Mercaptan-Induced Fragmentation of a Subunit-Like Proteolytic Fragment of Immunoglobulin M

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Limited papain hydrolysis of immunoglobulin M (IgM) produces a subunit-like proteolytic fragment designated IgM_p (Inman & Hazen, 1968). In the presence of mercaptans, IgM_p partially dissociated into Fc_μ-like and Fab_μ fragments. Treatment of residual IgM (that remaining after a papain digestion) with 2mM-mercaptoethylamine resulted in fragmentation of the same type that occurs in a routine limited digestion of IgM with papain, although exogenous enzyme was not added to the mixture. When IgM was hydrolysed with ¹⁴C-labelled papain, a small quantity of the enzyme was found to be associated with the residual IgM and IgM_p fractions. IgM and IgM 7S subunit (IgM_s) that had been exposed to papain in the absence of activating mercaptan and separated from the enzyme by gel filtration also fragmented when subsequently treated with 2mM-mercaptoethylamine. The fragments resembled those produced during a typical limited papain digestion of IgM. It was concluded that mercaptoethylamine induced fragmentation of IgM_p by activating adsorbed papain.

IgM* isolated from the plasma of persons with Waldenström's macroglobulinaemia has been used for investigations of the structure of this class of immunoglobulin. The intact molecule comprises five (Miller & Metzger, 1965a) similar subunits (IgM_s) each of which apparently consists of two μ-chains and two light (L) chains (Miller & Metzger, 1965a,b).

There have been several investigations of the proteolysis of IgM and IgM_s. Digestion with trypsin led to isolation of Fab_μ and F(ab')_{2μ} fragments (Miller & Metzger, 1966). High-molecular-weight Fc_μ fragment has been found after tryptic digestion at an elevated temperature (Plaut & Tomasi, 1970). When treated with chymotrypsin C, a fragment larger than F(ab')_{2μ} was noted in addition to the Fab_μ fragment (Chen *et al.*, 1969). Proteolysis with papain for a long time resulted in the formation of a papain-resistant Fab_μ fragment (Goodman & Inman, 1969). Although digestion with papain usually results in massive destruction of Fc_μ fragment, Onoue *et al.* (1968) and Mihaesco & Seligmann (1968) have reported the isolation of high-molecular-weight Fc_μ fragments after limited hydrolysis with papain.

In contrast with these results, when human IgM is digested with papain in the presence of a limiting quantity of mercaptoethylamine, a large fragment called IgM_p is liberated (Inman & Hazen, 1968). The fragment resembles the subunit (IgM_s) that is produced when IgM is gently reduced (Morris & Inman,

1968). IgM_p has a mol.wt. of approx. 180000, comprises μ- and L-chains and is antigenically identical with IgM and IgM_s. The major difference between IgM_p and IgM_s is that the former lacks the thiol residues involved in intersubunit interchain disulphide bonds. During digestion, the IgM_p is degraded to smaller fragments, primarily Fab_μ. Fc_μ-like fragments are also produced but they are extremely labile to enzyme action (Goodman & Inman, 1969).

In the present paper we describe the unexpected observation of mercaptan-induced dissociation of IgM_p. The phenomenon was investigated and an explanation found in terms of the activation, by mercaptans, of papain adsorbed to IgM_p.

Materials and Methods

Preparative methods

Isolation and purification of IgM. IgM was prepared from macroglobulinaemic plasma as described by Morris & Inman (1968). The procedure included euglobulin precipitation in deionized water to obtain impure IgM, which then was filtered through columns (2.5 cm × 125 cm) of Bio-Gel P-200 equilibrated with borate-saline buffer (0.32M-NaCl-0.001M-sodium borate buffer, pH8.0). All the protein was eluted from the column as one sharp peak; that in the tubes which had an *E*₂₈₀ of 2 or greater was pooled. The purity of the IgM was determined by immunoelectrophoresis.

When necessary, the IgM and its enzymic or reductive products were concentrated in a Diaflo

* Abbreviations: IgM, immunoglobulin M; IgG, immunoglobulin G. Fab_μ, F(ab')_{2μ} and Fc_μ are fragments of IgM corresponding to the fragments of IgG defined by the World Health Organization (1964).

ultrafiltration cell (Amicon Corp., Lexington, Mass., U.S.A.) operated under N_2 at 70lb/in² (470kN/m²) and with a UM-10 membrane.

Preparation of IgM_p. IgM was suspended in phosphate buffer (0.1M-sodium phosphate, pH7.0) or the protein was precipitated in 10vol. of deionized water and dissolved in phosphate buffer just before digestion. Proteolysis was performed with 2% by weight of crystalline papain (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) in 0.02M-EDTA-2mM-mercaptoethylamine under N_2 for 30min at 37°C. Hydrolysis was terminated by addition of iodoacetamide to a concentration of 0.02M. However, in some experiments involving inhibition of mercaptoethylamine-induced dissociation of IgM_p, the digestion mixture was treated with 1M-iodoacetamide. Alkylation usually was allowed to proceed in the dark for 1h at room temperature with subsequent dialysis against borate-saline buffer before further treatment.

Digestion mixtures were examined by analytical ultracentrifugation to determine the extent of conversion of IgM into IgM_p. IgM_p was isolated from the undigested IgM (residual IgM) and more slowly sedimenting components by filtering the digestion mixture through a column of Bio-Gel P-200 equilibrated with borate-saline buffer. Purity was determined by analytical ultracentrifugation.

In some experiments IgM was treated with papain but mercaptoethylamine was omitted. After 30min the mixture was filtered through Bio-Gel P-200 to separate the IgM from papain and other reagents. The IgM subsequently was exposed to 2mM-mercaptoethylamine and the other reagents normally used to prepare IgM_p with the exception of papain.

IgM_s was treated with papain for 5h in the absence of mercaptoethylamine and then was alkylated with 0.01M-iodoacetamide. IgM_s was separated from papain by gel-filtration through a column of Bio-Gel P-150 equilibrated with borate-saline buffer. Portions of the IgM_s were exposed to 0.05M-mercaptoethylamine for 6 or 24h. A control sample of IgM_s that had not been treated with papain was treated with 0.05M-mercaptoethylamine for 24h.

Mercaptan treatment of IgM_p. IgM_p was suspended in 0.2M-tris at pH8. The suspension was treated with mercaptoethylamine or 2-mercaptoethanol for 30min, 1h or 24h as indicated. Reactions were done under N_2 at 30°C and were terminated by addition of neutralized iodoacetic acid to a concentration in 10% excess of the mercaptan.

In subsequent experiments concerned with inhibition of dissociation and with reoxidation, IgM_p was suspended in phosphate buffer and treated with 0.01M-mercaptoethylamine, usually for 30min at 37°C. The reaction was terminated by addition of iodoacetamide to 0.02M.

The mixtures were resolved into individual com-

ponents by filtration through a column of Bio-Gel P-150 (2.5cm×35cm) equilibrated with borate-saline buffer. The identity of the components was determined by analytical ultracentrifugation and immunoelectrophoresis.

In some cases, IgM_p was first exposed to 0.5M-iodoacetamide in the presence of 6.7M-guanidine-HCl. After alkylation, the protein was dialysed against phosphate buffer and then was treated with 0.05M-mercaptoethylamine-0.02M-EDTA.

Reoxidation. IgM_p was dissociated by exposure to 2mM-mercaptoethylamine as described above. The resulting mixture was divided into two portions. The first acted as the control and was alkylated with 0.02M-iodoacetamide for 1h at room temperature. The excess of reducing and alkylating agents were removed by dialysis against borate-saline buffer.

The second sample was exhaustively dialysed for 24h in several large volumes of borate-saline buffer. During this period, the buffer solution was exposed to the atmosphere to facilitate reoxidation of any sulphhydryl groups that might have been present on the Fc_μ and Fab_μ molecules in the enzymic hydrolysate.

Mercaptan treatment of residual IgM. Residual IgM suspended in phosphate buffer was dissociated at 37°C under an atmosphere of N_2 in 2mM-mercaptoethylamine-0.02M-EDTA for various times ranging from 30min to 24h. Fragmentation occurred in the absence of any exogenous papain. The reaction was terminated by making the mixture 0.02M in iodoacetamide and then it was left at room temperature for at least 1h before dialysis or further treatment. Purification of the fragments was accomplished by using the conditions described above for the preparation of IgM_p.

Preparation of ¹⁴C-labelled papain. To 0.5ml (13.5mg) of the papain suspension was added an equal volume of a saturated solution of sodium acetate; the suspension was cooled in an ice bath to 0°C and 1mmol of [¹⁴C]acetic anhydride (116mCi/mmol) was added with constant stirring. After a reaction period of 1h, excess of ¹⁴C-labelled reagent was removed by exhaustive dialysis against large volumes of phosphate buffer. The specific radioactivity of the labelled papain was 0.65μCi/μmol.

The enzyme activity of acetylated papain was shown by Zappacosta *et al.* (1968) to be approx. 46% less than that of the unacetylated material. To compensate for this anticipated loss of activity, a 4% papain concentration (w/w), instead of the usual 2%, was utilized to digest IgM.

Radioactivity was measured with a model 3003 Packard Tri-Carb liquid-scintillation spectrometer. The scintillation fluid contained 4g of 2,5-diphenyl-oxazole, 0.1g of 1,4-bis-(5-phenyloxazol-2-yl)benzene dissolved in 632ml of toluene and 368ml of ethanol. To 10ml portions of the fluid, various amounts of

protein suspensions were added in volumes not exceeding 0.2 ml.

Preparation of IgM_s . Subunits (IgM_s) of IgM were prepared essentially as described by Morris & Inman (1968). IgM suspended in 0.05M-tris-0.5M-NaCl buffer, pH 8.0, was treated with 8mM-mercaptoethylamine for 30 min at 30°C in an atmosphere of N_2 . The reaction was terminated with 0.08M-iodoacetamide. IgM_s was isolated by gel filtration through Bio-Gel P-200. The purity of the IgM_s was determined by ultracentrifugation.

General methods

Analytical ultracentrifugation. Sedimentation was done in a Beckman Spinco model E analytical ultracentrifuge equipped with schlieren optics, electronic speed control and an An-H rotor. A speed of 68000 rev./min was used. Rotor temperature was 20°C. Sedimentation coefficients were determined by the method of Schachman (1957). Areas were calculated with use of a planimeter.

Determinations of protein concentrations. Concentrations of IgM, IgM_s and IgM_p were calculated by using $E_{280, 1cm}^{1\%} = 12$ (Miller & Metzger, 1965a).

Immunoelectrophoresis. Immunoelectrophoresis was performed on glass plates (11.5cm × 5.7cm) coated to a depth of about 2mm with 0.85% agarose in 0.05M-sodium barbital buffer, pH 8.2. Electrophoresis was done for 90 min at 160 V. Plates were developed with the following antisera used according to the specifications of the experiment: anti-(whole human serum), anti-IgG, anti-IgM (polyvalent), anti- μ -chain, anti-[light chain (kappa)] and anti-(Fc_μ fragment).

Results

Treatment of IgM_p

Dissociation of IgM_p . A portion of IgM_p , which subsequently was to be reduced, sedimented in the ultracentrifuge essentially as a single, symmetrical peak (Fig. 1a). Only a slight amount of a more slowly sedimenting contaminant was present. The IgM_p was treated with 0.1M-2-mercaptoethanol and then was alkylated. After overnight dialysis in borate-saline buffer at 4°C, the mixture was analysed by ultracentrifugation (Fig. 1b). Two peaks were clearly evident in the schlieren pattern; dissociating agents were not necessary to demonstrate these components. The more slowly sedimenting material constituted approx. 30% of the total area of the two peaks.

The components of the mercaptan-treated IgM_p mixtures could be resolved by gel filtration into two protein pools. The protein constituent eluted first was termed pool 1, and the more slowly eluted one, pool 2. Pool 1 was apparently a homogeneous pro-

Fig. 1. Schlieren patterns of IgM_p and IgM_p that had been treated with 0.1M-2-mercaptoethanol

(a) IgM_p ; (b) mercaptan-treated IgM_p . The schlieren patterns were photographed 40 min after reaching a rotor speed of 68000 rev./min. The concentration was 6.0 mg/ml for both proteins and centrifugation was done in borate-saline buffer. Sedimentation is from left to right.

tein suspension with a sedimentation coefficient of 6.7S. Pool 2 was almost homogeneous but contained a trace amount of a more rapidly sedimenting constituent, undoubtedly the same protein as in pool 1. The sedimentation coefficient of pool 2 was 3.6S.

The antigenic identification of the proteins in each of the pools was done immunoelectrophoretically.

Table 1. *Dissociation of IgM_p after exposure to 2-mercaptoethanol or mercaptoethylamine*

Samples of IgM_p suspended in 0.2M-tris buffer, pH8, were treated with 2-mercaptoethanol or mercaptoethylamine at 30°C for 30min or 24h. The reactions were terminated by addition of an excess of neutralized iodoacetic acid over the mercaptan. The samples were examined by ultracentrifugation. The dissociation was determined by planimetric measurements of schlieren patterns.

Experiment	2-Mercaptoethanol (M)	Mercaptoethylamine (M)	Time (min)	Dissociation (%)
1	0.05	—	30	41
	0.25	—	30	42
2	—	0.001	30	0
	—	0.002	30	25
	—	0.01	30	37
	—	0.1	30	37
			Time (h)	
3	—	0.001	24	0
	—	0.002	24	25
	—	0.01	24	37

Pool 1 (Plate 1) consisted of a single component that migrated slightly towards the cathode. It contained μ -chains and κ -chains. Although the κ -chain precipitin line did not appear in the photograph (Plate 1), it came up strongly with prolonged development of the immunoelectrophoresis plate. This protein undoubtedly was residual IgM_p.

Pool 2 comprised two antigenically different components. One of these migrated in the same position as pool 1 and carried the light chain (Plate 1) as demonstrated by its precipitation with anti- κ -chain antiserum. The other was electrophoretically fast. It precipitated with anti- μ -chain antiserum, but not with anti- κ -chain antiserum.

Preparations of IgM_p were treated with 2-mercaptoethanol for 30min. The % dissociation was the same whether 0.05M-mercaptan or five times that concentration was used (Table 1). When mercaptoethylamine was utilized as the thiol reagent, a concentration of 1mM was without effect, but 2mM was sufficient to cause 25% breakdown into fragments within 30min. Incubation for 24h did not result in more extensive fragmentation (Table 1). When the concentration of mercaptoethylamine was increased to 0.01M, the dissociation increased to 37%; ten times more mercaptoethylamine did not cause further fragmentation. Exposure to 0.01M-mercaptoethylamine for as long as 24h did not result in more fragmentation than could be attained within 30min. The actual amount of dissociation caused by 2-mercaptoethanol or mercaptoethylamine varied among the preparations of IgM_p; the data are typical of the upper limits of dissociation.

A large proportion of the IgM_p molecules did not fragment on exposure to the mercaptans. Pool 1 (Fig. 1b) could not be dissociated into fragments

when subsequently treated with 0.1M-mercaptoethylamine and alkylated.

Inhibition of mercaptoethylamine-induced dissociation of IgM_p. Several attempts to inhibit the mercaptan-induced dissociation of IgM_p were made. The papain digestion of IgM was terminated by increasing the concentration of iodoacetamide to 1M. The IgM_p, which was isolated from the alkylated mixture dissociated to the usual extent after exposure to 0.05M-mercaptoethylamine.

In another experiment, IgM_p suspended in guanidine-HCl was treated with iodoacetamide. A similar control was not alkylated. Upon exposure to mercaptoethylamine, both samples dissociated to approximately the same extent.

The data, up to this point, could be interpreted as indicating that papain had caused cleavage of the μ -chains of some IgM_p molecules. Dissociation into fragments might have been the result of subsequent reduction of intra- μ -chain disulphide bonds. The following experiments, however, suggested that this was not so.

Reoxidation of dissociated IgM_p. IgM_p was dissociated into a mixture of Fc μ -like and Fab μ fragments plus undissociated IgM_p. Comparison of the schlieren patterns observed in the ultracentrifuge showed that the area of the slowly sedimenting component for the alkylated sample represented 24% of the total under the peaks, whereas that of the re-oxidized sample represented 23%.

Treatment of IgM

Dissociation of residual IgM. The IgM remaining in a mixture from which IgM_p was isolated is termed residual IgM. When residual IgM was subjected to

EXPLANATION OF PLATE I

Immunoelectrophoresis of isolated components of IgM_p that had been treated with 0.1M-2-mercaptoethanol

The wells contain (from left to right), pool 1, pool 2, pool 1, pool 2. The antisera in the troughs are (a), anti-Fc_μ; (b), anti-IgM; (c), anti-κ-chain. The anode is at the top.

2mM-mercaptoethylamine, fragmentation occurred. The schlieren pattern of the mixture resembled that of a routine digestion of IgM with papain (Inman & Hazen, 1968), e.g., the mixture consisted of unfragmented IgM, a sizeable quantity of IgM_p, and some smaller fragments.

When IgM in a control experiment was treated with 2mM-mercaptoethylamine, only 1.5% of the protein sedimented as IgM_s in schlieren patterns (Table 2). Four or five times as much mercaptan would have been required to release IgM_s in the quantity observed (30–40%) for IgM_p in routine papain digestions.

To investigate further the disruptive effects of

mercaptoethylamine on residual IgM, samples of the IgM were subjected to the mercaptan for various times. The most rapid rate of fragmentation occurred within 30min (Table 3). IgM was slowly degraded after 3h so that it represented only 8% of the protein at the end of the experiment. A smaller product, with a sedimentation coefficient of 6.9S, was present in large amounts and apparently was IgM_p. Other fragments sedimenting at 3.5S were present in addition to IgM_p. The ratio of small fragments to IgM_p remained relatively constant after 3h of exposure to the mercaptan.

Digestion of IgM with ¹⁴C-labelled papain. IgM was hydrolysed with ¹⁴C-labelled papain. The mixture was fractionated on a column of Bio-Gel P-200, and the elution profile is shown in Fig. 2. IgM, IgM_p, and smaller fragments were separated well. Samples were analysed for the presence of radioactively labelled papain. A small quantity of the enzyme was found in IgM and IgM_p; most of it was eluted after one column volume. The amount of ¹⁴C associated with IgM and IgM_p was 0.212 and 0.024 μCi/μmol, respectively. This was equivalent to 1 mol of papain/3 mol of IgM and /26 mol of IgM_p. The ¹⁴C-bearing IgM_p was treated with mercaptoethylamine. Subsequent examination in the ultracentrifuge showed the presence of 3.8S fragments which amounted to 32% of the peak areas.

Papain treatment of IgM and IgM_s in the absence of activating mercaptan. IgM was exposed to papain in the absence of mercaptoethylamine and subsequently was separated from the enzyme by gel filtration. The protein then was treated with 2mM-mercaptoethylamine and fragmentation proceeded in a similar fashion to that described above for residual IgM.

In a comparable experiment, IgM_s was treated with papain for 5h in the absence of mercaptoethylamine. The mixture was made 0.01M in iodoacetamide. Only 15% dissociation had occurred when the mixture was analysed in the ultracentrifuge. The

Table 2. *Reductive dissociation of IgM by mercaptoethylamine*

Samples of IgM were suspended in a solution of 0.05M-tris–0.5M-NaCl buffer, pH8. Reduction with mercaptoethylamine was done for 30min at 30°C under N₂. The reaction was terminated by addition of iodoacetamide to a final concentration in slight excess over the mercaptoethylamine. The amount of IgM_s formed was determined by measuring the area of curves defined in schlieren patterns.

Mercaptoethylamine (mm)	IgM _s (%)
1	0
2	1.5
4	5
7	20
9	35
10	40
12	50
14	60
16	70
18	79
20	80

Table 3. *Mercaptan-induced dissociation of residual IgM*

IgM was digested with papain under limiting conditions. The residual, undigested IgM was isolated by gel filtration and exposed to 2mM-mercaptoethylamine for the times indicated in the table.

Time (h)	Components of mixture (%)			Ratio fragments:IgM _p
	IgM	IgM _p	Fragments	
0.5	54	34	12	0.354
1.5	42	41	17	0.415
3	32	47	21	0.447
6	27	50	23	0.460
12	11	61	28	0.459
24	8	63	29	0.460

Fig. 2. Elution profile of IgM digested with ^{14}C -labelled papain

The digestion mixture was filtered through Bio-Gel P-200 equilibrated with borate-saline buffer. ●, E_{280} ; the identity of the protein in the peaks is (left to right) IgM, IgM_p , and fragments. ·····, radioactivity in ^{14}C -labelled papain.

IgM_s was separated from the papain by gel filtration. Exposure of this IgM_s to 0.05M-mercaptoethylamine for 6h or 24h produced 22% and 48% dissociation, respectively. The IgM_s control not previously exposed to papain but which was treated with 0.05M-mercaptoethylamine for 24h showed only a slight amount of dissociation that resembled the reductive dissociation of IgM into Fc_μ and Fab_μ fragments and free light chains observed by Morris & Inman (1970).

Discussion

IgM molecules subjected to proteolysis with papain in the presence of sulphhydryl activating agents usually are degraded extensively to Fab_μ fragments. A small quantity of Fc_μ fragments may also be in the mixture. However, if the digestion is done with a minimal amount of papain-activating mercaptan (mercaptoethylamine), an intermediate IgM_p -like fragment (denoted IgM_p) is produced in good yield (Inman & Hazen, 1968).

When IgM_p was treated with 0.1M-2-mercaptoethanol, protein comprising about 30% of the total sedimented more slowly than the main material. Similar studies done with mercaptoethylamine indicated that a basic minimal concentration of thiol reagent was needed. At that concentration (2mM-mercaptoethylamine) 25% dissociation occurred and this was equal to about two-thirds of the maximum fragmentation which was obtained with 0.01M-mercaptoethylamine. As observed with 2-mercaptoethanol, additional dissociation could not be induced by increasing the concentration of mercaptoethylamine to 0.1M.

The dissociated protein consisted of two components. One of these was identified as Fab_μ fragment

by the following characteristics: (a) it sedimented at 3.6S. Fab_μ fragment has a similar sedimentation coefficient (Goodman & Inman, 1969). (b) It contained light chains and did not react with anti- Fc_μ antiserum. (c) On electrophoresis it migrated slightly towards the cathodes, like Fab_μ fragment (Goodman & Inman, 1969).

The other component had the characteristics of Fc_μ fragment. Since the mechanisms by which IgM_p is produced is enigmatic, the second component has been referred to as being Fc_μ -like, and has the following characteristics: (a) it sedimented with the Fab_μ fragment at 3.6S. Tryptic digestion of IgM produced a high-molecular-weight Fc_μ fragment (Plaut & Tomasi, 1970; Heyward, 1971) which in the monomeric form sedimented at 3.5S (Heyward, 1971). (b) The fragment reacted with anti- Fc_μ antiserum but not with anti- κ -chain antiserum. (c) The fragment migrated faster than Fab_μ on electrophoresis. Other investigators have reported similar relative positions on electrophoresis (Plaut & Tomasi, 1970; Onoue *et al.*, 1968; Heyward, 1971).

These data showed that a large proportion of the population of IgM_p molecules were subject to fragmentation induced by thiol reagents. Susceptible molecules dissociated within 30min and longer exposure to the reagent did not increase fragmentation. However, an even greater proportion of the IgM_p molecules were resistant to mercaptan-induced dissociation. On isolation, the latter did not fragment after a subsequent exposure to mercaptans.

One possible explanation for mercaptan-induced fragmentation was that the proteins were hydrolysed in the hinge region but that the Fc_μ and Fab_μ sections were not liberated because the sites of cleavage were between intrachain disulphide bonds. The observations supporting this included the following: (1)

fragmentation occurred only in the presence of mercaptans. (2) It did not seem likely to be enzyme-mediated because the original digestion mixture was alkylated with iodoacetamide and IgM_p was separated from soluble papain by gel filtration. (3) Residual IgM_s behaved in an analogous manner when it had been separated from papain by gel filtration. (4) Most of the protein did not dissociate, and this is in contrast with the observations of Zappacosta *et al.* (1968), who showed that papain adsorbed to IgG would completely degrade the IgG and exogenous IgG upon addition of a mercaptan. Further, attempts to inhibit enzyme action with an excessive quantity of iodoacetamide and by alkylation of enzyme adhering to isolated IgM_p suspended in guanidine-HCl failed.

Although reduction of intrachain disulphide bonds seemed to explain fragmentation, the failure of the mixture to reoxidize suggested that another alternative should be considered. The most convincing evidence against the reduced-disulphide-bond notion was obtained when residual IgM dissociated under the influence of mercaptoethylamine in a manner like that of papain digestion of IgM. The quantity of mercaptoethylamine used was absolutely inadequate for reducing even interchain disulphide bonds. Thus it was clear that IgM_s was not being mistakenly identified as IgM_p when residual IgM was treated with mercaptoethylamine. Further, fragmentation of IgM_s by mercaptans does not occur, whereas 3.6S fragments were evident in the mercaptan-treated residual IgM mixture. These observations certainly implicated papain.

When ¹⁴C-labelled papain was used to digest IgM, radioactivity clearly was absorbed to the residual IgM and to IgM_p. Only 4% of the IgM_p molecules had ¹⁴C-labelled papain adsorbed, but 33% of the IgM molecules contained papain. However, considerably more (35–40%) than 4% of the IgM_p molecules were degraded in the presence of mercaptoethylamine. In keeping with observations on the sequence of proteolytic events (Inman & Hazen, 1968), IgM_p was more resistant to hydrolysis than IgM. For example, although almost all the residual IgM was fragmented by mercaptoethylamine after 24h (Table 3), IgM_p could be induced to dissociate much less in the same time. If one assumes that five molecules of IgM_p arise from each molecule

of IgM, then one could presume the equivalent of 1 molecule of papain/5 molecules of IgM. Although IgM actually had 1.7 times as much enzyme adsorbed to it as the IgM_p equivalent, the former could be fragmented 2.7 times greater than the latter.

Additional evidence that rendered the reduced-disulphide-bond idea untenable was obtained by exposing IgM or IgM_s to papain in the absence of mercaptoethylamine. After separating the residual IgM or IgM_s from the free papain, both proteins fragmented on exposure to mercaptoethylamine. This left little doubt that papain was responsible for mercaptan-induced fragmentation of IgM_p and residual IgM.

The fact that it was impossible to inhibit the mercaptan-induced proteolysis of IgM, IgM_p and IgM_s even after alkylation suggests that, once papain is associated with its macroglobulin substrate, its active site must be well protected.

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