The thiol groups of mouse immunoglobulin A

Incomplete formation of the C α 1-domain disulphide bridge

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The BALB/c IgA (immunoglobulin A) myeloma protein M167 contained on average 5.7 free SH groups per IgA dimer. These groups were preponderantly on the heavy chains and comprised two distinct populations: 3.3 exposed SH groups per dimer in the Fc region, and 2.4 buried SH groups per dimer in the Fd region, detectable only after denaturation. To locate the cysteine residues involved, labelled peptides were purified from thermolysin digests of radioalkylated IgA by high-performance liquid chromatography. From the amino acid compositions of the peptides, the exposed thiol groups were assigned to Cys-307 in the C α 2 domain, which thus existed in the reduced form to an extent exceeding 80%. This residue may allow attachment of secretory component to dimer IgA in the mouse to proceed via thiol–disulphide exchange. The buried thiol groups were assigned to Cys-150 and Cys-208, in the C α 1 domain, each being in the reduced form to the extent of approx. 30%. This pair of residues would normally give rise to the characteristic intradomain disulphide bridge. It appears that disulphide formation is not a crucial event during folding of the C α 1 domain in IgA biosynthesis. The sequence in the region 140–151 was re-investigated, and residue 142 was shown to be serine, not cysteine, helping explain the lack of heavy-chain–light chain bonding in BALB/c mouse IgA. A disulphide-bond model for mouse IgA is proposed on the basis of these assignments and other features of the mouse α -chain sequence.

Cysteine residues in immunoglobulins participate in a number of well-characterized structurally important intrachain and interchain disulphide linkages. The incidence of free thiol groups is usually very low, e.g. 0.2 SH group per molecule of normal human IgG (Cecil & Stevenson, 1965) and less than 1 SH group per pentamer in human and mouse IgM (Miller & Metzger, 1965; Kaplan *et al.*, 1980). The H (or α -) chains of IgA contain several

cysteine residues peculiar to IgA, some of which give rise to further intradomain bridges (Liu *et al.*, 1976; Putnam *et al.*, 1978), whereas others form interchain bridges, including attachments to J chain (Hauptman & Tomasi, 1975; Chapuis & Koshland, 1975; Garcia-Pardo *et al.*, 1981) and to secretory component (Underdown *et al.*, 1977; Garcia-Pardo *et al.*, 1979). In contrast with IgG and IgM, significant amounts of free thiol groups have been found in IgAs from various species. The mouse myeloma protein M460 and a human myeloma IgA were reported to contain respectively 3.9 (Rosenstein *et al.*, 1972) and 2.0 (Hauptman & Tomasi, 1975) SH groups per dimer, and 6–9 SH groups per dimer were found for rabbit sIgA of various allotypes (Elliott *et al.*, 1980). The possible participation of unassigned thiol groups in the assembly of human sIgA from SC and J-chain-linked IgA dimer has been discussed (Lindh & Björk, 1976).

Despite these results, comparatively little attention has been given to the precise location of free

Abbreviations used: H, heavy chain, L, light chain, and J, joining chain of immunoglobulin; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; sIgA, secretory immunoglobulin A; SC, secretory component; M167, the myeloma protein from the tumour line MOPC 167; Nbs $_2$, 5,5'-dithiobis(2-nitrobenzoic acid); GdmCl, guanidinium chloride; h.p.l.c., high-performance liquid chromatography; ODS, octadecylsilyl; SDS, sodium dodecyl sulphate.

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thiol groups in IgA. Modification of a thiol group in M460 IgA was reported to influence the antigen-combining site (Rosenstein *et al.*, 1972), and the distance between a fluorescent thiol label and a bound hapten was estimated by resonance energy transfer (Manjula *et al.*, 1976). The amino acid sequence of a peptide bearing a free thiol group was also reported (Jackson & Richards, 1974). A competitive-labelling study of the average chemical properties of thiol groups in M167 IgA indicated that at least some of them were of high reactivity and hence exposed on the surface of the protein (Kaplan *et al.*, 1980). We now describe a more detailed investigation of the thiol groups in mouse IgA, leading to the identification of three specific reduced cysteine residues in the α chain of myeloma protein M167. Our results help explain the lack of H-L interchain disulphide bonds in BALB/c IgA, show that intrachain disulphide bond formation is not an obligatory step in the folding of immunoglobulin domains *in vivo*, and address the general problem of disulphide-bond arrangements in IgA.

Materials and methods

Isolation of IgA proteins

Serum was obtained from BALB/c mice carrying the plasmacytoma line MOPC 167, originally provided by Dr. M. Potter. Samples of J558, W3207, MOPC 167 and MOPC 511 ascites were kindly provided by Dr. B. Clevinger, Dr. L. Hood, Dr. S. Rudikoff and Dr. M. Potter respectively. MOPC 315 and TEPC 15 ascites were purchased from Litton Bionetics (Kensington, MD, U.S.A.).

M167, W3207, T15 and M511 IgAs were purified by affinity chromatography. To prepare the affinity medium, *p*-nitrophenylphosphocholine (Sigma Chemical Co.) was catalytically hydrogenated to *p*-aminophenylphosphocholine, which was coupled to Affi-Gel 10 (Bio-Rad Laboratories), a succinimido derivative of a cross-linked agarose gel (Cuatrecasas & Parikh, 1972). The binding capacity of freshly prepared medium was about 300 mg of IgA/25 ml of swollen gel. The IgA was eluted with 0.1 M-phosphocholine in 0.1 M-Tris/acetate buffer, pH 7.0, then dialysed into 0.1 M-sodium acetate buffer, pH 5.6, for storage. J558 IgA was purified by using the dextran affinity medium of Hiramoto *et al.* (1972), eluted with 1 M-methyl α -D-glucoside; M315 IgA was purified by using Affi-Gel 10 coupled to *N*^ε-2,4-dinitrophenyllysine, eluted with 2,4-dinitrophenylglycine.

The predominant dimer component of M167 IgA was isolated from the oligomeric mixture by gel filtration through a 2.5 cm $\times$ 90 cm column of Bio-Gel A-1.5 m (Bio-Rad Laboratories) in 0.1 M-sodium acetate buffer, pH 5.6. Protein

concentrations were measured spectrophotometrically by using the $A_{280}^{0.1\%}$ value 1.34 (Chesebro & Metzger, 1972).

SDS/polyacrylamide-gel electrophoresis

Electrophoresis was carried out in tubes as described by Weber & Osborn (1969). To obtain radioactivity profiles, 1.7 mm gel slices were dried at 40°C in scintillation vials, and digested with 100 μ l of 30% (v/v) H₂O₂ at 100°C for 1 h. The residues were dispersed in 10 ml of Aquasol (New England Nuclear) for scintillation counting.

Gel filtration through a 3 cm $\times$ 90 cm column of Sephacryl S-200 (Pharmacia) in 3 M-urea/1 M-acetic acid was also used to separate and quantify H- and L-chain species.

Determination of thiol groups

Mixtures containing 1–2 mg of protein/ml and 1 mM-Nbs₂ (Janatova *et al.*, 1968) in 2 mM-EDTA/0.1 M-Tris/HCl buffer, pH 7.5, with or without 4 M-GdmCl, were left to react for 20 min. The absorption coefficients used for the released thionitrobenzoate anion were respectively 14150 and 13700 M⁻¹·cm⁻¹ in the absence and in the presence of 4 M-GdmCl (Riddles *et al.*, 1979).

Alkylation of thiol groups

For modification with iodoacetic acid or iodoacetamide, M167 IgA (4 mg/ml) in 2 mM-EDTA/0.1 M-Tris/HCl buffer, pH 8.6, was allowed to react in the dark at room temperature with 1.5 mM recrystallized reagent for 2–8 h, or alternatively with 1 mM reagent in the presence of 4 M-GdmCl for 3 h. The ratios of reagent to protein thiol groups were about 40:1 and 15:1 respectively. Similar procedures were used with iodo[2-¹⁴C]acetic acid and iodo[1-¹⁴C]acetamide (Amersham Corp.) diluted with unlabelled reagent to 1200–22000 c.p.m./nmol. For complete reduction and alkylation of all cysteine residues, IgA (4 mg/ml) in 2 mM-EDTA/0.1 M-Tris/HCl buffer, pH 8.0, containing 4 M-GdmCl was incubated under N₂ with 10 mM-dithiothreitol for 3 h, then with 30 mM unlabelled iodoacetic acid or iodoacetamide for 2 h.

Before modification with *N*-ethylmaleimide, M167 IgA was carboxymethylated at the exposed thiol groups. *N*-[Et-1-¹⁴C]Ethylmaleimide (New England Nuclear) was diluted with unlabelled reagent (Sigma Chemical Co.) to a specific radioactivity of 1400 c.p.m./nmol. IgA (4 mg/ml) in 2 mM-EDTA/0.1 M-sodium acetate buffer, pH 4.8, containing 2 M-GdmCl was incubated at room temperature for 1 h with 15 mM reagent, a 400-fold excess over thiol groups.

Preparation of Fab and Fc fragments

IgA samples (2–3 mg/ml) in 2 mM-EDTA/0.1 M-Tris/acetate buffer, pH 7.9, were warmed to 37°C with 10 mM-cysteine and a trace of toluene, then treated with mercury-papain (2%, by wt.) (Fahey, 1963; Abel & Grey, 1968). After 4 h incubation, the enzyme was inactivated by addition of 20 mM-iodoacetamide, which also alkylated thiol groups introduced into the protein by the cysteine.

Digestion products were separated on an analytical scale by SDS/polyacrylamide-gel electrophoresis. For larger-scale purification, reaction mixtures were first dialysed against 2 mM-EDTA/0.1 M-Tris/HCl buffer, pH 7.5, concentrated, and passed through a Sephadex G-100 column (1.5 cm × 90 cm), the Fab and Fc fragments emerging as a single peak. The two fragments were then separated by affinity chromatography with the phosphocholine column described above. The unadsorbed fraction contained Fc together with inactive Fab and some albumin; active Fab was specifically adsorbed and was recovered in high purity by phosphocholine elution.

Preparation of radiolabelled cysteinyl peptides

M167 IgA samples, radioalkylated with the various reagents, were fully reduced and alkylated in 4 M-GdmCl with the unlabelled reagent, dialysed against water and freeze-dried. The alkylated protein was no longer very soluble in water. To obtain clear solutions, 5 mg/ml suspensions were treated at 0°C with 5 µl portions of citraconic anhydride to about 50 mM, with the pH maintained at 8.0 by using 5 M-NaOH. The citraconylated protein was dialysed into 0.1 M-NH₄HCO₃, and digested with thermolysin (2.5%, by wt.) at 37°C for 2.5 h.

Radiolabelled peptides were purified by h.p.l.c. on Beckman–Altex equipment, comprising model 100A and 110A pumps, model 420 gradient controller, model 210 injector and model 155 variable-wavelength u.v. detector. All separations were performed on a 5 µm Ultrasphere-ODS reverse-phase column (0.46 cm × 25 cm; Beckman Instruments) at 42°C with acetonitrile/aqueous buffer eluents at a flow rate of 1 ml/min.

Most peptides could be purified in three successive chromatographic steps, consisting of a gradient elution with 10 mM-sodium phosphate buffer, pH 7.4, a gradient elution with 10 mM-orthophosphoric acid, pH 2.3, and a final isocratic run at either pH 7.4 or pH 2.3. Pooled fractions were desalted between runs with the aid of Sep-Pak C₁₈ cartridges (Waters Scientific). A preliminary purification of peptides from IgA labelled at the exposed thiol groups was carried out by high-voltage paper electrophoresis at pH 6.5.

Amino acid analysis

IgA and IgA peptides were hydrolysed in 6 M-HCl *in vacuo* at 105°C for 24 h. Amino acid compositions were determined on a Durrum D500 analyser, with norleucine as an internal standard. Cysteine residues modified with *N*-ethylmaleimide generated ethylamine and *S*-(1,2-dicarboxy-ethyl)cysteine, which were also measured on the analyser (Guidotti & Konigsberg, 1964). *N*-Terminal residues were identified by dansylation (Gray, 1972).

Results

Characterization of myeloma proteins

Mouse IgAs display three types of heterogeneity that might influence their thiol-group contents: (a) IgA can exist in multiple molecular forms from monomer up to pentamer; (b) IgA from BALB/c mice has no H–L disulphide bridge, and its L chains are present as monomers and disulphide-bonded dimers (Abel & Grey, 1968); (c) albumin is commonly associated with serum IgA in non-stoichiometric amounts (Mannik, 1967; Hauptman & Sobczak, 1976).

Most of the present work was carried out with IgA from the tumour line MOPC 167 propagated in this laboratory. Gel filtration on Bio-Gel A-1.5 m revealed that the dimer form accounted for at least 85% of the total IgA in the samples used. According to SDS/polyacrylamide-gel electrophoresis, monomeric L chains formed 15–35% of the total L chain in six samples of M167 IgA, and comparable results were obtained for five other mouse IgA proteins. The gels also showed a weak band migrating slightly more slowly than H-chain monomer, with the same mobility as mouse albumin. Since this band was evident whether or not the protein had been previously reduced, this putative albumin contaminant was present mainly as non-covalently bonded monomer. Spectrophotometric scanning of gels from several M167 IgA samples indicated albumin contents in the range 0.4–0.6 mol/mol of IgA dimer.

Thiol content of IgA proteins

There was a marked effect of GdmCl on the accessibility of thiol groups to Nbs₂. Two gross populations of free thiol groups were distinguished, present in comparable amounts, but respectively reactive ('exposed') and unreactive ('buried') in the absence of denaturant. Total observed thiol contents were unaffected by varying the GdmCl concentration in the range 3–6 M, or by increasing the incubation time up to 45 min.

A sample of M167 IgA, from ascites provided by Dr. S. Rudikoff, and five other mouse IgAs were

assayed for thiol activity with Nbs₂. All six contained both buried and exposed thiol groups (Table 1), and their average content of 3.4 per IgA dimer is close to that of M460 IgA (Rosenstein *et al.*, 1972). Numbers of exposed thiol groups were appreciably higher in batches of our own MOPC 167 IgA and were higher still in samples of purified M167 dimer IgA (Table 1, bottom). The content of buried thiol groups was more constant, characteristically about 2 per IgA dimer.

Radiochemical modification methods confirmed the thiol contents determined with Nbs₂. Samples of M167 IgA alkylated with iodo[2-¹⁴C]-acetic acid in GdmCl had radioactivities corresponding to 94–112% of that predicted by the original thiol-group assays, and no residual reaction with Nbs₂ was detected. The label was shown to be associated almost exclusively with cysteine, by amino acid analysis of the radioalkylated protein. Iodo[2-¹⁴C]acetic acid also distinguished two populations of thiol groups in the same manner as Nbs₂. In the absence of GdmCl, the gradual uptake of radioactivity closely paralleled the loss of thiol groups detectable with Nbs₂. Reaction ceased after 2–3 h, leaving about 45% of the thiol groups intact, which could be quantified with Nbs₂ on denaturation. Analogous results were obtained with iodo[1-¹⁴C]acetamide. In the range 0–3 M GdmCl the buried thiol groups were gradually exposed, giving a curve resembling that of a typical co-operative unfolding transition with a midpoint at 1.5 M (Fig. 1).

Chain distribution of thiol groups

Three samples of M167 IgA were radiolabelled in GdmCl, then fully reduced and analysed by

Table 1. *Thiol-group contents of mouse myeloma IgAs*
Total thiol groups were determined with Nbs₂ in the presence of 4 M-GdmCl, and exposed thiol groups in the absence of GdmCl. Buried thiol groups were calculated as the difference between those two.

IgA	Thiol groups per dimer		
	Total	Exposed	Buried
M167*	3.6	1.5	2.1
M511	3.1	0.5	2.6
T15	3.6	1.7	1.9
W3207	4.8	2.1	2.7
M315	3.1	1.2	1.9
J558	2.5	0.8	1.7
M167†	5.0	3.0	2.0
M167 dimer†	5.7	3.3	2.4

* From ascites provided by Dr. S. Rudikoff.

† Average of four preparations, from serum raised in this laboratory.

SDS/polyacrylamide-gel electrophoresis and/or gel filtration in denaturant. Some 94–96% of the recovered radioactivity was located on H chain, whereas only about 5% was associated with L chain (Fig. 2a). Actual incorporation of [¹⁴C]-carboxymethyl groups into H chain purified by gel filtration was 1.3–1.5 per chain, equivalent to 5.1–5.8 per IgA dimer. The extent of L-chain labelling, about 0.06 group per chain, was far below the 0.2–0.3 expected if all the monomeric L chains had reacted, and hence the thiol group was already blocked on many of these molecules. There was no appreciable incorporation of radioactivity into J chain or albumin.

To locate the exposed thiol groups, IgA was [¹⁴C]carboxamidomethylated, then digested with papain into Fab and Fc fragments. The incorporation of [¹⁴C]alkyl groups was 2.4 per dimer, or 0.60 per H–L unit. SDS/polyacrylamide-gel electrophoresis (Fig. 2b) showed that radioactivity was predominantly associated with Fc (0.55 mol/mol), with slight labelling of L chain (approx. 0.05 mol/mol) and no labelling of Fd.

Samples of IgA in which the buried thiol groups had been alkylated in GdmCl and the denaturant removed by dialysis gave multiple cleavage fragments with papain. However, Nbs₂ assays indicated that the buried thiol groups were still intact after papain cleavage of native IgA, and moreover were restricted to the Fab fragment. To locate these groups, purified Fab with 0.67 SH groups per fragment was alkylated with iodo[1-¹⁴C]acetamide

Fig. 1. *Exposure of buried thiol groups of M167 IgA at various concentrations of GdmCl*

M167 IgA (4 mg/ml) was alkylated with 1.5 mM-iodo[2-¹⁴C]acetic acid for 3 h. For further experimental details see the text. The arrow indicates the original thiol-group content determined with Nbs₂.

Fig. 2. SDS/polyacrylamide-gel electrophoreses of radioalkylated M167 IgA and Fab fragments

For experimental details see the text. —, Absorbance (Coomassie Blue staining); ----, radioactivity. (a) IgA labelled with iodo[1- 14 C]acetamide in GdmCl. Incorporation of label was 93% in H chain, 6% in L chain and approx. 1% in J chain; albumin was not resolved from H chain in this gel. (b) Papain digest of IgA labelled at exposed thiol groups with iodo[1- 14 C]acetamide. Incorporation of label was 86% into Fc and 12% into L chain. The smaller peaks to the left are albumin and a large H-chain fragment. (c) M167 Fab labelled at the buried thiol groups with iodo[1- 14 C]acetamide. Incorporation of label was 72% into Fd and 28% into L chain.

intermolecular thiol–disulphide exchange during the radioalkylation (Fig. 2c).

Location of thiol groups

Radioactive peptides were isolated from thermolysin digests of radiolabelled IgA by h.p.l.c. and characterized by amino acid composition and *N*-terminal analysis. The positions of the labelled peptides in the sequence were established by comparison of the composition data with the published sequences of mouse IgA H and L chains. The specific radioactivity of each peptide was also determined, and, by comparison with that of the [14 C]alkylating agent itself, the proportion of cysteine residues at the identified position originally present in the reduced state could be established, irrespective of peptide yield. This should correspond with the thiol content of the native protein determined with Nbs₂.

To locate the exposed thiol groups, a sample of M167 IgA dimer containing 2.6 exposed SH groups per dimer (0.65 group per H chain) was alkylated with iodo[2- 14 C]acetamide under non-denaturing conditions. Fig. 3 illustrates the initial separation of thermolysin-digest peptides by h.p.l.c. The pools E1 and E2 on further fractionation yielded four peptides (three from E1 and one from E2), whose characteristics are given in Table 2. These peptides together accounted for 81% of the radioactivity of the crude peptide mixture, and their individual specific radioactivities were in reasonable agreement with the exposed thiol content of the H chain. Moreover, their compositions were consistent with the same site of labelling in each case, namely Cys-307 in the C $_{\alpha}$ 2 domain, according to the amino acid sequence of M511 α -chain (Robinson & Appella, 1980). The amino acid sequence in this region has been confirmed by nucleic acid sequencing (Auffray *et al.*, 1981; Tucker *et al.*, 1981).

To locate the buried thiol groups, M167 IgA was treated with unlabelled iodoacetamide to block the exposed thiol groups, followed by iodo[1- 14 C]acetamide in the presence of 4M-GdmCl. The original content of buried thiol groups was 2.6 per dimer, or 0.65 per H chain. Separation of the thermolysin digest by h.p.l.c. (Fig. 4), and additional h.p.l.c. steps on appropriate pools, ultimately yielded ten peptides representing five different cysteine residues, of which two of low specific radioactivity proved to belong to the papain-digest Fc segment (Cys-307 and Cys-365). In the Fd region, minor radiolabelling was detected at Cys-200 and Cys-224. The highest specific radioactivities as well as the highest individual recoveries of radioactivity were exhibited by two peptides found in pool B2 (Fig. 4). Both were

in GdmCl. As expected, the Fd contained most of the radiolabel (0.45 mol/mol), with L chain acquiring some label (0.17 mol/mol) presumably from

Fig. 3. *H.p.l.c.* separation of peptides from a thermolysin digest of M167 IgA labelled at exposed thiol groups with iodo[1- 14 C]acetamide

The sample contained 264000 c.p.m., corresponding to 25 nmol of label. The initial buffer was 10 mM-sodium phosphate, pH 7.4. For further experimental details see the text. The arrow indicates the point of injection. —, A_{225} , ----, concn. of acetonitrile; histogram, radioactivity.

attributed to the region around the invariant Cys-150 in the $C_{\alpha}1$ domain, as indicated in Table 3. However, peptide B23 appeared to contain only one cysteine residue, whereas peptide fragment 141–150 from the mouse α -chain sequence reported by Robinson & Appella, (1980) has a second cysteine residue at position 142.

To verify our amino acid composition, which implied the replacement of one cysteine residue in the published sequence by serine, peptide B23 was cleaved at the abnormally labile Asp–Pro linkage by warming with 1.7 M-acetic acid (10%, v/v) at 40°C for 96 h (Landon, 1977). Products were

collected by freeze-drying and fractionated by h.p.l.c. with a gradient of acetonitrile in aqueous trifluoroacetic acid, pH 2.3. Two subfragments were obtained, a non-radioactive leucine peptide B23A and a radioactive proline peptide B23B. Their amino acid compositions were fully consistent with residue 142 being serine rather than cysteine (Table 3).

In efforts to ensure that the above results had not been compromised by the scrambling effect of thiol–disulphide interchange in the denatured protein, a second experiment was carried out in which the buried thiol groups of MOPC 167

Table 2. Amino acid composition and assignment of labelled peptides from reaction of exposed thiol groups of MOPC 167 IgA with iodo[1-¹⁴C]acetamide

The integral values in parentheses and the assignments below are derived from the α -chain sequence (Robinson & Appella, 1980). Since tryptophan was not determined, peptide E2 may or may not contain Trp-311 as the C-terminal residue. Square brackets indicate residues assigned to an L-chain fragment Phe-Tyr-Pro-Lys (Rudikoff & Potter, 1978) contaminating E13, in almost equimolar proportion. Specific radioactivities were calculated relative to iodo[1-¹⁴C]acetamide. Abbreviations: Cys(Cm), S-carboxymethylcysteine; N.D., not determined.

Amino acid	Peptide ...	Amino acid composition (mol of residue/mol)			
		E11	E12	E13	E2
Cys(Cm)		0.2 (1)	0.7 (1)	0.7 (1)	0.8 (1)
Asx				1.3 (1)	
Ser				0.9 (1)	
Glx				1.3 (1)	1.1 (1)
Pro		1.2 (1)	1.0 (1)	1.8 (1) [1]	1.2 (1)
Gly		1.0 (1)	1.0 (1)	2.3 (2)	1.1 (1)
Ala			0.9 (1)	1.3 (1)	1.0 (1)
Val		0.8 (1)	0.8 (1)	0.9 (1)	0.9 (1)
Leu		1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)
Tyr				0.7 [1]	
Phe				0.8 [1]	
Lys				0.9 [1]	
Arg				0.6 (1)	1.1 (1)
Trp				N.D. (1)	N.D. (1)
Specific radioactivity		0.46	0.45	0.50	0.63
Recovery of ¹⁴ C radioactivity (%)		25	28	16	12

Assignments

IgA were modified with ¹⁴C-labelled *N*-ethylmaleimide. This reaction does not proceed via the thiolate anion, and hence the reagent can be used at pH values as low as 5 (Guidotti & Konigsberg, 1964), where thiol-disulphide exchange should be severely retarded owing to the very low concentration of thiolate anion. The two main peptides isolated from IgA labelled with this reagent were P1, assigned to the Cys-208 region, and P2, assigned to the Cys-150 region (Table 4); peptide P2 comprised the same fragment as peptide B23 above. Their specific radioactivities were both about 0.3 mol of radiolabel/mol of peptide, representing an even distribution of the original buried thiol content of 0.65 group per H chain. As discussed below, Cys-150 and Cys-208 normally constitute the C_α1 intradomain disulphide bridge. The buried thiol groups in this sample of IgA can thus be accounted for by a failure of bridge formation in about 30% of C_α1 domains.

Discussion

Thiol-group content of IgA

The thiol groups of BALB/c mouse IgA comprise two separate populations. The exposed groups are chiefly located at Cys-307 of the C_α2 domain of the H chain, 0.65–0.9 per chain, whereas the buried thiol groups are located at Cys-150 and Cys-208 of the C_α1 domain, 0.4–0.8 per chain. The small contribution of light-chain thiol groups to the exposed population (approx. 0.06 per chain) suggests that the C-terminal cysteine residues of most monomer L chains were blocked with small thiols such as cysteine or glutathione (Milstein, 1964).

For human myeloma IgA, values of 2 (Hauptman & Tomasi, 1975), 0.4 (Lindh & Björk, 1976) and 0 SH groups per dimer (Yang *et al.*, 1979) have been reported. Normal rabbit secretory IgA has 6–9 thiol groups per dimer (Elliott *et al.*, 1980), 95% of

Fig. 4. H.p.l.c. separation of peptides from a thermolysin digest of M167 IgA labelled at buried thiol groups with iodo[1- 14 C]acetamide

Hydrophilic peptides were removed in an initial run by elution with 10 mM-sodium phosphate buffer, pH 7.4. The sample contained 540 000 c.p.m., corresponding to 51 nmol of label. The initial buffer was 10 mM-sodium phosphate, pH 7.4. For further experimental details see the text. The arrow indicates the point of injection. —, A_{225} ; ----, concn. of acetonitrile; histogram, radioactivity.

which were associated with the Fab portion of the molecule, and only 1 SH group per dimer was titratable in the absence of denaturant. Mouse IgA has therefore more exposed thiol groups than rabbit sIgA or human IgA, but fewer buried thiols than rabbit sIgA.

Exposed thiol groups

As much as 80% of Cys-307 side chains are readily reactive with thiol-blocking reagents, and this was probably the residue of pK 9.2 and high reactivity with 1-fluoro-2,4-dinitrobenzene found in a competitive-labelling study (Kaplan *et al.*, 1980). The remaining Cys-307 residues may form disulphide bonds with each other or with other thiols such as free cysteine or glutathione, as discussed above for L-chain monomer, and with

the albumin present in IgA preparations. In rabbit sIgA, at least 0.6 SH group per molecule was detected in such mixed disulphides (Elliott *et al.*, 1980).

During studies of M460 IgA, Richards and co-workers alkylated a free thiol group that appeared to be close to the antigen combining site, though the labelled thiol group was not found in the Fab fraction after papain digestion (Rosenstein *et al.*, 1972). A labelled peptide was subsequently isolated from a thermolysin digest (Jackson & Richards, 1974) which resembles in its amino acid composition and first two residues the peptide E2 reported in the present paper. It seems probable that Cys-307 was in fact the residue labelled by these workers, but it is not evident how its modification would affect the combining site

Table 3. Amino acid composition and assignment of peptides resulting from labelling of buried thiol groups of MOPC 167 IgA with iodo[1-¹⁴C]acetamide

Integral values in parentheses and assignments are derived from the amino acid sequence (see the text). Specific radioactivities were calculated relative to iodo[1-¹⁴C]acetamide. Abbreviations: Cys(Cm), S-carboxymethyl-cysteine; N.D., not determined.

Amino acid	Peptide . . .	Amino acid composition (mol of residue/mol)			
		B21	B23	B23A	B23B
Cys(Cm)		0.9 (1)	0.9 (1)		0.9 (1)
Asx			1.1 (1)	1.0 (1)	
Ser			1.9 (2)	1.7 (2)	
Pro			1.0 (1)		1.0 (1)
Gly	1.0 (1)		1.0 (1)		1.0 (1)
Val			0.7 (1)		0.8 (1)
Ile	1.0 (1)		1.5 (2)		1.7 (2)
Leu			1.0 (1)	1.0 (1)	
Specific radioactivity		0.22	0.30	0.0	N.D.
Recovery of ¹⁴ C radioactivity		9	7		
N-Terminus		N.D.	Leu	Leu	Pro
Assignments	<div><div>141150</div><div>Ala-Leu-Ser-Ser-Asp-Pro-Val-Ile-Ile-Gly-Cys-Leu</div><div>←B21→</div><div>←B23→</div><div>←B23A→←B23B→</div></div>				

Table 4. Amino acid composition and assignment of peptides resulting from labelling of buried thiol groups of MOPC 167 IgA with N-[Et-1-¹⁴C]ethylmaleimide

Integral values in parentheses and assignments are derived from the sequence (see the text). Ethylamine is a hydrolysis product of N-ethylsuccinimidocysteine. Specific radioactivities were calculated relative to N-[Et-1-¹⁴C]ethylmaleimide. Abbreviation: Cys(Suc), S-(1,2-dicarboxyethyl)cysteine.

Amino acid	Peptide . . .	Amino acid composition (mol of residue/mol)	
		P1	P2
Cys(Suc)		0.6 (1)	0.3 (1)
Asx			1.1 (1)
Ser		0.8 (1)	1.7 (2)
Pro			1.2 (1)
Gly			1.0 (1)
Val	0.5 (1)		0.6 (1)
Ile			1.5 (2)
Leu			1.0 (1)
Lys	1.0 (1)		
Ethylamine	0.9 (1)		0.6 (1)
Specific radioactivity		0.29	0.30
Recovery of ¹⁴ C radioactivity (%)		12	16
N-Terminus		Val	Leu
Assignments	<div><div>206208</div><div>Ser-Val-Lys-Cys-Ser-Val</div><div>←P1→</div><div>141150</div><div>Ala-Leu-Ser-Ser-Asp-Pro-Val-Ile-Ile-Gly-Cys-Leu</div><div>←P2→</div></div>		

(Rosenstein *et al.*, 1972). Fluorescence energy-transfer experiments suggested distances of 2.2–4.8 nm (22–48 Å) between bound haptens and the thiol site (Manjula *et al.*, 1976), which is not consistent with its C_α2 position. These results might be explained by some long-range interactions occurring between the Fab and Fc arms of the IgA.

In models proposed for human IgA dimer, the cysteine equivalent to mouse Cys-307 has been tentatively assigned to an intrasubunit bond in the monomer, or to an intersubunit bond suspected in some dimer molecules (Tsuzukida *et al.*, 1979; Yang *et al.*, 1979). In mouse IgA, the large proportion of Cys-307 residues in the reduced state indicates that inter-H-chain linkages are not common. However, in myeloma sera, higher oligomers occur in which extra monomer units are directly disulphide-bonded to the (IgA)₂J molecule (Hauptman & Tomasi, 1975; Chapuis & Koshland, 1975). These intersubunit bridges are relatively labile and could well involve Cys-307 residues, forming bonds analogous to the intersubunit linkage in human pentameric IgM (Feinstein & Beale, 1977).

A major role for Cys-307 residues may lie in the covalent attachment of IgA dimer to secretory component. A double disulphide exchange between labile disulphide bridges in secretory component and in IgA has been proposed as the joining mechanism in the assembly of human sIgA (Lindh & Björk, 1976; Cunningham-Rundles & Lamm, 1975). In the mouse case, the Cys-307 thiol groups could insert directly into a secretory component disulphide by a non-enzymic thiol-disulphide exchange.

Absence of an H–L disulphide bridge

In most immunoglobulins, the H-chain cysteine participating in the H–L disulphide bond is located at the beginning of the C_α1 domain (Feinstein & Beale, 1977). The sequence proposed by Robinson & Appella (1980) for mouse α-chain includes a cysteine at residue 142, homologous to the human IgA1 case (Liu *et al.*, 1976; Yang *et al.*, 1979). This makes the lack of H–L bonding in BALB/c IgA (Abel & Grey, 1968) hard to understand. However, our data from the M167 peptides B23 and P2 reveal a difference from the primary structure of M511 IgA given by Robinson & Appella (1980), namely the presence of serine rather than cysteine at position 142. This assignment is supported by the nucleotide sequence obtained for cDNA complementary to α-chain mRNA from J558 tumour cells (Auffray *et al.*, 1981), but is contradicted by the α-gene sequence of genomic DNA from BALB/c liver tissue (Tucker *et al.*, 1981). All myeloma α-chains would be expected to have the same

constant-region sequence; however, somatic mutations such as Cys to Ser at residue 142 might possibly occur during B-lymphocyte differentiation. Kehry *et al.* (1982) reported that the amino acid sequence of M104E μ-chain also differed in two residues from that predicted from the nucleotide sequence of its germ-line gene. Another explanation is that BALB/c mice may have two α-chain genes, as proposed by Stanisz *et al.* (1983), giving rise to IgA variants with and without an H–L linkage. On this basis, the α-chain gene described by Tucker *et al.* (1981) could correspond to H–L-linked IgA, the type found in NZB mice.

Buried thiol groups

The ease with which thiol–disulphide exchange can take place when proteins are denatured at neutral or alkaline pH can greatly complicate the assignment of buried thiol groups. In the present work, we relied on two measures to avoid misassignments, one being the use of *N*-ethylmaleimide at low pH, and the other the determination of the specific radioactivity of the purified peptides relative to the alkylating agent. Ideally, the radioactivity should be the same as the molar proportion of buried thiol group per H chain in the initial proteins, or half that number in the case of two equally contributing cysteine residues. The values obtained for Cys-150 and Cys-208 (Tables 3 and 4) are satisfactory in this respect.

Sequence homologies among the C_H1 and other domains (Kabat *et al.*, 1983) indicate that Cys-150 and Cys-208 should form the buried disulphide bridge that is the hallmark of all immunoglobulin domains. It is therefore quite remarkable to find them even partially in the reduced state. About 30% of the C_α1 domains in the main M167 sample examined lacked this disulphide bridge, and 20–40% in other mouse IgAs. It is probable that the buried thiol groups of rabbit IgA Fab (Elliott *et al.*, 1980) are also in the C_α1 domain, since the V_H and L-chain portions are common to other rabbit immunoglobulins, which have not been reported to have free thiol groups.

The buried thiol groups almost certainly arise from incomplete oxidation during biosynthesis, rather than partial disulphide reduction subsequent to chain folding, although a reactive C_α2 domain disulphide bridge has been reported in human myeloma IgA1, capable of reduction at low concentrations (0.2 mM) of dithiothreitol (Fontaine *et al.*, 1981).

These findings raise the question of the precise role of the intrachain bridge in the acquisition and maintenance of the native conformation of immunoglobulins. The reduced C_α1 domains appear to be in a normal folded condition, insofar as the thiol groups are inaccessible to chemical

reagents unless GdmCl is added at concentrations known to cause denaturation of immunoglobulin domains. The reduced $C_{\alpha}1$ domains could be stabilized strongly by interaction with apposed C_L domains (Lapanje & Dorrington, 1973; Rowe, 1976). The denaturation curve (Fig. 1) indicates a structural transition at 1.5M-GdmCl. An unusual β -structure-forming transition around this concentration was observed in c.d. studies of M167 IgA (Young & Williams, 1982) and M167 Fab (N. M. Young & R. E. Williams, unpublished work), and this may be connected with the presence of reduced $C_{\alpha}1$ domains.

Reduced immunoglobulin domains have been shown to fold successfully *in vitro*, giving rise to a pair of buried thiol groups of low reactivity (Goto & Hamaguchi, 1979, 1981). Other evidence suggests that formation of the disulphide bridge is a central event along a co-operative folding pathway, ensuring that later stages are carried through rapidly and efficiently (Isenman *et al.*, 1979; Goto & Hamaguchi, 1982). Undoubtedly disulphide-bond formation is an important biosynthetic step with both thermodynamic and kinetic implications, but, at least in the case of mouse $C_{\alpha}1$ domains, it is evidently not a mandatory event in the establishment of native structure. For a particularly efficient folding pathway, the final tertiary structure could be reached before thiol oxidation can take place. The domain cysteine residues would then be trapped in the reduced form by the cage of β -structure, and be carried forward to the next stage of assembly. As a class, the buried disulphide bridges of immunoglobulins are

unusual in having a much more extended conformation than all other intramolecular bridges so far investigated by X-ray crystallography (Richardson, 1981; Thornton, 1981). As well as implying a degree of strain, this feature would perhaps allow a pair of free thiol groups to be accommodated with minimal perturbation of the domain structure.

A disulphide-bond model for mouse IgA

Aside from the question of H-L linkage, the arrangement of disulphide bonds in mouse IgA has been less thoroughly studied than those of human and rabbit IgAs, though none can be regarded as definitively characterized. The sequence of the constant portion of mouse α -chain contains 12 undisputed cysteine residues, at positions 150, 200, 208, 224 ($C_{\alpha}1$), 238 (hinge), 262, 295, 297, 307, 319 ($C_{\alpha}2$), 365, 428 ($C_{\alpha}3$) and 467 (tail-piece), as adapted from the M511 numbering (Robinson & Appella, 1980). Fig. 5 illustrates a possible disulphide-bond model for dimeric mouse IgA derived from the present work on thiol groups, the various α -chain sequences, and a model deduced from a thorough primary structural study of human IgA1 (Yang *et al.*, 1979).

A second disulphide bridge in mouse $C_{\alpha}1$ would be formed by Cys-200 and Cys-224, analogous to that in human $C_{\alpha}1$. In contrast with the buried intradomain bridge from Cys-150 to Cys-208, it is completely formed in M167 IgA, as judged by the failure to radiolabel Cys-200 and Cys-224 to a significant extent.

The hinge region of human α -chains contains

Fig. 5. Proposed disulphide bonding in α -chain constant regions of IgA dimer

The upper diagram is of mouse IgA, numbered in accordance with Robinson & Appella (1980), but corrected in the $C_{\alpha}3$ domain (Auffray *et al.*, 1981; Tucker *et al.*, 1981). The lower diagram is of human IgA1, adapted from Yang *et al.* (1979) and Tsuzukida *et al.* (1979). Normal intradomain loops are shown below the chains and additional loops above the chains. Some alternative bonding arrangements are shown by broken lines (see the text). Arrows to H_s represent intersubunit disulphide bonds, and arrows to H represent intrasubunit disulphide bonds.

two consecutive cysteine residues, one of which belongs to an inter-H-chain bridge, while the other bonds to a cysteine residue in C_α2 (Yang *et al.*, 1979). The latter occurs in an unusual tripeptide sequence Cys-Gly-Cys, of which the first cysteine residue forms another disulphide bridge to the adjacent H chain (Yang *et al.*, 1979). All four are predicted to be close together on the grounds of domain structural homology (Feinstein & Beale, 1977). Mouse α -chain contains the same Cys-Gly-Cys sequence (residues 295–297), but has only one hinge cysteine residue at residue 238 (Robinson & Appella, 1980). If the same intrachain bond from the hinge to the C_α2 domain is present as in the human IgA model, the mouse IgA would be unique among immunoglobulins in lacking a hinge disulphide bridge between the two H chains. The presence of a hinge disulphide bridge is indeed suggested by a report that an (Fab)₂ fragment was obtained by pepsin digestion of mildly reduced and alkylated McPC 603 IgA, in which any L–L disulphide bridges should have been destroyed (Rudikoff *et al.*, 1972). Alternative arrangements permitting a normal hinge disulphide bridge would require either two more inter-H-chain bonds through Cys-295 and Cys-297 or an intrachain link between them as depicted in Fig. 5. Both schemes are geometrically possible, and no evidence presently exists to distinguish between them. The short loop advocated here is structurally quite feasible according to molecular-model-building, and creates a fold similar to a γ -turn. Such a loop has been proposed in human chorionic gonadotropin (Mise & Bahl, 1980) for the sequence Cys-His-Cys.

Cys-262 and Cys-319 residues and Cys-365 and Cys-428 residues can safely be assigned to the C_α2 and C_α3 intradomain bridges respectively. The two remaining cysteine residues are Cys-307, the position of the exposed thiol groups, and Cys-467, which occurs in a non-domain tail-piece and is the penultimate α -chain residue. The latter plays a central role in IgA polymerization. In serum IgA dimer, two penultimate cysteine residues, one from each subunit, are disulphide-bonded to the J chain (Mestecky *et al.*, 1974; Hauptman & Tomasi, 1975; Chapuis & Koshland, 1975). Their counterparts on the other two α -chains could either be joined together in an intersubunit link or attached to cysteine residues elsewhere in their respective subunits, as suggested for one monomeric myeloma IgA (Prah *et al.*, 1971). In mouse IgA, the only realistic candidate for participation in the latter intrasubunit bond (which could be intra- or inter-chain) is Cys-307, all other potentially available cysteine residues being much more distant, on the hinge side of C_α2. Besides confirming that Cys-467 in mouse IgA dimer is not present in the free thiol form, our data demonstrate that a disulphide

bond from Cys-307 to Cys-467 could not be present in every molecule, since this would permit a maximum of only 2 free thiol groups per dimer at Cys-307, considerably below the observed amounts. In Fig. 5 the intersubunit bond between two Cys-467 residues is shown, although some Cys-307–Cys-467 bonds could exist in the population.

This model-building exercise emphasizes that, unlike the more familiar immunoglobulins such as human IgG, mouse IgA does not conform to a single disulphide-bond pattern; instead it comprises a population of molecules varying in their thiol and possibly disulphide arrangements.

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