The occurrence of disulphide bonds in purified clathrin light chains

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Three forms of clathrin light chain contain two cysteine residues. These are the predominant brain-specific forms of LC_a and LC_b, and the non-brain form of LC_b. After purification in the absence of thiols they contain intramolecular disulphide bonds. The reduced and the oxidized forms show differences in electrophoretic mobility, explaining the variable and heterogeneous patterns observed on electrophoresis. Accessibility of the thiol groups in the free light chains is greater than when they are associated with the heavy chain. In contrast the cysteine residues of the clathrin heavy chain are completely inaccessible in the absence of denaturants and are not found in disulphide bonds. The antigenic properties of the oxidized and the reduced forms of the clathrin light chains are similar, as is their capacity to bind to the clathrin heavy chain. After isolation in the presence of 10 mM-iodoacetamide, the light-chain cysteine residues are fully alkylated. The results are consistent with the reduced form being the native state and the light-chain disulphide bonds an artifact of isolation.

INTRODUCTION

Clathrin is the major protein of coated pits and vesicles (Pearse, 1976). Clathrin triskelions, comprising three heavy chains and three light chains (Ungewickell & Branton, 1981; Kirchhausen & Harrison, 1981), have a characteristic three-armed structure that polymerizes *in vivo* and *in vitro* to form the regular hexagonal and pentagonal faces of the clathrin coat (Woodward & Roth, 1978; Keen *et al.*, 1979). In mammalian cells there is a single species of clathrin heavy chain (Kirchhausen *et al.*, 1987a) and multiple forms of the clathrin light chain (Holmes *et al.*, 1984). Analysis of cDNAs shows that bovine and rat clathrin light chains are encoded by two homologous genes, those for LC_a and LC_b, which are expressed in many cell types and undergo tissue-specific differential mRNA splicing in the brain (Jackson *et al.*, 1987; Kirchhausen *et al.*, 1987b). Thus in addition to the shared property of binding to the heavy chain there may be functional diversification among the clathrin light chains. Monoclonal antibodies have defined one region involved in heavy-chain binding and a second that is accessible to the cytoplasm and may contribute to such functional diversity (Brodsky *et al.*, 1987). All light-chain forms contain one or two cysteine residues in three distinct patterns that are localized to the exposed antigenic region (Fig. 1). In the present paper we describe the accessibility and oxidation state of these cysteine residues.

EXPERIMENTAL

Coated vesicles and clathrin triskelions were purified from bovine brain and adrenal gland, sometimes with omission of 2-mercaptoethanol in the buffers used for clathrin extraction and Sepharose 4B chromatography

(Pearse & Robinson, 1984). Light chains were prepared by boiling these preparations for 10 min (Lisanti *et al.*, 1982; Brodsky *et al.*, 1983). After centrifugation at 100 000 *g* for 1 h, the supernatant was dialysed against 0.2 mM-EDTA/0.02% NaN₃/10 mM-Tris/HCl buffer,

Fig. 1. Diagram showing the five forms of the clathrin light chains and the arrangement of their cysteine residues

The brain forms are found exclusively in coated vesicles isolated from brain, whereas the non-brain forms are found in all tissues thus far examined, including brain. Regions with similar shading are homologous, the brain-specific inserts being represented by the largest boxes. The numbering of the amino acid residues is given at the bottom of the Figure and is in accordance with Jackson *et al.* (1987).

Abbreviations used: LC, clathrin light chain; DTT, dithiothreitol; PAGE, polyacrylamide-gel electrophoresis; BPNS-skatoles, 3-bromo-3'-methyl-2-(2-nitrobenzenesulphenyl)indolenine.

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pH 7.5 (DEAE-cellulose buffer), then applied to a 10 ml pipette column of DEAE-cellulose (Whatman DE-52) equilibrated in the same buffer. A 280 ml gradient of 0–0.3 M-NaCl in the same buffer at a flow rate of 8 ml/h resulted in elution of two major peaks corresponding to LC_a and LC_b as described by Ungewickell (1983). These preparations were over 90% pure as judged by SDS/PAGE. Separation on DEAE-cellulose was best for preparations of 1 mg or more of light chains. For small amounts separation by reverse-phase h.p.l.c. on a C₃ RSPC column (0.46 cm × 7.5 cm) (Beckman) was used. The column was equilibrated in 0.1% (v/v) trifluoroacetic acid, run at a flow rate of 0.4 ml/min and eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid: 0–44% (v/v) in 5 min and then 44–52% (v/v) in 40 min. The light chains were eluted at approx. 48% (v/v) acetonitrile.

Clostripain digestion was for 8 h at 37 °C in 0.1 M-sodium phosphate buffer, pH 7.8, containing 2.5 mM-DTT with a substrate/enzyme ratio of 20:1 (w/w) (Mitchell & Harrington, 1968). Trypsin digestion was for 4 h at 37 °C in 0.1 M-NH₄HCO₃ with an initial substrate/enzyme ratio of 40:1 (w/w), an equivalent portion of trypsin being added after 2 h of digestion. For BNPS-skatole fragmentation, 100–250 µg samples of dried LC_b were redissolved in 50 µl of 66% (v/v) acetic acid containing 0.1 mg of BNPS-skatole/ml and incubated for 24 h in the dark (Fontana, 1972). Loss of ³H radioactivity from the carboxy[³H]methylcysteine residues was observed that was dependent on the concentration of BNPS-skatole. The conditions used represent a compromise that gave sufficient cleavage and retention of radioactivity.

Complete reduction and alkylation of light chains was carried out in 6 M-guanidinium chloride/1 mM-EDTA/0.1 M-Tris/HCl buffer, pH 8.0. Incubation was with 4 mM-DTT for 1–2 h at 37 °C followed by 4–8 h at room temperature with 10 mM-iodo[³H]acetic acid (150 mCi/mmol; Amersham). Samples were desalted on a Sephadex G-50 column (2 cm × 25 cm) in 10% (v/v) acetic acid and then dried in a Speedvac (Savant).

For heavy-chain-binding studies, oxidized light chains were iodinated (Brodsky *et al.*, 1987), then incubated with 10 mM-DTT for 3 h at 37 °C followed by 25 mM-iodoacetamide for 1–2 h at 20 °C, or incubated identically without reduction and alkylation. Preparations were then dialysed and analysed by SDS/PAGE to confirm their oxidized or reduced state.

Peptides were purified on C₃ and C₁₈ reverse-phase h.p.l.c. columns equilibrated in 0.1% (v/v) trifluoroacetic acid and eluted with gradients of acetonitrile in 0.1% trifluoroacetic acid. Amino acid sequencing used a gas-phase automated sequencer (Applied Biosystems).

SDS/PAGE, fluorography, preparation of monoclonal antibodies, solid-phase radioimmunoassays and the binding of clathrin light chains to heavy chains were as described previously (Bonner & Laskey, 1974; Brodsky *et al.*, 1987).

RESULTS

Chromatographic separation of clathrin light chains

Preparations of clathrin light chains LC_a and LC_b show heterogeneity on SDS/PAGE that has been attributed to proteolysis and the presence of different isoforms (Keen *et al.*, 1981; Winkler & Stanley, 1983; Holmes

Fig. 2. Separation of bovine brain LC_a and LC_b by reverse-phase h.p.l.c.

Detergent-extracted coated vesicles were purified as described by Pearce & Robinson (1984) and then resuspended in the minimum volume of 0.2 mM-EDTA/0.2 mM-DTT/0.02% NaN₃/10 mM-Tris/HCl buffer, pH 7.5. The suspensions were incubated in a boiling-water bath for 10 min and then centrifuged at 100 000 *g* for 2 h. A 500 µl portion of this supernatant, containing approx. 0.5 mg of light chains, was applied to a C₃ RSPC column (0.46 cm × 7.5 cm) (Beckman) equilibrated in 0.1% (v/v) trifluoroacetic acid (Pierce Chemical Co.). The column was run at a flow rate of 0.4 µl/min with a gradient of acetonitrile (Burdick and Jackson) in 0.1% trifluoroacetic acid: 0–44% (v/v) in 5 min and 44–52% (v/v) in 40 min. The light chains were eluted at between 48% and 49% (v/v) acetonitrile. The profile of material eluted with absorbance at 280 nm is shown in (a). Fractions (200 µl) were collected and 25 µl samples were analysed by SDS/PAGE (12% polyacrylamide) as shown in (b).

et al., 1984). Differential splicing of the transcripts of the LC_a and LC_b genes accounts for five distinct protein species (Jackson *et al.*, 1987; Kirchhausen *et al.*, 1987b). However, the electrophoretic heterogeneity cannot be fully explained by the differential splicing alone. Of particular concern has been the presence of material with mobility similar to LC_b in preparations of LC_a. To understand this complexity we first sought methods for better separation of the light chains obtained after boiling and centrifugation of coated vesicles or clathrin triskelions. One method is reverse-phase h.p.l.c. on a C₃ column (Fig. 2), and a second is DEAE-cellulose chromatography with a smaller column and lower flow rate than described by Ungewickell (1983). H.p.l.c. on a C₃ column is faster and the light chains are eluted in a volatile solvent. DEAE-cellulose chromatography is more suited to larger sample sizes. Both methods give

equivalent separation of LC_a and LC_b, as judged by SDS/PAGE, resolving one major and several minor bands from each light-chain peak. Although some separation of the major and minor species of LC_a and LC_b occurred during both chromatographic procedures, it did not obviously correlate with the fine structure in the elution profiles.

Since Schook *et al.* (1983) reported that clathrin light chains bind nucleotides, we considered this as one possible source of light-chain heterogeneity. However, the absorption spectra of column fractions obtained from DEAE-cellulose-chromatographic separation of light chains provided no evidence for bound nucleotide. Fractions containing light chains had spectra typical of proteins and had no specific absorption in the nucleotide region. In contrast, the early-eluted material that was devoid of light chains did show increased absorption in this region. Thus if the light chains contained bound nucleotide it was removed during chromatography and could not account for the observed heterogeneity.

Isolation procedures for coated vesicles and clathrin have generally not included thiols in the buffers (Pearse, 1976; Keen *et al.*, 1979). In contrast, methods for subsequent separation of light chains and heavy chains or separation of the two light chains have often used DTT. Ungewickell (1983) and Winkler & Stanley (1983) found it was essential for obtaining good recoveries of light chains, especially of LC_a from DEAE-cellulose columns. The possibility that the oxidation state of the cysteine residues contributes to light-chain heterogeneity was explored. Clathrin was prepared in the presence of 0.1% (v/v) 2-mercaptoethanol. The light chains prepared by boiling and centrifugation were dialysed against DEAE-cellulose buffer containing DTT. One half of the preparation was chromatographed on DEAE-cellulose in the presence of DTT and the other half in its absence. The yields of LC_b and LC_a in the absence of DTT were approx. 40% and 10% respectively of those obtained in its presence, confirming the results reported by Winkler & Stanley (1983). However, if all isolation and purification procedures were carried out in the absence of thiols then good yields of both light chains were obtained. In addition, the fine structure in the elution profile was considerably less complex. These observations prompted further investigation of the oxidation state of the cysteine residues of the clathrin light chains.

Electrophoretic heterogeneity of clathrin light chains

When brain light chains are purified in the absence of thiols and analysed by SDS/PAGE under reducing and non-reducing conditions they show differences in electrophoretic mobility. On reduction LC_a and LC_b have apparent molecular masses of 39 kDa and 36 kDa respectively; under non-reducing conditions LC_a has a mobility that is similar to that of reduced LC_b, and that of LC_b is slightly faster. Similar differences in mobility between brain LC_a and LC_b are seen if reduced and alkylated samples are compared with untreated samples on non-reduced gels (Fig. 3).

Mixtures of the reduced and non-reduced forms were often observed with reduction conditions commonly used in SDS/PAGE, and this additional complexity could be eliminated with reduction and alkylation. The minor species of 34 kDa for LC_a and of 32 kDa for LC_b seen in the brain preparations in Fig. 3 are identical in mobility with the non-brain forms of the two light

Fig. 3. Reduction and alkylation changes the mobility of clathrin light chains on SDS/PAGE

Brain LC_a and LC_b were reduced and alkylated by incubation with 5 mM-DTT/0.1% SDS/10 mM-Tris/HCl buffer, pH 8.0, for 30 min at 37 °C followed by addition of 12.5 mM-iodoacetamide and incubation for 30 min at room temperature. Samples were then analysed by SDS/PAGE (10% polyacrylamide). Samples designated 1 were incubated with buffer alone. Samples designated 2 were reduced and alkylated as described above, and samples designated 3 were alkylated without prior reduction.

chains (Holmes *et al.*, 1984). Their presence in the brain is consistent with the isolation of cDNAs encoding these forms from brain libraries (Jackson *et al.*, 1987; Kirchhausen *et al.*, 1987b). In common with brain light chains, non-brain LC_b shows a decreased mobility on reduction and alkylation (Fig. 3), whereas non-brain LC_a exhibits a slightly increased mobility. An intermediate species is seen in reduced and alkylated preparations of brain LC_a. This may represent a second form of brain-specific LC_a corresponding to the product of a cDNA characterized by Kirchhausen *et al.* (1987b). This protein is predicted to have an 18-residue instead of a 30-residue brain-specific insert and one instead of two cysteine residues.

Reduction and alkylation improves resolution of clathrin light chains on SDS/PAGE. Alkylation alone had no effect, and when iodo[³H]acetic acid was incubated with either brain or adrenal clathrin light chains little incorporation of radioactivity was detected without previous incubation with DTT. Similar results were obtained in the presence of 6 M-guanidinium chloride, showing that inaccessibility of the thiol groups of brain light-chain cysteine residues was not due to burial within the protein structure. It was in fact due to intramolecular disulphide bonds, as demonstrated by the following experiments.

Disulphide bonds in LC_b

The cDNA sequence of brain and non-brain LC_b predicts cysteine residues at positions 213 and 223 according to the numbering given by Jackson *et al.* (1987) (Fig. 1). To confirm these predictions, carboxy-[³H]methylated LC_b was digested with clostripain, and the CI fragment encompassing residues 158–228 was purified on a C₃ reverse-phase h.p.l.c. column. This fragment was treated with BNPS-skatole, and the peptide corresponding to residues 207–228 was purified on a C₁₈ reverse-phase h.p.l.c. column. Sequence analysis local-

Fig. 4. Disulphide-linked tryptic-digest peptides from brain LC_a .

A tryptic digest of non-reduced brain LC_a was fractionated by C_{18} reverse-phase h.p.l.c. Two peptides (IV and VII) that were sensitive to DTT were identified and rechromatographed on the C_{18} column. Panels (a) and (c) show rechromatography of these purified peptides without prior reduction. Panels (b) and (d) show rechromatography of peptides IV and VII respectively after treatment with 2.5 mM-DTT. —, A_{219} ; ----, acetonitrile gradient.

ized the carboxy[3H]methylated cysteine residues to positions 213 and 223. Identical results were obtained with a BNPS-skatole-cleavage fragment corresponding to residues 207–243 of the non-brain form of LC_b purified from adrenal gland.

The accessibility of the two cysteine residues in the clostripain-digest CI fragment of brain LC_b was examined by alkylation with iodo[3H]acetic acid. Incorporation of radioactivity required prior reduction with DTT and was unaffected by the presence of denaturants. This result is consistent with the presence of an intramolecular disulphide bond in LC_b and predicts that tryptic digestion of non-reduced LC_b should yield two peptides cross-linked by a disulphide bond. To test this possibility, the clostripain-digest CI fragment of brain LC_b was digested with trypsin. Duplicate portions of the digest were either treated or not treated with DTT, then analysed on a C_{18} reverse-phase h.p.l.c. column. A single peptide peak was lost on addition of DTT and two new peaks were seen. The DTT-sensitive peptide peak from the non-reduced digest was isolated and sequenced as also were the two peaks from the reduced digest. The DTT-sensitive peak contained cross-linked peptides QCK (residues 221–224) and VAQLCDFNPK (residues 209–218), which were resolved upon reduction. Similar results were obtained with adrenal-gland LC_b . Thus in both brain and non-brain forms of purified LC_b the two cysteine residues are stoichiometrically linked by an intramolecular disulphide bond.

Disulphide bonds in LC_a

The cDNA sequence predicts that brain LC_a has one cysteine residue at position 213, in common with LC_b , and a second at position 182, within the brain-specific insertion sequence (Fig. 1). Tryptic digests of brain LC_a were fractionated on a C_8 reverse-phase h.p.l.c. column with and without addition of 2.5 mM-DTT (Fig. 4). One DTT-sensitive peptide was observed (peak IV). On reduction this peak gave rise to two peptides (peaks V and VI). Sequence analysis of peptide IV and of its reduced components (V and VI) showed that cysteine residues at positions 182 and 213 form an intramolecular disulphide bond. Peptide VI comprised residues 166–208, of which QPFADVIGYVTNINHPCYSLEQA was obtained by sequencing, and peptide V comprised residues 212–218, giving the predicted sequence of LCDNFNPK.

A minor DTT-sensitive peak (VII) also yielded peptide V upon reduction and had the sequence of residues 212–218. It may derive from non-brain LC_a and/or the minor brain-specific LC_a species, both of which only have cysteine-213. Alkylation of brain and adrenal-gland clathrin light chains with iodo[3H]acetic acid did not result in preferential incorporation of radioactivity into non-brain LC_a , as would be expected if the single cysteine residue had a free thiol group. This result, combined with the observed shift in elution position of peptide VII on treatment with DTT, suggests that this cysteine residue may be bonded to a small cellular thiol such as GSH.

In conclusion, we find that the thiol groups of purified clathrin light chains are not accessible to alkylating agents. For the major brain LC_a and LC_b forms and for non-brain LC_b they are involved in intramolecular disulphide bonds. Reduction causes a decrease in electrophoretic mobility, the magnitude of which correlates with the number of residues between the two cysteine residues. For brain LC_a it is 30 residues and the change

Fig. 5. Alkylation of bovine brain clathrin light chains with and without prior reduction

Brain coated vesicles (lanes 1, 4, 7 and 10), clathrin trimers (lanes 2, 5, 8 and 11) and clathrin light chains (lanes 3, 6, 9 and 12) were purified in the presence of 1 mM-DTT (lanes 1–3 and 7–9) and in the absence of DTT (lanes 4–6 and 10–12). Samples were then treated with 10 mM-iodo[^3H]acetic acid after pretreatment with 3 mM-DTT (lanes 7–12) or no pretreatment (lanes 1–6). After addition of excess 2-mercaptoethanol to stop the alkylation reaction, the samples were analysed by SDS/PAGE. Panel (a) shows the staining of the gel with Coomassie Brilliant Blue, panel (b) the fluorograph of the dried gel. The highly radioactive bands at about 50 kDa are probably tubulin polypeptides.

in apparent molecular mass is approx. 3000 Da, whereas for brain and adrenal-gland LC_b it is nine residues and the change in molecular mass is approx. 1000 Da. For non-brain LC_a , with its single cysteine residue, reduction and alkylation gives a slight increase in mobility, presumably due to the addition of a negative charge.

Disulphide bonds in coated vesicles and clathrin triskelions

Do the light-chain disulphide bonds exist *in vivo* or are they an artifact of purification *in vitro*? To address this question, clathrin light chains were purified in the presence of an alkylating agent. Inclusion of 10 mM-iodoacetamide in all buffers throughout the purification produced light chains with electrophoretic mobilities characteristic of the reduced and alkylated forms. In contrast, a control preparation made in the absence of iodoacetamide gave electrophoretic mobilities corresponding to the disulphide-bonded forms. These observations, indicating that complete alkylation of the cysteine

residues had occurred in the presence of iodoacetamide, were confirmed by reducing and alkylating the two preparations with iodo[^3H]acetic acid. Incorporation of radioactivity and a change in electrophoretic mobility were only seen for preparations made in the absence of iodoacetamide. Thus *in vivo* the cysteine residues exist in the reduced state and disulphide bonds are a product of isolation.

The light chains of clathrin triskelions and coated vesicles exhibited the same electrophoretic patterns under reducing and non-reducing conditions as did free light chains. Thus light-chain disulphide bonds were either present in the coated vesicles and triskelions or rapidly formed on dissociation of the heavy chains and light chains in the gel sample buffer. To examine the accessibility of the thiol groups, preparations of coated vesicles, clathrin triskelions and free light chains were made in the absence and in the presence of 1 mM-DTT (Fig. 5). These preparations were then alkylated with iodo[^3H]acetic acid in the presence and in the absence of

Table 1. Binding of iodinated light chains to clathrin heavy chain

Clathrin heavy chain was prepared by elastase digestion of triskelions, which removes light chains (Kirchhausen & Harrison, 1981). Light-chain binding was tested by solid-phase radioimmunoassay (Brodsky *et al.*, 1987), 10^6 c.p.m. of each light chain being added at 'neat' concentration. Values are the means for duplicate determinations of radioactivity (c.p.m.) bound per well.

Twofold dilution of light chain	Light-chain binding (c.p.m. bound)			
	Reduced and alkylated LC _a	Oxidized LC _a	Reduced and alkylated LC _b	Oxidized LC _b
Neat	48 195	44 249	74 267	68 954
1	27 055	25 838	40 821	39 863
2	12 596	13 459	21 373	18 742
3	7 747	7 286	11 380	11 002
4	4 013	3 839	5 753	6 022
5	2 332	2 280	3 410	3 438
6	1 240	1 221	1 818	1 936
7	683	698	970	980
8	454	442	556	612
9	278	249	338	345
10	247	245	225	204
11	243	205	266	246

DTT. As expected, the free light chains prepared in the absence of DTT incorporated no radioactivity (Fig. 5b, lane 6) unless DTT was added (lane 12). Incorporation of radioactivity into the light chains of triskelions and coated vesicles prepared without DTT was poor and not greatly increased by the addition of DTT. Similarly the incorporation of radioactivity into triskelions and coated vesicles made in the presence of DTT was poor compared with free light chains. In none of the coated-vesicle or triskelion preparations was radioactivity incorporated into bands corresponding to alkylated LC_b. Incorporation was only seen in bands corresponding to the alkylated brain and non-brain forms of LC_a. Therefore the thiol groups of light chains bound to clathrin heavy chains are considerably less accessible to alkylation than are those of the free light chains.

No incorporation of radioactivity into the clathrin heavy chain was seen unless 6 M-guanidinium chloride or 1 % SDS was present, and the extent of labelling was not affected by the presence of DTT. Thus the clathrin heavy chain contains no disulphide bonds and all the free thiol groups are inaccessible.

Antigenicity and heavy-chain binding of reduced and oxidized light chains

When samples of clathrin light chains were reduced with DTT and extensively dialysed against physiological buffers without reducing agents, stoichiometric formation of disulphide bonds occurred. This reversibility of the reduction and oxidation facilitated a comparison of the antigenic properties of light chains having or lacking the disulphide bonds.

The capacity of reduced and alkylated and fully oxidized light chains to inhibit the binding of 16 monoclonal antibodies with specificity for either LC_a (X16,

X49, CVC.1, CVC.6 and CVC.7) or LC_b (X43, X45, X50, LCB.1, LCB.2, LCB.3, LCB.4, LCB.5, LCB.6, LCB.72 and LCB.74) was measured. For each antibody a complete and titratable inhibition was seen. Of the antibodies 14 gave superposable inhibition curves for reduced and oxidized light chains. For antibodies X49 and LCB.1 increased inhibition was seen with oxidized compared with reduced and alkylated light chains. However, the difference was only a factor of 2-fold. Thus no major loss or gain of the epitopes recognized by these antibodies occurs on reduction or on oxidation of the cysteine residues.

To examine the effect on the binding of clathrin light chains to the heavy chain, oxidized preparations of LC_a and LC_b were radioiodinated and divided into two portions. One half was reduced and alkylated and the other underwent 'mock' treatment. Both preparations showed equal binding to the clathrin heavy chain, demonstrating that the presence or absence of the disulphide bond has no effect upon this interaction (Table 1).

DISCUSSION

Purification of clathrin light chains in the absence of thiols produces brain LC_a, brain LC_b and non-brain LC_b having specific intramolecular disulphide bonds (Fig. 1). These bonds can be stoichiometrically reduced and re-formed by addition or removal of thiols. The disulphide bonds in brain LC_b and non-brain LC_b are more readily formed and more stable once formed than is the brain LC_a bond. This is probably a function of the larger number of residues separating the two cysteine residues in brain LC_a compared with LC_b.

It has been repeatedly observed that SDS/PAGE of light-chain preparations under reducing conditions reveals a polypeptide with mobility close to that of L_b but having antigenic determinants and primary structure in common with LC_a (Ungewickell, 1983; Winkler & Stanley, 1983; Holmes *et al.*, 1984). This polypeptide varies in amount and has been attributed to proteolysis. It is now clear that this band is related to intact LC_a not by a proteolytic cleavage but by the formation of a disulphide bond. Although not systematically studied, we find that the amount of the disulphide-bonded form on reducing gels increases with the protein load and that reversible breakage and formation can take place during sample preparation and electrophoresis.

Non-brain LC_a and the shorter form of brain LC_a migrate similarly to brain LC_b, and also produce a heterogeneity in brain LC_a preparations that does not result from proteolysis. Electrophoretic heterogeneity of LC_b can also arise from disulphide-bond formation, and in fact the reaction is faster and the bond more stable than for LC_a.

The similarity in mobility of disulphide-bonded brain LC_a and reduced brain LC_b can lead to underestimates of the effectiveness of schemes to separate LC_a and LC_b. For example we found that monoclonal-antibody-purified preparations of LC_a appeared to contain about 50 % LC_b when assessed by SDS/PAGE. However, peptide mapping showed that the contamination was 10 % or less (Holmes *et al.*, 1984). Similar heterogeneity was described by Winkler & Stanley (1983) for preparations of LC_a produced by DEAE-Sephacel chromatography. These investigators also noted that when clathrin

triskelions were extracted from coated vesicles in the presence of DTT subsequent separation of heavy chains and light chains required the presence of DTT 'to obtain reproducible yield of light chains and in particular of LC_a'. We have had a similar experience with the separation of LC_a and LC_b by DEAE-cellulose chromatography. However, if all procedures are carried out in the absence of thiols then good yields of both light chains can be obtained on DEAE-cellulose chromatography. The selective loss of LC_a is presumably related to the lower rate at which the disulphide bond is formed on removal of the thiol.

An important question is whether disulphide bonds exist in the clathrin light chains *in vivo*. Experiments in which light chains were purified in the presence of an alkylating reagent show that the disulphide bonds are most probably an artifact of isolation. However, their formation had no significant effects upon various immunological epitopes or the heavy-chain-binding site. Despite these negative results it will be important in future analysis to consider the possible effects of disulphide-bond formation on other functional properties of the clathrin light chains.

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