Disulfide Bond Assignment in Human J Chain and Its Covalent Pairing with Immunoglobulin M†

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ABSTRACT: The assignment of disulfide bonds in human J chain and its covalent pairing with immunoglobulin M was determined under conditions which minimize disulfide bond interchange. We show that in J chain the three intradisulfide bridges are formed between Cys 12 and 100, Cys 71 and 91, and Cys 108 and 133. Previous reports [reviewed by Koshland, M. E. (1985) *Annu. Rev. Immunol. 3,* 425–453] have proposed that cysteines 12, 14, or 68 were linked to the penultimate cysteine 575 of two μ chain tails. In this work, we demonstrate that cysteines 14 and 68 are disulfide-bridged to μ chains. A revised, albeit putative, model of J chain folding is presented which takes into account the correct disulfide pairing and the predictive secondary structure assignment.

J chain is an acidic 15-kDa polypeptide found exclusively in polymeric immunoglobulins (Igs)[1] in covalent association with dimeric IgA and pentameric IgM molecules (Koshland, 1985). There is one molecule of J chain per molecule of Ig polymers, regardless of its size (Koshland, 1985; Zikan et al., 1986), although this stoichiometric ratio has been challenged by Brandtzaeg (1985), who reported, on the basis of immunochemical estimates, two J chain molecules per IgA dimer and at least three per IgM pentamer.

Amino acid sequence comparisons of mouse, human, frog, and rabbit J chains show that this polypeptide is highly conserved (Cann et al., 1982; Max & Korsmeyer, 1985; Mikoryak et al., 1988; Hughes et al., 1990). J chains comprise eight cysteine residues, out of which Cys 14 appears to be disulfide-linked to the penultimate Cys 575 of at least one μ chain of pentameric IgM (Mendez et al., 1973a; Mestecky & Schrohenloher, 1974; Mole et al., 1976). It has been demonstrated that J chain binds to the μ chain of two adjacent monomers. Pairings of other IgM subunits are formed by disulfide bonds between the remaining cysteines 575 (Wilde & Koshland, 1973, 1978; Chapuis & Koshland, 1974). In IgA also, J chain was postulated to be disulfide-linked to the penultimate Cys 471 of α chains (Mestecky et al., 1974; Chapuis & Koshland, 1975; Garcia-Pardo et al., 1981).

The exact role of the J chain is unclear. Some studies suggest that polymerization of IgM requires J chain (Della Corte & Parkhouse, 1973; Eskeland, 1974; Koshland, 1985). In contrast, direct evidence that J chain is not required in this process originates from mutant IgM in which Cys 414 and 575 have been substituted by Ser; such mutant IgMs were assembled as pentamers and hexamers that lacked J chain (Davis et al., 1988, 1989). In addition, J chain is not needed for secretion of IgM, nor is it necessary for complement activation (Cattaneo & Neuberger, 1987; Davis et al., 1988).

The usual reducing conditions used to release J chain from Ig polymers also reduce all intradisulfide bridges (Morrison & Koshland, 1972; Mendez et al., 1973b; Elliott & Steiner, 1984; Mikoryak et al., 1988; Hughes et al., 1990), and presumably because of this, the three-dimensional structure of native J chain has not been determined. Two hypothetical models for the folding of J chain have been predicted from its amino acid sequence. One of these (Cann et al., 1982) suggests a two-domain structure while the other (Zikan et al., 1985) predicts a single Ig-like β-barrel domain. The validity of such predicted models should be taken with caution. No significant sequence homology has been observed between J chain and any other proteins including Ig heavy and light chains, and thus, J chain cannot be placed into a superfamily of structurally related molecules. In addition, the correct disulfide bond pairing in J chain associated with polymeric Igs has not yet been determined.

The aim of the present investigation was to determine the arrangement of disulfide bonds in human J chain as well as its disulfide pairing with μ chains in a pool of Waldenström IgMs. Chemical fragmentation of IgM containing J chain by cyanogen bromide was carried out and J-chain-containing material was identified by Western blotting with a J-chain-specific antiserum. Further extensive proteolytic digestions under conditions which minimize disulfide interchange allowed us to isolate all pairs of peptides connected by one disulfide bridge. The data are reported below.

EXPERIMENTAL PROCEDURES

Materials

Nitrocellulose membranes were purchased from Bio-Rad Laboratories, Richmond, CA. Endoproteinase Asp-N (sequencing grade) was from Boehringer, Mannheim, Germany, and endoproteinase Lys-C was from Wako Chemicals, Neuss, Germany. L-1-Tosylamino-2-phenylethyl chloromethyl ketone-treated porcine trypsin was a generous gift from Professor R. E. Offord, and sera from Waldenström patients were kindly provided by Professor A. Cruchaud. Urea, cyanogen bromide, and solvents for HPLC were purchased from Merck, Darmstadt, Germany. Sephacryl S-300 was from Pharmacia, Uppsala, Sweden, and Ultrogel AcA 22 was from IBF, Villeneuve-la-Garenne, France. Guanidine hydrochloride was

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[1] Abbreviations: CD, circular dichroism; Cys, half-cystine; Ig, immunoglobulin; RP-HPLC: reversed-phase high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

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from Sigma, St Louis, MO. The antisera (goat anti-human J chain and peroxidase-conjugated rat anti-goat IgG) were obtained from Nordic Immunological Laboratories, Tilburg, The Netherlands. Solvents used for sequencing were from Fluka and were further purified as described (Hunkapiller et al., 1983). Water used for the preparation of buffers was obtained from a Milli-Q water system (Millipore Corp., Bedford, MA.). All other chemicals were analytical grade.

Methods

Purification of Waldenström IgM. Twenty-four samples (2.5 mL each) of sera from Waldenström patients were analyzed in this study. Four sets, each consisting of six different sera, were purified according to the following procedure: after dialysis at 4 °C against water for 48 h, the sample was centrifuged (10000*g*, 30 min) and the pellet, which consisted of partially pure IgM, was dissolved in 10 mL of 50 mM sodium phosphate (pH 6.5) containing 0.5 M NaCl and was fractionated by gel filtration on an Ultrogel AcA 22 column (2.8 × 180 cm) equilibrated with the same buffer. Typically, 100–150 mg of pure IgM, as judged by SDS–PAGE performed under reducing and nonreducing conditions, was obtained.

CNBr Digestion of Waldenström IgM. After dialysis against 1% ammonium bicarbonate and lyophilization, 100 mg of purified IgM was dissolved in 10 mL of 70% formic acid and sonicated. Cyanogen bromide (0.5 g) was added and the mixture was left in the dark for 48 h. Water (150 mL) was added and the solution was then lyophilized. This procedure was repeated twice.

Immunoblotting. An aliquot (70 μg) of the CNBr digest was run in a SDS gel (Laemmli, 1970) under a nonreducing condition and then electroblotted onto a nitrocellulose membrane (Towbin et al., 1979). After staining with Ponceau S, the blot was incubated with a goat anti-human J chain antiserum (previously absorbed with pooled human Ig heavy chains) and developed with a peroxidase-conjugated rat anti-goat IgG antiserum according to classical procedures (Towbin et al., 1979). A unique band of apparent M_r 23 000 became visible.

Purification of the 23-kDa Fragment Reacting with the Anti-J Chain Antiserum. One hundred milligrams of CNBr-digested IgM, dissolved in 10 mL of 1 M acetic acid containing 4 M guanidine hydrochloride, was fractionated by gel filtration on a Sephacryl S300 column (1.5 × 120 cm). Fractions reacting with the antiserum were pooled, dialyzed against 1% ammonium bicarbonate, and dried by vacuum centrifugation. This sample, dissolved in 200 μL of 0.1% TFA, was applied to a reversed-phase phenyl column (2.1 × 100 mm) of Aquapore PH 300 (Applied Biosystems, Foster City, CA) equilibrated with the same buffer. Elution was performed over 50 min with a gradient of 0–60% 2-propanol at a flow rate of 300 μL/min. The first peak, eluting at 34 min, contained the 23-kDa polypeptide and was judged pure by SDS–PAGE. N-Terminal sequencing indicated that this polypeptide was the J chain covalently bound to the C-terminal octapeptide of the μ chain (see Results and Discussion).

Proteolytic Digestions. Digestion of 5-μg aliquots of the J chain–μ chain complex with endoproteinase Asp-N (0.5 μg) was performed overnight at 37 °C in 50 μL of 50 mM sodium phosphate (pH 7.0) containing 20 mM methylamine hydrochloride. Subdigestions with trypsin were done overnight at ambient temperature in 50 mM sodium phosphate, pH 6.5, containing 6 M deionized urea. Endoproteinase Lys-C cleavage was carried out overnight at 37 °C in 20 mM sodium phosphate, pH 8.0.

Purification of Peptides by RP-HPLC. Separation of the peptides from the endopeptidase Asp-N cleavage of the J chain–μ chain complex was performed on a column (2.1 × 100 mm) of Aquapore RP300 (Applied Biosystems) equilibrated in 20 mM sodium phosphate, pH 6.5. A linear gradient, 0–40% in 50 min, of increasing acetonitrile concentration was applied at a flow rate of 0.3 mL/min. Each fraction was further purified by a second RP-HPLC using a 1-mm column and 0.1% TFA as the aqueous buffer. Subdigestions were typically separated on columns (1 × 100 mm) of Aquapore RP300 equilibrated with 0.1% TFA and elution was at 50 μL/min using a 60-min gradient of 3–30% acetonitrile.

Peptide Characterization. N-Terminal sequence determination was carried out with Models 477A or 473A pulsed liquid-phase sequencers from Applied Biosystems. In the pairs of peptides connected by disulfide bonds, the identification of the PTH half-cystine residue is possible only when the second half-cystine partner is cleaved off. In our hands, PTH-cystine could not be identified and this residue is indicated by X in Tables I and II.

Structure Prediction. The amino acid sequences of human, mouse, and rabbit J chains were aligned and a sequence profile was established (Gribskov et al., 1987; 1990). A search in the Swiss-Prot database (Bairoch & Boeckman, 1991) revealed no significant similarity of this profile to that of any other protein.

Prediction of the secondary structure was made using both the Garnier–Robson (Garnier et al., 1978) and the Chou–Fasman (Chou & Fasman, 1978) methods as implemented in the GCG sequence analysis package (Devereux et al., 1984). The predictions for the three known J chain sequences were combined and a model was made taking into consideration the disulfide pairings determined in this work and the previously reported CD spectrum of J chain (Zikan et al., 1985).

RESULTS AND DISCUSSION

It has been known for almost two decades that, upon cyanogen bromide treatment of human IgM, J chain is released together with the C-terminal octapeptide from the μ chain. The existence of a disulfide bridge between Cys 14 of J chain and the penultimate Cys 575 of μ chain was demonstrated (Mendez et al., 1973a; Mestecky & Schrohenloher, 1974; Mestecky et al., 1974; Mole et al., 1976). However, recent studies on the effects of interchanging cysteine residues in IgM μ chains by site-directed mutagenesis led to the observation of the absence of J chain from mutant IgM in which Cys 414 was replaced by Ser, suggesting that J chain might also bind to Cys 414 in normal IgM (Davis et al., 1989, Davis & Shulman, 1989).

In our attempt to determine the inter- and intradisulfide pairings, pooled Waldenström IgMs were treated with cyanogen bromide and the products were separated into four peaks by gel filtration under acidic and denaturing conditions. All fractions were analysed by SDS–PAGE (under nonreducing conditions) and by immunoblotting using a specific anti-human J chain antiserum. A unique band with an apparent M_r of 23 000 was revealed. No higher M_r J-chain-containing fragments were apparent in the blot, as would be expected if J chain were bonded to Cys 414. The 23-kDa peptide was purified to homogeneity by RP-HPLC on a phenyl column. Microsequencing data (not shown) clearly demonstrated the presence of only two sequences, one originating

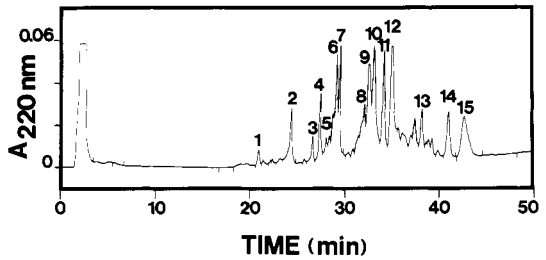

FIGURE 1: Elution profile of an endoproteinase Asp-N digest of the J chain/μ chain complex obtained from cyanogen bromide treatment of human IgM. Five micrograms of the J chain–μ chain complex was digested with endoproteinase Asp-N and applied to a narrow-bore RP300 reversed-phase column equilibrated at pH 6.5. The organic eluant was acetonitrile. Each fraction numbered 1–15 was further purified by RP-HPLC at pH 2 on a microbore column, before N-terminal sequencing was performed. The sequences of peptides containing cystines, i.e., 6, 7, 9, 10, 11, and 12, are shown in Table I.

Table I: Amino Acid Sequences of Peptides Containing Cystine Obtained after Digestion of the J Chain–μ Tail Complex with Endoproteinase Asp-N

Fraction 6	
DNQIVTATQSNI[X]	position 79–91, X = Cys 91
DL[X]KK[X]DPTEVEL	position 66–78, X = Cys 68 and 71
SDTAGT[X]Y	μ chain C-terminal peptide, X = Cys 575
Fraction 7	
sequence similar to fraction 6	
Fraction 9	
DNK[X]K[X]ARITSRIIRSSE	position 9–26, X = Cys 12 and 14
DSATET[X]YTY	position 94–103, X = Cys 100
SDTAGT[X]Y	μ chain C-terminal peptide, X = Cys 575
Fraction 10	
sequence similar to fraction 9	
Fraction 11	
DNK[X]YTAVVPLVYGGETK	position 104–122, X = Cys 108
DA[X]YPD	position 131–136, X = Cys 133
Fraction 12	
sequence similar to fraction 11	

from the carboxy-terminal octapeptide of μ chain at position 569 and the other one following Met 123 in J chain, the amino-terminal residue of human J chain being blocked as pyrrolidonecarboxylic acid (Meinke & Spiegelberg, 1972). It cannot be formally excluded that some minor proportion of J chains may be linked via Cys 414 alone or utilizing both Cys 414 and Cys 575. However, our data based on pooled Waldenström IgMs rules out the disulfide bonding of at least 90% of J chain to Cys 414.

Human J chain contains eight half-cystine residues in positions 12, 14, 68, 71, 91, 100, 108, and 133 [Max & Korsmeyer, 1985; numbering according to Hughes et al. (1990)]. An endoproteinase Asp-N digest of the 23-kDa J chain–μ chain complex was separated by RP-HPLC at pH 6.5. The elution profile is shown in Figure 1. Each fraction was further purified by RP-HPLC at pH 2.0 and sequenced in order to determine the peptides containing cystines. As shown in Table I, fractions 6 and 7 were similar and both exhibited three sequences corresponding to residues 79–91 and 66–78 of the J chain and to the C-terminal μ octapeptide. At this stage we could surmise that either Cys 68 or 71 was bound to the μ octapeptide. If this were not the case, Cys 68 and 71 should form an intradisulfide bond, and therefore, peptide 66–78 should not be disulfide-linked to its two partners in fraction 6. Similarly, fractions 9 and 10 gave three sequences encompassing residues 9–26 and 94–103 of the J chain and the μ octapeptide. Accepting that, as previously shown, Cys 14 is linked to μ chain (Mole et al., 1976), it could be deduced that Cys 12 forms an intramolecular disulfide bridge with Cys 100. Fractions 11 and 12 exhibited two sequences corresponding to residues 104–122 and 131–136. This result demonstrates that Cys 108 and Cys 133 are disulfide-bridged within the J chain molecule. Why seemingly identical peptides (6 and 7, 9 and 10, and 11 and 12) should elute as twin peaks is unclear. Nevertheless, random scrambling of disulfide bonds can be essentially ruled out. Four endoproteinase Asp-N digests were performed on four batches of six pooled IgM samples and identical sequencing results were obtained (data not shown).

Table II: Sequences of Peptides Obtained after Subdigestion with Trypsin or Lys-C of Fractions 6, 7, 9, and 10 from Figure 1

Digestion of Pooled Fractions 6 and 7 with Endoproteinase Lys-C	
pair 1:	
SDTAGT[X]Y DL[X]KK	μ chain C-terminal peptide, X = Cys 575 position 66–70, X = Cys 68
pair 2:	
DNQIVTATQSNI[X] K[X]DPTEVEL	position 79–91, X = Cys 91 position 70–78, X = Cys 71
Digestion of Pooled Fractions 9 and 10 with Trypsin	
pair 1:	
SDTAGT[X]Y [X]AR	μ chain C-terminal peptide, X = Cys 575 position 14–16, X = Cys 14
pair 2:	
DSATET[X]YTY [X]K	position 94–103, X = Cys 100 position 12–13, X = Cys 12

In order to complete the assignment of disulfide bonds, fractions 6 and 7 were pooled, subdigested with endoproteinase Lys-C, and chromatographed on a reversed-phase column at pH 2.0. Two pairs of peptides were obtained and their sequences are shown in Table II. Clearly, the J chain peptide encompassing residues 66–70 forms a disulfide bond with the μ octapeptide (pair 1). Pair 2 contains positions 70–78 and 79–91, thus proving that Cys 71 and 91 form an intramolecular disulfide bond. A tryptic subdigest of fractions 6 and 7 (performed at pH 6.5) gave identical results, suggesting that random scrambling of disulfide bonds was not taking place.

The subdigestion of fractions 9 and 10 presented more of a problem. Endoproteinase Lys-C was unable to cleave the Cys-Lys-Cys sequence (residues 12–14 of J chain). However, trypsin in the presence of 6 M urea allowed us to isolate two pairs of peptides containing cystine. Their sequences are shown in Table II. Pair 1 encompasses positions 14–16 of the J chain and the μ octapeptide. We thus confirmed that, as previously claimed, Cys 14 is linked to the μ chain. Pair 2 contains residues 12–13 and 94–103, proving that Cys 12 and Cys 100 are bound.

The assignment of disulfide bond pairing, as determined in this work, is a pertinent piece of information, which should help validate two hypothetical published models of J chain folding. In the first model (Cann et al., 1982), a two-domain structure was consistent with the predictions of Chou–Fasman

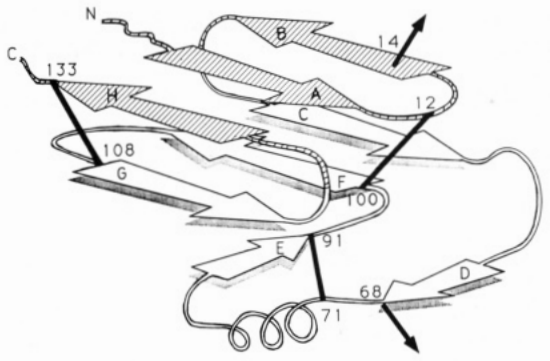

FIGURE 2: Predicted model for the three-dimensional structure of human J chain. This schematic drawing of a two-domain model, made according to the Garnier–Robson and Chou–Fasman methods, accommodates the disulfide pairings determined in this work. The large arrows designated A–H indicate β strands and the thick black lines represent the intradisulfide pairings. Black arrows indicate the interdisulfide bonds with Cys 575 of μ chain.

algorithms (Chou & Fasman, 1978); i.e., the amino-terminal half had a high propensity for forming β-sheets while the carboxy-terminal half had no apparent conformational symmetry. In that model, the surmised bridging of J chain to μ chain with two opposing half-cystine residues, 14 and 68, is correct, as is also one of three intradisulfide bonds, 108–133. The second model (Zikan et al., 1985) was based upon the predictions of the secondary structure assignments by the method of Novotny and Auffray (1984) and on CD measurements of J chain. It looks like a single domain in the shape of an eight-stranded antiparallel β-barrel; although attractive because it fits better the principles of protein folding than the first model, none of the assigned disulfide bonds were found to be correct.

A search in the Swiss-Prot sequence database with a sequence profile (Gribskov et al., 1987) of the J chain did not reveal any other protein with a significant score. Many of the proteins with Z scores (Gribskov et al., 1990) above 4.5 were plant virus coat proteins. Several three-dimensional structures of coat proteins from plant viruses have been determined (Rossmann et al., 1983) and found to contain antiparallel β-barrels. Among the highest scoring proteins found with the profile search, however, there were none of known three-dimensional structure. Thus no known structure could be used as a basis for building a model.

We would like to propose an alternative, albeit putative, model based on predictive methods for secondary structure profiles according to Garnier et al. (1978) and Chou and Fasman (1978). The model is shown in Figure 2 as a two-domain-like structure. It is consistent with (a) the known disulfide bond pairing, (b) the susceptibility of J chain to mild reduction (Morrison & Koshland, 1972; Koshland, 1985; Mikoryak et al., 1988; Hughes et al., 1990), (c) the selective proteolysis of the polypeptide with subtilisin (Koshland et al., 1977), and (d) the CD data of reoxidized J chain (Zikan et al., 1985). Domain 1 consists of two antiparallel β-sheets that are held together by two disulfide bridges located in opposite loops connecting β-strands. This arrangement is compatible with the high degree of lability of disulfide bonds to reduction in the absence of denaturing conditions. The β-barrel-like structure is also compatible with the apparent resistance of the N-terminal region toward proteolytic degradation (Koshland et al., 1977) and is associated with a second domain displaying a mixture of α-helical and β-strand propensities. Because of the predictions of β-turns between β-strand segments C and D as well as in the region around residue 95, this domain may bend under the β-barrel domain. The region around residue Cys 71 is quite accessible to proteolytic attack, as it was reported to be hypersensitive to subtilisin (Koshland et al., 1977). The linkage of J chain to μ chains involves Cys 14 and Cys 68 (shown by arrows in Figure 2), which would protrude from each domain in opposite direction, assuming that the domains fold back against each other, between two IgM subunits (Davis et al., 1989).

We should stress that our hypothetical model is one of many folding schemes and that the three-dimensional structure of J chain should be studied by X-ray crystallography. In that regard, the results presented here may help isolate J chain in its native conformation by mild reduction followed by reoxidation in the presence of reduced μ chain octapeptide and of a protein disulfide isomerase (Hillson et al., 1984; Tang et al., 1988). The reformed dioctapeptide–J chain complex, once checked for correct disulfide pairing, could be used for crystallization assay and eventually X-ray diffraction studies or NMR investigations.

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