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The protein component of scrapie-associated fibrils is a glycosylated low molecular weight protein

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Scrapie-associated fibril protein (SAF-protein) extracted from infectious scrapie-associated fibrils (SAF) isolated from scrapie hamster brains is not infectious. SAF-protein is composed of various mol. wt. species of glycoproteins differing in carbohydrate content rather than amino acid composition. The N-linked carbohydrate chains represent ~40–60% of the mol. wt. of SAF-protein. The deglycosylated SAF-protein has a surprisingly low mol. wt. of ~7 kd, representing ~55 amino acid residues. This size and chemical analyses indicate that SAF-protein is an amyloid-type of protein. The simplest explanation for the available data is that SAF-polypeptide is very likely not to be part of the scrapie agent but that it is, like other amyloid proteins, derived from host-encoded proteins and not infectious. It is suggested that the infectivity of fractions rich in SAF is due to co-purification of scrapie virus and SAF caused by the high carbohydrate content of SAF-protein.

Key words: subacute spongiform virus encephalopathy/SAF/SAF-proteins/amyloid protein

Introduction

Scrapie-associated fibrils (SAF) are disease-specific morphologic structures in subacute spongiform virus encephalopathies (Merz *et al.*, 1981, 1983, 1984). They are composed of a major, structural protein (Diringer *et al.*, 1983a; Prusiner *et al.*, 1983). Prions and prion protein (Prusiner *et al.*, 1984) have been used as synonymous expressions for SAF and SAF-protein, respectively. SAF either represent a slow unconventional virus or a pathological by-product (Merz *et al.*, 1981; Diringer *et al.*, 1983a). In electron micrographs (Merz *et al.*, 1981; Diringer *et al.*, 1983a; Prusiner *et al.*, 1983), or by Congo red staining (Prusiner *et al.*, 1983) SAF closely resemble amyloids, a family of proteins involved in various diseases including some degenerative diseases of the central nervous system (Glenner, 1980). Here we describe an efficient method for the isolation of the SAF-glycoprotein isolated from scrapie brains of inbred and outbred hamsters, and we report the overall amino acid composition, the mol. wt. and N-terminal sequence of the deglycosylated SAF-protein. The 7-kd polypeptide constituting the major SAF-protein is compared with amyloid proteins isolated from brain material.

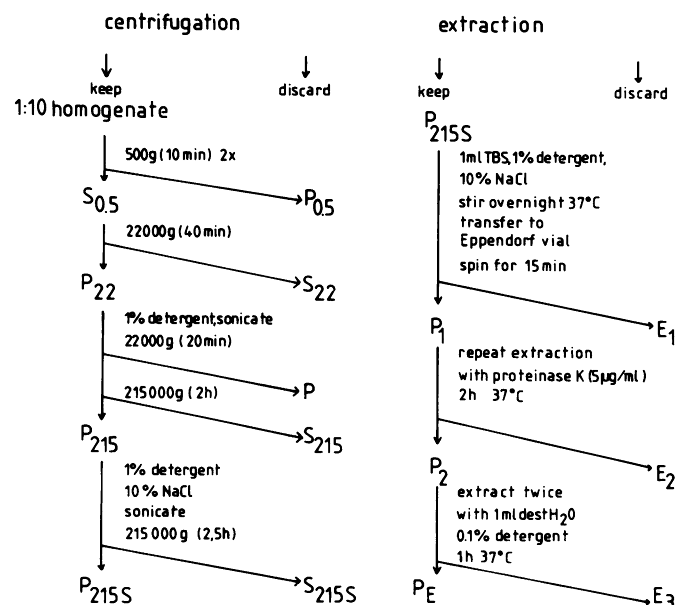
Results

Purification procedure

The isolation of SAF-protein can be divided into three distinct parts: (i) differential centrifugation to obtain a SAF-enriched fraction, designated as P_{215S}; (ii) buffer extraction to remove contaminants from SAF in P_E employing a mild proteolytic treatment; (iii) extraction of SAF with formic acid to obtain SAF-protein (see Scheme 1).

Pellet P_E has been assayed for recovery of infectivity by comparing the incubation period of 10^{-3} and 10^{-4} dilutions of this fraction with that of the homogenate. In three independent preparations the incubation period in P_E increased by ~10 days indicating that ~10% of the starting infectivity was recovered in P_E. The P_E fraction was measured as containing 40–70 μg of protein per brain, compared with 100 mg initial protein content in the homogenate, and less than 1 μg of nucleic acid (RNA + DNA) also based on one brain, compared with ~3.5–4 mg in the initial homogenate. Control preparations from normal brains only contained 10–40 μg of protein in P_E. Thus, the specific infectivity has increased ~1000-fold with respect to protein and at least 400-fold with regard to nucleic acid. Aliquots of seven independent P_E preparations from inbred CLAC or outbred AURA hamsters have been analyzed by gel electrophoresis (Figure 1). All preparations contained one major protein in the region of 26 kd with a smearing component in the range below 25 kd. Only minor amounts of protein were represented by other bands in the gel and some materials tended to stay at the top of the gel.

A single extraction with formic acid quantitatively extracted SAF-protein from P_E leaving a good portion of undissolved material behind. A second extraction, as checked by PAGE, did not solubilize any additional SAF-protein (Figure 1). OD_{280}-measurements of the formic acid extracts of eight independent



Scheme 1. Scheme for preparation of P_E (infectious material) from 10 scrapie hamster brains. The final extraction with HCOOH as given in the text yields uninfectious SAF-protein.

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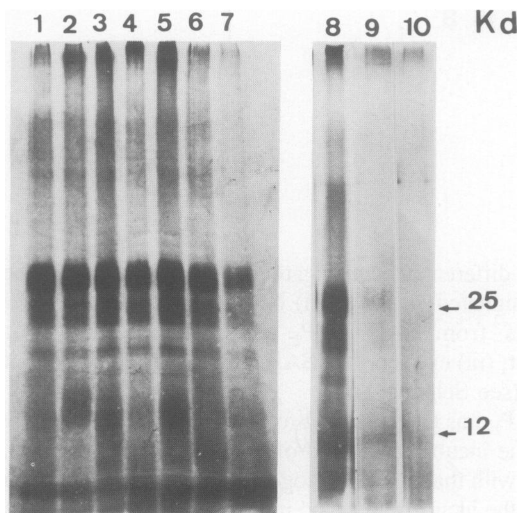

Fig. 1. PAGE of the P_E fraction of seven independent preparations from scrapie hamster brains and HCOOH-extracts. 5 μl of a P_E-suspension of 20 scrapie brains in 2 ml were applied per lane. The gels were stained with silver (Bürk *et al.*, 1983). **Lanes 1–4:** P_E from CLAC hamsters. **lanes 5–7:** P_E from AURA hamsters. **Lanes 8** and **9:** first and second formic acid extracts of P_E, respectively.

preparations and quantitative amino acid analysis in six of them indicated that we extracted 0.064 ± 0.022 OD_{280} representing 7.5 ± 1.4 μg of SAF-protein per scrapie brain. The protein in the formic acid extract after removal of HCOOH and re-dispersing in buffer was injected i.c. into hamsters at a 10^{-2} dilution (1 brain equivalent per 100 ml buffer). The material was not infectious, although P_E itself is infectious (Hilmert and Diringer, 1984).

SAF-protein is a glycoprotein

SAF contains a single major protein species of apparent mol. wt. 26 kd (Figure 1). Extraction of purified SAF by formic acid solubilized the same protein species as did 0.1% SDS (Prusiner *et al.*, 1983; Hilmert and Diringer, 1984), i.e., a main protein of 26–30 kd could be detected on SDS-polyacrylamide gels. A mol. wt. of 24–26 kd for SAF-proteins was also obtained by gel permeation chromatography using formic acid-extracted material and I-125 protein columns (Figure 2). These values are in agreement with estimates obtained by gel permeation chromatography of prion-protein in TSK-2000 SW columns (Prusiner *et al.*, 1984). In addition, we also found material with an apparent mol. wt. of 6–8 kd to be a constituent of the formic acid extracts of SAF preparations. The corresponding peaks emerged at 102 and 93 min for CLAC and AURA SAF-proteins, respectively (Figure 2). These fractions, as well as the front peaks centered at 60–65 min in Figure 2, were analyzed for amino acid content (Table I) and protein (Figure 3).

The amino acid composition of the SAF-protein obtained from CLAC or AURA hamsters, which were analyzed after gel permeation chromatography, did not show significant differences. They differed, however, in their glucosamine content, indicating differences in N-linked carbohydrates (Table I). Under the conditions of HCl hydrolysis, N-acetylglucosamine of N-glycosylated proteins is converted to glucosamine and the latter can be quantitated by amino acid analysis. The 24-kd species of the CLAC preparation (Figure 2a and Figure 3, slot 7) contained only 50% of the glucosamine content of the 25-kd protein species isolated from CLAC or AURA scrapie brains. SAF-proteins from AURA were found to lack a 24-kd species (Figure 2). The

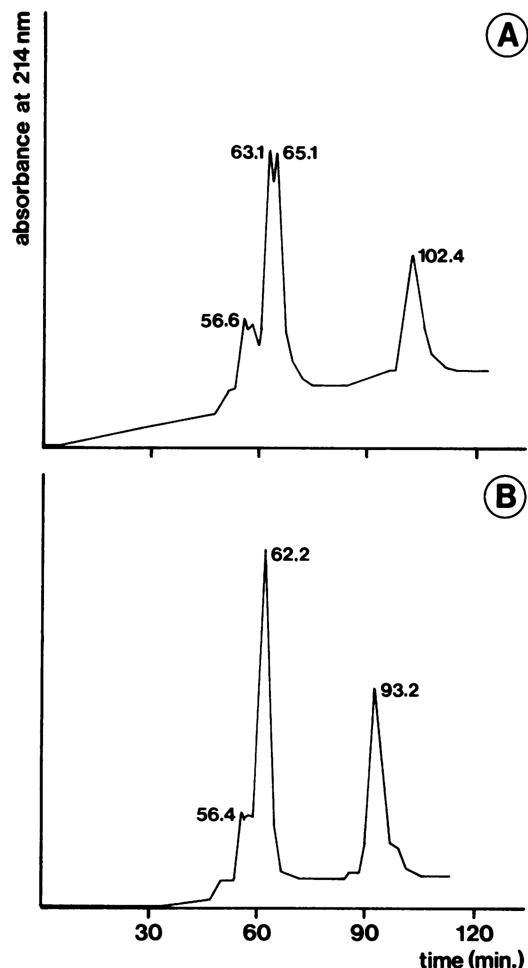

Fig. 2. H.p.l.c. separation of SAF-proteins (vertical axis are OD at 214 nm) on 125-I protein columns. **(A)** SAF-proteins (10 μg) of scrapie-infected CLAC inbred hamster strains. Fractions at 56.6 min contain proteins of mol. wt. ~30 kd; at 63.1 min, of ~26 kd; at 65.1 min of ~24 kd; and at 102.4 min of ~6.5 kd. **(B)** SAF-protein (10 μg) of scrapie-infected AURA outbred hamster strains. Fractions at 56.4 min contain proteins of mol. wt. ~30 kd; at 62.2 min of ~26 kd and at 93.2 min of ~6.5 kd.

6–8 kd species contained almost no carbohydrate (i.e., glucosamine). However, their amino acid compositions were very similar to those of the 26-kd and 24-kd protein fractions (Table I), except in the content of glycine and the basic amino acids. Since the resolutions of the gel permeation column are rather poor for proteins of mol. wt. >6 kd the high content of glycine might be caused by a contamination of free glycine or the unavoidable contamination with peptides derived from collagen, whereas the reduced content of basic amino acids is most likely due to proteolysis. Proteolysis could also account for the absence of carbohydrate in the 6–8 kd SAF-protein fraction, suggesting that both proteolytic removal of basic amino acids and carbohydrate occur by the same cuts at the same site of the polypeptide. Below we will present evidence for a C-terminal location of this putative cutting site.

To find out whether the 26–24 kd protein fractions isolated by gel permeation chromatography are true monomers or oligomers of 8–6 kd species we re-analyzed the peak fraction of SDS-polyacrylamide gel electrophoresis in the presence of 6 M urea (Figure 3). The apparent mol. wt. determined by this technique is in agreement with the values derived from the gel permeation chromatography. However, the peak eluting at 65.1 min in Figure 2a clearly includes protein species co-migrating with

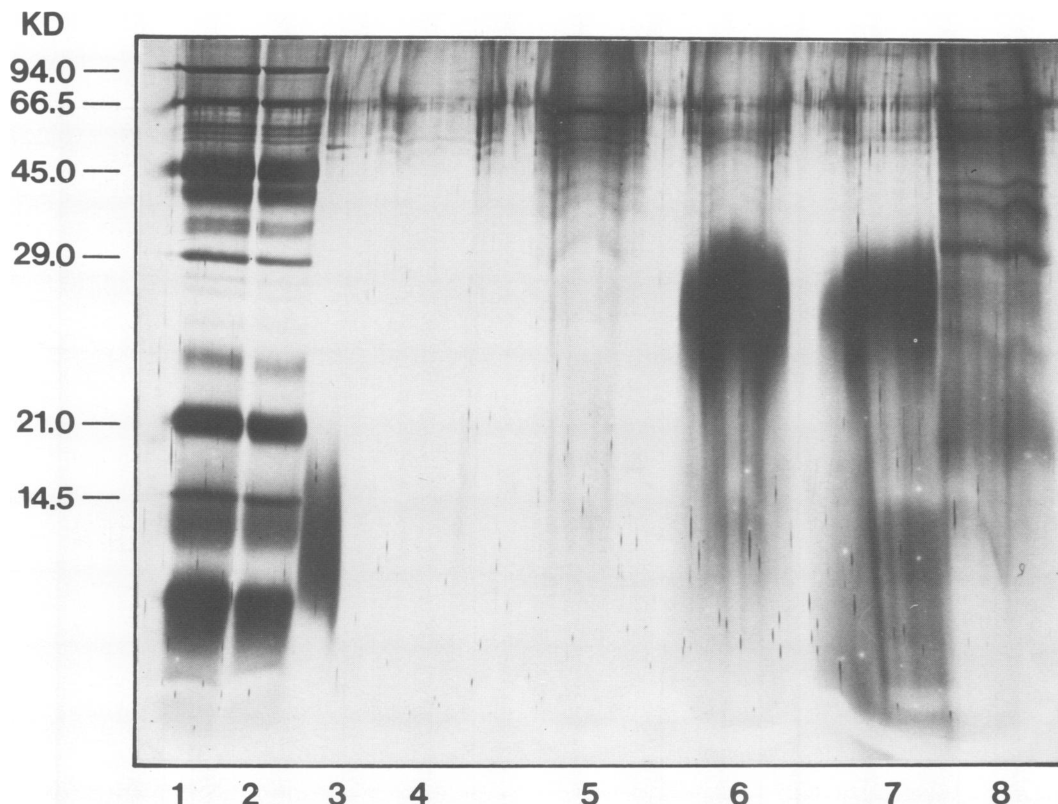

Fig. 3. SDS-PAGE in the presence of 6 M urea of fractions from gel permeation chromatography of CLAC SAF-proteins. **Lanes 1–3** contain marker proteins [100 ng of aprotinine (mol. wt. 6.5 kd) was applied to **lane 3**]. **Lane 4** is sample buffer. **Lane 5** corresponds to material eluting before 56.6 min shown in Figure 2A. **Lane 6** corresponds to the peak centered around 63.1 min, **lane 7** to 65.1 min, **lane 8** to 56.6 min of Figure 2A. The gel was stained with silver nitrate.

Table I. Amino acid composition of SAF-proteins from scrapie brains of CLAC and AURA hamster strains

Amino acid	Nanomolar percent					No. of residues (CLAC)		Nanomolar percent (CLAC)	No. of residues (CLAC)	Nanomolar percent					No. of residues (AURA)	
	Total CLAC	H.p.l.c. of SAF-proteins (CLAC)								Total AURA	H.p.l.c. of SAF-proteins (AURA)					
		Peak 1	Peak 2	Peak 3	Peak 4	Peak 2	Peak 3	CLAC, HF	CLAC, HF		Peak 1	Peak 2	Peak 3	Peak 4	Peak 3	Peak 4
Asp	13.70	12.53	11.89	14.94*	8.97	6.4	8.2*	15.90*	8.0	9.14	8.46	9.20	10.57	7.92	5.3	4.4
Thr	6.28	5.72	7.00	6.47	4.39	3.9	3.6	5.65	3.1	8.14	4.92	5.78	7.39	4.32	3.9	2.4
Ser	6.22	7.03	5.10	5.39	13.36	2.8	3.0	6.28	3.4	5.10	8.32	8.30	4.97	10.20	2.7	5.6
Glu	12.19	9.12	12.33	10.04	18.70	6.8	5.5	10.46	5.7	10.55	10.80	11.19	11.42	11.64	6.1	6.4
Pro	1.92	4.82	4.50	4.65	tr.	2.5	2.5	4.71	2.6	4.28	3.07	0.27	3.89	nd	2.1	nd
Cys	1.85	–	–	–	–	(?)	(?)	–	–	0.44	–	–	–	+	(?)	(?)
Gly	13.79	12.69	11.81	11.12	21.56	6.5	6.2	13.60	7.5	19.34	19.70	18.41	15.65	33.85	8.4	18.6
Ala	4.71	9.18	6.86	8.04	7.06	3.8	4.4	7.11	3.9	6.88	9.05	9.57	7.16	6.96	3.8	3.8
Val	4.02	5.93	5.07	5.53	4.58	2.8	3.0	5.65	3.1	4.72	6.13	5.60	5.09	5.28	2.7	2.9
Met	3.21	2.62	4.23	*	*	2.3	*	*	*	3.54	1.31	1.90	3.66	nd	2.0	nd
Ile	2.50	3.83	3.13	3.49	2.86	1.7	1.9	3.87	2.1	3.45	3.50	3.52	3.41	2.52	1.8	1.2
Leu	4.11	8.18	4.45	6.33	6.11	3.5	3.4	7.10	3.9	6.27	7.44	7.40	5.17	5.28	2.8	2.9
Tyr	4.47	3.09	4.31	3.34	2.10	2.5	1.2	4.18	2.3	3.08	2.04	2.53	4.00	1.20	2.1	0.7
Phe	2.66	3.30	2.95	3.21	3.24	1.6	1.8	3.14	1.7	3.78	3.50	3.07	2.96	3.12	1.6	1.7
His	2.66	1.99	2.87	2.65	1.91	1.6	1.5	2.93	1.6	2.55	1.90	2.17	2.81	1.68	1.5	0.9
Lys	5.08	5.30	5.20	5.96	2.86	2.9	3.3	6.60	3.7	5.74	4.96	5.78	6.11	3.48	3.3	1.9
Arg	4.23	4.67	4.76	5.86	2.29	2.6	3.2	2.72	1.5	3.10	4.82	5.32	5.74	2.52	3.1	1.4
Trp	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
$GlcNH_2$	6.66	+	3.5	2.1	tr.	8	4	tr.	tr.	3.6	+	+	3.69	+	8	+
$GalNH_2$	0	0	0	0	0	0	0			0	0	0	0	0	0	0

*Met included as MetSO; nd, not determined; tr., trace amounts; CLAC, HF, after deglycosylation with HF.

lysozyme (14.5 kd), already to be seen in P_E and the HCOOH-extract (Figure 1), and aprotinine (6.5 kd) whereas the peak at 63.1 min includes very little of the 14.5–6.5 kd protein species. The peak at 65.1 min observed with scrapie-protein fractionations on I-125 protein columns includes low mol. wt. species which behave as dimers and tetramers on the columns (cf. Figures 2a and 3). This behaviour resembles that of the amyloid proteins isolated from the plaques of Alzheimer's disease and aged Down's syndrome brains. These amyloid plaque core proteins have a mol. wt. of ~4 kd and migrate as stable dimers and tetramers on the same I-125 protein column under identical conditions as described for this analysis (Glenner and Wong, 1984; Masters *et al.*, 1985). The similarity of the amino acid compositions of the 26-kd, 24-kd and 8–6 kd SAF-protein species was so striking that we decided

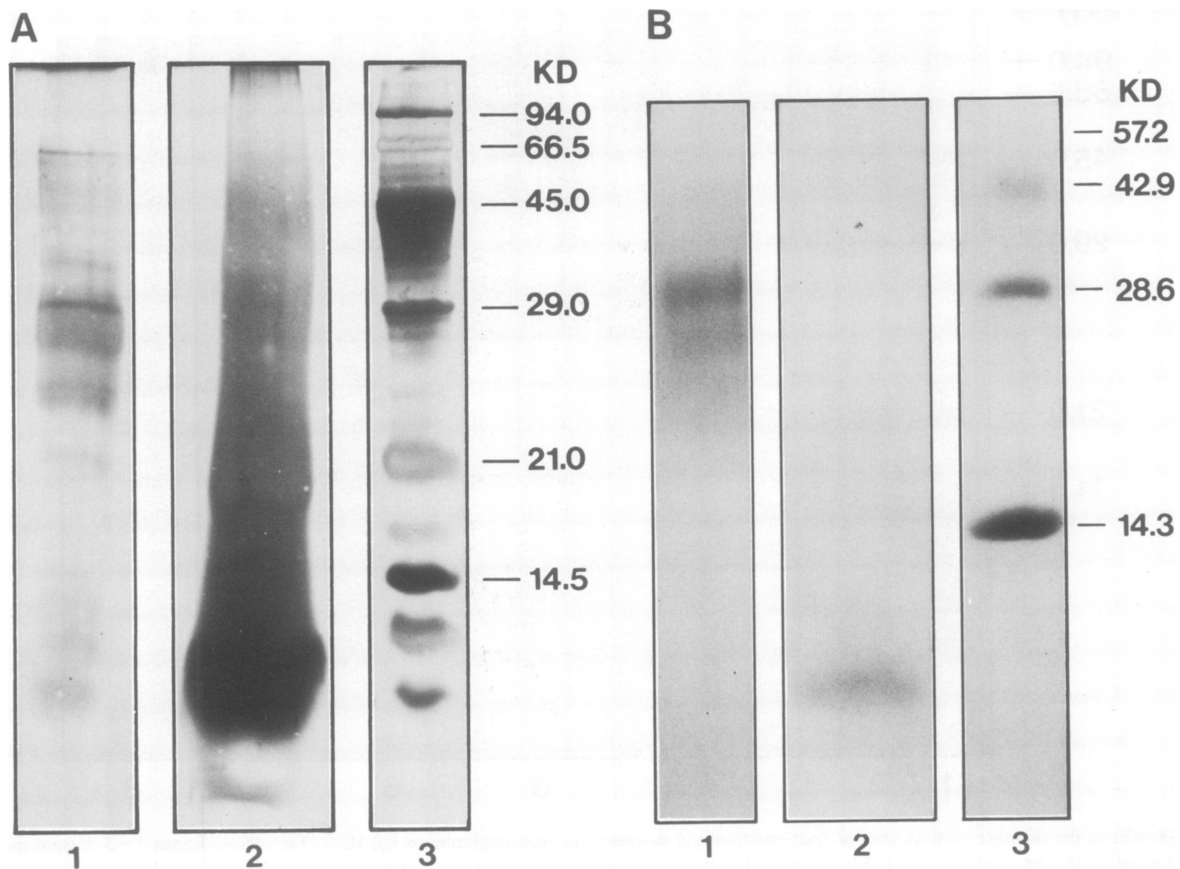

Fig. 4. SDS-PAGE of SAF-proteins before and after deglycosylation treatment. **(A) Lane 1** corresponds to 4 μg of SAF-protein from CLAC, **lane 2** to 1.25 μg of HF-treated SAF-protein and **lane 3** to marker protein. The gel was stained with silver nitrate. **(B)** The same gel as in **A** but with 10 μg SAF-protein from CLAC strains (**lane 1**), 4 μg of HF-treated SAF-protein (**lane 2**) and marker protein (**lane 3**) and after staining with Coomassie brilliant blue R.

to determine the mol. wts. of deglycosylated SAF-protein to find out whether the differences are solely due to the different content of carbohydrate. The carbohydrate content of the different species would be in agreement with two N-linked carbohydrate chains for the 26-kd species and one N-linked carbohydrate chain for the 24-kd fraction, assuming an average content of five N-acetyl glucosamine residues per N-linked carbohydrate of rodents (hamster) (Sauter and Glick, 1979). We could not detect galactosamine in SAF-protein samples suggesting that these proteins do not contain O-linked sugars.

SAF-proteins are glycosylated 7-kd polypeptides

Hydrofluoric acid treatment is known to cleave glycosidic bonds but not N-glycosidic linkages between asparagine and N-acetyl-glucosamine thus leaving single glucosamine residues attached to the protein at these sites (Mort and Lamport, 1977). Peptide bond cleavage does not occur under the conditions employed. SAF-proteins were subjected to such deglycosylation treatment with hydrofluoric acid. HF-treated SAF-proteins from CLAC and AURA hamsters analyzed by SDS-PAGE migrated as single bands at positions corresponding to an apparent mol. wt. of 8–6 kd, i.e., deglycosylated SAF-proteins co-migrated with aprotinine (mol. wt. 6.5 kd) as shown in Figure 4. The heterogeneity of the starting material (lane 1 in Figure 4) disappeared after HF treatment and was converted to a relatively sharp band indicating the presence of a single protein species. The deglycosylated SAF-proteins had the same apparent mol. wt. as the proteins eluting at ~100 min from the I-125 column (Figure 2a and b).

	1				5					10
major sequence:	H-Ser-	Gly-	Pro-	Trp-	Gly-	Gln-	Gly-	Gly-	Gly-	Thr-
pmol residue:	111-	509-	188-	75-	531-	212-	419-	473-	507-	97

minor sequence:	H-Gly-	Gln-	Gly-	Gly-	? -	Thr-	His-	Asn-	Gln
pmol residue:	100-	154-	75-	110-	-	20-	24-	53-	38

Fig. 5. N-terminal amino acid sequence of deglycosylated SAF-protein (7-kd SAF-polypeptide). The amount of SAF 7-kd polypeptide loaded onto the gas-liquid solid phase sequence was 12 μg.

The amino acid compositions of deglycosylated SAF-proteins were almost indistinguishable from that of the 26-kd and 24-kd proteins eluted from the h.p.l.c. gel permeation column (Table I). Therefore, we conclude that the major SAF-protein is a glycoprotein composed of a polypeptide of ~55 residues (Table I) and a carbohydrate moiety of ~10 kd or 5 kd including sialic acid (Prusiner *et al.*, 1984). The high carbohydrate content of SAF-proteins interferes with SDS binding and results in an

apparent mol. wt. on SDS-PAGE which is about twice as high as calculated.

The deglycosylated major SAF-proteins are typical amyloid proteins with respect to size and aggregation properties (Glenner, 1980). Amyloid proteins are reported to be small proteins derived by proteolysis of precursor proteins (Glenner, 1980; Glenner and Wong, 1984; Masters *et al.*, 1985). Often they are polypeptides with β-pleated sheet structure formed by anti-parallel β-strands, containing only 40−50 residues (Glenner, 1980). Rod-like preparations containing SAF-proteins were shown recently to bind Congo red in a fashion typical of amyloid proteins (Prusiner *et al.*, 1983).

Partial sequence of the 7-kd polypeptide

SAF-proteins derived from CLAC or AURA scrapie hamster brains are either N-terminally blocked or are not suited for gas-liquid solid phase protein microsequencing, since in several trials no N-terminal sequence could be obtained using >10 μg of untreated SAF-protein. HF-treated SAF-proteins from CLAC, however, yielded the sequence shown in Figure 5. This sequence matches that of a minor sequence published recently (Prusiner *et al.*, 1984). Since HF-treatment leads to deglycosylation without impairment of peptide bonds and the N-terminal sequence presented in Figure 5 does not include N-glycosylation sites, the two postulated Asn-X-Thr/Ser sites are expected at the more distal sequence region of the 55-residues SAF-polypeptide.

Discussion

Purification of SAF-proteins by a three-step procedure involving a mild proteolysis step and a final formic acid solubilization procedure allows the isolation of sufficient amounts of homogeneous protein for amino acid analyses, sequence analyses and mol. wt. determinations of glycosylated and deglycosylated forms. Careful mol. wt. determinations of SAF-proteins by gel permeation chromatography reveals protein heterogeneity due to glycosylation differences for inbred and outbred hamster strains (CLAC and AURA, respectively). These variations could be interpreted, on the basis of the present chemical analyses of SAF-proteins, as differences in post-translational processing or as products of different alleles. SAF-proteins are accordingly proteins specified, or encoded, by the host since both hamster strains employed for these studies produce similar SAF-polypeptides. Glycosylation of a 7-kd polypeptide results in SAF-proteins of apparent mol. wt. of 26−24-kd. These proteins may form filaments, but with the exception of some mouse models (Dickinson *et al.*, 1983) do not result in the extracellular deposition of amyloid as plaques. Extracellular protein depositions of a variety of different disease processes are thought to result from aggregation of β-pleated sheet structures (Eanes and Glenner, 1968). They are formed from various proteins but only rarely from glycoproteins (Glenner, 1980). The anti-parallel configuration of the twisted β-pleated sheets of amyloid depositions is due to aggregation of paired filaments in a manner reminiscent of silk (Eanes and Glenner, 1968). Alternating side chains extending out on either side of the hydrogen bonded β-pleated sheet plane pack the pleated sheets by homologous interaction (identical side chains are faced and held together) and lead to a very stable sheet package (Cooper, 1974). Glycosylation of asparagine residues located at the turn regions of sheets is expected to interfere with packing of filaments but not with formation of paired filaments. This is indeed the dominant structure seen in electron micrographs of SAF (Merz *et al.*, 1981, 1983, 1984; Diringer *et al.*, 1983a; Prusiner *et al.*, 1983). It will be interesting to see whether the mouse models with scrapie agent-associated amyloidosis do produce carbohydrate-free SAF-proteins.

Deglycosylation of SAF-proteins from scrapie-infected hamster brains results in a single major polypeptide species of 7 kd. It is composed of ~ 55 residues and resembles in size amyloid fibril proteins of acquired systemic amyloidoses, organ-limited amyloidoses and localized depositions (Glenner, 1980; Glenner and Wong, 1984; Masters *et al.*, 1985). Among these diseases are Alzheimer's disease and Down's syndrome. None of these diseases has been shown to be infectious and the amyloid fibril proteins found in them are only host proteins (Masters *et al.*, 1985) which have been partially degraded: we have no reason to assume that SAF-proteins are fundamentally different. As on the one hand, all viruses containing glycoproteins that we are aware of, are sensitive to organic solvents and strong detergent treatment whereas the scrapie virus is not, and, on the other hand, SAF are composed of a highly glycosylated low-mol. wt. protein, we disagree with the proposal put forward recently (Bolton *et al.*, 1984) that SAF is a constituent of the scrapie virus.

SAF-protein extracted with HCOOH is not infectious. The P_E fraction containing SAF and some uncharacterized material, however, is infectious prior to formic acid treatment, suggesting that the agent co-purifies with the fibrils formed by the highly glycosylated 7-kd polypeptide. Since we know partial sequences of amyloid plaque core proteins and of cerebrovascular amyloid in Alzheimer's disease and Down's syndrome (Masters *et al.*, 1985; Glenner and Wong, 1984) their differences from the 7-kd SAF-polypeptide described here are obvious. Neither the amino acid compositions nor the partial sequences show great similarities. They are simply the promoters of proteinaceous twisted β-pleated sheet fibrils and chemically not identical. However, it should be stressed again that all these proteins, i.e., amyloid core proteins in Alzheimer's disease and aged Down's syndrome and the SAF-polypeptide from scrapie hamster brains, are small polypeptides of 4 kd and 7 kd, respectively. This size is shared with 'amyloid' fibrils created *in vitro* from Bence-Jones proteins (Glenner *et al.*, 1971) and other amyloid-fibril proteins (Glenner, 1980; Glenner and Wong, 1984; Masters *et al.*, 1985). The inability of these small proteins to form β-barrel structures, i.e., closed-end β-pleated sheets requiring six β-strands and at least 100 residues, may lead to an uncontrolled polymerization resulting in the various forms of amyloids.

Since all known amyloid-like proteins are host-coded, we expect that SAF will also prove to be host-coded. Further, since the amyloids from numerous diseases are not infectious, the concept of an 'infectious amyloid' should not be accepted in the case of SAF-protein, until it is supported by much more rigorous data than any so far produced. Similarly, there would have to be very clearcut evidence that the SAF-polypeptide is a necessary part of the scrapie agent before rejecting simpler alternatives. The simplest is that scrapie infectivity co-purifies with SAF in this type of protocol, possibly due to the unusually high carbohydrate content of SAF-protein.

Materials and methods

Infection of animals with the 263-K strain of scrapie agent (Kimberlin and Walker, 1983), to obtain scrapie brain material and chemical analyses of protein and nucleic acids, has been described (Diringer *et al.*, 1983b).

Purification of SAF-protein

The procedure is a modification of a method described recently (Hilmert and Diringer, 1984), and is outlined in Scheme 1. It can be divided into three parts: (i) differential centrifugation to obtain the SAF-enriched pellet P_{215S}; (ii) extraction of contaminants, resulting in SAF of higher purity in P_E; and (iii) extraction of SAF-protein.

(i) Differential centrifugation. A 1:10 homogenate (100 ml) prepared from 10 brains of clinically diseased hamsters was centrifuged for 10 min at 500 *g*. [This low-speed centrifugation instead of the 3000 *g* treatment used recently (Hilmert and Diringer, 1984; Diringer *et al.*, 1983b) was suggested by Dr L.Manuelidis, Yale University, New Haven, USA.] The supernatant was filtered through glass wool. The pellet was resuspended in 50 ml of buffered saline and centrifuged as before. The combined supernatants represent supernatant $S_{0.5}$. This supernatant was centrifuged for 40 min at 22 000 *g* and the pellet P_{22} was dissolved in 50 ml of buffered saline containing 1% sarcosyl using careful ultra-sonication with a sonic cell disrupter. After another run at 22 000 *g* for 20 min the supernatant was spun for 120 min at 215 000 *g* to obtain pellet P_{215S}. this was again resuspended in 50 ml of buffered saline, this time containing 1% sarcosyl and 10% NaCl. A final run at 215 000 *g* for 150 min resulted in pellet P_{215S} which subsequently was used for extraction. For centrifugation at 22 000 *g* and 215 000 *g* the Beckman R 60 Ti was used at 18 000 and 55 000 r.p.m., respectively.

(ii) Extraction of contaminants. P_{215S} was dissolved in 1 ml of buffered saline containing 1% sarcosyl and 10% NaCl, stirred overnight at 37°C, transferred to an Eppendorf vial, and spun for 15 min. Pellet P_1 was extracted for another 2 h at 37°C with the same amount of buffer containing 5 µg/ml proteinase K. Pellet P_2 was extracted twice for 1 h at 37°C with 1 ml of 0.1% sarcosyl in distilled water to remove a considerable portion of RNA. Pellet P_E was kept at 4°C until further use.

(iii) Extraction of SAF-protein. [This procedure was suggested by Dr J.Heukeshoven, Heinrich Pette-Institut, Hamburg, derived from his and Dr R.Dernick's experience with poliovirus proteins (Heukeshoven and Dernick, 1983).]

P_E obtained from 10 hamster brains was extracted for 10–15 min with 0.5 ml of anhydrous formic acid in a Branson sonication bath. After centrifugation in the Eppendorf centrifuge at 10 000 *g* for 10 min the pellet was discarded. In the supernatant the optical density at 280 nm was measured. Subsequently the formic acid was removed in a desiccator over NaOH pellets. The dried residue represents SAF-protein with minor impurities.

Amino acid analysis

SAF-protein (1–3 µg) was dissolved in 100 µl of 70% formic acid by adding 30 µl of distilled water and then 70% formic acid, transferred to hydrolysis tubes (100 x 10 mm) and dried. All tubes were rinsed carefully with analysis-grade methanol (Merck AG, Darmstadt, FRG) and dried at 150°C prior to use in order to minimize proteinaceous contaminations. Hydrolysis was carried out in the presence of 200 µl of 6 M HCl, 0.1% phenol (w/v) for 24 h at 110°C after sealing the tubes under aspirator vacuum. Amino acid analyses were performed on a Beckman 121 M amino acid analyzer run in ninhydrin mode at a sensitivity of 1 nmol full scale.

Determination of carbohydrate

N-linked carbohydrate was determined indirectly by amino acid analysis measuring the glucosamine content derived from N-acetylglucosamine after hydrolysis with 6 M HCl, 0.1% phenol (110°C, 24 h). The values obtained are corrected for destruction. The correction factor of 1.56 corresponds to 64% recovery of glucosamine from free N-acetylglucosamine after HCl hydrolysis. The galactosamine content was determined by a subtractive method involving amino acid anlaysis of oxidized and non-oxidized samples (W.Dahr and K.Beyreuther, in preparation). Glucosamine elutes between Tyr and Phe and galactosamine co-elutes with Phe on the Beckman 121 M amino acid analyzer using the single column program for protein hydrolysates provided by the manufacturer.

Removal of the carbohydrate moiety

SAF-protein was glycosylated with anhydrous hydrogen fluoride (Mort and Lamport, 1977). SAF-protein (20 µg) was dissolved in 100 µl of 70% formic acid, transferred to the 4 ml reaction chamber of a HF-apparatus and thoroughly dried. The apparatus is a closed, all Kel-F system (Peninsula Laboratories, San Carlos, CA) designed for deprotection of peptides and cleavage of peptides from polystyrene resins. The unit contains a HF cylinder, a reservoir for HF, two reaction chambers (miniaturized, non-commercial version with total volume of 4 ml, used throughout), a second reservoir for HF, and a trap filled with calcium hydroxide for neutralization of HF after removal from the reaction vessel. To the dried sample 1 ml of anisole was added as scavenger to prevent transfer of newly formed glycosylfluoride to the side chains of aromatic amino acids. The entire HF-apparatus with the exception of the HF reservoir was evacuated and the reaction vessel was filled with 1 ml of HF by cooling in a dry ice acetone bath. The reaction was carried out at 0°C for 60 min. At the end of the reaction the HF was removed *via* the calcium oxide trap by slow evacuation of the lines until all visible HF (60 min) had disappeared. The residue was re-dissolved in 70% formic acid, transferred to an Eppendorf tube (rinsed as described for hydrolysis tubes), lyophilized and precipitated with chloroform-methanol (Wessel and Flügge, 1984). The HF-treated SAF-proteins were then subjected to automated Edman degradation SDS-PAGE and HCl hydrolysis and subsequent amino acid analysis.

SDS-PAGE

Proteins obtained after formic acid extraction of SAF (SAF-protein) and HF-treated SAF-proteins were dissolved in 70% formic acid, lyophilized in a Speed Vac (Savant Instruments, Hicksville, NY), dissolved in PAGE sample buffer (Laemmli, 1970) and heated for 10 min at 110°C before loading the gels. PAGE was carried out in 15% (w/v) polyacrylamide slab gels, 1 mm thick.

If SDS-PAGE was performed in the presence of 6 M urea, samples were re-dissolved in sample buffer containing 6 M urea and heated for 30 min at 37°C before loading. The slab gels were prepared from solutions containing 6 M urea.

After electrophoresis (at 28 mA constant current), the protein bands were stained with either Coomassie brilliant blue R or with silver nitrate as described by Bürk *et al.* (1983).

HPLC

Proteins (2–25 µg) dissolved in formic acid extraction of SAF were lyophilized and re-dissolved in 50 µl of h.p.l.c. buffer (0.1% SDS, 150 mM ammonium bicarbonate, pH 8.0). The sample was loaded onto two analytical I-125 protein columns (Waters, Eschborn, FRG) (30 cm x 7.8 mm) connected in tandem. The columns were attached to a guard column (3 cm x 2 mm) filled with I-125 T-bulk-packing phase (Waters). The columns were equilibrated and developed with 0.1% SDS, 150 mM ammonium bicarbonate, pH 8.0 at a flow-rate of 0.2 ml/min. The effluent was monitored at 214 nm. Protein peaks were pooled, lyophilized and precipitated with the chloroform-methanol technique (Wessel and Flügge, 1984). The h.p.lc. systems employed were a DuPont 850 or a Shimadzu LC-5A liquid chromatograph with a SPD-2 AM photometer and a CR-2 integrator operated at AT 4 (8 mV full scale for recorder settings at 10 mV).

Protein sequence analysis

SAF-protein (15 µg by amino acid analysis) or HF-treated SAF-protein (12 µg) was dissolved in 30 µg of 70% formic acid and dried on the glass fiber discs of a gas-liquid solid phase protein sequencer (Model 470A, Applied Biosystems, Foster City, CA). Prior to sample loading the discs were pre-loaded with 1.5 mg of Polybrene (Aldrich-Chemie, Steinheim, FRG) and 1.5 nmol of the dipeptide alanine-isoleucine and subjected to five cycles of Edman degradation. The 2-anilino-5-thiazoline amino acids were converted to phenylthiohydantoin derivatives (PTH) using 25% (v/v) trifluoroacetic acid at 52°C for 25 min and/or 2 M HCl in methanol at 40°C for 7 min. The PTH-samples were identified by reverse-phase h.p.l.c. on Zorbax CN columns (25 cm x 4.6 mm). Complete separation of all PTH-amino acid derivatives was achieved with the isocratic buffer system composed of 23.6% tetrahydrofuran, 6.6% acetonitrile, 2.8% methanol in 8–20 mM Na acetate, pH 5.2 (Beyreuther *et al.*, 1983). The flow-rate was 1.25 ml/ min at 30°C and the PTH-derivatives were detected at 269 nm. In order to achieve optimal separations of PTH-His from PTH-Gly and PTH-Arg from PTH-Trp, adjustment of the Na acetate concentration was essential (Beyreuther *et al.*, 1983).

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