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The major polypeptide of scrapie-associated fibrils (SAF) has the same size, charge distribution and N-terminal protein sequence as predicted for the normal brain protein (PrP)

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Scrapie-associated fibrils (SAF) are unique structures characteristic of the group of unconventional slow infections which includes scrapie and Creutzfeldt–Jakob disease. A major component of hamster fibrils has been described as a protease-resistant glycoprotein with an apparent mol. wt of 27 000–30 000 (PrP27–30). However, we report here that if fibrils are prepared by procedures designed to minimise proteolysis the PrP proteins co-purifying with hamster SAF have mol. wts of 33 000–35 000 (PrP33–35) and 26 000–29 000 (PrP26–29). We find a Lys-Lys-Arg-Pro-Lys sequence at the amino terminus of these SAF proteins, that is absent from PrP27–30, and which has recently been predicted to be the N-terminal sequence of the native PrP protein of uninfected brain. The major SAF protein (PrP33–35) and its normal brain homologue are shown to have the same apparent mol. wt and ionic charge distribution by two-dimensional gel analysis, silver staining and immunoblotting. These results support our view that PrP33–35 and the normal brain PrP protein may have the same covalent structure, and that the PrP protein is recruited into these amyloid-like SAF or into association with a non-protein component of SAF by an irreversible event initiated directly or indirectly by scrapie infection.

Key words: scrapie/scrapie-associated fibrils (SAF)/SAF protein sequence/cerebral amyloidosis

Introduction

Scrapie-associated fibrils (SAF) are unique structures characteristic of the group of unconventional slow infections which includes scrapie and Creutzfeldt–Jakob disease (Merz *et al.*, 1981, 1983a, 1983b, 1984). A major component of hamster fibrils has been described as a protease-resistant glycoprotein with an apparent mol. wt of 27 000–30 000 (PrP27–30) (Diringer *et al.*, 1983; Bolton *et al.*, 1982, 1984, 1985; McKinley *et al.*, 1983; Prusiner *et al.*, 1982, 1983). The partial amino acid sequence of this fibril protein (PrP) has been determined by protein microsequencing (Prusiner *et al.*, 1984; Multhaup *et al.*, 1985; Oesch *et al.*, 1985) and a possible complete primary structure inferred from the cDNA sequence of its mRNA (Oesch *et al.*, 1985; Chesebro *et al.*, 1985). PrP is the product of a highly conserved cellular gene (Oesch *et al.*, 1985; Westaway and Prusiner, 1986) and similar amounts of PrP mRNA are found by *in situ* hybridisation almost exclusively in the neurones of both infected and uninfected animals (Kretzschmar *et al.*, 1986).

Scrapie infectivity appears to be relatively resistant to proteinase K under non-denaturing conditions (McKinley *et al.*, 1983). This finding has led to the inclusion of a proteinase K digestion step in all recent methods that have been used in attempts to purify scrapie infectivity (Diringer *et al.*, 1983; Hilmert and Diringer, 1984; Bolton *et al.*, 1982; Prusiner *et al.*, 1983). SAF and infectivity co-purify, at least in part, during the subcellular fractionation of scrapie-infected brain (Diringer *et al.*, 1983; Somerville *et al.*, 1986) and these fibrils, and their major protein component (PrP27–30) (Diringer *et al.*, 1983; Bolton *et al.*, 1982; McKinley *et al.*, 1983; Prusiner *et al.*, 1982, 1983), have also been described as protease resistant (Diringer *et al.*, 1983; Bolton *et al.*, 1982, 1984, 1985; Prusiner *et al.*, 1984). The association of PrP27–30, SAF and infectivity has led to the speculation that SAF are a form of the scrapie agent (Merz *et al.*, 1983a; Diringer *et al.*, 1983; Somerville *et al.*, 1986), and as a scrapie-specific nucleic acid has yet to be found in SAF, that PrP27–30 is, in itself, the infectious entity (Bolton *et al.*, 1982, 1984, 1985; McKinley *et al.*, 1983; Prusiner *et al.*, 1982, 1983). This latter notion appears to be incompatible both with the occurrence of >15 distinct strains of scrapie (Dickinson *et al.*, 1984) which indicates it has a host-independent genome (Kimberlin, 1986), and with the evidence that the PrP sequence is encoded by a single gene in the host genome (Oesch *et al.*, 1985).

Although the initial protein product of the PrP gene remains to be defined (but see below), PrP27–30 has been reported to account for 143 amino acids in the central part of a possible 240 amino acid protein (Oesch *et al.*, 1985). Affinity-purified immunoglobulins from animals immunised with PrP27–30 detect a form of this protein in extracts of scrapie-infected brain and uninfected brain which has an apparent mol. wt of 33 000–35 000, significantly larger than PrP27–30 (Oesch *et al.*, 1985; Manuelidis *et al.*, 1985; Rubenstein *et al.*, 1986). This PrP 33–35 protein in homogenates of uninfected hamster brain is completely degraded by proteinase K (Oesch *et al.*, 1985; Rubenstein *et al.*, 1986) and neither fibrils nor protein are found in fractions of uninfected brain prepared by the methods developed to isolate SAF. This evidence can be used to support a model for the pathogenesis of SAF in which scrapie infection induces the proteolysis of PrP33–35 to PrP27–30 and that it is this latter, abnormal by-product of infection which then polymerises into the characteristic disease-specific fibrils (Figure 4A). This pathway is similar to that proposed for the formation of systemic amyloid fibrils following the proteolytic cleavage of precursor proteins (Glenner, 1980). The morphology of SAF does resemble that of amyloid fibrils (Merz *et al.*, 1981, 1983b; Prusiner *et al.*, 1983; DeArmond *et al.*, 1985) but these structures can be distinguished by electron microscopy (Merz *et al.*, 1981, 1983b, 1984).

However, the possibility that the complete conversion of PrP to PrP27–30 is an artefact of the use of proteinase K during the purification of SAF has led us to purify fibrils by procedures designed to minimise proteolysis. We report here that the PrP proteins co-purifying with hamster SAF have mol. wts of 33 000–35 000 (PrP33–35) and 26 000–29 000 (PrP26–29).

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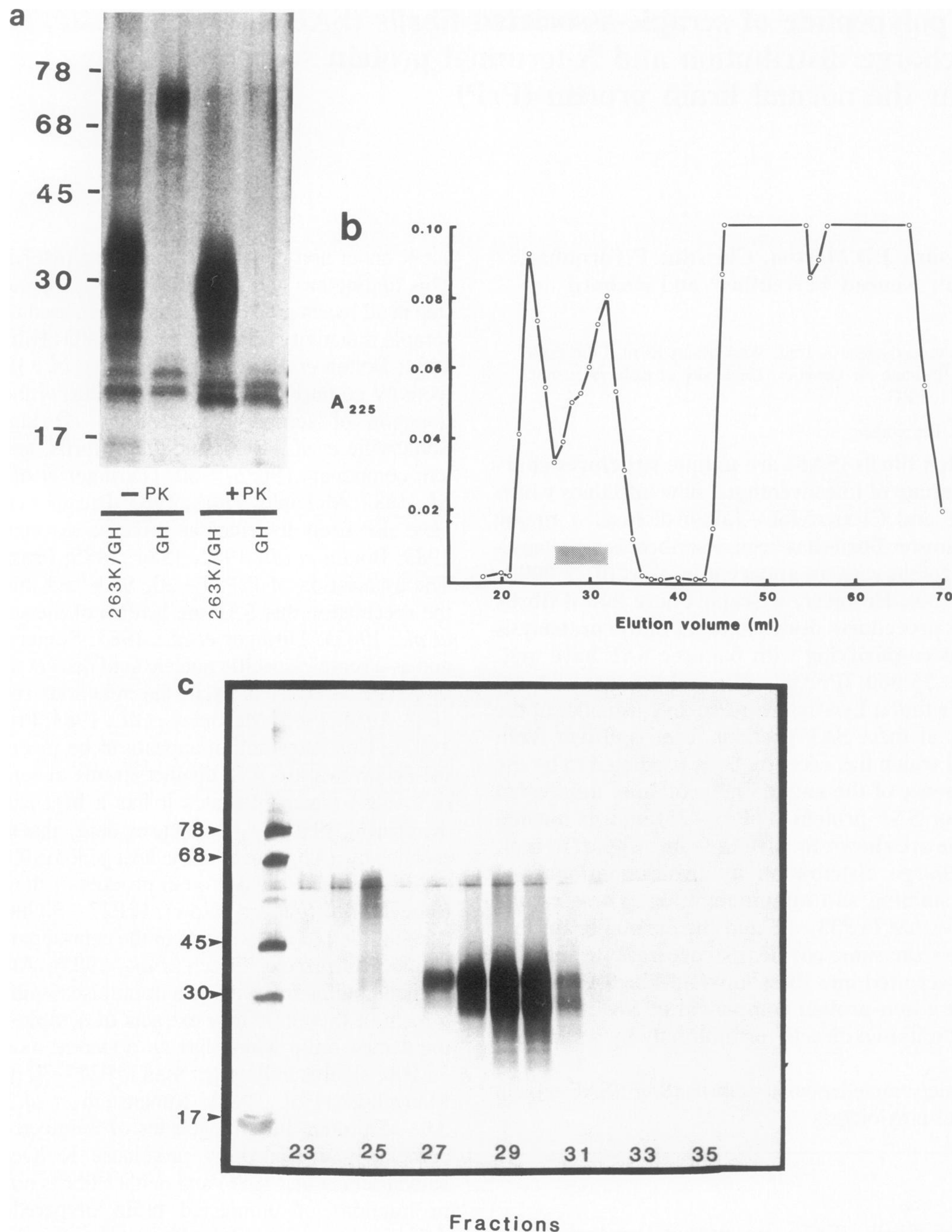

Fig. 1. Purification of hamster (GH) PrP: **(a)** SDS−PAGE analysis and silver staining of proteins extracted from SAF-containing fractions and control fractions purified from scrapie (263 K/GH) and non-infected (GH) brain with (+PK) and without (−PK) proteinase K. Each lane corresponds to 0.00167 hamster brain equivalents. **(b)** Gel filtration chromatography and u.v. absorbance profile at 225 nm of proteins extracted from SAF-containing fractions purified without proteinase K. The profile shows the equivalent of 1.5 hamster brains. ▩ indicates the elution volume of PrP-related proteins. **(c)** SDS−PAGE analysis and silver staining of proteins in fractions 23−35 of the gel filtration chromatography experiment shown in **(b)**, following their precipitation from solution by the chloroform−methanol method (Wessel and Flugge, 1984). One-hundredth of each column fraction was used for this SDS−PAGE analysis, corresponding to 0.015 hamster brain equivalents. The apparent molecular mass is shown in kilodaltons.

We have sequenced the 20 amino-terminal residues of these SAF proteins and found that they contain a region of PrP rich in basic amino acids which is encoded by the cDNA sequence but absent from PrP27−30. By implication, previous results (Diringer *et al.*, 1983; Bolton *et al.*, 1982, 1984, 1985; McKinley *et al.*, 1983; Multhaup *et al.*, 1985; Prusiner *et al.*, 1982, 1983, 1984) were based in part on N-terminal proteolysis *in vitro* produced by the use of proteinase K. This leads us to a major reappraisal of the relationship of these SAF proteins to the normal cellular product of the PrP gene, and of ideas about the pathogenesis of these amyloid-like fibrils.

Results and discussion

Nomenclature

Our methods purify aggregates of PrP which have the morphology of SAF and so we regard SAF protein and PrP as synonyms. We use PrP to denote proteins with sequences of

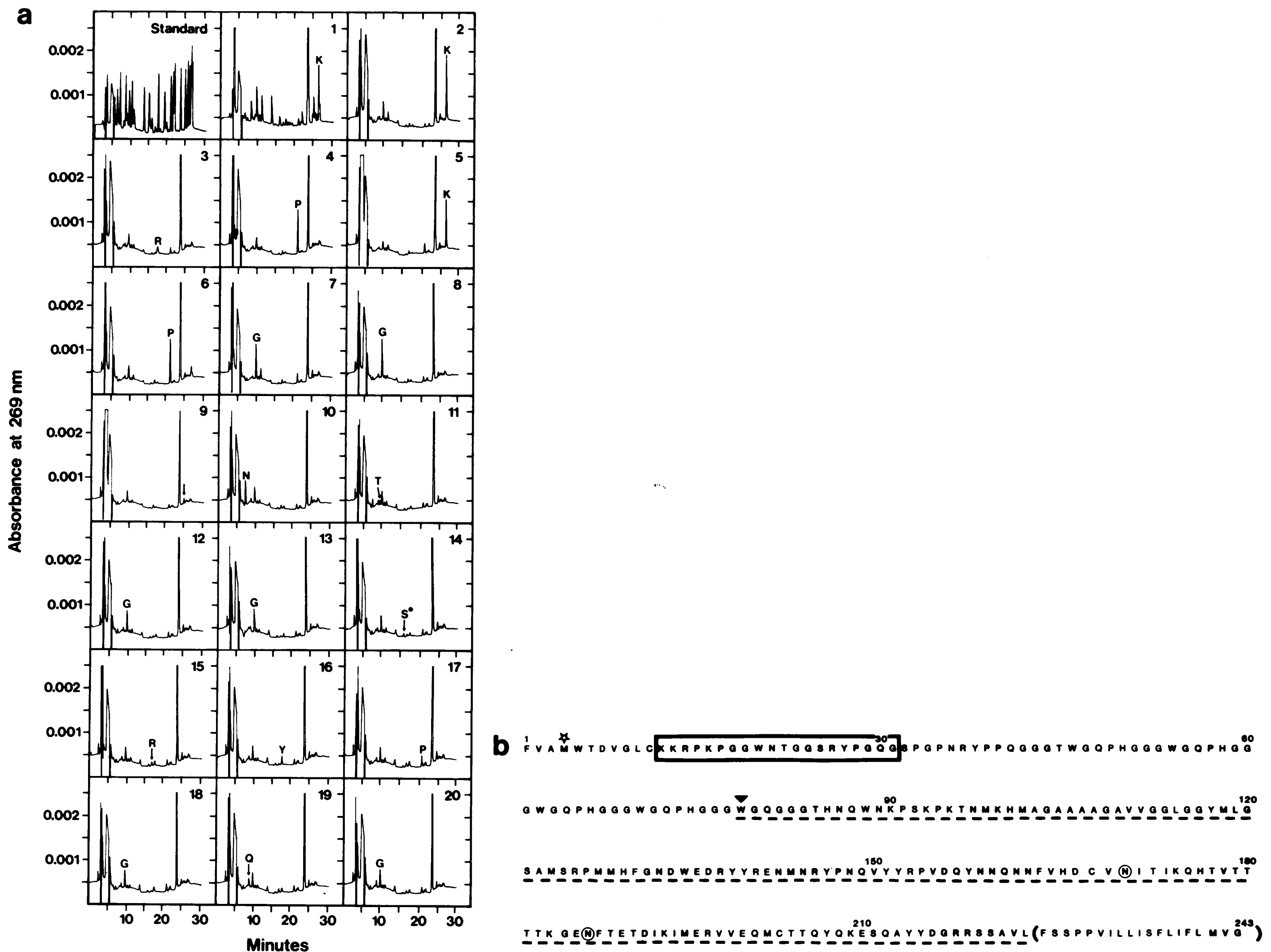

Fig. 2. Sequencing of PrP33−35: comparison of the amino acid sequences of PrP33−35, PrP27−30 and the predicted structure of PrP. **(a)** The h.p.l.c. analyses of PTH amino acids released at each of the first 20 cycles of automated Edman degradation on a gas-phase microsequencer (Hewick *et al.*, 1981) during the sequencing of PrP33−35 (fraction 27, Figure 1c). The order of elution of the PTH-amino acids, dimethylaminophenylthiourea (DMPTU) and diphenylthiourea (DPTU) (12.5 pmol each) in the standard mixture (upper left panel) is Asp (D), Asn (N), Ser (S), Gln (Q), DMPTU, Ala (A), His (H), DDT adduct of Ser (*S), Tyr (Y), Arg (R), Thr (T), DPTU, Trp (W), Phe (F), Ile (I), Lys (K), Leu (L). During the sequencing run shown (fraction 27), the elution of the PTH-amino acids tyrosine and arginine inverted and arginine eluted before tyrosine (see cycles 3, 15 and 16). This sequence could be assigned to residues 12−31 of the PrP structure deduced by Oesch and his colleagues (Oesch *et al.*, 1985) (see **b**). **(b)** The relationship between PrP, PrP27−30 and PrP33−35 protein: □, the amino-terminal sequence of the PrP33−35 protein (see above); - - - -, the proposed sequence of PrP27−30 (Oesch *et al.*, 1985); ▼, the amino-terminal end of PrP27−30, found to be heterogeneous by protein sequencing (Multhaup *et al.*, 1985; Prusiner *et al.*, 1984); Ⓝ, possible sites for asparagine-linked glycosylation (Oesch *et al.*, 1985; Struck *et al.*, 1978); ( ), the carboxyl end of PrP27−30 has not been sequenced directly but has been inferred from the cDNA sequence and the empirical, amino acid composition of PrP27−30 (Oesch *et al.*, 1985); ☆, the methionine residue corresponding to the site first proposed for the initiation of translation on the PrP mRNA (Oesch *et al.*, 1985) (see text).

amino acids corresponding to those predicted for the SAF protein (Oesch *et al.*, 1985). Usually, this designation is used to prefix the molecular mass (in Kilodaltons) of a fibril-associated protein; for example, the PrP-related protein which co-purifies with fibrils prepared using proteinase K methodologies has a molecular mass of 27 000−30 000 daltons and is usually referred to as PrP27−30.

Purification of SAF proteins: effect of proteinase K

We have modified published methods (Hilmert and Diringer, 1984; Manuelidis *et al.*, 1985) to minimise artefactual proteolysis. Our method produces fibrils containing a major protein of mol. wt, 33 000−35 000, and a minor component of mol. wt 26 000−29 000 (Figure 1, lane 1). These proteins, and SAF (data not shown), are found in the fraction of purified SAF from scrapie-infected brains and not in the corresponding fractions from non-infected brain (Figure 1a, lane 2). The inclusion of proteinase K in this procedure leads to the purification of PrP27−30 as the major protein component of SAF fractions (Figure 1a, lane 3) as described by others (Diringer *et al.*, 1983; Hilmert and Diringer, 1984; Bolton *et al.*, 1982; Prusiner *et al.*, 1983; Manuelidis *et al.*, 1985). We have used an antiserum to hamster PrP27−30 (Bode *et al.*, 1985) to verify that the 33 000−35 000 and 26 000−29 000 proteins and PrP27−30 have common epitopes (data not shown) and confirmed their structural relatedness by amino acid sequencing of the 33 000−35 000 protein alone or in mixtures with the 26 000−29 000 protein (see below).

The mol. wt of the PrP protein found in non-infected brain

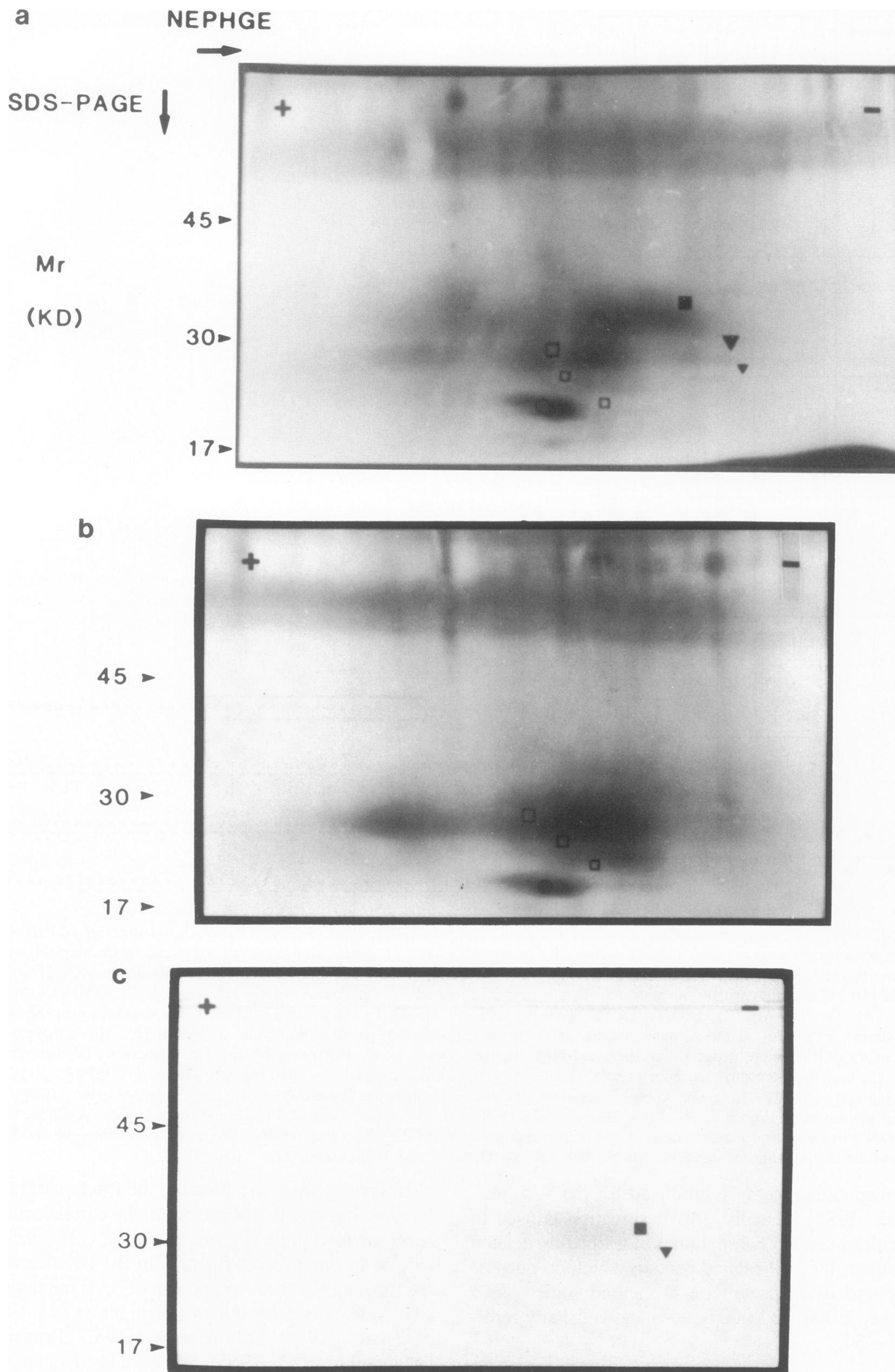

Fig. 3. Two-dimensional gel analysis and silver staining of SAF proteins, and immunoblotting of detergent extracts of non-infected brain. The hamster SAF proteins purified in the **(a)** presence or **(b)** absence of proteinase K using NEPHGE gel analysis and silver staining. ■,▼, the major PrP33−35 protein and minor PK-sensitive variants. □,□, denotes the more acidic PrP-related proteins of SAF in both **(a)** and **(b)**; in **(b)** the topmost smudge is PrP27−30. These silver-stained gels are of the P285 pellet (see Figure 1) before chromatography and therefore unrelated protein contaminants (●,○) can be seen in both **(a)** and **(b)**. **(c)** The immunoreactive PrP-related proteins in detergent extracts of non-infected brain. ■, the normal cellular PrP33−35 and ▼, a minor variant which is also seen in **(a)**. Each gel analysis represents the equivalent of 0.01 hamster brains.

was originally reported as 33 000−35 000 (Oesch *et al.*, 1985) and we also estimate the normal and proteinase K-sensitive fibril protein to be of this weight, if we use carbonic anhydrase A or B as our 30 000 marker (data not shown). However, this estimate depends on which standard proteins are used. For example, chymotrypsinogen (25 700) runs on our polyacrylamide gradient gels in the same position as carbonic anhydrase and using it as a reference leads to a lower estimate of the mol. wt of PrP33−35 (mol. wt ~29 000) and PrP27−30 (mol. wt ~26 000). This observation possibly explains the discrepancy between our mol. wt estimates and those of Cho who recently reported the mol. wt of the protein in scrapie- and uninfected mouse or hamster brain to be 28 500 (Cho, 1986).

Protein sequencing

To resolve the structural difference between PrP33−35 and PrP27−30 we have purified PrP33−35 from scrapie-infected hamster brain by gel-filtration chromatography (Figure 1b,c) and sequenced the amino-terminal region of this proteinase K-sensitive protein (fraction 27; Figure 1c). Apart from one position we could not assign (see Figure 2a), the first 20 amino acids of this protein correspond exactly to residues 12−31 of the PrP sequence deduced by Oesch and his colleagues. The protein appeared to have a single N-terminal amino acid and the heterogeneous N termini described by two of us (K.B., G.M.) (Multhaup *et al.*, 1985) and others (Oesch *et al.*, 1985; Prusiner *et al.*, 1984) when sequencing PrP27−30 were not found. The initial yield of the major phenylthiohydantoin (PTH) amino acid lysine seen at the first cycle was 35−40 pmol; the other derivatives seen in this cycle (serine, glycine, glutamic acid and alanine) are probably major contaminations by free amino acids since only a single major residue is detected in the subsequent cycles. At cycle 9, which corresponds to a predicted tryptophan residue (Oesch *et al.*, 1985), no amino acid derivative could be assigned and there was a significantly lower yield at each subsequent cycle. This may be due to oxidative degradation of the tryptophanyl indole ring *in vivo* (Wolff *et al.*, 1986) or during purification, producing a derivative which interferes with the sequencing procedure. Residue 14 (serine) could be assigned as the DTT adduct of serine (*S). The yield of PTH-amino acids from this fraction (27; Figure 1c) was 70−100 pmol which is equivalent to 2.1−3.0 μg of a protein of mol. wt, 30 000. We estimate a yield of 10−15 μg of the PrP-related protein per hamster brain, similar to that found by Diringer and his colleagues (Multhaup *et al.*, 1985; Hilmert and Diringer, 1984).

The region of the protein which is hydrolysed from PrP27−30 by proteinase K contains a relatively high proportion of basic amino acids and, by comparison to the predicted protein, a series of four exact heptapeptide repeats between residues 46 and 77 (see Figure 2b). The structural homology of this repeat to the N-terminal domain of some members of another class of filament proteins, the epidermal keratins, has been noted (Oesch *et al.*, 1985) and our results provide the first evidence that this domain is also part of the SAF-associated proteins.

Importantly, sequencing of proteins in fractions 29 and 31 (Figure 1c) gave essentially the same results as those shown in Figure 2a for the sequencing of fraction 27; for example, all minor PTH-amino acid signals accounted at most for <5% of the protein sequenced in fraction 29 and <1% in fraction 31. We also found this homogeneity of N-terminal sequence between the size variants of SAF protein from certain mouse scrapie models (87V scrapie in MB mice) and from Cheviot sheep ex-

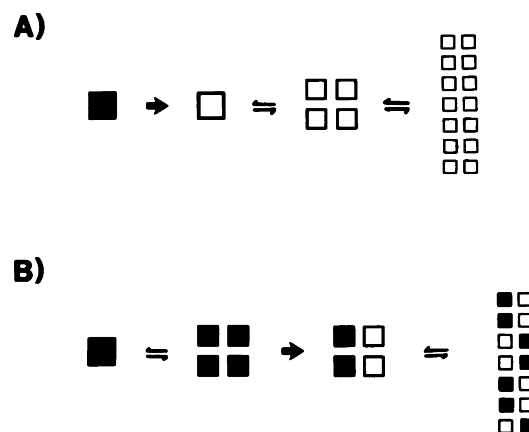

Fig. 4. Different pathways for the aggregation of PrP: ■, the normal cellular PrP protein; □, covalently modified form(s) of PrP; ➔ the irreversible event initiated directly or indirectly by scrapie infection; ⇌, denotes equilibrium between monomeric and oligomeric forms of PrP.

perimentally infected with the SSBP/1 source of scrapie (Hope *et al.*, in preparation). We estimate that >95% of the protein in these fractions was sequenced and hence do not think the homogeneity of our results is due to the chemical blocking of the N-terminal amino acid residue of one or other of these SAF proteins.

Diversity of SAF

Differences in the morphology and physicochemical properties of SAF have been found in at least three scrapie models (263 K in hamsters, ME7 or 139A in mice) (Kascsak *et al.*, 1985). This diversity may reflect the timing and location of some specific interactions between different strains of scrapie and host cells. Our finding is that the size diversity of hamster SAF proteins and those found in certain scrapie mouse models is not due to variation in N-terminal amino acid sequence. This diversity may be due to any degree or permutation of other possible post-translational changes in the structure of PrP33−35. For example, lectin binding (Manuelidis *et al.*, 1985) and amino—sugar analyses (Multhaup *et al.*, 1985) have already provided evidence for differences in the number and type of carbohydrate chains attached to hamster SAF proteins.

Similarities between normal brain PrP and SAF protein

The idea that the major SAF protein may have the same covalent structure as the normal, cellular PrP is supported by two-dimensional gel analysis and silver staining of SAF proteins and immunoblotting of the protein in detergent extracts of non-infected brain. The major PrP protein (Figure 3a) and its non-infected brain counterpart (Figure 3c) have the same apparent mol. wt (33 000−35 000) and ionic charge distribution. These proteinase K-sensitive proteins have a more basic mean isoelectric point than PrP27−30 (Figure 3b). Bolton and his colleagues have published a charge pattern for PrP27−30 similar to that seen in Figure 3b (Bolton *et al.*, 1985).

The cleavage of a leader peptide sequence from the nascent PrP protein has recently been predicted on theoretical grounds to take place after a cysteine residue (residue 11 in Figure 2b) to produce a mature protein with the lysyl-lysyl-arginyl sequence which we find at the amino terminus of these SAF proteins (Robakis *et al.*, 1986). This supports our view, based on size

and charge similarities, that the major SAF protein and its normal cellular counterpart may have the same covalent structure. We suggest that the recruitment of the host PrP protein into SAF or into association with a non-protein component of SAF may involve only a conformational change in the structure of the protein. This physical alteration could be induced by a purely chemical change in the cellular environment (pH, divalent cation concentration) (Figure 4B). Although abnormal covalent changes may be involved, perhaps represented by the minor PrP26 – 30 component of SAF, we find that quantitatively such changes must be much more subtle than previously supposed.

Mechanisms for the formation of SAF

The determination of the complete protein and polysaccharide structures of the 33 000 – 35 000 proteins of uninfected brain and SAF is needed to test this suggestion of their identity. If differences in the amino acid or sugar sequences of these proteins are found the question will remain of whether such differences in structure precede or follow the aggregation of these proteins. Mechanisms for the formation of SAF which require such modifications of PrP to be prior to or co-incident with protein aggregation (see Figure 4A) predict that all fibril-associated molecules will differ covalently from their non-infected brain counterpart and our results make this less plausible. Our evidence is that at least a proportion of the molecules in SAF seem to be identical to the normal protein and this supports schemes, such as that shown in Figure 4B, in which PrP does not require a covalent change in its structure in order to aggregate. PrP could exist *in vivo* as a functional oligomeric structure (Figure 4B) and scrapie infection might then irreversibly alter a fraction of the PrP molecules in this complex so that either *in vivo* or *in vitro* during purification (Meyer *et al.*, 1986) it further polymerises into or in association with (Manuelidis *et al.*, 1985) the disease-specific SAF.

Materials and methods

Hamsters were injected intracerebrally with 1% (w/v) brain homogenate (0.03 ml) prepared in 0.15 M sodium chloride using hamster brain from terminal cases after infection with the 263 K strain of scrapie (Kimberlin and Walker, 1978). Brain from non-injected, age-matched hamsters was used to prepare control fractions. Donor brains had previously been irradiated (3.8 Mrads; by Irradiation Products Ltd., Bradford, UK) to destroy non-scrapie pathogens. Animals with clinical signs of the disease were killed when judged to be within 1 week of natural death, brain tissue was frozen at −70°C and stored at −20°C. The mean incubation period (±S.E.M.) of cases of scrapie used in this experiment was 84 ± 1 days.

Purification of SAF

SAF and equivalent fractions of uninfected brain were prepared by a modification of published methods (Hilmert and Diringer, 1984; Manuelidis *et al.*, 1985). A 5% (w/v) brain homogenate was prepared in 0.01 M sodium phosphate, pH 7.4, 10% (w/v) N-lauryl sarcosinate containing phenylmethylsulphonyl chloride (1 mM), N-ethyl maleimide (1 mM) and diisopropyl fluorophosphate (0.1 mM) and incubated at 22°C for 30 min. The suspension was centrifuged at 22 000 *g* for 30 min at 10°C and the pellet (P22) discarded. The supernatant (S22) was centrifuged at 215 000 *g* for 150 min at 10°C and the supernatant (S215) discarded. The pellet was suspended in de-ionised water (3 ml/g of brain), ribonuclease A (Sigma: final concentration 2 μg/ml) and Tris-HCl, pH 8.5 (final concentration 50 mM) were added, shaken at 37°C for 1 h (100 r.p.m.; New Brunswick Shaker). The volume and ionic composition of the solution were adjusted to 9 ml/g of brain and 0.6 M potassium iodide, 6 mM sodium thiosulphate, 1% (w/v) N-lauryl sarcosinate, and 10 mM sodium phosphate, pH 8.5, respectively. To produce proteinase K (PK)-digested fractions, the enzyme (Sigma: final concentration 10 μg/ml) was added at this stage and proteolysis carried out by shaking at 37°C for 1 h. Non-PK and PK-treated fibrils and control fractions were sedimented from this solution by centrifugation at 285 000 *g*, for 90 min at 10°C through a cushion of 20% (w/v) sucrose in iodide (0.6 M), thiosulphate (6 mM), sarcosinate (1% w/v), 10 mM sodium phosphate, pH 8.5 buffer (sucrose/sample ratio; 1:4; v/v). The pellet (P285) was either resuspended in water and sonicated briefly (3 × 10 s: Branson Sonifier with microprobe) before taking a sample for negative staining and electron microscopy or the pellet was dissolved in 23.4 M formic acid (0.2 ml/brain).

SDS – PAGE analysis

For SDS – PAGE analysis, formic acid was removed under vacuum from extracts and the residue dissolved by heating at 80°C for 10 min in SDS – PAGE sample buffer [1% SDS, 0.2% (v/v) β-mercaptoethanol, 10% (w/v) sucrose, 10 mM Tris sulphate, pH 6.1]. The samples were cooled and run on a 7.5 – 20% (w/v) polyacrylamide gradient gel containing 0.1% (w/v) SDS using the Neville gel buffer system (Neville, 1971). Proteins and standards [BDH; mol. wt range, 12 300 – 78 000, and carbonic anhydrase A and B (mol. wt 30 000); Sigma] were visualised by the colour-silver stain method (Sammons *et al.*, 1981).

Gel filtration chromatography

Formic acid was removed under vacuum from extracts of P285 and the residue dissolved by heating at 80°C in 5% (w/v) SDS, 100 mM Tris-HCl, pH 7.5, (0.5 ml/brain equivalent), cooled and loaded onto a column (1 cm × 83 cm) of Sephacryl S-200 (Pharmacia) equilibrated and eluted in 0.05 M ammonium formate, pH 6.6, 0.1% (w/v) SDS, 0.2% (w/v) sodium azide at a flow-rate of 20 ml/h. Fractions (1 ml) were collected and their u.v. absorbance measured using a Beckman DU-6 spectrophotometer.

Protein sequencing

The PrP proteins were precipitated from chromatography fractions (Figure 1c) by the chloroform/methanol method (Wessel and Flugge, 1984) and re-dissolved in formic acid (0.06 ml/fraction). Each of fractions 27, 29 and 31 (see Figure 1c) (60 – 100 pmol) were sequenced once (29,31) or twice (27) using the ABI A470 protein sequenator with on-line h.p.l.c. analysis (120A PTH-analyser, Applied Biosystems, CA, USA). The separation of the PTH-amino acids was according to the manufacturer's protocol.

Two-dimensional gel electrophoresis and immunoblotting

Non-equilibrium pH gradient gels (NEPHGE) were prepared and run according to published methods (O'Farrell *et al.*, 1977) and Laemmli SDS – PAGE gels were used for the analysis of proteins in the second dimension (Laemmli, 1970). Proteins were stained using the colour silver staining method (Sammons *et al.*, 1981). Detergent extracts of non-infected hamster brain equivalent to fraction S215 of the fibril preparation (see above) were used as the source of non-scrapie material. Blots were prepared by standard methods (Towbin *et al.*, 1979). The PrP-related proteins were detected using a rabbit anti-hamster SAF antiserum (Ab 537; 1 in 500 final dilution) (Bode *et al.*, 1985) and visualised using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin and Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Sigma) by a procedure similar to that used to detect biotinylated DNA (Manuelidis *et al.*, 1982).

To ensure authentic alignment of SAF proteins and the normal PrP protein, samples and pI standards (Serva) were run in parallel on NEPHGE gels. Each pair of NEPHGE samples for comparison were run on a single SDS gel in the second dimension. Duplicate two-dimensional gels were prepared to allow both silver staining and immunoblotting of each pair of samples. Pre-stained protein mol. wt standards (BRL) were used to calibrate the immunoblots.

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