Detection of disulphide bonds and localization of interchain linkages in the third (C3) and the fourth (C4) components of human complement

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Disulphide bonds contribute significantly to the maintenance of structural/functional integrity of many proteins. Therefore it was of interest to study the distribution and the effect of disulphides on conformation of complement components C3 and C4. These proteins are precursors of several fragments with various binding sites and distinct physiological functions. The constituents of C3c (β , α 27, α 43) and those of C4c (β , α 27, α 16, γ) were investigated, since other fragments of C3 or C4 do not participate in interchain linkages. Inter- and intra-chain disulphide bonds in C3c and C4c were localized by using a modification of conventional SDS (sodium dodecyl sulphate)/polyacrylamide-gel electrophoresis such that the change in mobility of disulphide-bond-containing proteins can be detected throughout the transition from a non-reduced to a fully reduced state. Several forms of the α 43 fragment from C3, and of the γ -chain of C4, with different mobilities can exist, depending on the number of intra-chain disulphide bonds reduced. The intermediates (heterodimers) generated by a partial reduction of C3c or C4c were characterized by two-dimensional SDS/polyacrylamide-gel electrophoresis performed in the absence, then in the presence, of β -mercaptoethanol. The inter-chain linkages in C3c were determined to be β - α 27 and α 27- α 43, thus indicating the presence of only one interchain bond in C3. The two interchain bonds in C4c are β - α 27 and α 16- γ . The third interchain bond in C4 (α 27- γ , tentative) remains to be determined.

INTRODUCTION

The C3 and C4 proteins are two components of the complement system (Müller-Eberhard & Schreiber, 1980; Reid & Porter, 1981). They are synthesized as single polypeptide chain pro-forms (Morris *et al.*, 1982; Goldberger *et al.*, 1980), but exist in plasma as multiple-chain structures, β and α in C3, β , α , and γ in C4.

Both C3 and C4 are the precursors of several biologically active fragments. The first cleavage of C3 or C4 generates two fragments each, a and b. The larger cleavage product, disulphide-bridged two-chain C3b or three-chain C4b, has the ability in its nascent state to bind covalently to cell surfaces or immune complexes and serve as a non-catalytic subunit of C3 and/or C5 convertases (reviewed by Janatova, 1983). Unlike native C3 or C4, the C3b or C4b fragment is susceptible to cleavage by factor I in the presence of the appropriate cofactor. C3b or C4b with fragmented α -chain can no longer participate in the formation of C3/C5 convertase, but it can interact with the complement receptors that are present on a variety of blood or tissue cells (see, e.g., Arnaout & Colten, 1984; Fearon, 1984).

In C3b the first two cleavages by factor I occur in the presence of factor H at the distance of 46 and 43 kDa from the C-terminus of the α -chain, thus yielding a

three-chain iC3b (β , α 68, α 43) and a single-chain C3f of M_r 3000 (Sim *et al.*, 1981; Davis & Harrison, 1982). The third cleavage by factor I is mediated by C3b receptor (CR1) present on erythrocytes. As a result of this proteolytic reaction, the C3d,g fragment (M_r 40000), the part of C3 molecule that remains covalently attached to cell surfaces or immune complexes, is removed from the middle of the C3b α chain (Medof *et al.*, 1982; Ross *et al.*, 1982). The larger C3c is released into the fluid phase and consists of β -chain (M_r 75000) and two α -chain fragments, N-terminal C3 α 27 (M_r 27000) and C-terminal C3 α 43 (M_r 43000). The recent results of N-terminal sequence analyses of the constituents of the C3c fragment (Davis *et al.*, 1984) allowed the localization of the cleavage sites in the α -chain by both C3 convertases and factor I within the proposed amino acid sequence of human C3 (de Bruijn & Fey, 1985).

The two cleavages of C4b mediated by factor I in the presence of C4bp (C4b-binding protein) result in two fragments: C4d (M_r 46000) and C4c (Fujita *et al.*, 1978; von Zabern *et al.*, 1982a). The constituents of the larger C4c, i.e. β -chain (M_r 75000), two α -chain fragments, N-terminal C4 α 27 and C-terminal C4 α 16 and γ -chain (M_r 33000), were isolated, characterized (Press & Gagnon, 1981), and positioned within the primary structure of human C4 (Belt *et al.*, 1984).

Disulphide bonds are structural as well as functional

Abbreviations used: NHS, normal human serum; CVF, cobra venom factor; SDS, sodium dodecyl sulphate; SH, a free thiol group; NEM, N-ethylmaleimide; NTCB, 2-nitro-5-thiocyanobenzoic acid; S-S, disulphide bond(s); DTT, dithiothreitol; C3 and C4, third and fourth protein components of the serum complement system; C3b or C4b, major fragment of C3 or C4 activation; factor I, an enzyme that cleaves the α -chain in both C3b and C4b; iC3b or iC4b, C3b or C4b cleaved at one site by factor I in the presence of factor H (see below) or C4bp; C4bp, cofactor for cleavage of C4b α ; factor H and/or CR1, cofactors for factor I-mediated cleavages of C3b α ; CR1, a C3b receptor on erythrocytes and other blood cells; C3c, a three-chain fragment (β , α 27, α 43) generated from iC3b by factor I in the presence of CR1 (erythrocytes); C4c, a four-chain fragment (β , α 27, α 16, γ) generated from C4b by factor I in the presence of C4bp.

components of many proteins, conferring additional stability to the active forms of the molecule. It is almost axiomatic that locations of disulphide bonds remain invariant in homologous proteins (Thornton, 1981). Several lines of evidence exist that indicate a possible common ancestry for C3 and C4. We here present results of a study on the distribution of disulphide bonds in C3 and C4 and their effects on conformation as monitored by SDS/polyacrylamide-gel electrophoresis. Since C3a, C3f, and C3d,g fragment of C3, and C4a and C4d fragments of C4 do not participate in interchain bonds in these proteins, we chose to investigate disulphide bonds between and within the constituents of C3c (β , $\alpha 27$, $\alpha 43$) and those of C4c (β , $\alpha 27$, $\alpha 16$, γ). A preliminary report of these studies has been presented (Janatova, 1985).

EXPERIMENTAL

Materials

Reagents for polyacrylamide-gel electrophoresis analyses, including high- and low- M_r protein standards, were purchased from Bio-Rad Laboratories. SDS was from BDH. Trizma Base and Na_2EDTA were obtained through Sigma; DTT, β -mercaptoethanol, and NEM were from Pierce Chemical Co. NTCB was a gift from Dr. H. S. Wiley of this University. All chemicals and reagents used were of the highest grade available, or as appropriate.

Generation and isolation of C3c and C4c

Physiologically relevant fragments C3c and C4c were generated in NHS in the presence of ≥ 2.5 mM-NEM to prevent anticipated $\text{SH} \rightleftharpoons \text{S-S}$ interchange. A quantitative conversion of C4 into C4c (β , $\alpha 27$, $\alpha 16$, γ), and C3 into iC3b (β , $\alpha 68$, $\alpha 43$) was achieved in fresh NHS after its incubation at 37 °C with aggregated human IgG (0.76 mg/ml) and CVF (4.15 $\mu\text{g}/\text{ml}$). After 4 h the reaction mixture was incubated for an additional 20 h at 30 °C in the presence of erythrocytes (5×10^{10} cells/10 ml of NHS). Both C3c and C4c were isolated from complement-activated NHS by a two-step procedure involving (i) an adsorption to, and (ii) electrophoretic desorption from, the respective immunoabsorbent beads. The proteins were eluted at the bottom of the resolving gel with pH 8.8 buffer (378 mM-Tris/60 mM-HCl). Detailed conditions for generation and isolation of C3c and C4c, including materials used, have been described elsewhere (Janatova & Gobel, 1985). The sample of elastase-generated C3c, which was used to establish experimental conditions in the present study, was prepared by using a previously described protocol (Tack *et al.*, 1981) and was a gift from Dr. B. F. Tack (Scripps Clinic and Research Foundation, La Jolla, CA 92037, U.S.A.).

Analyses of disulphide linkages by SDS/polyacrylamide-gel electrophoresis

To detect inter- and intra-chain or fragment disulphide bonds in C3c and C4c we have used a simple modification (Lam & Kasper, 1980; Allore & Barber, 1983) of conventional one-dimensional SDS/polyacrylamide-gel electrophoresis. Buffers and gels were prepared as described by Laemmli (1970). Electrophoresis was carried out at 22 °C in a water-cooled slab-gel apparatus (Protean cell from Bio-Rad Laboratories). Unless stated otherwise, a 30 μl aliquot of each sample containing

~ 10 μg of the protein material was diluted with 15 μl of 0.18 M-Tris/HCl buffer, pH 6.8, containing 9% (w/v) SDS, 30% (v/v) glycerol, and either 1.5% (v/v) or no β -mercaptoethanol. The samples were then applied to individual wells (25 mm high, 6 mm wide and 1.5 mm thick), separated by a 3 mm-wide gel partition. Unless stated otherwise, samples were not heated before analysis, except for a standard mixture of M_r markers (for composition, see Fig. 4 below). The samples containing 0.5% β -mercaptoethanol were applied last. It took 60 min at 15 mA/gel before all proteins entered the stacking gel. During the migration through a 2 cm-high stacking gel (50 min at 25 mA), β -mercaptoethanol from the reduced samples continued to diffuse in the direction of the non-reduced samples forming a thiol gradient. The resolving gel of 10% (w/v) polyacrylamide (115 mm high, 140 mm wide) was used to separate molecules according to size for 128 min at 35 mA. Bromophenol Blue was used as a tracking dye. Gels were stained with 0.25% Coomassie Brilliant Blue R-250 in methanol/acetic acid/water (5:1:4, by vol).

Characterization of partially reduced forms and intermediates by two-dimensional SDS/polyacrylamide-gel electrophoresis

Stock solutions of 90 mM-DTT and 500 mM-iodoacetamide were prepared immediately before use in pH 8.8 buffer (378 mM-Tris/60 mM-HCl). The buffer had been filtered through 0.2 μm -pore-size filter, evacuated and purged with N_2 twice. Portions of C3c or C4c (80 μl) in pH 8.8 buffer as described above were allowed to react with 10 μl of appropriate concentrations of DTT for 30 min at 20 °C. A 10 μl portion of iodoacetamide giving a 10-fold molar excess over the concentrations of DTT was then added, and alkylation was allowed to proceed for 30 min at 37 °C in the dark. After alkylation, 50 μl of the sample buffer [0.18 M-Tris/HCl (pH 6.8)/9% (w/v) SDS/30% (v/v) glycerol] without β -mercaptoethanol was added to each of the 100 μl samples. The volume of each partially reduced sample applied into the well was 60 μl , and electrophoresis was carried out as described above. Results are presented in Fig. 3 (below).

Partially reduced forms and intermediates

These were analysed as follows. A sample of C3c or C4c that had been treated with 0.5 mM-DTT and alkylated with 5 mM-iodoacetamide was electrophoresed in the absence of a reducing agent in a 1.5 mm-thick slab gel. Individual tracks were then excised and treated much as described by Lam & Kasper (1979). Each slice was incubated for 15 min at 22 °C in 10 ml of sample buffer (diluted three times), containing 5% (v/v) β -mercaptoethanol. The gel slices were then immersed in the diluted sample buffer that was layered over the stacking gel (2 cm high) polymerized on the top of a 10% (w/v) resolving gel (11.5 cm high). The slab gel used for the second dimension was 3 mm thick; the current was therefore increased twice over the values that were described above for 1.5 mm-thick slab gel.

Preparation and cleavage of S-cyanylated constituents of C3c or C4c

The C3c or C4c protein solutions (~ 2 μM) were treated at pH 8.8 with 5 mM-DTT for 30 min at 37 °C. After the reduction of disulphides, cysteine residues were S-cyanylated directly with 10 mM-NTCB (Degani *et al.*,

Fig. 1. Electrophoretic analysis of disulphide linkages in C3c

C3c, physiologically relevant (a) and elastase-generated (b), was analysed by one-dimensional SDS/polyacrylamide-gel electrophoresis as described in the Experimental section. A gradient of reducing agent was established (lanes 3 and 7; 3' and 7') between adjacent slab gel tracks, one containing (lanes 2 and 8; 2' and 8') and the other not containing β -mercaptoethanol. C3c fragments (lane 5 or 5') consist of the intact β -chain of M_r 75000 and two α -chain fragments each: one of M_r 27000 or 25000 ($\alpha 27$ or $\alpha 25$) and the other a C-terminal one of M_r 43000 or 40000 ($\alpha 43$ or $\alpha 40$). The electrophoretic patterns of C3c samples fully reduced with 0.5% (v/v) β -mercaptoethanol can be observed in lanes 1, 2, 8 and 9, or in lanes 2' and 8'. As seen in lanes 3 and 7 or 3' and 7', several forms of $\alpha 43$ or $\alpha 40$ with different mobilities can exist, depending on the number of intrachain disulphides reduced.

1970) for 15 min at 22 °C. Next, the treated solutions were mixed with the sample buffer without β -mercaptoethanol (2:1, v/v), and were immediately heated for 15 min at 95 °C to accomplish a denaturant-induced cleavage of the peptide bond N-terminal to the S-cyanylated cysteine residue (Vanaman & Stark, 1970).

RESULTS AND DISCUSSION

Polyacrylamide-gel electrophoresis can be a useful method for studying the conformational properties of proteins (Goldenberg & Creighton, 1984). The effect of reduction of intramolecular disulphide bonds on the mobility of proteins in SDS/polyacrylamide-gel electrophoresis was recognized many years ago (Griffith, 1972). An excellent example is the difference in the mobilities of non-reduced and reduced serum albumin (Fig. 7 in Allore & Barber, 1983). When one uses the gradient of reducing agent established between adjacent slab gel tracks, the resulting pattern gives a graphic illustration of the conformational changes in the protein, as reflected in the change in mobility of disulphide-bond-containing proteins throughout the transition from a non-reducing to a reducing environment (Lam & Kasper, 1980; Allore & Barber, 1984).

Detection of inter- and intra-chain disulphide bonds in physiologically relevant C3c

We tested the effect of several concentrations of β -mercaptoethanol in the range of 0.05–5.0% (v/v). The

intermediate β - $\alpha 25$ from elastase-generated C3c was detected in all lanes adjacent to any lane containing β -mercaptoethanol over the range of 0.05–0.5% (v/v). The use of 5% (v/v) β -mercaptoethanol as recommended by Allore & Barber (1983) resulted in full reduction of the C3c molecules. The best results were obtained with 0.5% (v/v) β -mercaptoethanol (Fig. 1). Under these conditions we could observe several discrete forms of $\alpha 43$ or $\alpha 40$, rather than only the 'diagonal' effect. The mobilities of these forms corresponded to the ones generated by the partial reductions (Fig. 3 below). The two-band pattern of unheated C3c samples (Fig. 1, lanes 5 and 5') could be changed into a single band corresponding to the one with a lower mobility on boiling (results not shown). This phenomenon was not due to the heterogeneity manifested by C3 $\alpha 27$ (Davis *et al.*, 1984).

Except for unreduced C3c, there was no difference in the mobilities of the elastase-generated C3c constituents (β , $\alpha 25$, $\alpha 40$) or β - $\alpha 25$ intermediates, when the heated samples were compared with unheated ones, whether treated with 0.5 or 5% (v/v) β -mercaptoethanol or whether DTT-reduced and alkylated. Consequently, all the following experiments were performed with unheated samples.

The behaviour of C3c during SDS/polyacrylamide-gel electrophoretic analyses in a thiol gradient indicated that the C-terminal $\alpha 43$ from C3, or $\alpha 40$ from elastase-generated C3c must contain a relatively large number of intrachain disulphide bonds (Fig. 1). The observed difference in the mobilities of $\alpha 43$ between 'unreduced'

Fig. 2. Comparison of inter- and intra-chain disulphides of C4c with those of C3c

Both C4c (a) and C3c (b) were generated in, and isolated from, NHS containing NEM. This electrophoretic analysis was performed under the same experimental conditions as those used in Fig. 1. These results demonstrate once more the homology between C4 and C3. Noteworthy are the similarities between the β -chains, $\alpha 27$ fragments, or high- M_r intermediates β - $\alpha 27$. The low- M_r intermediate $\alpha 16$ - γ in C4c is apparently the counterpart of C-terminal $\alpha 43$ in C3c. The $\alpha 16$ fragment of M_r 16000 did not stain under the given conditions.

Fig. 3. Effect of a reducing agent (DTT) on the electrophoretic pattern of (a) C3c and (b) C4c

Each sample of C3c and C4c was treated with DTT at the concentration indicated, and was subsequently alkylated with iodoacetamide. The protein concentration during the reduction step was approx. $2 \mu\text{M}$. The treated C3c and C4c samples were analysed under the same conditions as those used in Fig. 1 and Fig. 2, in the absence, however, of a reducing agent. The electrophoretic patterns in lanes 7 and 7' show the results of the attempt to cleave the constituents of C3c and C4c at their cysteine residues after *S*-cyanylation with thiocyanate (SCN).

Fig. 4. Apparent sizes of the constituents of C3c and C4c after partial or complete reduction of disulphides

This Figure summarizes data obtained from the experiments represented in Fig. 1, 2, and 3 and those not shown. The mixture of M_r markers containing (1) myosin (200 000), (2) β -galactosidase (116 250), (3) phosphorylase *b* (92 500), (4) bovine serum albumin (66 200), (5) ovalbumin (45 000), (6) carbonic anhydrase (31 000), and (7) soya-bean trypsin inhibitor (21 500) was used as a standard in every electrophoretic analysis.

(M_r 30 000) and fully reduced (M_r 43 000) states suggests that this part of C3 molecule is highly cross-linked by disulphide bridges.

We have never been able to observe any difference in the mobility of the β -chain. Such an observation implies that the number of intrachain disulphide bonds in the β -chain, if any, is either small, or their location does not influence the overall shape of that part of C3 molecule, and/or that cysteine residues could be involved in the interchain disulphide linkages.

Homology between physiologically relevant C3c and C4c

The results presented in Fig. 2 emphasize once more the homology between C3 and C4. The similarities between β -chains, $\alpha 27$ fragments, or high- M_r intermediates (β - $\alpha 27$) between the two proteins are remarkable. A striking similarity also exists between the electrophoretic pattern of human C3c (present data) and that of CVF (von Zabern *et al.*, 1982b), under both partially and fully reducing conditions. If the low- M_r intermediate with M_r 43 000 from C4c comprised the $\alpha 16$ fragment with a single cysteine residue and the γ -chain, then the apparent size of the γ -chain in that complex could be estimated to be M_r 27 000, a value smaller than the one obtained after a partial reduction of γ -chain (M_r 32 000 was determined for the fastest form of γ -chain). These results taken together suggest that intrachain disulphide bonds in the γ -chain are either more susceptible to the reduction and/or their effect on the overall shape of the γ -chain is less when compared with the C3 $\alpha 43$ domain.

Analyses of heterodimers and intermediate forms generated from C3c and C4c by partial reduction

To test the susceptibility to reduction of inter- and intra-chain disulphide bonds in C3c or C4c, individual samples were treated with several concentrations of DTT. As Fig. 3 (lanes 1 and 1') shows, only a few, if any, C3c or C4c molecules were affected by the treatment with 0.01 mM-DTT. With respect to intermediates and partially reduced forms, perhaps most informative are the electrophoretic patterns of the C3c or C4c sample treated with 0.5 mM-DTT (Fig. 3, lanes 4 and 4').

To confirm our predictions that: (i) the high- M_r intermediates in both C3c and C4c do consist of the intact β -chains and the $\alpha 27$ fragments, (ii) the lower- M_r intermediate from C4c is a heterodimer of the γ -chain and the $\alpha 16$ fragment, and that (iii) several forms within the range of M_r from 30 000 to 43 000 do correspond to the same part of the C3 molecule, we analysed the same C3c and C4c samples as depicted in lanes 4 and 4' (Fig. 3) (i.e. treated with 0.5 mM-DTT) by two-dimensional SDS/polyacrylamide-gel electrophoresis. The first dimension was in the absence, and the second dimension in the presence, of β -mercaptoethanol. The conditions are described in the Experimental section. Indeed, as expected, all C3 $\alpha 43$ forms, as well as C4 γ forms, migrated in the second dimension as the fully reduced $\alpha 43$ or γ -chain respectively. The mobilities of the $\alpha 27$ -fragments and those of the β -chains were unchanged, i.e. they were located on the diagonals. The high- M_r intermediates were separated into β -chains and $\alpha 27$ fragments, and the low- M_r heterodimer into γ -chain and $\alpha 16$ fragment.

Analysis of S-cyanylated constituents of C3c and C4c

Peptide chains can be selectively cleaved at the cysteine residues after S-cyanylation (Degani *et al.*, 1970; Vanaman & Stark, 1970).

After thiocyanate-induced cleavage of the β -chain, three high- M_r species were detected by SDS/polyacrylamide-gel electrophoresis (Fig. 3, lane 7). In most C3c molecules the β -chain was cleaved at the most interior cysteine residue (no. 559 in de Bruijn & Fey, 1985), yielding the fragment of an apparent M_r 62 000.

It is rather surprising that the N-terminal $\alpha 27/\alpha 26$ fragment remained uncleaved, although it was clearly separated by reduction from both $\alpha 43$ and β -chain. One of the explanations could be that $\alpha 27$ assumed a conformation after treatment with DTT such as to make the cysteine residues inaccessible to S-cyanylation. The mobility of $\alpha 27$ remained unaffected by the above treatment.

Only a limited number of thiocyanate-induced cleavages occurred within the $\alpha 43$ domain. The pattern and the apparent sizes of generated polypeptides would suggest that this part of C3 molecule was cleaved at two/three cysteine residues, one of which is possibly involved in the $\alpha 43$ - $\alpha 27$ linkages, and another from the disulphide linkage that was most susceptible to the treatment with 5 mM-DTT under non-denaturing conditions.

Similar results were obtained with the C4c sample, at least as far as the $\alpha 27$ fragment is concerned. Also, most of the C4 γ chain was cleaved after reduction and S-cyanylation. The pattern of the C4 β chain cleavage products was more complex, apparently due to the presence of two additional cysteine residues located close to the N-terminus (Belt *et al.*, 1984). It is the peptide bond

Fig. 5. Interchain disulphide bridges in C3c and C4c molecules

The two cysteine residues that are known with certainty to be involved in the interchain bonds are the ones present in the C3 β chain (residue no. 559 in de Bruijn & Fey, 1985) of C3 and in the C4 $\alpha 16$ fragment from C4; they are denoted by continuous lines. The other disulphides, represented by broken lines, are also indicated in the present work; however, it is not certain as yet which of the two cysteine residues of the $\alpha 27$ fragments is linked to the respective β -chain. See the text for further discussion.

at the third cysteine residue from the C-end of the β -chain, likewise in C3, that seems to be preferentially cleaved.

Interchain disulphide bridges in C3 and C4

The sequence data show a total of 26 cysteine residues present in both C4 (Belt *et al.*, 1984) and C3 (de Bruijn & Fey, 1985). An additional cysteine residue is present in the form of a thiol ester and is located within C4d or C3d domain of the respective protein. All other cysteine residues are presumed to exist as disulphides. Three disulphide linkages form a knot in the activation peptides, C4a and C3a. One intrachain disulphide bond is located in the C3d fragment. The remaining cysteine residues are present in C4c and C3c. The $\alpha 27$ domain from both proteins contains only two cysteine residues each. The three cysteine residues in the β -chain of both C4 and C3 (the only ones in C3 β) are located close to the C-termini. The C3 β chain contains one intrachain disulphide bond (M. S. Strickland, W. R. Gray & J. Janatova, unpublished work). Since only one cysteine residue from the C3 β chain remains available for the interchain linkage with $\alpha 27$, the second cysteine residue from C3 $\alpha 27$ must thus be linked to C3 $\alpha 43$. This implies that the two chains in C3 molecule, α and β , are linked by only one interchain disulphide bond, as indicated in Fig. 5 (below). These conclusions are also supported by recent independent evidence showing that the β - $\alpha 27$ heterodimer contains one SH group detectable by alkylation with monoiodo[14 C]acetic acid; the radiolabel was localized in the $\alpha 27$ fragment (Matsuda *et al.*, 1985). Further work is required to determine which of the two cysteine residues of $\alpha 27$ is linked to the β -chain, and which of the 13 cysteine residues present in C3 $\alpha 43$ is involved in the $\alpha 43$ - $\alpha 27$ linkage. This particular bond seems to be the one most susceptible to reduction (the present data; Matsuda *et al.*, 1985), and it could be the same one that was reduced in native C3 by treatment with 0.5 mM-DDT (Isenman *et al.*, 1980). An additional one or two

intra- α -chain disulphide bonds were split when native C3 was incubated with 1 or 2 mM-DDT in the absence of denaturants, whereas the interchain bond remained unaffected. The comparison of these results with our data (Fig. 3, lanes 4 and 5) would suggest that the interchain β - $\alpha 27$ bond is more accessible to reduction with DTT in C3c than in native C3.

The two interchain bonds in C4 are the ones bridging the β -chain to the $\alpha 27$ domain, and the γ -chain to the C-terminus of the α -chain. Since the $\alpha 16$ fragment contains only one cysteine residue that is linked to the γ -chain, it is highly probable that one of the remaining eleven cysteine residues from the γ -chain is involved in the third interchain bridge in C4, the one connecting γ -chain to $\alpha 27$. This particular disulphide would be the most labile one, and it would link the two heterodimers, β - $\alpha 27$ and γ - $\alpha 16$ in C4, analogous to the bridge between β - $\alpha 27$ and $\alpha 43$ in C3. It would then follow that the C4 β chain contains two intrachain disulphides, one at the C-terminus (by analogy to C3 β) and the other one at its N-terminus. Confirmation of this remains to be determined.

A summary giving the apparent molecular sizes of C3c, C4c and their constituents under a range of reducing conditions (non-reduced to fully reduced) is presented in Fig. 4. The disulphide linkages discussed in the present paper are shown in Fig. 5.

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REFERENCES

- Allore, R. J. & Barber, B. H. (1983) *Mol. Immunol.* **20**, 383–395
- Allore, R. J. & Barber, B. H. (1984) *Anal. Biochem.* **137**, 523–527
- Arnaout, M. A. & Colten, H. R. (1984) *Mol. Immunol.* **21**, 1191–1199
- Belt, K. T., Carroll, M. C. & Porter, R. R. (1984) *Cell* **36**, 907–914
- de Bruijn, M. H. L. & Fey, G. H. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 708–712
- Davis, A. E., III & Harrison, R. A. (1982) *Biochemistry* **21**, 5745–5749
- Davis, A. E., III, Harrison, R. A. & Lachmann, P. J. (1984) *J. Immunol.* **132**, 1960–1966
- Degani, Y., Neumann, H. & Patchornik, A. (1970) *J. Am. Chem. Soc.* **92**, 6969–6971
- Fearon, D. T. (1984) *Immunol. Today* **5**, 105–110
- Fujita, T., Gigli, I. & Nussenzweig, V. (1978) *J. Exp. Med.* **148**, 1044–1051
- Goldberger, G., Abraham, G. N., Williams, J. & Colten, H. R. (1980) *J. Biol. Chem.* **255**, 7071–7074
- Goldenberg, D. P. & Creighton, T. E. (1984) *Anal. Biochem.* **138**, 1–18
- Griffith, I. P. (1972) *Biochem. J.* **126**, 553–560
- Isenman, D. E., Sundsmo, J. S. & Cooper, N. R. (1980) *J. Immunol.* **125**, 1798–1805

- Janatova, J. (1983) *Ann. N.Y. Acad. Sci.* **421**, 218–234
- Janatova, J. (1985) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44**, 990 (abstr.)
- Janatova, J. & Gobel, R. J. (1985) *J. Immunol. Methods*, in the press
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lam, K. S. & Kasper, C. B. (1979) *J. Biol. Chem.* **254**, 11713–11720
- Lam, K. S. & Kasper, C. B. (1980) *J. Biol. Chem.* **255**, 259–266
- Matsuda, T., Seya, T. & Nagasawa, S. (1985) *Biochem. Biophys. Res. Commun.* **127**, 264–269
- Medof, M. E., Iida, K., Mold, C. & Nussenzweig, V. (1982) *J. Exp. Med.* **156**, 1739–1754
- Morris, K. M., Goldberger, G., Colten, H. R., Aden, D. P. & Knowles, B. B. (1982) *Science* **215**, 399–400
- Müller-Eberhard, H. J. & Schreiber, R. D. (1980) *Adv. Immunol.* **29**, 1–53
- Press, E. M. & Gagnon, J. (1981) *Biochem. J.* **199**, 351–357
- Reid, K. B. M. & Porter, R. R. (1981) *Annu. Rev. Biochem.* **50**, 433–464
- Ross, G. D., Lambris, J. D., Cain, J. A. & Newman, S. L. (1982) *J. Immunol.* **129**, 2051–2060
- Sim, E., Wood, A. B., Hsiung, L.-M. & Sim, R. B. (1981) *FEBS Lett.* **132**, 55–60
- Tack, B. F., Janatova, J., Thomas, M. L., Harrison, R. A. & Hammer, C. H. (1981) *Methods Enzymol.* **80**, 90–91
- Thornton, J. M. (1981) *J. Mol. Biol.* **151**, 261–287
- Vanaman, T. C. & Stark, G. R. (1970) *J. Biol. Chem.* **245**, 3565–3573
- von Zabern, I., Bloom, E. L., Chu, V. & Gigli, I. (1982a) *J. Immunol.* **128**, 1433–1438
- von Zabern, I., Przyklenk, H. & Vogt, W. (1982b) *Scand. J. Immunol.* **15**, 357–362

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