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Assembly of Immunoglobulin M

BLOCKED THIOL GROUPS OF INTRACELLULAR 7S SUBUNITS

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We have shown previously that immunoglobulin M (IgM) is present within IgM-forming cells mainly in its 7S subunit form (IgMs), whereas only fully assembled IgM pentamers are secreted. There is no spontaneous polymerization of intracellular IgMs in cell lysates, suggesting that the 7S subunits had blocked cysteine residues. This suggestion was explored and confirmed in the present paper. Radioactive IgM (secreted) and IgMs (intracellular) were prepared by sucrose-density-gradient centrifugation after incubation of cells of the IgM-producing mouse myeloma MOPC 104E with [^{3}H]leucine. We investigated the susceptibility to reduction of fully assembled mouse IgM and its reconstitution from subunits by analysis by polyacrylamide-gel electrophoresis under dissociating conditions. With increasing concentrations of dithioerythritol, interchain disulphide bonds were cleaved in the following order: inter-IgMs subunit, intra-IgMs subunit H-H, intra-IgMs subunit H-L. Removal of the reducing agent from IgM-reduction mixtures by filtration through Sephadex G-25 caused partial reconstitution of IgM at low protein concentrations (5–100 μg/ml) and total reconstitution at higher protein concentrations (300μg/ml or more). Isolated radioactive intracellular IgMs showed no tendency to polymerize unless first treated with a reducing agent; under optimum conditions removal of the reducing agent caused 70% of the subunits to be assembled into IgM. Similar assembly occurred when IgMs was isolated from cells that had been lysed in the presence of an irreversible alkylating reagent (iodoacetamide). The intracellular IgMs cysteine residues responsible for inter-IgMs linkage therefore appear to be reversibly blocked within the cells. Assembly into IgM is thus controlled by removal of this block during secretion.

It is now accepted that IgM* generally consists of five 7S subunits (IgMs) linked by disulphide bonds located on the Fc part of the molecule. Each IgMs subunit comprises two heavy and two light chains, disulphide-bonded (for review of IgM structure see Metzger, 1970).

In our study of assembly and secretion of IgM by mouse plasma-cell tumour MOPC 104E (Parkhouse & Askonas, 1969) we found that within the cells IgM was present mainly in its 7S subunit form (IgMs), whereas the secreted product of the cell was the fully assembled 19S IgM. In 'chase' experiments the intracellular 7S subunits were shown to be precursors of the secreted IgM (Parkhouse, 1971). This indicated that polymerization and disulphide bonding of the subunits into the circular pentamer occurs shortly before, or at the time of, secretion of the molecules. It was particularly striking that IgMs persisted in stored cell lysates even in the absence of alkylating agents. This raised the questions whether IgMs subunits within the cells were incomplete, possibly lacking important carbohydrate moieties, or whether thiol groups responsible for inter-IgMs-disulphide bonding were blocked.

We have studied the question of possible blocked thiol groups in IgMs in the following way. First of all we established conditions for the reconstitution of subunits derived from secreted IgM by partial reduction. Partial reduction of the IgM yielded 7S or H-L subunits which, on removal of thiol reagent, reassembled easily into IgM on standing in air, provided that IgM subunits were present at a sufficient concentration.

Since there was no difficulty in polymerization of artificially prepared IgMs, we attempted to assemble IgM from intracellular IgMs isolated from MOPC 104E cells pulsed with [^{3}H]leucine *in vitro*. We observed that IgMs could only form IgM after mild reduction of the subunits. This process was not inhibited by prior treatment of intracellular IgMs

* Abbreviation: IgM, immunoglobulin M (macroglobulin).

with an alkylating reagent. The results suggest that IgMs molecules within the cells have blocked thiol groups, preventing polymerization into IgM.

MATERIALS AND METHODS

The MOPC 104E plasma-cell tumour line was kindly sent to us by Dr M. Potter and maintained in Balb/c mice by subcutaneous transfer of 1 mm^3 tumour pieces every 2–3 weeks after transplantation. The cells secrete IgM and light chains of the λ type (McIntire, Asofsky, Potter & Kuff, 1965).

Preparation of serum IgM (MOPC 104E). IgM in the serum of mice bearing the MOPC 104E tumour was prepared by sucrose-density-gradient centrifugation followed by preparative polyacrylamide-gel electrophoresis (Brownstone, 1969), as previously described (Parkhouse & Askonas, 1969).

Preparation and standardization of antisera to the MOPC 104E IgM. This was described by Parkhouse & Askonas (1969).

Preparation of radioactive secreted IgM and intracellular IgMs. Cell suspensions of MOPC 104E tumour were incubated with [4,5-^{3}H]leucine (50 μCi/ml, at 1 Ci/mmol) for labelling of secreted IgM, and 100 μCi/ml (at 19 Ci/mmol) for labelling of intracellular IgMs; the cells and culture supernatant were harvested as described below.

(*a*) Extracellular protein. The supernatant from 4 h cultures was adjusted to contain L-leucine at 1.0 mg/ml and carrier IgM at 50–100 μg/ml, clarified by centrifugation (19000*g* for 10 min), and the free radioactive leucine was removed by passage through a column of Sephadex G-25 (Pharmacia, Uppsala, Sweden), 15 times the sample volume, equilibrated with 0.05 M-sodium phosphate buffer, pH 7.4. The radioactive extracellular IgM was purified by sucrose-density-gradient sedimentation in either the Spinco SW 50 rotor [10–30% (w/v) linear sucrose gradient; 100000*g* for 16–18 h] or the Spinco 41 rotor [7–25% (w/v) linear sucrose density gradient layered over 1.0 ml of 30% (w/v) sucrose and 1.0 ml of 40% (w/v) sucrose; 200000*g* for 24 h]. All sucrose solutions were made up in phosphate-buffered saline (130 mM-NaCl–4 mM-KCl–10 mM-sodium phosphate, pH 7.4).

(*b*) Intracellular protein. The washed cell pellet from a 1 h incubation mixture was resuspended in phosphate-buffered saline containing 0.25 M-sucrose, 1% (w/v) Nonidet P.40 (Shell Chemicals Ltd., London W.C.2, U.K.) (Borun, Scharff & Robbins, 1967) and L-leucine at 1 mg/ml. Iodoacetamide was either absent or present at a concentration of 0.2 M as indicated in the text. After centrifugation at 100000*g* for 90 min, the solution was passed through Sephadex G-25 as described above. For the reconstitution experiments the protein eluate was collected and once more passed through Sephadex G-25 to remove the last traces of iodoacetamide.

Isolation of IgMs from the cell lysate was accomplished by sucrose-density-gradient centrifugation in the Spinco SW 41 rotor (as described above for preparation of extracellular IgM).

Reduction and alkylation of MOPC 104E IgM isolated from serum. Solutions of IgM (1–2 mg/ml) in 0.3 M-tris–HCl buffer, pH 8.0, were treated with dithioerythritol at the concentrations indicated in the text for 1 h at room temperature in air. The mixtures were alkylated at 0°C with freshly prepared iodoacetamide (50% molar excess over added thiol). Reduced and alkylated samples were analysed by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis.

Reduction and reconstitution of radioactive extracellular IgM and intracellular IgMs. Freshly prepared radioactive IgM and IgMs were reduced in 0.5 mM-dithioerythritol for 1 h at room temperature in air. Carrier IgM was added at the concentrations indicated in the text. To determine the extent of reduction to subunits, a sample of the reduction mixture was alkylated immediately with iodoacetamide (50% molar excess over added thiol). Reducing agent was removed from the remaining sample by filtration through Sephadex G-25 equilibrated with 0.05 M-sodium phosphate buffer, pH 7.4. Approximate protein concentrations were estimated on the basis of radioactivity. In some experiments the eluted protein peak was recycled through Sephadex G-25 to remove possible traces of reducing agent. The resulting sample was left in air and analysed for reconstitution into IgM by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis.

Polyacrylamide-gel electrophoresis. This was carried out in the dissociating sodium dodecyl sulphate system (Summers, Maizel & Darnell, 1965) with 4.25% (w/v) gels as described by Parkhouse & Askonas (1969).

For protein analysis the gels were prepared by using *NN*-methylenebisacrylamide as the cross-linking reagent, and protein was detected by staining with Amido Black. For analysis of radioactive samples, the gels were identical except that ethylene diacrylate was used as the cross-linking agent (Choules & Zimm, 1965). The gels were sliced into 1 mm slices, dissolved and counted for radioactivity at 10% efficiency in Kinard's (1957) scintillation fluid as described by Choules & Zimm (1965). Fractions were numbered from the negative to the positive electrode.

RESULTS

Partial reduction of IgM. To obtain IgM subunits (IgMs) from fully assembled IgM, we isolated IgM either from serum of mice bearing the MOPC 104E tumour, or in radioactively labelled form from the incubation medium of a tumour-cell suspension pulsed with [^{3}H]leucine for 4 h. Reduction-susceptibility of the inter-subunit disulphide bonds was tested by varying the concentration of several reducing agents. Fig. 1 shows an analysis of IgM reduction products (3 mg/ml) obtained with dithioerythritol by using the sodium dodecyl sulphate–polyacrylamide-gel system to ensure dissociation of non-covalently bonded subunits. At the lowest concentration of reducing agent (0.1 mM) the IgM is partially degraded to a mixture of the IgMs subunit and polymers of size intermediate between the monomer and pentamer (IgMs polymers). Noteworthy was the absence of HL, H or L fragments. With an increased concentration of dithioerythritol (0.5 mM) the major products were IgMs and HL fragment, although polymers of IgMs were still apparent. At still higher concentrations of reducing agent the IgM molecule was converted into heavy

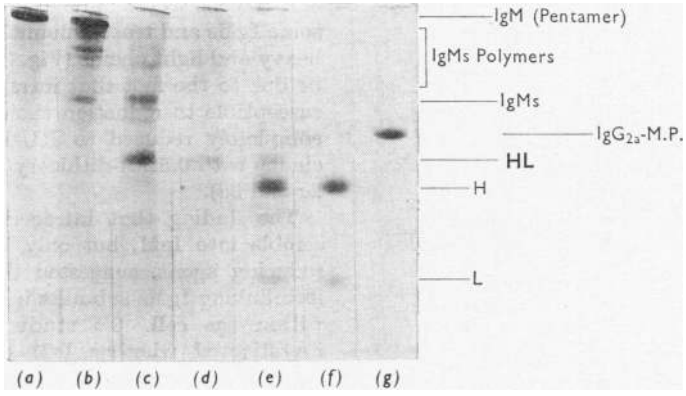

Fig. 1. Sodium dodecyl sulphate–polyacrylamide-gel analysis of MOPC 104E serum IgM reduced at various concentrations of dithioerythritol and alkylated. Conditions are given in detail in the Materials and Methods section. Heavy chains, light chains and the disulphide-bonded heavy chain–light chain subunit are indicated by H, L and HL respectively in the figure. (*a*) Unreduced IgM; (*b*) IgM+0.1 mM-dithioerythritol; (*c*) IgM+0.5 mM-dithioerythritol; (*d*) IgM+1.0 mM-dithioerythritol; (*e*) IgM+5.0 mM-dithioerythritol; (*f*) IgM+10.0 mM-dithioerythritol; (*g*) IgG_{2a} mouse myeloma protein 5563 (IgG_{2a}–M.P.).

and light chains. In order of decreasing lability the order of reduction sensitivity of the various disulphide bonds is as follows: inter-IgMs bonds, intra-subunit H-H bond, intra-subunit H-L bond. With 0.5 mM-dithioerythritol there was an optimum yield of 7S and H-L subunits with no IgM left intact, and this concentration was chosen for further experiments. Other reducing agents, such as cysteine, gave a greater overlap in splitting the different interchain disulphide bonds. Since no special precautions were taken to exclude air in the reductive step, it is possible that the extent of reduction at the lowest concentrations of reducing agent is influenced by the rate of reoxidation of the reducing agent.

Reconstitution of IgM reduction products. Fig. 2(*a*) shows IgMs, HL subunits, and smaller quantities of heavy chain, light chain and IgMs polymers resulting from partial reduction of radioactive IgM with 0.5 mM-dithioerythritol. The pattern of subunits revealed by polyacrylamide-gel analysis on immediate alkylation was identical for IgM concentrations ranging from 5–1000μg/ml. After removal of the reducing agent by gel filtration on Sephadex G-25 the samples were left at room temperature for various time-intervals. Alkylation with iodoacetamide (final concentration 10 mM) terminated the time-period allowed for reoxidation of subunits into their polymeric form; the degree of reconstitution was then assessed by sodium dodecyl sulphate–polyacrylamide-gel analysis.

The polymerization showed some concentration dependence. At high concentrations of IgM (1 mg/ml) there was almost complete reconstitution into IgM immediately after removal of the reducing agent. However, with lower concentrations of IgM, reconstitution was more gradual and less complete, reaching an equilibrium within 2h. In Fig. 2 we present the results of a series of reconstitution experiments in which the concentration of IgM in the reduction step was varied to yield protein concentrations in the Sephadex G-25 eluate of 5μg/ml (Fig. 2*b*), 90μg/ml (Fig. 2*c*) and 300μg/ml (Fig. 2*d*); 2h at room temperature was allowed for reoxidation. The results show that the degree of polymerization is less at lower protein concentrations. Similar conclusions may be drawn from the results of another experimental series (Table 1). Here, the approximate proportions of different subunits after 2h reoxidation at different protein concentrations are presented in tabular form. Reconstitution into IgM was entirely reproducible, and electron microscopy showed the preferred conformation of reassembled IgM to be the usual pentamer ring structure of the original IgM (Parkhouse, Askonas & Dourmashkin, 1970).

Assembly of intracellular IgMs. Having ascertained appropriate conditions to reassemble artificially prepared IgM subunits, we tried to polymerize intracellular radioactive IgMs. As isolated by sucrose-density-gradient centrifugation from a cell lysate after a 1h radioactive pulse (Fig. 3*a*), IgMs did not assemble into IgM on storage at 4°C or room temperature in air. Moreover, IgMs persisted whether cells were lysed in the presence or absence of iodoacetamide. Also, there was no polymerization of intracellular IgMs when partially reconstituted IgM molecules (prepared by using dilute protein solutions as described above) were added to radioactive cell lysates. Cell lysates did not interfere with reconstitution of IgM subunits prepared from IgM.

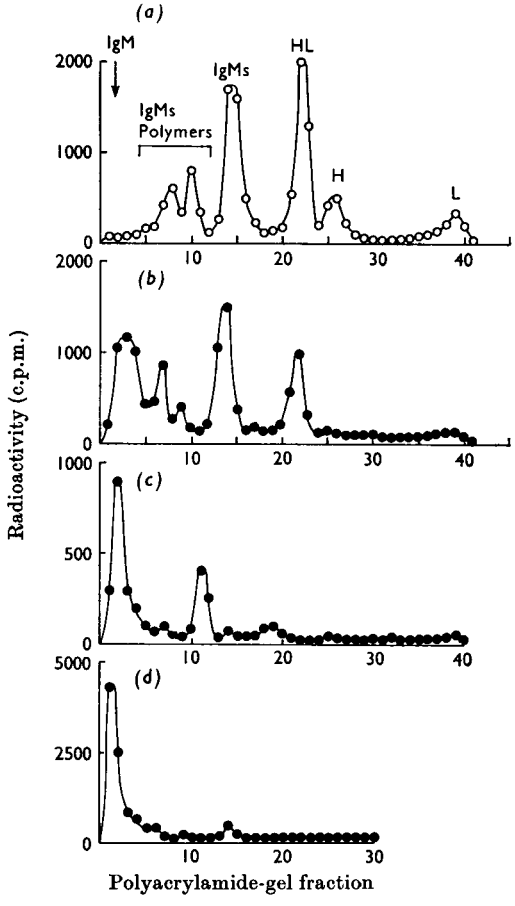

Fig. 2. Concentration dependence of IgM assembly from partially reduced subunits: sodium dodecyl sulphate–polyacrylamide-gel analysis of radioactive secreted IgM after reduction with 0.5 mM-dithioerythritol and reconstitution at different protein concentrations by removal of the reducing agent on Sephadex G-25. Protein eluates from the Sephadex G-25 columns were left at room temperature for 2h and alkylated (see the Materials and Methods section and the text). (*a*) IgM (5 μg/ml), reduced and immediately alkylated; (*b*) IgM (5 μg/ml) reduced and reconstituted; (*c*) IgM (90 μg/ml) reduced and reconstituted; (*d*) IgM (300 μg/ml), reduced and reconstituted.

However, after reduction of IgMs with 0.5 mM-dithioerythritol in the presence of added carrier IgM (Fig. 3*a*), and removal of reducing agent on Sephadex G-25, intracellular IgMs polymerized into IgM (Fig. 3*b*). At least two-thirds of the label was present as IgM after the samples had been left in air overnight at 4°C. In these experiments the added carrier IgM was present at 400 μg/ml in the Sephadex G-25 eluate. At this concentration of protein IgM would have been expected to reassemble by about 94% after reduction (Table 1). The reduced and reconstituted intracellular IgMs contained some IgMs and trace amounts of HL fragment and heavy and light chains (Fig. 3*b*). This could partly be due to the fact that intracellular IgMs is more susceptible to reduction than secreted IgM, being completely reduced to HL and heavy and light chains with 0.5 mM-dithioerythritol (compare Figs. 2*a* and 3*a*).

The finding that intracellular IgMs could assemble into IgM, but only after the addition of reducing agent, suggested that the thiol groups interlinking IgMs subunits were reversibly blocked within the cell. To study this possibility, we investigated whether IgMs prepared from cells lysed in the presence of an alkylating agent (0.2 M-iodoacetamide) could still assemble into IgM after reduction. Free thiol groups would then have been irreversibly alkylated and on reduction could not have yielded covalently bonded IgM. However, we observed that IgMs isolated from alkylated lysates assembled into IgM after reduction as efficiently as IgMs isolated in the absence of alkylating agent (Fig. 3*c*). Thus, the thiol groups responsible for interlinking the 7S subunits appear to have been unavailable to the iodoacetamide.

DISCUSSION

In mouse myeloma IgM, selective reduction of the inter-IgMs disulphide bonds only occurred under mild reducing conditions, as previously noted for human IgM (Miller & Metzger, 1965*a*,*b*; Morris & Inman, 1968; Beale & Feinstein, 1969). These authors reported few or no polymers of size intermediate between IgMs and IgM even under mild reducing conditions. Their method of analysis, however, was ultracentrifugation of reduced protein samples in non-dissociating aqueous solvents. Our analysis, obtained with a system where non-covalent interactions do not occur, shows that under appropriate conditions partial reduction can yield significant quantities of intermediate-sized polymers of IgMs (see Fig. 2*a*). This apparent discrepancy is readily explained by our observation (R. M. E. Parkhouse, unpublished work) that such mixtures of polymeric forms apparently associate through non-covalent forces and sediment in the same position as IgM in a neutral sucrose gradient.

The results of partial reduction show the most labile disulphide bond to be inter-IgMs subunit and the most resistant to be heavy–light chain. The inter-subunit H-H disulphide bridges are of intermediary sensitivity to reducing agents. Following an earlier suggestion (Askonas, Williamson & Awdeh, 1969) we would therefore predict the intracellular order of disulphide bond formation in biosynthesis to be H + L → HL → IgMs → IgM, and in fact this proved to be the case (Parkhouse, 1971).

Table 1. *Concentration dependence of IgM assembly from partially reduced subunits*

The procedure was as described in the legend to Fig. 2, and the relative proportions of different components were obtained by estimating the total radioactivity of the various radioactive protein peaks separated by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. The concentration of IgM given for the reconstitution experiments refers to the concentration of IgM present in the Sephadex G-25 eluates.

		Approximate relative percentage of IgM and subunits					
	Concn. of IgM (μg/ml)	IgM	$(IgMs)_{2-4}$	IgMs	H-L	H	L
Partial reduction (immediate alkylation)	5	—	17	35	31	10	7
Reconstitution	5	12	41	32	15	Trace	Trace
Reconstitution	90	66	5	23	8	0	0
Reconstitution	300	94	1	5	0	0	0

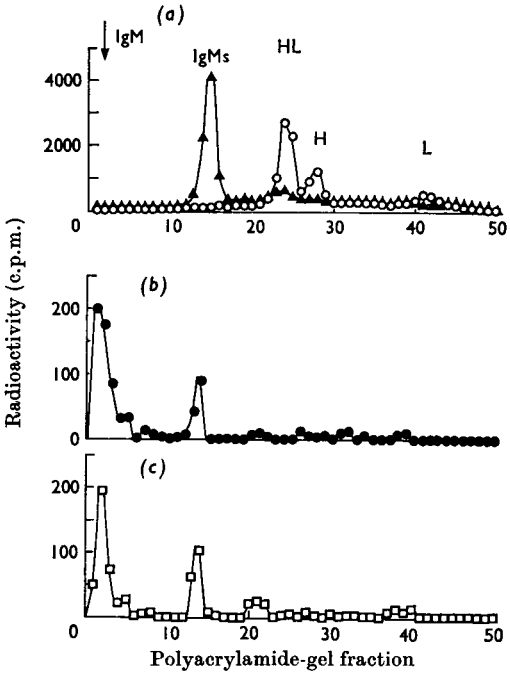

Fig. 3. Isolation of intracellular IgMs, its reduction and assembly into IgM: analysis by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. Intracellular IgMs was isolated from cells lysed in the absence or presence of 0.2M-iodoacetamide by sucrose-density-gradient centrifugation, reduced and reconstituted as described in the Materials and Methods section or in the text. Carrier IgM was added before reduction to yield a concentration of 400μg of IgM subunits/ml of Sephadex G-25 eluate. Samples were kept for 18h at 4°C to allow maximal assembly into IgM. (*a*) Isolated intracellular IgMs before (▲) and after (○) reduction with 0.5mM-dithioerythritol and alkylation. (*b*) ●, Intracellular IgMs isolated in the absence of iodoacetamide, reduced with 0.5mM-dithioerythritol and reconstituted to IgM by removal of reducing agent on Sephadex G-25. (*c*) □, Intracellular IgMs isolated in the presence of 0.2M-iodoacetamide, reduced with 0.5mM-dithioerythritol and reconstituted to IgM as described above.

In common with human IgMs prepared from IgM by partial reduction (Miller & Metzger, 1965*a*,*b*; Morris & Inman, 1968; Beale & Feinstein, 1969), intracellular IgMs sedimented at approximately 7S with no tendency to aggregation into higher-molecular-weight forms, and thus only weak non-covalent interaction can occur between subunits. This, and the fact that IgMs could be isolated from cell lysates prepared in the absence of an alkylating agent, suggested that the cysteine residues responsible for inter-subunit linkage within the cell might be blocked. If such a block did exist, it might be released by reduction. Subsequent removal of the reducing agent should then result in assembly to the pentamer form.

As a preliminary to this approach, we first investigated the reassembly of subunits prepared by reduction of isolated serum IgM. The reduced protein was readily converted into IgM on removal of the thiol reagent by gel filtration. In the experiments described above, identification was based on polyacrylamide-gel electrophoresis, but electron microscopy has ascertained that the reassembled IgM displayed the characteristic radial symmetry of the starting IgM (Parkhouse *et al.* 1970). Although reconstitution was only partial at low protein concentrations, with 300μg of reduced IgM/ml there was a 90–100% conversion into IgM after removal of the reducing agent.

We then attempted the conversion of intracellular IgMs into IgM by using the same methodology. To be sure that cell lysis did not cause the formation of interchain disulphide bonds or mixed disulphides, cells were lysed in the presence of 0.2M-iodoacetamide so that free thiol groups would be alkylated. The intracellular IgMs was isolated from such lysates, mildly reduced and tested for assembly into IgM after removal of the reducing agent. As shown, 70% of the isolated IgMs could be converted into IgM by this procedure. Under the conditions employed a 90–100% reconstitution of subunits prepared from secreted IgM would have occurred. A similar result was obtained by using IgMs isolated

from cells lysed in the absence of iodoacetamide. We therefore conclude that the cysteine residues responsible for inter-subunit linkage are blocked in intracellular IgMs. It is not clear why about 30% of the IgM subunits, found mostly as IgMs after reduction and subsequent removal of the reducing agent, are unable to assemble into IgM; this could be due to stereochemical reasons, possibly arising from the lack of critical carbohydrate residues. Perhaps related is the greater reduction-sensitivity of intracellular IgMs compared with secreted IgM. Further experiments are required to clarify these points.

The actual nature of the cysteine block is not known. Likely candidates are a mixed disulphide involving cysteine or glutathione, or formation of a disulphide bond linking the two heavy chains of the IgMs molecule. In either case, we assume that removal of the block controls the final polymerization of the molecule, perhaps with the participation of the enzyme catalysing disulphide exchange, or with changes in oxidation–reduction potential in different subcellular compartments through which the molecules pass during secretion. Other factors such as addition of carbohydrate or, in the case of antigen-sensitive cells, contact with antigen might also play a role.

Mixed disulphide derivatives of proteins have been described for human serum albumin (King, 1961), haemoglobin (Taylor, Antonini & Wyman, 1963), urinary light chain (Milstein, 1965) and a streptococcal protease (Ferdinand, Stein & Moore, 1965). In these examples, however, it is difficult to exclude the formation of such derivatives as a result of 'aging' or the method of purification, whereas in our study we are looking at freshly synthesized IgMs. It would be particularly interesting to see in the case of the streptococcal enzyme, and other examples of proteolytic enzymes with active-site thiol groups, if the intracellular precursor contains a blocked thiol group. This would provide a simple mechanism for proteolytic enzymes to be contained within the cell in an inactive form.

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