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SP-40,40, a protein involved in the control of the complement pathway, possesses a unique array of disulphide bridges

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SP-40,40 is a two-chain serum protein which acts in vitro as a potent inhibitor of the assembly of the membrane attack complex of human complement. It contains 10 cysteine residues, the numbers and locations of which are conserved in several mammalian species. Evidence is presented that all the cysteine residues are involved in interchain (α–β) disulphide bonds. There are no free cysteine residues. The disulphide bond motif established in this study for SP-40,40 is unique and bears no obvious homology to those complement components whose disulphide bonds have been assigned, nor is there any homology apparent between SP-40,40 and other multi-chain proteins containing disulphide bonds.

SP-40,40; Complement; Disulphide bridge; Protein sequencing; Apolipoprotein A-I; Sequence homology

1. INTRODUCTION

The human serum protein, SP-40,40, was shown to be a disulphide-bonded heterodimer in which both chains are encoded by a single mRNA molecule as a single chain precursor which is cleaved post-synthetically into the 2 constituent chains [1,2]. The normal biological function of SP-40,40 is unknown but it acts in vitro to prevent the formation of the membrane attack complex (MAC) by binding to C5b-6 or C5b-7 intermediates, and preventing the insertion event into plasma membranes which results in the addition of C8 and C9 complement components to the membrane bound C5b-9 (MAC) complex [3–5]. SP-40,40 also occurs in human semen [2] and indeed its rat counterpart, known as sulphated glycoprotein-2 (SGP-2), was initially isolated as the major product of Sertoli cells [6,7].

Neither SP-40,40 nor SGP-2 exhibit extensive sequence homology to any protein other than homologues in other species [8–12]. The presence in SP-40,40 and SGP-2 of discrete regions with homology to myosin heavy chain has been noted [12]. Marginal levels of sequence homology have been noted between SGP-2 and apolipoprotein 1 [7], and between SP-40,40 and complement components, C7, C8 and C9 [2]; the significance of both sequence comparisons is as yet unclear. Recent observations in this laboratory [13] and elsewhere [14] have revealed that SP-40,40 exists in human blood associated with apolipoprotein A-I in high density lipoprotein (HDL) particles and a role in lipid traffic between tissues has been suggested. Purified apolipoprotein A-I and SP-40,40 exhibit measurable specific affinity for one another [13], accounting for the presence of SP-40,40 in HDL particles.

The earlier work from this laboratory left 2 major aspects of the covalent structure of SP-40,40 open to further investigation. Firstly, the location of the disulphide bonds within SP-40,40 could not be inferred on the basis of sequence homology to any protein of known structure. Secondly, the number and exact location of the proteolytic cleavage events which are responsible for separating the α and β polypeptide chains were not known.

In this communication we report the location of the disulphide bridges of SP-40,40, confirm the presence of carbohydrate (CHO) at all six *N*-linked CHO addition sites, and define the carboxyl-terminus of the mature β chain within narrow limits.

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Abbreviations: SGP-2, sulphated glycoprotein-2; HDL, high density lipoproteins; CHO, carbohydrate; CNBr, cyanogen bromide; DTT, dithiothreitol; E:S, enzyme:substrate; TFA, trifluoroacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate.

2. MATERIALS AND METHODS

2.1. *Purification of SP-40,40*

Human serum was subjected to immunoaffinity chromatography essentially as described [1,2] except that the monoclonal antibody–Sepharose conjugate, after the passage of human serum, was washed with 0.5% (w/v) sodium deoxycholate dissolved in 20 mM Tris, pH 8.0 (HCl), to remove apolipoprotein A-I attached to serum SP-40,40 [13].

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2.2. *Determination of thiol groups in SP-40,40*

Free thiol groups in proteins were determined using the Ellman reaction [15]. In brief, the protein concentrations of small samples of lysozyme and SP-40,40 were determined using the Bio-Rad Protein Assay Kit (Product No. 500-0001). Known amounts of both proteins were dissolved in 0.1 M Tris, 1.0% sodium dodecyl sulphate (SDS), pH 8.0 (HCl), and exhaustively reduced by the addition of dithiothreitol (DTT) to a final concentration of 100 mM, boiling for 2 min and then incubating for 1 h at ambient temperature. Acetone (9 vols.) was then added to each reaction mixture and protein precipitation conducted at −20°C for 1 h. Protein pellets were recovered by centrifugation and washed 3-times in ice-cold acetone (9 vols.) to remove excess reducing agent. Samples were then dissolved in 20 μl 6 M guanidine hydrochloride; 100 μl of water was added followed by 10 vols. of Ellman's reagent (0.13% (w/v) dithiobis nitrobenzoic acid (Sigma) dissolved in 40 mM Na_2HPO_4, pH 8.1) to each protein sample.

Known concentrations of both dithiothreitol and β-mercaptoethanol were used to construct the (linear) curve relating A^{412}_{1cm} and [-SH]. Absorbance measurements were made immediately after addition of Ellman's reagent.

2.3. *Enzymatic and chemical cleavages*

Proteins were dialysed against 1% acetic acid (v/v) and lyophilised. Known amounts of sample were digested with cyanogen bromide (CNBr) by dissolving aliquots (100–500 μg protein) in 100 μl of 70% (v/v) aqueous formic acid containing CNBr (Pierce, 25 mg/ml). Digests were incubated overnight at room temperature and then rotary evaporated to dryness several times, adding 200 μl–1 ml of H_2O between dry-down steps. Samples were dissolved in 6 M guanidine hydrochloride, 100 mM Tris, pH 8.0 (HCl), for chromatography, and in Laemmli SDS-lysis buffer for SDS-PAGE.

Digests with trypsin (Worthington), endoproteinase glu-C (Boehringer-Mannheim) and endoproteinase lysine-C (Boehringer-Mannheim) were conducted as previously described [2]. Pepsin digestions were conducted by dissolving protein in 70% formic acid at a concentration of 50 mg/ml. 20 vols. of 1 mM HCl containing enzyme (1% enzyme:substrate (E:S) ratio) were added for overnight digestions. Thermolysin (Boehringer-Mannheim) digests of SP-40,40 were conducted in a buffer of 2 mM $CaCl_2$, 100 mM ammonium bicarbonate, pH 6.8 (CO_2), at a maximum E:S ratio of 5:100. Digests were rotary evaporated to dryness and dissolved in 100 μl volumes of 6 M guanidine hydrochloride, 100 mM Tris, pH 8.0 (HCl). Aliquots were then either reduced with dithiothreitol (DTT, 10 mM) and treated with iodoacetamide (22 mM) or iodoacetamide alone without prior reduction. Samples were subjected to reverse-phase HPLC within 2 h of alkylation.

2.4. *Peptide separation*

Reverse-phase HPLC was conducted using a complete Pharmacia FPLC equipped with A^{214}_{1cm} detection unit. Acetonitrile gradients were used for peptide elution as indicated. All peptides to be sequenced were purified by 2 rounds of chromatography, one using 20 mM formic acid, pH 4.0 (NH_3), as a buffer and the second round using 0.1% (v/v) trifluoroacetic acid (TFA) as a buffer. The matrices used to conduct chromatography were Pharmacia Pep-RPC HR5/5 (C_2C_{18}) or Pro-RPC HR5/10 (C_1C_8) columns as indicated.

2.5. *Sequence analysis*

An Applied Biosystems (ABI) Model 471A Protein Sequencer was used to perform the automated Edman degradation on relevant peptide samples. The system was equipped with a Brownlee Laboratories microgradient delivery system which was electronically interfaced with the Protein Sequencer. The separation of PTH amino acids was conducted using an ABI PTH column eluted using 2 buffers, the compositions of which were optimised according to the manufacturer's recommendations [16]. Detection of PTH amino acid peaks was by absorbance at 269 nm. All sequencing reagents used were obtained from ABI.

2.6. *Gel electrophoresis and electrophoretic transfer*

Polyacrylamide gel electrophoresis (PAGE) in the presence of SDS was conducted by the method of Laemmli [17]. Transfer of proteins from slab gels to polyvinyl difluoride (PVDF) membranes (Immobilon, Millipore) was conducted at 60 V for 1–1.5 h using 0.1 M 3-cyclohexylamino-1-propane-sulphuric acid (CAPS, Sigma), pH 11 (NaOH); filters were stained with Coomassie brilliant blue and destained with 7% (v/v) aqueous acetic acid.

3. RESULTS

3.1. *SP-40,40 contains no free thiol group*

The results of Table I reveal that non-reduced SP-40,40 contains much less than 1 free -SH group per molecule. Material reduced before titration of thiol groups yielded values of about 8 mol of thiol group per mol of the SP-40,40 protein and the corresponding value for lysozyme was approximately 9. These values concord fairly well with the known values of 10 and 8 cysteine residues per molecule for SP-40,40 and lysozyme, respectively. Without reduction neither lysozyme nor SP-40,40 were found to contain significant free thiol groups. The experiment with reduced and exhaustively carboxamidomethylated lysozyme rules out any contribution from carry over reducing agent (DTT).

3.2. *CNBr produces a 40-kDa fragment of SP-40,40 containing all 10 cysteine residues*

Gel electrophoresis of a CNBr digest of SP-40,40 produced a single fragment which was reducible by DTT (Fig. 1). The approximate mol. wt. of the CNBr-1 fragment (40 kDa) suggested that multiple segments of the SP-40,40 dimer must be crosslinked by disulphide bonds. Fig. 2 depicts the location of methionine, half-cysteine and glycosylated asparagine residues in both α and β chains of SP-40,40 and assigns mol. wts. to each

Table I

Determination of cysteine and cystine residues of SP-40,40 using Ellman's reagent

	Thiol groups per mol of protein	
Protein	Reduced	Non-reduced
SP-40,40	7.9	0.4
	8.2	0.3
Lysozyme	9.2	0.4
	8.6	0.3
CAM lysozyme	0.4	0.1
	0.2	0.2

Duplicate measurements of thiol groups are shown. Protein concentrations were determined using the Bio-Rad Protein Assay (Materials and Methods). 260 μg of SP-40,40 (3.25 nmol), 200 μg of intact lysozyme and reduced and carboxamidomethylated (CAM) lysozyme (13.8 nmol) were used in relevant experiments. Assumed mol. wt. for SP-40,40 and lysozyme were 80,000 and 14,400 Da, respectively.

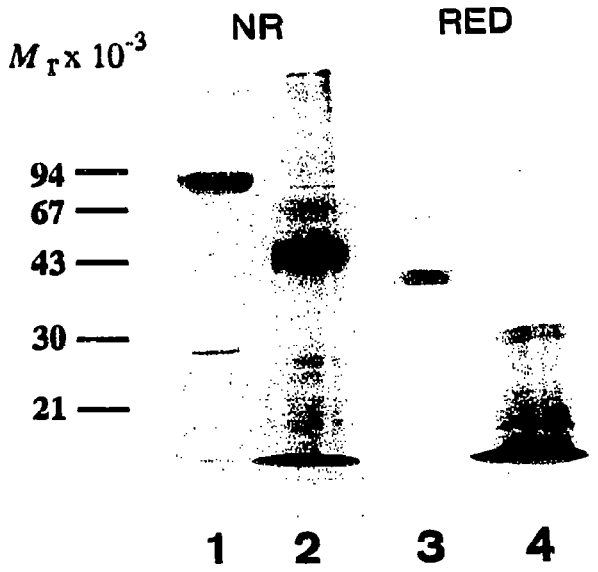
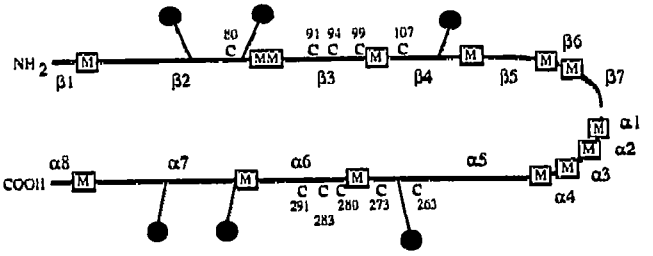


Fig. 1. Denaturing polyacrylamide gel electrophoresis of affinity purified intact SP-40,40 (lanes 1,3) and CNBr-cleaved SP-40,40 (lanes 2,4). Approximately 3 μg of both intact and CNBr-cleaved SP-40,40 were subjected to SDS-PAGE without prior reduction on a 12.5% gel (NR, lanes 1 and 2, respectively) while 3 μg of intact as well as CNBr-cleaved SP-40,40 were reduced prior to electrophoresis on the same gel (RED, lanes 3 and 4, respectively). Migration positions for standard marker proteins (Pharmacia) and their relative mol. masses are indicated. The gel was stained with Coomassie brilliant blue.



Peptide	Amino acid residues	Half cystine residues	glycosylation sites	Mr*
β1	1–12	0	0	1440
β2	13–85	1	2	14760
β3	86–100	3	0	1800
β4	101–132	1	1	6840
β5	133–150	0	0	2160
β6	151–154	0	0	480
β7	155–205	0	0	6120
α1	206–208	0	0	360
α2	209–221	0	0	1560
α3	222–228	0	0	840
α4	229–236	0	0	960
α5	237–276	2	1	7800
α6	277–330	3	1	9480
α7	331–409	0	1	12480
α8	410–427	0	0	2160

Fig. 2. Schematic and tabular representation of the location of methionine, half-cysteine and glycosylated asparagine residues of SP-40,40. Approximate mol. mass of each peptide was calculated assuming 120 Da per amino acid and a contribution of 3,000 Da for every *N*-linked carbohydrate moiety. Amino acid residues are numbered according to the strict order in which they appear in the β–α precursor molecule [2].

predicted CNBr fragment [2]. Clearly, if all the half-cystine-containing fragments occur in the peptides of Fig. 2 (i.e. fragments β2, β3, β4, α5 and α6), then equimolar amounts of 5 distinct sequences should occur in this fragment. Electrophoretic transfer to an Immobilon membrane followed by sequence analysis of CNBr-1 was consistent with the presence of all 5 predicted sequences (Table II). Similarly, addition of the mol. wts. of the predicted half-cystine-containing peptides produced a value of 40,680 Da (assuming an *N*-linked carbohydrate chain contributes 3,000 Da); the agreement between predicted and measured mol. wts. of CNBr-1 is well within the experimental limitations of SDS-PAGE.

3.3. *Enzymatic proteolysis of intact SP-40,40 and CNBr-1*

A number of enzymes were used to digest intact SP-40,40 or its fragment CNBr-1. After each digest, an aliquot of material was reduced and then both reduced and non-reduced digest samples were alkylated and subjected to reverse-phase HPLC. Peptide fragments which occurred in the non-reduced but not the reduced digests were repurified, and small samples subjected to reduction and alkylation to confirm that they contained at least 1 disulphide bond, and finally the purified non-reduced material was subjected directly to sequence analysis or further digested with a second cleavage reagent prior to re-chromatography and sequence analysis. The chromatograms of Fig. 3a and b depict this analysis using the enzyme trypsin. From this particular digest 2 relevant peptides, T-2 and T-21, were recovered and their sequences are shown in Table II. Chromatography of pepsin-digested peptide T-21 resulted in the recovery of a single major fragment T/P-16 (Fig. 4). The amino acid sequence of peptide T/P-16 is shown in Table II, which contains sequence data on all the peptide sequences relevant to this investigation.

The following points emerged from analysis of the data presented in Table II:

(i) Cysteine residues 80 (β) and 291 (α) are bonded. The peptides which evidence this are CN/V8-24 and CN/P-21.

(ii) Cysteine residues 107 (β) and 263 (α) are bonded as indicated by peptide T-2 and CN/V8-23.

(iii) Peptide CN/T-30 contains 2 sequences in similar amounts. One sequence corresponds to residues 277–286(α) and encompasses cysteine 280 and 283 (α). This peptide terminates at 286 and thus cysteine 291 (α) is not present. The other sequence corresponds to residues 86–95 (β) and contains cysteine residues 91 and 94 (β) but terminates at residue 95 and thus does not contain cysteine residue 99 (β). Thus cysteine 91 (β) is joined to either cysteine 280 or 283 (α), and cysteine 94 (β) is

Table II

Amino acid composition of peptides confirming the location of disulphide bridges, the carboxyl-terminus of the mature β-chain and *N*-linked glycosylation sites of the SP-40,40 molecule

Cycles where no PTH-amino acid derivative was assigned are indicated with (–) where the cDNA of SP-40,40 predicts a cysteine residue or (*) where asparagine is the predicted residue. (→) Sequence continued on the next line.

PEPTIDE	SEQUENCE	POSITION IN SP-40,40
CNBr-1	[S][N][-][G][S][K].... [A][L][W][E][E][-].... [K][F][Y][A][R][V].... [D][I][H][F][H][S].... [K][D][Q][-][D][K]....	β 13–18 β 86–91 β 101–106 α 237–242 α 277–282
T-21	[L][K][E][L][P][G][V][-][-][E][T][M].... [E][L][P][G][V][-][-][E][T][M][M][A].... [M][K][D][Q][-][D][K][-][R][E][I][L] [H][*][S][T][G][-][L][R]	β 73–84 β 75–86 α 276–287 α 268–275
CN/V8-24	[T][K][L][K][E][L][P][G][V][-][*][E] [I][L][S][V][D][-][S][T][N][N][P][S]QAKLRRE	β 71–82 α 286–304
CN/P-21	[K][E][L][P].... [S][V][D][-]....	β 74–77 α 288–291
T-2	[V][-][R] [T][V][-]R	β 106–108 α 261–264
CN/V8-23	[K][F][Y][A][R][V][-][R][S]GSGLVGRQLE [G][D][D][D][R][T][V][-][R]	β 101–119 α 256–265
CN/T-30	[A][L][W][E][E][-][K][P]-LK [K][D][Q][-][D][K][-][R]	β 86–96 α 277–284
T/P-16	[W][E][E][-][K][P][-][L][K][Q][T]-MK [M][K][D][Q][-][D][K][-][R][E][I] [H][*][S][T][G][-][L][R]	β 88–101 α 276–286 α 268–275
Th/L-53	KKEDAL*ETRES	β 58–69
CNβ7	QDHFSRASSIIDELFQDRFFTREPQDTYHYL→ PFSLPHRRPHFFFPKSRIVR	β 155–201

joined to the remaining cysteine residue (α). Attempts to sub-digest peptide CN/T-30 with several enzymes were unsuccessful and an unambiguous assignment of its disulphide bonds was not possible.

(iv) Cysteine residues 99 (β) and 273 (α) are bonded. A disulphide-bonded peptide containing 3 sequences (T/P-16) was isolated. Peptide T/P-16 contained residues 88–101 (β) and included cysteine residues 99, 94 and 91 (β) and residues 276–286 (α) which includes cysteines 280 and 283 but not cysteine 291 (α). It also contained residues 268 to 275 including cysteine 273 (α). Since cysteine residues 94 and 91 (β) were known to be bonded to cysteines 280 and 283 (α), the only way to account for the existence of the peptide spanning residues 268–275 is to invoke the existence of a disulphide bond between cysteines 273 (α) and 97 (β). Despite repeated attempts using cleavage reagents of differing specificities, no fragments corresponding to a disulphide-bonded doublet containing only cysteine residues 273 (α) and 97 (β) were isolated. The reason(s) for this are unclear.

The locations of the disulphide bonds of SP-40,40 are depicted in Fig. 5 along with schematic representations

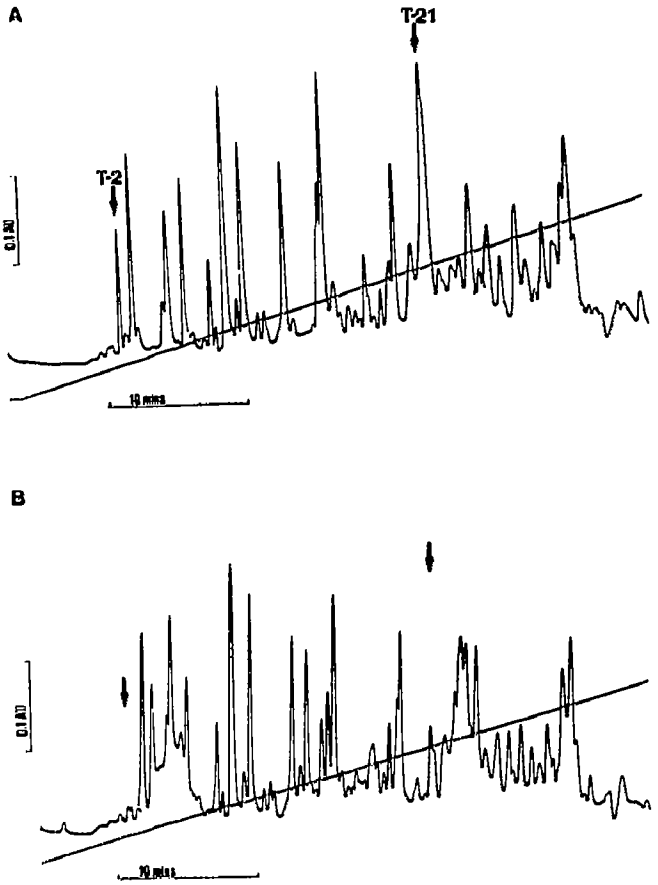


Fig. 3. Isolation of cystine-containing SP-40,40 peptides. Parallel tryptic digests of (A) non-reduced and (B) reduced and alkylated SP-40,40 were subjected sequentially to reverse-phase HPLC using a complete Pharmacia FPLC with a Pep-RPC (C_{18}) HR 5/5 column as the stationary phase. Digests were carried out as described in Materials and Methods. Briefly, peptides were eluted by a linear acetonitrile gradient composed of buffer A (20 mM formic acid, pH 4 (NH_3)) and buffer B (15 mM formic acid, pH 4 (NH_3), 92.5% acetonitrile) and delivered in 3 segments: (i) 0% B, 0–5 min; (ii) 0–40% B, 5–55 min; and (iii) 40–60% B, 55–75 min at a constant flow rate of 1 ml/min throughout the gradient. Elution of peptides was monitored at a wavelength of 214 nm. Arrows indicate the presence (in A) or absence (in B) of unique non-reduced SP-40,40 peptides.

of all the peptides in Table II which enabled the establishment of the model.

One peptide which did not conform to the model of Fig. 3 was isolated (CN/V8-6). This peptide consisted of a single sequence extending from residue 277–285 (α). No sequence assignments were possible at positions corresponding to cysteines 280 and 283 (β) indicating that this fragment contained an intrachain disulphide bond. The peptide was isolated as a minor peak; its yield was low (<1%) as determined by quantification of its PTH amino acids after sequencing. If this disulphide bond were to exist in native SP-40,40 then the locations of essentially all of the other disulphide bonds independently assigned in this work would be in error. Indeed the existence of peptide CNBr-1 (Fig. 1) would be impos-

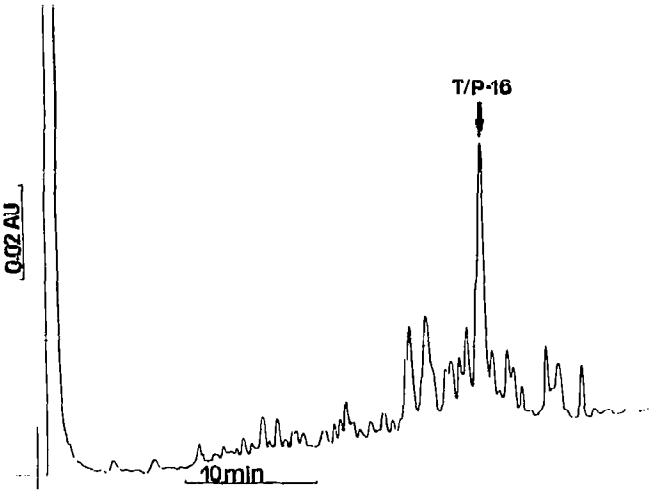


Fig. 4. Pepsin digest of tryptic peptide T-21. Digests were carried out as described in Materials and Methods. HPLC was carried out as described in the legend to Fig. 3.

sible without multiple changes to the proposed model. It seems much more likely that a low level of disulphide interchange may have occurred during the digestion or chromatography steps which resulted in the isolation of peptide CN/V8-6, that is, an experimental artifact. Such events have been reported by other investigators to result from the use of high pH buffers such as were used in this investigation [18].

3.4. *The location of the carboxyl terminus of the SP-40,40 β chain*

Previous investigations did not reveal the precise length of the β chain of SP-40,40. At least one proteolytic cleavage event must occur in vivo to cleave the bond between β Arg-205 and α Ser-1 but additional cleavage events may shorten the β chain further. A peptide was isolated corresponding in amino-terminal sequence to that predicted for CNBrβ7 of Table II. Sub-digestion of this fragment with endoproteinase lysine-C followed by direct sequence analysis of the unfractionated mixture, revealed 2 sequences: one, the original amino-terminus, the other commencing at serine residue 201 which extended through to Ile-203 which is within 2 residues of the commencement of the SP-40,40 α chain. The failure to detect the remaining 2 residues Val-204 and Arg-205 may be their removal in vivo due to endogenous carboxypeptidase activity or to the technical problem of sequencing small amounts of peptide material to completion.

3.5. *All potential* N-*linked glycosylation sites in SP-40,40 are used*

Asparagine residues within NX^S_T motifs occur at residues 64, 81, 123, 269, 332 and 352 [2]. Conventional PTH amino acid detection techniques do not allow the identification of glycosylated asparagine residues and no positive assignment of such residues can be made. If the complete amino acid sequence is known, however, the absence of an assignable asparagine residue within an NX^S_T motif establishes that the missing residue must be glycosylated. Previous work has established that at least 4 potential glycosylation sites of SP-40,40, namely 123, 269, 332 and 352, are actually used [2]. Peptides Th/L-53 and CN/V8-24 of Table II correspond to sequences flanking asparagine residues 64 and 81. In neither case was an assignable PTH-asparagine detected at the expected sequencing cycles, indicating that both of these potential *N*-linked glycosylation sites carry carbohydrate (CHO) chains in SP-40,40.

4. DISCUSSION

This work has resulted in the assignment of the disulphide bonds of SP-40,40 and in the completion of its covalent structure other than for that of the *N*-linked CHO side chains. The structure is conveniently depicted as a two-dimensional hairpin in which the α and β chains are covalently joined by 5 disulphide bonds (Fig. 5, upper panel). The assignment of half-cystine residues 91 (α) to either 280 or 283 (β) leaves a single degree of freedom which could not be eliminated due to the difficulty in obtaining convenient cleavages between cysteine pairs 91 and 94 and between 280 and 283. Thus the 2 alternative and experimentally indistinguishable models for the disulphide motif of SP-40,40 are combined in Fig. 5, upper panel. Both of these motifs are unusual and no disulphide bonded protein known to the authors exhibits substantial similarity to either motif. Specifically, members of the Ig superfamily contain mainly tandemly linked intrachain disulphide bonds with a single inter-chain disulphide connecting H and L chains. The human complement component, C1s, was recently shown to contain 13 disulphide bonds, 7 of which consist of tandem joints between cysteine residues which are 'next in line' within the primary structure (1–2 bonds). The remaining 8 disulphide bonds were organised into 1–3, 2–4 motifs interspersed between simple 1–2 motifs [19]. Similarly, bovine factor X consists of a mosaic of 1–2 and 1–3, 2–4 motifs within the light chain, 1–2 motifs within the heavy chain and a single interchain disulphide bond [20].

To date no data on the disulphide bonding pattern of any terminal complement protein is available. Components C6, C7, C8 and C9 are homologous in amino acid sequence [21,22] and all contain multiple cysteine residues. We noted previously that sequence homology may exist between a cysteine-containing segment of SP-40,40 and corresponding segments in several terminal complement components. If this sequence homology is significant it may underly a more obvious similarity in tertiary structure between terminal complement components and SP-40,40. The demonstration of similar

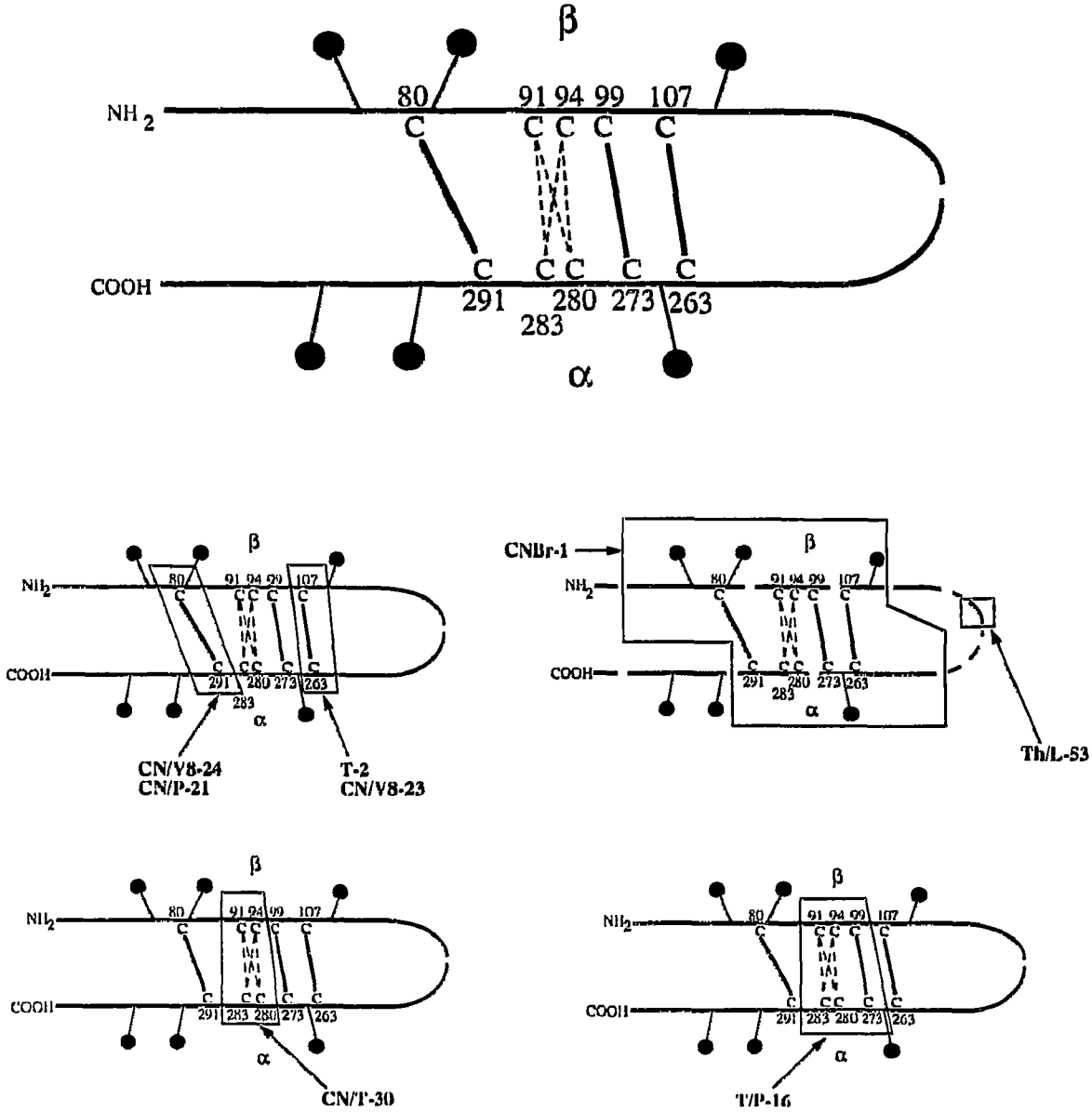


Fig. 5. Complete covalent structure of SP-40,40. Assignment of the disulphide bonds and determination of the carboxyl terminus of the mature β-chain are summarised schematically. (Upper panel) The covalent structure of SP-40,40 showing both possibilities for the disulphide bridges has been deduced from the peptide isolates depicted in the middle and lower panels. All peptides indicated are listed in Table II. ●— represent *N*-linked glycosylation sites.

disulphide bond motifs in terminal complement components would support a distant ancestral relationship to SP-40,40 which extensive amino acid sequence divergence has obscured.

Publication of the complete sequence of SP-40,40 from human, rat, cow, dog and quail [2,7–9,11] plus partial sequence from hamster [10] have allowed a comparison to be made of the alignment of these species homologues in the key areas of their cysteine rich regions. All cysteine residues are conserved in the mature chains of the SP-40,40 homologues where sequence data for such a comparison is available. The high degree of homology and the precise alignment of the cysteine residues between all species (including phylogenetically distant quail) must reflect the importance of this structural motif in the functional and structural integrity of the SP-40,40 molecule.

Finally, Tsuruta et al. [12] have proposed a speculative model for the secondary structure of SP-40,40 and homologues whereby 4 myosin tail-like arms form a cross, the core of which is stabilised by disulphide bonds. The location of the disulphide bonds elucidated in this study is compatible with this proposal. More precise structural studies are required to define the salient features of this molecule at the levels of secondary and tertiary structure.

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