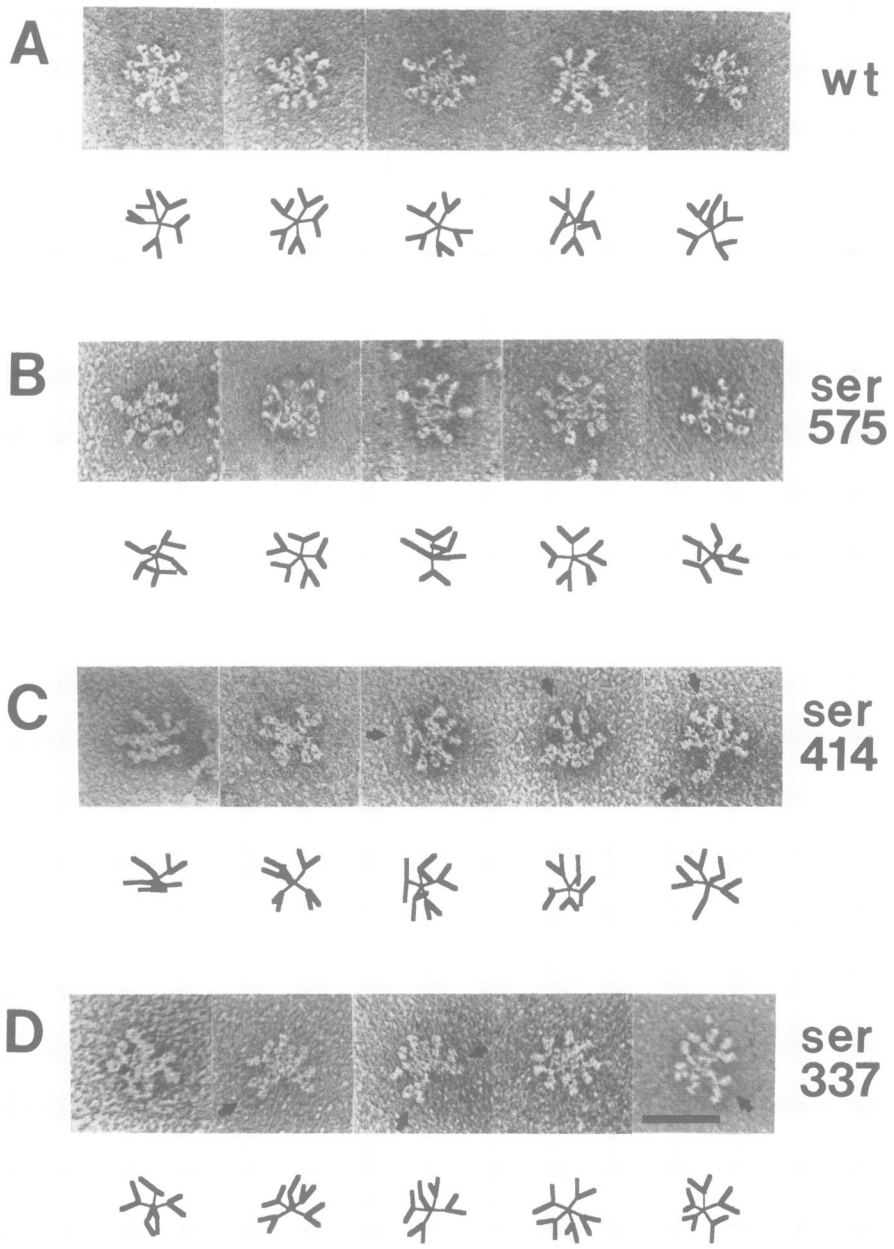

Fig. 3. Electron micrographs of mutant and wild-type pentameric IgM. Arrows indicate monomer subunits with exposed Fc regions, as described in the text. The bar is 40 nm. See Davis *et al.* (1988) for comparisons of hexameric and pentameric IgM.

Electron microscopy of IgM-wt and IgM mutants

As described in Materials and methods, 4 mg (non-radiolabeled) IgM was prepared and fractionated on sucrose gradients for electron microscopy. The samples were analyzed on SDS−PAGE and gave results similar to the radiolabeled samples (data not shown). Electron micrographs (Figure 3) of IgM-wt and the IgM mutants from various fractions across the polymer peak were examined and scored for the number of apparent subunits in each molecule (Table I). A molecule was judged to be unscorable if it did not possess discernible protruding arms about the entirety of its circumference. In most cases subunits could be identified by the presence of two adjoining Fab arms. However, it was often possible to discern only one Fab arm. The remaining Fab arm is presumed to be folded back onto the Fc disk (Davis *et al.*, 1988), or the plane of the Fab 'V' might be perpendicular to the plane of the Fc such that the two Fab appear as one (Feinstein *et al.*, 1986). Such cases were scored as subunits if (i) the Fab arm was located between two subunits in each of which the two Fab arms were apparent; or (ii) the relative angles and distances between Fab arms indicated that these arms could not extend to join in a 'V' of reasonable size. This analysis tends to underestimate the number of subunits, as both Fab arms of some subunits might sometimes be obscured. However, as all samples were treated in a similar manner, comparisons between fractions of the wild-type and the mutants are likely to represent real differences.

The majority of IgM-wt molecules from each fraction are circular pentamers (Table I). However, there is a substantial number of hexamers in the more rapidly sedimenting fractions, suggesting that the second peak contains some

Table I. Summary of electron microscopy of wild-type and mutant IgM

IgM type	Fraction no.	No. of molecules with a specific number of subunits						
		1	2	3	4	5	6	Undetermined
IgM-wt	25			2(7)	5(17)	20(69)	2(7)	26(47)
	32			2(4)	10(18)	31(56)	12(22)	45(45)
	37			0(0)	2(4)	29(59)	18(26)	22(31)
IgM-Ser575	27			1(1)	7(10)	29(42)	32(46)	27(28)
	36			2(3)	4(5)	26(35)	37(50)	24(26)
IgM-Ser414	28			1(2)	22(45)	24(49)	2(4)	40(45)
	31			2(4)	23(40)	31(54)	1(2)	28(33)
IgM-Ser337	31	16(26)	4(7)	12(21)	18(31)	8(14)	0(0)	44(43)
	35	2(2)	3(3)	1(1)	19(19)	72(73)	2(2)	43(30)
	39	12(10)	3(3)	2(2)	16(13)	79(66)	7(6)	67(36)
	43	0(0)	0(0)	0(0)	13(12)	83(80)	8(8)	63(38)

IgM was fractionated on sucrose gradients and negatively stained for electron micrographs as described in Materials and methods. Individual molecules from the indicated fractions were scored for the number of subunits as described in the text. Numbers in brackets indicate the percentage of determined structures with that number of subunits. The number in brackets after the undetermined molecules are expressed as the percentage of the total number of molecules counted.

Table II. Cytolytic activity of wild-type and mutant IgM

Sample	Cell line	Fraction no.	ng/ml to give 50% lysis
IgM-wt	Sp6/HL	36	15.0
		38	15.0
		40	10.5
		42	3.4
		44	3.1
IgM-wt	T/Sp6-1.4	36	9.6
		38	4.6
		40	3.0
		42	2.1
		44	2.2
IgM-Ser575	T/Ser575	41	1.0
		43	1.8
IgM-Ser337	T/Ser337	36	15.5
		38	11.8
		40	10.0
		42	3.3
		43	4.3
IgM-Ser414	T/Ser414	36	>400.0

Polymeric IgM fractions from the sucrose gradients shown in Figure 2 were tested for their efficiency in mediating complement-dependent cytolysis, as described in Materials and methods.

hexameric IgM. For the IgM-Ser575, ~50% of the determined molecules are hexamers, and the numbers are similar regardless of whether the leading (fraction 36) or trailing edge (fraction 27) of the peak is examined. IgM-Ser414, which co-sedimented with pentameric IgM-wt, shows a predominance or circular pentamers and tetramers. Most IgM-Ser337 molecules show five subunits. Smaller species are evident in the IgM-Ser337 polymeric peak; these might be due to disruption of non-covalently assembled polymers during storage or preparation for electron microscopy.

Comparison of the different fractions of the gradient provides a way of gauging whether we have miscounted the number of subunits. For IgM-wt the relative frequency of hexamers is greater in the more rapidly sedimenting fractions, suggesting that at least these fractions contain *bona fide* hexamers. The frequency of hexamers in IgM-Ser575 is unchanged across the polymer peak and is higher than for IgM-wt. The high frequency suggests that these fractions include *bona fide* hexamers, and the uniform distribution suggests that the preparation is homogeneous, i.e. nearly all hexameric IgM. In the case of IgM-Ser337, molecules with six subunits occur at the same low frequency in all parts of the polymer peak. Thus these results argue that this preparation of IgM-Ser337 contains few if any hexamers, or alternatively, that hexameric IgM-Ser337 is less stable than pentameric IgM-Ser337.

The wild-type and mutant IgMs differ slightly in the central

part of the molecules. Compared to IgM-wt, IgM-Ser575 appears to have a more uniformly dense central Fc disk, in which only the N-terminal portion of the C_H region of each subunit was clearly distinguished from that of the neighboring subunits. Compared to IgM-wt, IgM-Ser414, and to a lesser extent IgM-Ser337, has a more open, disordered Fc disk, often with pronounced gaps between subunits. The subunits in IgM-Ser414 and IgM-Ser337 appear to attach to neighboring subunits only at the center (C-terminal end) (arrows in Figure 3C and D).

Effect of serine substitutions on cytolytic activity of IgM

Activation of complement component C1 requires multivalent binding of the $C1_q$ arms to Ig. A single molecule of polymeric IgM can activate C1, whereas several molecules of (monomeric) IgG are needed. The mutant IgMs are in each case assembled into polymers, so it is meaningful to compare their cytolytic activity. We have therefore tested the ability of the polymeric fractions of the wild-type and mutant IgMs shown in Figure 2 to mediate complement dependent cytolysis by measuring the quantity of IgM required to lyse a standard number of TNP−SRBC (Table II). IgM-Ser337 and IgM-Ser575 were effective in cytolysis, whereas for IgM-Ser414, no cytolysis was evident even at IgM concentrations 25-fold higher than for IgM-wt.

We have previously described the heterogeneity in cytolytic activity of IgM-wt, i.e. that the faster sedimenting, hexamer containing fractions are 5- to 20-fold more efficient at cytolysis (Davis *et al.*, 1988). Since the heavy chains have the same V region, their affinity for TNP−SRBCs is expected to be the same, but the assembly of the molecules might affect their avidity for antigen. We measured the avidity of the pentamer and hexamer containing fractions of IgM-wt and found them to bind to TNP−SRBCs with equilibrium constants of 2.0×10^{10} M^{-1} and 2.9×10^{10} M^{-1} respectively (Figure 4). We consider that this small difference in avidity cannot account for the 5- to 20-fold increase in cytolytic activity. IgM-Ser575 is as efficient in mediating complement dependent cytolysis as the most active wild-type fractions and on the basis of only two data points, has comparable avidity (Figure 4). IgM-Ser414, which is non-cytolytic, has 4-fold higher avidity: 8.5×10^{10} M^{-1}. The difference in the *X*-intercepts of the Scatchard transformation of the binding data (Figure 4) shows that there are significantly fewer binding sites on these (identical) TNP−SRBC targets for mutant IgMs than for IgM-wt. As in the case of IgM-wt, the faster sedimenting fractions of polymeric IgM-Ser337 were more cytolytic than the slower fractions; the faster sedimenting material also contained a higher fraction of covalent polymers (Figure 2f).

J chain analysis

IgM contains an additional polypeptide chain, J chain, which is thought to be disulfide bonded to the Cys575 residue (Mestecky and Schrohenloher, 1974). We analyzed our mutants for the presence of J chain by alkaline−urea gel electrophoresis (Figure 5). The IgM-Ser337 polymer contained J chain. However, J chain was not detected in the IgM-Ser575 or IgM-Ser414 polymers; reconstruction experiments indicated that we would have detected J chain if it were present at 10% of the wild-type level. These results show that the covalent incorporation of J chain into IgM is

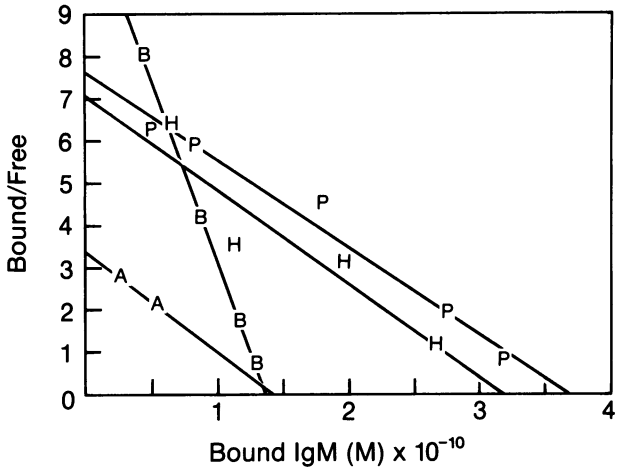

Fig. 4. Scatchard transformation of the binding of IgM-wt, IgM-Ser575 and IgM-Ser414 to TNP−SRBC. Radiolabeled IgM was incubated with TNP−erythrocytes; the bound and free radioactivity were then separated by centrifugation (Wright *et al.*, 1988). 'A' is IgM-Ser575 from fraction 44 of the gradient in Figure 2a. 'B' is IgM-Ser414 from fraction 35 of the gradient in Figure 2b. 'P' and 'H' represent pentameric and hexameric IgM-wt which were isolated by sucrose gradient centrifugation (gradient profile not shown). Lines were determined by the least squares method of linear regression.

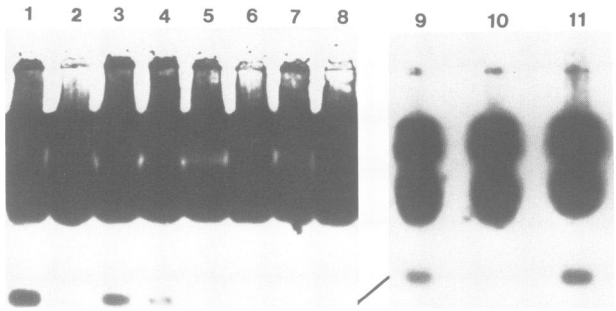

Fig. 5. J chain in IgM-wt and IgM cysteine mutants. Biosynthetically labeled, affinity-purified IgM was sedimented through a sucrose gradient. The polymeric material was then electrophoresed in an alkaline−urea polyacrylamide gel. **Lane 1**: unfractionated IgM-wt. **Lanes 2−4**: IgM-wt monomer, pentamer and hexamer. **Lanes 5** and **6**: IgM-Ser414 monomer and pentamer. **Lanes 7** and **8**: IgM-Ser575 monomer and hexamer. **Lane 9**: IgM-wt pentamer. **Lane 10**: IgM-Ser414Ser575 (double mutant) monomer. **Lane 11**: IgM-Ser337 pentamer.

not required for the assembly of hexameric cytolytic IgM-Ser575, as reported previously (Davis *et al.*, 1988), nor for the assembly of pentameric (non-cytolytic) IgM-Ser414. The same recipient cell line was used to express the mutant and wild-type μ genes, so we suppose that J chain is present in all cases. We conclude, therefore, that J chain is not incorporated into IgM which lacks either Cys414 or Cys575.

We did not detect J chain in monomeric mutant or wild-type IgM.

Discussion

The serine-for-cysteine substitutions were designed to study the importance of disulfide bonding in IgM assembly and function. The substitution of a single serine residue at

position 337, 414 or 575 affects both the fraction of IgM which is assembled as a polymer, as well as the structure and cytolytic activity of the polymeric IgM. Amino acid substitutions have the potential for altering Ig domain folding, and might therefore have effects unrelated to disulfide bonding. The likelihood of this complication can be estimated if the μ chain domains follow the typical Ig pattern (Beale and Feinstein, 1976). Thus, Cys337 and Cys575 would lie between the domains in areas of undefined secondary structure and would be unlikely to disrupt domain structure. Cys414, on the other hand, is expected to lie within the $C_\mu 3$ domain. However, it is likely to be on a β-turn, and the analysis of many V region structures suggests that changes in the amino acid sequence of such turns do not grossly affect domain structure.

While the specificity of disulfide bonding must be directed by non-covalent protein−protein interactions, we do not know for IgM whether these interactions permit more than one type of pairing. However, if Cys−Cys pairs form in only one way, and if this pairing, where it occurs, is the same in mutant and normal IgM molecules, our finding of polymeric, disulfide linked IgM-Ser414 and IgM-Ser575 indicates that Cys337−Cys337 is in series with Cys414−Cys414 and Cys575−Cys575 (Davis and Shulman, 1989). Figure 1 (c and d) shows two ways of arranging Cys414−Cys414 and Cys575−Cys575 in series with Cys337−Cys337. In (c) Cys414−Cys414 and Cys575−Cys575 are in parallel with each other while in (d) they are in series. Model (c) predicts that the IgM-Ser337 polymers would be held together by non-covalent interactions and migrate as monomers on SDS−PAGE. Approximately 50% of IgM-Ser337 appears to be of this type. Model (d) predicts that IgM-Ser337 should assemble covalent polymers and, in fact, ~50% of the IgM-Ser337 is assembled into material which is larger than monomers. Perhaps model (d) is correct but with some of the cysteine residues not forming disulfide bridges. Alternatively, in as much as models (c) and (d) pair comparable cysteines, we consider it a logical possibility that both these pairings occur within individual IgM molecules, thus leading to covalently assembled polymers of intermediate size. Whilst the consistent use of pattern (d) in a pentamer would covalently link all 10 μ chains, this same bonding applied to a hexamer links the μ chains into two groups of six chains, which are likely to be catenated and therefore might migrate as a hexamer in SDS−PAGE.

That IgM-Ser337 is (in part) a non-covalently assembled polymer indicates that non-covalent interactions occur in series with Cys414−Cys414 and Cys575−Cys575. The evidence that these interactions are mediated by the same $C_\mu 2$ domains which are normally disulfide linked has been reviewed (Davis and Shulman, 1989).

Polymeric IgM-wt and IgM-Ser337 are each heterogeneous in their sedimentation rate, electrophoretic mobility, pentamer/hexamer composition and cytolytic activity. By contrast, IgM-Ser414 and IgM-Ser575 are relatively homogeneous, suggesting that the heterogeneity of IgM-wt and IgM-Ser337 might reflect variations in the way that Cys414 and Cys575 disulfide bonds form.

It is worth considering the possibility that cysteines bond as hetero pairs in polymeric IgM. Of particular interest is the possibility suggested by Pumphrey (1986) that the hetero pair Cys414−Cys575 can form. Such pairs in series could also account for the formation of covalently assembled polymeric IgM-Ser337. We also wish to raise the possibility that J chain is disulfide linked directly to four μ chains, such that denaturation yields a $\mu_4L_4J_1$ molecule, which would migrate as an apparent dimer.

While polymeric IgM-Ser575 is assembled predominantly as hexamers, polymeric IgM-Ser414 is predominantly pentamers, with some smaller forms. The explanation of this difference in polymer size might be simply related to the location of the available cysteine residues. The inter-subunit disulfide bonds of IgM-Ser414 must be formed between two adjacent Cys575 residues, whereas in IgM-Ser575 the inter-subunit bond is formed between Cys414 residues. Cys575 is the penultimate amino acid of the heavy chains and thus in a circular molecule these residues are relatively close to each other, and five monomers might be sufficient to circularize. On the other hand, Cys414, in the third constant domain, might be separated from its partner Cys414 by a considerable distance and thus, to bring two Cys414 residues close enough for disulfide bonding, six subunits might be required.

IgM-Ser414 and IgM-Ser575 are synthesized predominantly in the monomeric form. The monomers might have accumulated because the serine substitutions make them less reactive. For example, the abnormal tail structure in IgM-Ser575 might impede non-covalent interactions which would otherwise hold the monomers together while Cys414−Cys414 forms. Alternatively, the occurrence of covalent (intermediate-size) polymers of IgM-Ser337 suggests that Cys414−Cys414 can be in series with Cys575−Cys575, and thus suggests that these disulfide bonds might sometimes form in parallel with Cys337−Cys337 (e.g. see Figure 1b). If this parallel arrangement occurs in IgM-Ser414 or IgM-Ser575, it would result in monomeric IgM. Half of the polymeric IgM-Ser337 is covalently assembled to at least some extent, so this mechanism might account for a substantial fraction of the monomeric IgM-Ser414 and/or IgM-Ser575. Earlier investigations of this question which analyzed the exposed cysteines in partially reduced polymeric and monomeric IgM led to opposite conclusions, namely that Cys337−Cys337 and Cys575−Cys575 are in parallel (human IgM; Beale and Feinstein, 1969) and in series (mouse IgM; Milstein *et al.*, 1975). Perhaps both patterns occur, or perhaps the analysis was confounded by disulfide interchange.

While J chain has been reported to be associated with polymeric Ig only, the absence of J chain in IgM polymers is correlated with the presence of hexamers (Eskeland and Harboe, 1973; Kownatzki, 1973; Eskeland and Christensen, 1975; Cattaneo and Neuberger, 1987; Davis *et al.*, 1988). J chain is absent from polymeric IgM-Ser575, which is predominantly hexameric, and polymeric IgM-Ser414, which is predominantly pentameric. J chain is present in IgM-wt and IgM-Ser337, although the amount decreases with increasing size of the polymer in IgM-wt (Davis *et al.*, 1988). The absence of J chain in hexameric IgM-Ser575 is not surprising, as J chain was reported previously to be bound to Cys575 (Mestecky and Schrohenloher, 1974). This might be the reason that IgM-Ser575 lacks J chain, and similarly the absence of J chain in IgM-Ser414 might indicate that J is also bound by Cys414 in normal IgM, perhaps as shown in Figure 1. However, the decreased J chain content of hexameric IgM argues that the J chain content is related

to the size of the IgM. We propose that J chain is incorporated into molecules where its incorporation is sufficient to permit circularization and that the requirements for circularizing IgM are different depending on the state of Cys414. Where Cys414−Cys414 does not form, as in IgM-Ser414, we hypothesize that the molecule is sufficiently flexible to be circularized by five monomer subunits without J chain. We further suppose that formation of Cys414−Cys414 rigidifies the molecule so that pentamers cannot circularize unless J is also incorporated. If only some of the Cys414 are paired, as might be the case in IgM-wt, the incorporation of J chain might be sufficient to allow circularization of pentamers. In the extreme case where all Cys414−Cys414 are formed, as in IgM-Ser575, the molecule might be so rigid that the gap remains too large to be spanned by J chain, and circularization might require six subunits.

Some of these postulated effects of Cys414−Cys414 on IgM structure might be visible in the electron micrographs, although the differences between the mutant and wild-type IgMs are subtle and their perception is sometimes subjective. The subunits of IgM-Ser414 are sometimes seen to be attached only at the center of the polymer. By contrast, the subunits of IgM-Ser575, where we anticipate that all Cys414−Cys414 have formed, remain associated in the central part of the polymer, i.e. only the N-terminal region of each Fc can be clearly discerned. The central area of IgM-wt is more dense than IgM-Ser414 and less dense than IgM-Ser575, a result which argues that some, but not all, Cys414−Cys414 are formed in IgM-wt. Work is in progress to digitize the electron micrographs to obtain a more rigorous analysis of these differences.

Why does the substitution of Ser414 in the $C_\mu3$ domain abrogate cytolytic activity? Other substitutions in $C_\mu3$ depress cytolytic activity (Shulman *et al.*, 1986, 1987), and it is reasonable to consider that the Ser414 substitution might distort the binding site for the complement component C1. On the other hand, the mutation might affect the accessibility of an otherwise normal C1 binding site. The C1 activating conformation of IgM is thought to resemble a multi-legged table, or 'staple', with the Fab arms bent perpendicular to the central Fc disk; the Cys414−Cys414 disulfide bond might be necessary to give the central disk of the molecule sufficient rigidity to assume the staple conformation (Feinstein *et al.*, 1986). Such a mechanism might underlie the unusually high cytolytic activity of (hexameric) IgM-Ser575. That is, all Cys414−Cys414 bonds must be formed in polymeric IgM-Ser575, whereas polymeric IgM-wt might include some unpaired Cys414, as suggested by the comparison of IgM-wt and IgM-Ser575 by electron microscopy. By measuring C1 binding to monomeric IgM (Swanson *et al.*, 1988) it might be possible to test whether the Ser414 substitution distorts the C1 binding site or restricts its availability.

Materials and methods

Construction of mutant μ genes and transfer vectors

The plasmid pR-Sp6, which includes the μ gene from Sp6/HL (Ochi *et al.*, 1983), was remade using a derivative of pSV2neo from which the *Bam*HI site had been removed. The 2.5 kb *Bam*HI−*Kpn*I fragment encoding $C_\mu3$ and $C_\mu4$ from pR-Sp6 was cloned into M13mp18 and oligonucleotide directed mutagenesis (Zoller and Smith, 1983) was used to convert the nucleotides TGT and TGC to AGT and AGC, respectively, at the appropriate sites. Plaques were screened by differential hybridization to mutant and wild-type oligonucleotides and the entire *Bam*HI−*Kpn*I fragments of the selected mutant phage were sequenced to confirm the presence of the desired mutation and the absence of any extraneous mutations. Plasmids for transfer into mammalian cells were constructed by substituting the *Bam*HI−*Kpn*I fragment from M13 mutant for the wild-type fragment in pR-Sp6. Transformants were screened by differential hybridization of duplicate colony lifts to the mutant and wild-type oligonucleotide.

Normal and transformed cell lines

Tissue culture conditions and gene transfer techniques have been previously described (Shulman *et al.*, 1982, 1986). Sp6/HL is a mouse hybridoma which secretes polymeric IgM specific for the haptens dinitrophenyl (DNP) and trinitrophenyl (TNP) (Kohler *et al.*, 1978). The mutant cell line igm10, which has lost the μ heavy chain gene but produces the TNP-specific κ chain, was used as a recipient cell to express the wild-type or mutant μ genes and produce the corresponding TNP specific IgM. Transformants were selected in 0.6 mg/ml G418 and screened for immunoglobulin production by ELISA. The transformant T/Sp6-1.4 (Shulman *et al.*, 1986) was derived by transfer of the plasmid pR-Sp6, which contains the normal μ heavy chain gene, into igm10. Other transformants are named according to the mutation in their μ heavy chain gene. For instance, T/Ser414 is a cell line produced by transfer to igm10 of a mutated μ heavy chain gene in which the codon for Cys414 has been replaced by a codon for serine. The IgM secreted by these transfectants is named according to the mutation. Thus T/Sp6-1.4 and Sp6/HL secrete IgM-wt and T/Ser414 secretes IgM-Ser414.

Analysis of IgM

IgM was labeled biosynthetically with [^{35}S]methionine and purified by binding to DNP−Sepharose and sucrose density gradient centrifugation (Davis *et al.*, 1988). Polymeric and monomeric IgM were then analysed by J chain content by alkaline urea gels (Reisfeld and Small, 1966), for size by electron microscopy (Roux and Metzger, 1982; Roux *et al.*, 1987) and SDS−PAGE, and for cytolytic activity by lysis of TNP−erythrocytes (Davis *et al.*, 1988).

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