

## Articles

Molecular Characteristics of the Major Scrapie Prion Protein<sup>†</sup>

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**ABSTRACT:** A major protein was identified that purifies with the scrapie agent extracted from infected hamster brains. The protein, designated PrP 27-30, was differentiated from other proteins in purified fractions containing the scrapie agent by its microheterogeneity ( $M_r$  27 000-30 000) and its unusual resistance to protease digestion. PrP 27-30 was found in all fractions enriched for scrapie prions by discontinuous sucrose gradient sedimentation or sodium dodecyl sarcosinate-agarose gel electrophoresis. It is unlikely that PrP 27-30 is a pathologic product because it was found in fractions isolated from the brains of hamsters sacrificed prior to the appearance of histopathology. If PrP 27-30 is present in normal brain, its concentration must be 100-fold lower than that found in equivalent fractions from scrapie-infected hamsters. Three protease-resistant proteins similar to PrP 27-30 were found

in fractions obtained by discontinuous sucrose gradient sedimentation of scrapie-infected mouse brain. These proteins were not evident in corresponding fractions prepared from normal mouse brain. One-dimensional peptide maps comparing PrP 27-30 and normal hamster brain proteins of similar molecular weight demonstrated that PrP 27-30 has a primary structure which is distinct from these normal proteins. Heating substantially purified scrapie fractions to 100 °C in sodium dodecyl sulfate inactivated the prion and rendered PrP 27-30 susceptible to protease digestion. Though the scrapie agent appears to be hydrophobic, PrP 27-30 remained in the aqueous phase after extraction with organic solvents, indicating that it is probably not a proteolipid. PrP 27-30 is the first structural component of the scrapie prion to be identified.

The structure and biochemical composition of the scrapie agent have not been established despite intensive research efforts for more than 2 decades. The scrapie agent causes a slow, degenerative neurological disease of sheep and goats in which the clinical and pathologic features closely resemble those of two human diseases, kuru and Creutzfeldt-Jakob disease (CJD) (Gajdusek, 1977; Hadlow et al., 1980). The etiologic agents of these three diseases exhibit many unusual properties which distinguish them from other infectious pathogens (Alper et al., 1966, 1967; Hunter, 1972; Gajdusek, 1977; Diener et al., 1982; Prusiner, 1982). The unusual properties and inferred presence of a protein within the scrapie agent led to introduction of the term "prion" to describe and identify this group of infectious pathogens (Prusiner, 1982). Though most of the data characterizing the biochemical properties of these pathogens have been obtained from studies of the scrapie agent, evidence to date indicates that it is prototypic of the entire group (Gajdusek, 1977; Hadlow et al., 1980; Prusiner, 1982).

Although early studies suggested that a protein component might be required for expression of scrapie infectivity (Hunter et al., 1969; Millson et al., 1976; Cho, 1980), these results were inconclusive. Convincing evidence that the scrapie agent contains a protein was obtained following development of improved purification methods. In particular, inactivation of the scrapie agent after protease digestion and reversible

chemical modification with diethyl pyrocarbonate (DEP)<sup>1</sup> was demonstrated only with substantially purified preparations (Prusiner et al., 1981; McKinley et al., 1981). Although no specific protein component was identified, the results presented in those studies and later confirmed by others (Lax et al., 1983) demonstrated the requirement for a functional protein within the scrapie agent.

Improvements in the methodology for purifying the scrapie agent led to the identification of a unique protein (Bolton et al., 1982; Prusiner et al., 1982a). The protein exhibited size microheterogeneity upon electrophoresis in SDS-polyacrylamide gels, showing an apparent relative molecular weight ( $M_r$ ) of 27 000-30 000, and was unusually resistant to digestion by proteinase K under nondenaturing conditions (Bolton et al., 1982). The close association of this protein with the scrapie prion suggested, but did not establish, that it was a structural component.

Subsequent data indicated that the protein, designated PrP 27-30, is a component of the prion (McKinley et al., 1983). In extensively purified fractions, the concentration of PrP 27-30 correlated with the titer of the scrapie agent over a 10 000-fold range. In addition, prolonged digestion of these fractions with proteinase K resulted in concomitant decreases in the concentration of PrP 27-30 and scrapie agent titer. Similar digestions using trypsin or SV-8 protease degraded most other proteins but did not alter the concentration of PrP 27-30 or the prion titer. No other protein exhibited these characteristics.

In this paper, we present some biological and biochemical characteristics of PrP 27-30. The data presented here in

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<sup>1</sup> Abbreviations: DEP, diethyl pyrocarbonate; DOC, sodium deoxycholate; SDS, sodium dodecyl sulfate; PEG, poly(ethylene glycol); Sarkosyl, sodium dodecyl sarcosinate; SV-8, *Staphylococcus aureus* V-8; Cl<sub>3</sub>CCOOH, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)amino-methane hydrochloride.

conjunction with other evidence (McKinley et al., 1983) support the hypothesis that PrP 27-30 is a component of the scrapie prion.

### Experimental Procedures

**Materials.** All chemicals were of the highest grades commercially available. Sarkosyl, sodium cholate, DOC, Triton X-100, PEG, and micrococcal nuclease were purchased from Sigma. Enzyme-grade ammonium sulfate and sucrose were purchased from Schwarz/Mann. Proteinase K was purchased from Merck. SV-8 protease and  $\alpha$ -chymotrypsin were obtained from Miles Laboratories. SDS was obtained from BDH Chemicals. Acrylamide and *N,N'*-methylenebis(acrylamide) were purchased from Bio-Rad Laboratories.  $\text{Na}^{125}\text{I}$  was obtained from Amersham. *N*-Succinimidyl 3-(4-hydroxyphenyl)propionate was purchased from Calbiochem.

**Source of Scrapie Prions and Bioassay.** A hamster-adapted isolate of the scrapie agent was passaged and prepared as described (Prusiner et al., 1980a, 1982a). The Chandler isolate of the mouse-adapted scrapie agent was passaged as described (Chandler, 1961; Prusiner et al., 1977, 1978a,b) and, following an incubation period of 126-131 days, was isolated from infected brain homogenates by sedimentation in discontinuous sucrose gradients (Prusiner et al., 1982a). Scrapie agent titers were determined by measuring the incubation time intervals from inoculation to the onset of neurological illness and to death (Prusiner et al., 1980a, 1982b).

**Purification of Scrapie Prions.** (A) *Sedimentation through Discontinuous Sucrose Gradients.* The procedure was described in detail by Prusiner et al. (1982a). Briefly, homogenates of infected brain (10% w/v) were prepared in 320 mM sucrose and clarified by two low-speed centrifugations. Triton X-100 and DOC were added, and the scrapie agent was precipitated from the suspension upon addition of PEG. The resuspended pellet was sequentially digested with micrococcal nuclease (12.5 units/mL for 16 h) and proteinase K (100  $\mu\text{g}/\text{mL}$  for 8 h). Sodium cholate was added, and the prions were removed from the suspension by precipitation with ammonium sulfate. Triton X-100 and SDS were added to the resuspended pellet, and the scrapie agent was sedimented through a 25% (w/v) sucrose solution onto a 60% sucrose cushion. Fraction 2 at the 25%/60% sucrose interface of this gradient contained scrapie prions purified approximately 1000-fold with respect to protein in the homogenate (Prusiner et al., 1982a).

(B) *Electrophoresis in Sarkosyl-Agarose Gels.* The procedure was described in detail by Prusiner et al. (1981). Briefly, homogenates of infected brain (10% w/v) were prepared as described above and clarified by two low-speed centrifugations. Prions were removed from the suspension by sedimentation to a pellet in a zonal rotor (32 000 rpm for 16 h). The resuspended pellet was made 0.5% with DOC, and the scrapie agent was again sedimented to a pellet (50 000 rpm for 2 h). The resuspended pellet was sequentially digested with micrococcal nuclease and proteinase K as described above. Sodium cholate was added and the scrapie agent precipitated from the digested suspension by addition of ammonium sulfate. Following dialysis to remove residual ammonium sulfate, the scrapie agent was electrophoresed into an 0.8% agarose gel having a 2-cm path length. The scrapie agent was removed from this gel by electroelution or by pulverizing the gel with a polytron device. The prions in these fractions ( $E_6$ ) were purified approximately 100-fold with respect to protein in the homogenate (Prusiner et al., 1981).

**Purification of Normal Brain Protein Fractions.** Fractions corresponding to those containing the substantially purified

scrapie agent were prepared by the two procedures described above. The animals used were uninoculated or inoculated with normal hamster brain homogenates analogous to the scrapie-containing inocula. The control animals were held for comparable periods of time as specified in Table II.

**Radioiodination of Proteins.** Protein fractions were concentrated by precipitation with SDS and quinine hemisulfate (Durbin & Manning, 1984) and the pellets washed with 80% acetone. For Bolton-Hunter labeling, the protein pellets were resuspended in 0.1 M sodium borate and 0.1% SDS (pH 8.5) and radioiodinated with *N*-succinimidyl 3-(4-hydroxy-5- $^{125}\text{I}$ )iodophenyl)propionate as described (Bolton & Hunter, 1973). The radioiodination reaction was terminated by addition of 0.5 mL of 0.2 M glycine in 0.1 M sodium borate (pH 7.5). The iodinated proteins were removed from residual glycine and radioiodinated glycine by reprecipitation with SDS and quinine hemisulfate.

Labeling of the precipitated proteins resuspended in 50 mM sodium phosphate and 0.1% SDS (pH 7.5) with  $\text{Na}^{125}\text{I}$  was performed by using chloramine-T according to a modification of the procedure described by Hunter & Greenwood (1962). Radioiodination of the concentrated proteins resuspended in 30 mM sodium phosphate was performed by using Iodobeads as described (Markwell, 1982). The efficiency of labeling could be enhanced by addition of 2% SDS and 8.0 M urea to the buffer, though proteins were undoubtedly denatured under those conditions. In some cases, the labeled proteins were reprecipitated by using SDS-quinine hemisulfate prior to separation by electrophoresis.

**Polyacrylamide Gel Electrophoresis.** Radiolabeled proteins in electrophoresis sample buffer (62.5 mM Tris-HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.002% bromphenol blue, pH 6.8) were heated to 100 °C for 2 min. The proteins were separated by electrophoresis through vertical slab polyacrylamide gels using the discontinuous pH buffer system described by Laemmli (1970). The gels were stained and destained, and autoradiographic exposures were made as described previously (Bolton et al., 1982; Prusiner et al., 1982a).

**One-Dimensional Peptide Mapping.** Radioiodinated proteins were pretreated with proteinase K (100  $\mu\text{g}/\text{mL}$ ) in 100 mM Tris-HCl (pH 7.4) or with Tris buffer alone for 30 min. The digestions were terminated upon addition of an equal volume of 2 $\times$  concentrated electrophoresis sample buffer and boiling at 100 °C for 2 min. The denatured proteins were separated by SDS-polyacrylamide gel electrophoresis as described above. The region containing PrP 27-30 (migration range of approximately 27 000-30 000 daltons), or the corresponding region of gels containing normal brain proteins, was identified by using an autoradiograph of the gel and cut from the gel with a razor blade. The proteins were eluted from these gel fragments into sterile 62.5 mM Tris-HCl buffer (pH 6.8) containing 0.2% SDS at room temperature over 24-48 h. The eluted proteins were concentrated by precipitation with quinine hemisulfate and the pellets washed with 80% acetone. The pellets were resuspended in digestion buffer containing 0.125 M Tris-HCl, 0.5% SDS, 10% glycerol, and 0.001% bromphenol blue (pH 6.8) and heated to 100 °C for 2 min. Aliquots of the denatured proteins were treated with digestion buffer only or with SV-8 protease or  $\alpha$ -chymotrypsin at final concentrations of 133  $\mu\text{g}/\text{mL}$  (Cleveland et al., 1977). The digestions were conducted at 37 °C for the time periods indicated in the legends to Figures 6 and 7. The peptide fragments were separated on 20% polyacrylamide gels, and autoradiography was performed as described (Bolton et al., 1982; Prusiner et al., 1982a).

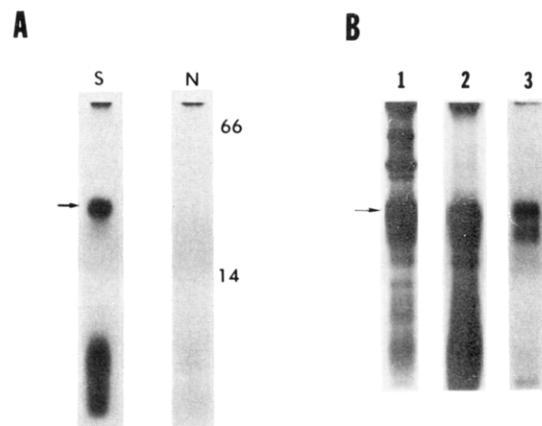
**Concentrating PrP 27–30.** Six methods for concentrating PrP 27–30 from 200- $\mu$ L aliquots of scrapie sucrose gradient fractions were studied. These were the following: (1) The suspension was made 0.2% with SDS, and the protein–SDS complexes were precipitated upon addition of 40  $\mu$ L of 0.1 M quinine hemisulfate and 0.1 N HCl. The suspension was diluted with 1.0 mL of double-distilled water and cooled to 0 °C for 20 min prior to sedimentation at room temperature for 20 min in a microcentrifuge. The pellets were vigorously washed with 80% acetone and repelleted as before (Durbin & Manning, 1984). (2) The suspension was made 0.015% with DOC and the protein precipitated upon addition of 40  $\mu$ L of 50%  $\text{Cl}_3\text{CCOOH}$ . The suspension was diluted with 1.0 mL of 10%  $\text{Cl}_3\text{CCOOH}$  and cooled to 0 °C for 20 min prior to sedimentation as described above. The pellets were vigorously washed with 80% acetone and repelleted as before. (3) The suspension was made 80% by volume with acetone and cooled to 0 °C for 20 min. The precipitated proteins were sedimented as described above. (4) The suspension was made 80% by volume with ethanol and treated as described above for acetone. (5) The suspension was made 80% by volume with methanol and treated as described above for acetone. (6) The suspension was diluted with 4 volumes of double-distilled water and treated as described above for acetone. The precipitated proteins were radioiodinated with the Bolton–Hunter reagent and analyzed by SDS–polyacrylamide gel electrophoresis.

**Organic Solvent Extractions.** Three different organic solvent combinations were used in an attempt to extract PrP 27–30 from the aqueous phase. To each 200- $\mu$ L aliquot of scrapie sucrose gradient fraction 2 was added 800  $\mu$ L of chloroform–methanol (2:1), chloroform, or toluene–ethyl acetate (1:1). The two-phase systems were vigorously mixed and cooled to 0 °C for 20 min prior to separation of the phases by centrifugation in a microcentrifuge. The control sample consisted of a 200- $\mu$ L aliquot of fraction 2 which was diluted with 800  $\mu$ L of double-distilled water and treated in an identical manner. The phases were collected separately, and the pellets were resuspended in 20  $\mu$ L of 0.1 M sodium borate (pH 8.5) containing 0.1% SDS. Proteins in the aqueous phase were concentrated by precipitation with SDS and quinine hemisulfate as described above. Proteins in the organic phase were concentrated by evaporation to dryness with nitrogen. The concentrated proteins were radioiodinated and analyzed by SDS–polyacrylamide gel electrophoresis as described above.

## Results

**Detection of PrP 27–30.** Two unusual properties of PrP 27–30 served to distinguish it from other proteins obtained from scrapie-infected or normal hamster brain (Bolton et al., 1982; Prusiner et al., 1982a). First, PrP 27–30 exhibited size microheterogeneity which was readily evident upon separation by SDS–polyacrylamide gel electrophoresis. Second, PrP 27–30 showed resistance to digestion by a variety of proteases under nondenaturing conditions. In this report, we used these two properties as the criteria for establishing the presence and absence of PrP 27–30 in fractions prepared from scrapie and normal hamster brains, respectively.

Figure 1 illustrates the application of these criteria for detecting PrP 27–30. Protease digestion for 30 min under nondenaturing conditions hydrolyzed all proteins in normal brain fractions. Under these conditions, cellular proteins in scrapie fractions also were hydrolyzed, but PrP 27–30 was left intact (Figure 1B). Thus, the protease resistance of PrP 27–30 did not result from the presence of a protease inhibitor in the scrapie fraction. The protein band which migrated slightly ahead of PrP 27–30 in these gels is probably a cleavage product



**FIGURE 1:** Identification of the scrapie prion protein (PrP 27–30). (A) Aliquots of sucrose gradient fraction 2 from scrapie-infected (S) or normal (N) hamster brain were concentrated 10-fold and radio-labeled with [ $^{14}\text{C}$ ]DEP (Bolton et al., 1982). The radiolabeled proteins were resuspended in 10 mM Tris–HCl and 0.2% Sarkosyl (pH 7.4) and digested with proteinase K (100  $\mu\text{g}/\text{mL}$ ) for 30 min prior to electrophoresis in SDS–polyacrylamide gels. The autoradiograph was exposed for 720 h. The arrow indicates the position of PrP 27–30. (B) Aliquots of scrapie sucrose gradient fraction 2 were concentrated and radioiodinated with *N*-succinimidyl 3-(4-hydroxy-5- $^{125}\text{I}$ iodophenyl)propionate. The radioiodinated fractions were incubated at 37 °C for 30 min in 10 mM Tris–HCl and 0.2% Sarkosyl (pH 7.4) alone (lane 1) or containing 500  $\mu\text{g}/\text{mL}$  SV-8 protease (lane 2) or 100  $\mu\text{g}/\text{mL}$  proteinase K (lane 3) prior to electrophoresis in SDS–polyacrylamide gels. The autoradiograph was exposed for 24 h. The arrow indicates the position of PrP 27–30.

**Table I:** Scrapie Prions Were Unaltered by Iodination or Protease Digestion

(A) Chemical Modification by $^{125}\text{I}$ Incorporation		
procedure	log titer ( $\text{ID}_{50}$ units/mL $\pm$ SE)	
	before labeling	after labeling
Bolton–Hunter	8.3 $\pm$ 0.2	8.2 $\pm$ 0.2
chloramine-T	10.2 $\pm$ 0.1	10.1 $\pm$ 0.1
(B) Protease Digestion of $^{125}\text{I}$ -Labeled Bolton–Hunter-Labeled Fractions		
protease	log titer ( $\text{ID}_{50}$ units/mL $\pm$ SE)	
none	8.1 $\pm$ 0.2	
SV-8	8.4 $\pm$ 0.2	
proteinase K	8.7 $\pm$ 0.2	

of PrP 27–30. Possibly, this protein was generated during purification since prolonged digestion with proteinase K was utilized. The lower molecular weight protein has been observed frequently in purified fractions prior to additional protease treatment and has not been seen uniformly following secondary proteinase K digestion of the purified fractions.

Several methods for detecting PrP 27–30 have been employed. The most sensitive methods involved chemically labeling the protein with  $^{125}\text{I}$ . In general, the chloramine-T method was slightly more efficient than the Iodobead procedure, and both gave significantly greater incorporation of radioiodine than the Bolton–Hunter method. No change in the titer of samples iodinated by two of these methods was observed (Table IA). In addition, PrP 27–30 has been detected in polyacrylamide gels by two different silver staining techniques (Merril et al., 1981; Morrissey, 1981). Detection of PrP 27–30 by staining with Coomassie blue has rarely been successful due to the low concentrations of PrP 27–30 available for electrophoretic analysis.

**Denaturation of PrP 27–30 and Reduction of Prion Titer.** Digestion of substantially purified fractions with proteinase K under the conditions described above did not alter the prion

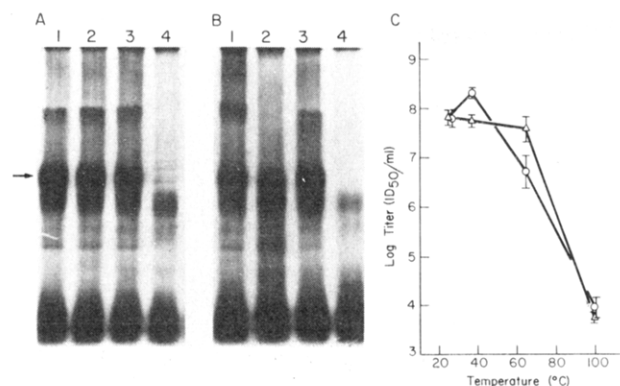


FIGURE 2: PrP 27-30 is denatured under conditions which reduce infectivity. Sucrose gradient purified scrapie suspensions were radioiodinated by using the Iodobead procedure and concentrated by sedimentation to a pellet in a microcentrifuge. The pellets were resuspended in electrophoresis sample buffer containing (A) 0.25% SDS and 1.25% 2-mercaptoethanol or (B) 1.25% SDS and 1.25% 2-mercaptoethanol. Ten-microliter aliquots of each suspension were heated to 25, 37, 65, and 100 °C for 2 min (lanes 1, 2, 3, and 4, respectively) and cooled to room temperature prior to the addition of 1  $\mu$ L of proteinase K (1 mg/mL). The digestion was terminated after 30 min by adding 1  $\mu$ L of phenylmethanesulfonyl fluoride (1 mM) followed by 12  $\mu$ L of 2 $\times$  concentrated electrophoresis sample buffer. Immediately, the samples were boiled for 5 min and the proteins separated on a 15% acrylamide gel. The autoradiograph was exposed for 17 h. The arrow indicates the position of PrP 27-30. (C) Sucrose gradient purified scrapie suspension was concentrated and the pellet resuspended in electrophoresis sample buffer containing 0.25% SDS and 1.25% 2-mercaptoethanol ( $\Delta$ ) or 1.25% SDS and 1.25% 2-mercaptoethanol (O). Aliquots were heated for 2 min as indicated, then diluted 100-fold in phosphate-buffered saline containing 5% bovine serum albumin, frozen in dry ice, and stored at -70 °C prior to bioassay. The vertical bars indicate standard errors.

titer. Identical samples iodinated with *N*-succinimidyl 3-(4-hydroxy-5-[<sup>125</sup>I]iodophenyl)propionate and digested with SV-8 protease or proteinase K showed no significant change in titer (Table IB).

PrP 27-30 remained resistant to digestion with proteinase K after being heated to 65 °C in 0.25% SDS and 1.25% 2-mercaptoethanol (Figure 2A) or in 1.25% SDS and 1.25% 2-mercaptoethanol (Figure 2B). However, heating fractions to 100 °C in SDS and 2-mercaptoethanol denatured PrP 27-30 and rendered it protease sensitive. Infectivity also was stable after heating to 65 °C but was reduced by a factor of  $\sim 10^4$  after incubation at 100 °C (Figure 2C). Thus, PrP 27-30 was denatured under conditions which inactivated the prion.

**PrP 27-30 in Sucrose Gradient Scrapie Fractions.** We have detected PrP 27-30 in 28 preparations of the scrapie agent purified by sucrose gradient sedimentation (Table II). PrP 27-30 was uniformly found in fractions prepared from hamsters inoculated 47-83 days prior to sacrifice. In general, we have found that the concentration of PrP 27-30 in purified fractions from these preparations was useful in predicting the titer.

We have not detected PrP 27-30 in analogous fractions prepared from normal hamster brain by the sucrose gradient sedimentation protocol (Table II). Fractions obtained from brains of uninoculated hamsters ranging in age from weanlings to approximately 120 days were analyzed for the presence of PrP 27-30. In addition, we searched for PrP 27-30 in analogous fractions prepared from weanlings inoculated with a 10% suspension of normal hamster brain and held for comparable incubation periods. By the criteria established above, PrP 27-30 was not present in any normal brain fraction examined (Table II).

Table II: PrP 27-30 and the Scrapie Prion Copurify by Two Different Methods

inoculum <sup>a</sup>	sex <sup>b</sup>	no. of purified prepn	time from inoculation to sacrifice (days)	PrP <sup>c</sup>
(A) Purification by Discontinuous Sucrose Gradient Sedimentation				
S	M	2	68, 70	+
S	F	4	68 (2), <sup>d</sup> 71, 76	+
S	M/F	22	47, 63, 64, 66 (2), 67, 68 (6), 69, 70 (3), 73, 77, 73-78, 75-77, 76-78, 69-83	+
none	M/F	5	0, <sup>e</sup> 41-90 (2), 57-65, 68-90	-
N	M	1	66	-
N	M/F	3	63, 73, 75	-
(B) Purification by Sarkosyl-Agarose Gel Electrophoresis				
S	M	2	67 (2)	+
S	M/F	3	63 (2), 65	+
none	M/F	4	0 <sup>e</sup> (4)	-

<sup>a</sup>Ten percent homogenate of scrapie-infected (S) or normal (N) brain. <sup>b</sup>Males (M), females (F), or males and females together (M/F). <sup>c</sup>Presence (+) or absence (-) of PrP 27-30 in purified fractions. <sup>d</sup>Number of preparations in parentheses if greater than 1. <sup>e</sup>Equivalent number of days from time of inoculation.

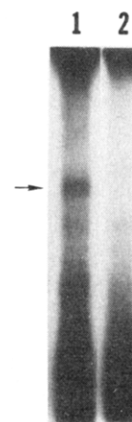


FIGURE 3: PrP 27-30 and the scrapie prion copurify by Sarkosyl-agarose gel electrophoresis. Purified fraction E<sub>6</sub> was obtained from preparations of scrapie-infected hamster brain (lane 1) or normal hamster brain (lane 2). Aliquots were concentrated, radioiodinated with the Bolton-Hunter reagent, and then digested with proteinase K as described in Figure 1. The autoradiograph was exposed for 72 h. The arrow indicates the position of PrP 27-30.

**PrP 27-30 in Sarkosyl-Agarose Gel Electrophoresis Fractions.** Our success in detecting PrP 27-30 in sucrose gradient preparations highly enriched for the scrapie agent prompted a search for the protein in fractions purified by another protocol. The final purification step used in that protocol, Sarkosyl-agarose gel electrophoresis, separated macromolecules on the basis of different physical principles than the sucrose gradient sedimentation procedure. Therefore, although the procedure was less efficient in concentrating and purifying the scrapie agent, the presence of PrP 27-30 in scrapie fractions prepared by that method would provide evidence against artifactual copurification.

PrP 27-30 was found in scrapie fractions from five different preparations purified by this method (Table II, Figure 3). The concentration of the scrapie agent in these fractions was 50-100-fold less than that generally found in sucrose gradient fractions and varied over a 20-fold range between the five fractions tested. In general, we found that the concentration of PrP 27-30 in each of these fractions corresponded with the titer. Analysis of equivalent fractions prepared from normal hamster brain by the Sarkosyl-agarose gel electrophoresis

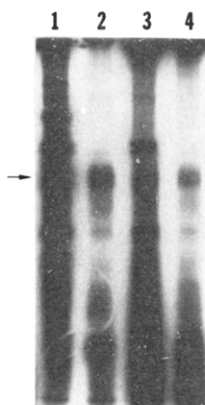


FIGURE 4: Identification of PrP 27-30 in fractions prepared from brains of hamsters sacrificed 47 days after inoculation. Aliquots of fractions 2 and 15 from a sucrose gradient prepared from hamsters inoculated with the scrapie agent 47 days previously were radioiodinated with the Bolton-Hunter reagent. The samples were digested with proteinase K as described in Figure 1 for 30 min prior to denaturation in electrophoresis buffer at 100 °C for 2 min. The samples were analyzed by electrophoresis on a 15% polyacrylamide gel. The autoradiograph was exposed for 69 h. Lane 1, fraction 2, buffer only; lane 2, fraction 2, proteinase K; lane 3, fraction 15, buffer only; lane 4, fraction 15, proteinase K. The arrow indicates the position of PrP 27-30.

technique failed to show PrP 27-30 (Table II, Figure 3).

**Identification of PrP 27-30 in Brains prior to Pathology.** We looked for PrP 27-30 in the brains of hamsters at 47 days postinoculation because it would establish the presence of the protein in the brain prior to the appearance of histopathology (Baringer et al., 1983). By use of the discontinuous sucrose gradient method, the scrapie agent was substantially purified from a brain homogenate obtained from hamsters sacrificed 47 days postinoculation. Analysis of the fractionated sucrose gradient suggested that PrP 27-30 was present in those fractions (F-2 and F-15) which normally contain the highest concentrations of the scrapie agent when purified from hamsters sacrificed 65 days postinoculation (Bolton et al., 1982; Prusiner et al., 1982a). Treatment of these two fractions with proteinase K demonstrated that the protease-resistant protein, PrP 27-30, was present (Figure 4). Bioassay of the gradient fractions confirmed these findings. Fractions 2 and 15 were found to contain the highest titers,  $10^{8.0}$  and  $10^{7.9}$  ID<sub>50</sub>/mL, respectively.

**Protease-Resistant Proteins in Scrapie-Infected Mouse Brains.** Three proteins resembling PrP 27-30 were found in fractions prepared from scrapie-infected mouse brain by discontinuous sucrose gradient sedimentation (Figure 5). These proteins were remarkably resistant to protease digestion, a characteristic of PrP 27-30. In addition, two of these proteins migrated with considerable size heterogeneity ( $M_r$  29 200–32 000 and  $M_r$  23 800–27 400) which is also characteristic of PrP 27-30. The third protein had an apparent molecular weight of 21 000. We could not detect these protease-resistant proteins in analogous fractions prepared from normal mouse brain (Figure 5, lane 4).

**Search for PrP 27-30 in Normal Hamster Brain.** The inability to detect PrP 27-30 in fractions purified from normal brain by either sucrose gradient sedimentation or Sarkosyl-agarose gel electrophoresis led us to define the limits of sensitivity of this analytical method. Aliquots of sucrose gradient fractions prepared from the brains of scrapie-infected and normal brain inoculated hamsters were radiolabeled with <sup>125</sup>I-labeled, Bolton-Hunter reagent. The radioiodinated samples were then digested with proteinase K (100 µg/mL) for 30 min at room temperature. Upon termination of the



FIGURE 5: Protease-resistant proteins in fractions isolated from scrapie-infected mouse brain. Sucrose gradient fraction 2 was prepared from homogenates of mouse brain infected with the Chandler strain of the scrapie agent (lanes 1 and 2) or homogenates of normal mouse brain (lanes 3 and 4). The proteins were concentrated and radioiodinated as described (Bolton et al., 1982) and then incubated for 30 min with 10 mM Tris-HCl and 0.2% Sarkosyl (pH 7.4) alone (lanes 1 and 3) or with this buffer containing 100 µg/mL proteinase K (lanes 2 and 4). The digestion was terminated by heating to 100 °C for 2 min after addition of an equal volume of 2× concentrated electrophoresis sample buffer, and the samples were separated by electrophoresis in a 15% polyacrylamide gel.

digestion, the samples were analyzed by SDS-polyacrylamide gel electrophoresis, and a series of autoradiographic exposures of these gels was used to determine the limits of detection. PrP 27-30 was visible on the autoradiograph of one proteinase K digested scrapie fraction after an exposure for only 0.5 h was seen in the autoradiograph of both proteinase K digested scrapie fractions after exposure for 6 h.

Identically purified normal brain fractions contained significant amounts of protein including numerous proteins with molecular weight values similar to that of PrP 27-30. However, proteinase K digestion of these fractions degraded virtually all of these proteins. PrP 27-30 was not detected in the autoradiograph of any of the proteinase K digested normal brain fractions following exposure for 532 h. We conclude that if PrP 27-30 is present in normal brain fractions its concentration must be <1% of that found in the equivalent scrapie fractions.

**One-Dimensional Peptide Mapping of PrP 27-30 and Normal Brain Proteins.** PrP 27-30 was not present in purified normal brain fractions as defined by the criteria of molecular size and resistance to proteolysis. However, several protease-sensitive proteins which migrated with apparent molecular weights comparable to that of PrP 27-30 were often found in normal brain preparations. We asked if these protease-sensitive proteins were related to PrP 27-30. The resistance of native PrP 27-30 to degradation by proteases could result from conformational alterations of an existing host protein. For example, an unusual secondary or tertiary structure of the polypeptide chain, or a quaternary structure such as aggregation, could confer protease resistance. One-dimensional peptide mapping studies comparing PrP 27-30 to normal brain proteins of similar molecular weight were conducted to resolve this question.

PrP 27-30 and the corresponding normal brain proteins were prepared from sucrose gradient fraction 2 and isolated by SDS-polyacrylamide gel electrophoresis following treatment with proteinase K (100 µg/mL) or Tris-HCl buffer alone. The denatured proteins were analyzed by a one-dimensional peptide mapping technique using SV-8 protease (Figure 6). Figure 6A shows the results of a control digestion



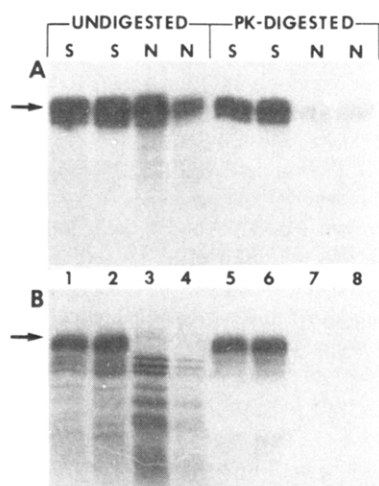


FIGURE 6: One-dimensional peptide mapping of PrP 27-30 and similar normal brain proteins. PrP 27-30 (S) and normal (N) brain proteins of similar molecular weight were isolated from sucrose gradient fraction 2 by SDS-polyacrylamide gel electrophoresis after incubation in Tris-HCl buffer alone ("UNDIGESTED") or proteinase K ("PK-DIGESTED"). The isolated proteins were denatured and incubated with digestion buffer alone (A) or with 133  $\mu$ g/mL SV-8 protease (B) for 30 min. The digested samples were separated on 20% polyacrylamide gels. The arrows indicate a position 2 cm from the top of each gel.

in which the isolated proteins were electrophoresed again after incubation in digestion buffer alone. The proteins migrated as broad bands and showed little evidence of proteolytic degradation. As anticipated, almost no detectable protein remained in the normal brain sample digested with proteinase K prior to isolation (Figure 6A, lanes 7 and 8).

Digestion of the samples with SV-8 protease resulted in cleavage of a small peptide fragment from PrP 27-30, as shown by the small but significant increase in the electrophoretic mobility of the broad protein band (Figure 6B, lanes 1, 2, 5, and 6). SV-8 protease digestion of normal brain proteins produced discrete fragments migrating with a wide range of molecular weights. Most of these fragments could be found in the scrapie samples not pretreated with proteinase K (lanes 1 and 2), indicating contamination of these preparations with normal brain proteins. The slight amount of protein detected in the control digestion of proteinase K pretreated normal brain proteins (Figure 6A, lanes 7 and 8) was completely removed following digestion with SV-8 protease (Figure 6B, lanes 7 and 8). Similar results were obtained following digestion with  $\alpha$ -chymotrypsin (data not shown). The molecular size and diversity of fragments generated by SV-8 protease and  $\alpha$ -chymotrypsin digestion of the normal brain proteins distinguished them from PrP 27-30.

Kinetic studies confirmed that PrP 27-30 was cleaved by SV-8 protease. PrP 27-30 was isolated as described above and treated with SV-8 protease for periods ranging from 0 to 240 min. As shown in Figure 7A, a small peptide was cleaved from PrP 27-30, converting it to a faster migrating species. This process was evident as early as 5 min and was essentially complete within 60 min of digestion. The portion of PrP 27-30 remaining after this cleavage was stable to further digestion for 240 min.

SV-8 protease digestion of normal brain proteins of molecular weight similar to that of PrP 27-30 produced peptide fragments resembling those described in Figure 6. The fragments were not stable and were almost completely degraded within 180-240 min (Figure 7B). Peptide maps were produced from normal brain proteins predigested with proteinase K. No peptide fragments were found after an equiv-

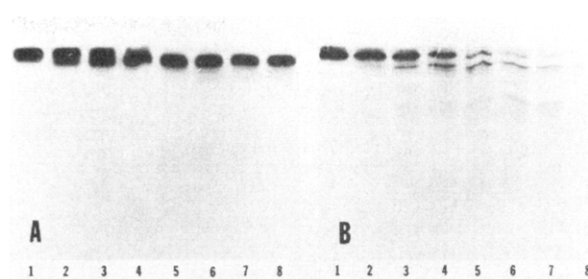


FIGURE 7: Kinetics of PrP 27-30 digestion by SV-8 protease. (A) PrP 27-30 was labeled with  $^{125}$ I-labeled Bolton-Hunter reagent and digested with proteinase K as described in Figure 1 and then denatured by boiling in SDS and isolated by polyacrylamide gel electrophoresis. Denatured PrP 27-30 was then digested with SV-8 protease for 0, 5, 15, 30, 60, 120, 180, and 240 min as shown in lanes 1, 2, 3, 4, 5, 6, 7, and 8, respectively. The digestions were terminated upon addition of an equal volume of 2 $\times$  concentrated electrophoresis sample buffer and heating to 100  $^{\circ}$ C for 2 min. The samples were electrophoresed in a 20% polyacrylamide gel. The autoradiograph was exposed for 22 h. (B) Normal brain proteins were labeled with  $^{125}$ I-labeled Bolton-Hunter reagent and denatured by boiling in SDS. Proteins having molecular weight values of 27 000-30 000 were isolated from a polyacrylamide gel after electrophoresis. The denatured proteins were then digested with SV-8 protease, as described in (A). The autoradiograph was exposed for 22 h.

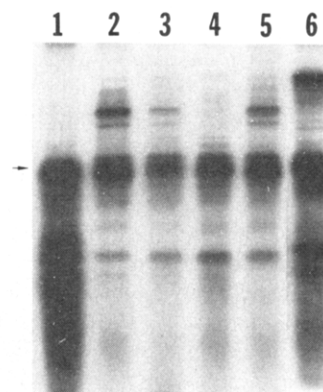


FIGURE 8: Concentration of PrP 27-30 from sucrose gradient fractions. Lane 1, SDS-quinine hemisulfate; lane 2, DOC-Cl<sub>3</sub>CCOOH; lane 3, acetone; lane 4, methanol; lane 5, ethanol; lane 6, distilled water. Samples were analyzed by electrophoresis on a 15% polyacrylamide gel. The autoradiograph was exposed for 17 h. The arrow indicates the position of PrP 27-30.

alent period of autoradiographic exposure of the gel (data not shown). However, prolonged exposure of the gel revealed patterns similar to those shown in Figure 7B (data not shown). These results clearly demonstrated primary structural differences between PrP 27-30 and normal brain proteins of similar molecular weight.

**Concentrating PrP 27-30.** We found that the efficiency of radiolabeling and subsequent analysis of radiolabeled samples by SDS-polyacrylamide gel electrophoresis were enhanced by use of several concentration methods. These methods were employed to concentrate dilute protein suspensions and remove the protein(s) from buffers containing compounds which would inhibit the radiolabeling reaction. Following radiolabeling, the proteins were removed from unreacted reagents by a second precipitation. PrP 27-30 was found to be concentrated efficiently from sucrose gradient fractions by six different methods (Figure 8). The aggregated nature of the proteins in sucrose gradient fractions was shown by their sedimentation to a pellet by centrifugation at moderate speed in a microcentrifuge after dilution of sucrose with distilled water (lane 6).

Infectivity was preserved to varying degrees in samples concentrated by using these methods (Table III). In most

Table III: Concentration of Scrapie Prions

treatment	infectivity (log ID <sub>50</sub> ) recovered in each fraction		total infectivity recovered	
	pellet	aqueous phase	sucrose phase	log ID <sub>50</sub> %
DOC-	1.3	2.8		2.8 <0.001
Cl <sub>3</sub> CCOOH <sup>a</sup>				
SDS-QS <sup>b</sup>	6.4	5.0		6.4 2
acetone	6.4	2.1	6.3	6.7 3
methanol	6.8	3.4		6.8 4
ethanol	7.5	4.3		7.5 20
H <sub>2</sub> O	8.0	7.6		8.2 100

<sup>a</sup>DOC-Cl<sub>3</sub>CCOOH = deoxycholate-trichloroacetic acid. <sup>b</sup>SDS-QS = sodium dodecyl sulfate-quinine hemisulfate.

cases, the majority of the recovered infectivity was found in the pellet. Infectivity was quantitatively recovered by sedimentation after dilution with distilled water alone. In this case, approximately 72% of the infectivity was found in the pellet.

**Extraction of Sucrose Gradient Fractions with Organic Solvents.** The size heterogeneity of PrP 27-30 and apparent hydrophobicity of the scrapie agent suggested that PrP 27-30 might be a proteolipid. Extraction of sucrose gradient fraction 2 with organic solvents demonstrated that PrP 27-30 was not soluble in these solvents. Extraction with chloroform-methanol (2:1), chloroform alone, or toluene-ethyl acetate (1:1) failed to partition PrP 27-30 into the organic phase (lanes 2, 4, and 6) to any significant extent. PrP 27-30 and most other proteins were found both in the pellet in and the aqueous phase following organic solvent extraction. Exclusion of PrP 27-30 from the organic solvent was not due to the density of the medium because toluene-ethyl acetate is less dense than water. Since PrP 27-30 was not extracted from the aqueous phase by organic solvents, it does not exhibit the properties characteristic of a proteolipid as originally defined by Folch & Lees (1951). PrP 27-30 might fulfill the criteria for proteolipids recently suggested by Schlesinger (1981), but this determination awaits the demonstration of fatty acid residues covalently bound to the protein.

## Discussion

The studies presented here and in two other communications (McKinley et al., 1983; Prusiner et al., 1983) support our hypothesis that PrP 27-30 is the major protein component of the scrapie agent (Bolton et al., 1982; Prusiner et al., 1982a). PrP 27-30 is the first structural component of the scrapie prion to be identified.

PrP 27-30 and the prion were found to copurify by two different methods. Different techniques were used in each protocol to concentrate the prions from clarified supernatants. In the sucrose gradient procedure, PEG was added to precipitate prions from a suspension containing 4% Triton X-100 and 2% DOC (Prusiner et al., 1982a). For the electrophoretic procedure, prions were sedimented from the suspension by prolonged centrifugation in a zonal rotor and then repelleted by centrifugation at high speed in a fixed-angle rotor after extraction with 0.5% DOC (Prusiner et al., 1981).

The final steps of the two purification protocols utilized either discontinuous sucrose gradient centrifugation or Sarkosyl-agarose gel electrophoresis. In the sucrose gradient procedure, advantage was taken of the apparent hydrophobic behavior of the prions by sedimenting them from a suspension containing 0.2% Sarkosyl, 2% Triton X-100, and 0.8% SDS into a sucrose layer containing no detergents. It is likely that the scrapie agent and contaminating normal brain proteins aggregated as they sedimented into the sucrose layer lacking

detergents and these aggregates rapidly sedimented to the interface of the 25% and 60% sucrose layers (Prusiner et al., 1982a). This behavior would be similar to that of the (Mg<sup>2+</sup> + Ca<sup>2+</sup>)-ATPase, a membrane protein which has been shown to aggregate when sedimented into sucrose gradients devoid of detergents (Warren et al., 1974a,b). In the electrophoresis protocol, the scrapie agent was separated from contaminants by electrophoresis into a porous agarose gel under nondenaturing conditions. Negatively charged, rapidly migrating molecules including <5% of the recoverable prions were electrophoresed through the gel and eluted at the anode. Those prions which migrated less rapidly and thus remained in the gel under these conditions were eluted after electrophoresis by mechanical or electrophoretic procedures (Prusiner et al., 1980b, 1981).

A final point regarding differences between these two purification protocols concerns the kinds of detergents used in each. In the electrophoresis procedure, only the anionic detergents Sarkosyl, sodium cholate, and DOC were used (Prusiner et al., 1981). The sucrose gradient sedimentation procedure employed, in addition to these, the nonionic detergent Triton X-100 and the anionic detergent SDS (Prusiner et al., 1982a). It is difficult to determine what effects, if any, these differences had on the behavior of prions during purification because the use of detergents in the purification of macromolecules is largely an empirical process.

Copurification of PrP 27-30 and the scrapie agent throughout these two procedures indicates that the protein and the prion exhibit similar physicochemical properties. This behavior would be expected if PrP 27-30 were a component of the scrapie agent but would be unlikely if it were a product of the disease process. The presence of PrP 27-30 in the brains of infected hamsters prior to appearance of histopathology provides additional evidence against PrP 27-30 being a pathologic product. While normal brain proteins do purify with the scrapie agent by these two procedures, the proteins do not exhibit the resistance to protease digestion exhibited by PrP 27-30 and the prion (McKinley et al., 1983). If PrP 27-30 is present in normal brain fractions, it must exist at a concentration less than 1% of that in scrapie fractions, i.e., beyond our limits of detection.

Substantially purified fractions purified from scrapie-infected mouse brains contain protease-resistant proteins which resemble PrP 27-30 in their molecular size; thus, these proteins exhibit physicochemical properties similar to those of the scrapie agent and PrP 27-30. We could not detect these proteins in equivalent fractions prepared from normal mouse brain. We do not know whether the two lower molecular weight proteins represent distinct species or cleavage products of the largest protein. Further studies will be required to establish whether or not any of these three protease-resistant proteins found in scrapie-infected mouse brain are structural components of the murine scrapie prion.

Digestion of sucrose gradient fractions with proteinase K for 30 min under nondenaturing conditions degraded virtually all proteins in normal brain fractions and left only PrP 27-30 intact in scrapie fractions. Under these same conditions of proteolytic digestion, no change in infectivity was observed. Conversely, denaturation of scrapie prions by boiling in SDS and 2-mercaptoethanol rendered PrP 27-30 susceptible to degradation by proteases and diminished the infectivity by a factor of 10<sup>4</sup>. These data demonstrate a correlation between infectivity and the conformation of PrP 27-30, thus providing additional evidence for PrP 27-30 being the major protein component of the prion. In another report, we demonstrated

that prolonging the time of digestion with proteinase K to 30 h under nondenaturing conditions significantly reduced scrapie infectivity concomitant with degradation of PrP 27-30 (McKinley et al., 1983). In contrast, digestion of scrapie prions with the amino acid specific proteases trypsin and SV-8 protease under these conditions degraded most other proteins but had no effect on infectivity or PrP 27-30. These properties are consistent with the behavior predicted for a macromolecular component of the scrapie agent.

The observation that normal brain fractions contained protease-sensitive proteins with electrophoretic properties similar to PrP 27-30 suggested that PrP 27-30 might represent a modified form of one or more of these proteins. An unusual secondary or tertiary structure in otherwise identical polypeptides could influence their susceptibility to digestion by proteases (Hilz et al., 1975; Rossmann & Argos, 1981; Ott et al., 1982), as could aggregation to form a protected multimeric structure (Tanford, 1961; Holzer & Heinrich, 1980). However, these noncovalent modifications would not affect the digestion patterns produced by cleaving the denatured proteins with amino acid specific proteases (Cleveland et al., 1977). We have shown that PrP 27-30 has a primary structure that is unique from these normal brain proteins when compared by one-dimensional peptide mapping.

Chemical modification and radiolabeling studies have shown that native PrP 27-30 probably has Lys, Tyr, and His residues on its surface or in positions accessible to the solvent (McKinley et al., 1981; Bolton et al., 1982). Chemical labeling by the method of Bolton and Hunter provides an efficient means of incorporating radioiodine into  $\alpha$ - and  $\epsilon$ -amino groups (Bolton & Hunter, 1973). It is not uncommon for proteins modified by this reagent to retain their biological activity (Langone, 1980). We have used this reagent to label PrP 27-30 under conditions which did not affect infectivity. This property made it possible to demonstrate, through proteolytic digestions studies, a correlation between degradation of PrP 27-30 and reduction of infectivity (McKinley et al., 1983).

At this time, the evidence implicating PrP 27-30 as the major protein component of the scrapie prion seems convincing. PrP 27-30 purifies with the scrapie agent by two different techniques and appears in the brain during the early phase of infection when prion titers are high but prior to clinical and pathologic manifestations of the disease. PrP 27-30 has not been observed in any fraction purified from normal hamster brain. The amount of PrP 27-30 in extensively purified scrapie fractions correlates with the titer of prions (McKinley et al., 1983). Treatments which inactivate the scrapie agent, such as thermal denaturation, chemical inactivation with DEP (McKinley et al., 1981; Bolton et al., 1982), or prolonged digestion with proteinase K (McKinley et al., 1983), effect corresponding changes in PrP 27-30. Conversely, treatments which degrade other proteins present in sucrose gradient fractions, such as digestion with proteinase K for 30 min or prolonged digestion with trypsin or SV-8 protease (McKinley et al., 1983), affect neither PrP 27-30 nor the prion. In our most purified fractions of the scrapie agent, PrP 27-30 is the major protein and accounts for 70-85% of the total detectable protein (Prusiner et al., 1983). We are aware of no data which contradict the hypothesis that PrP 27-30 is a structural component of the scrapie prion.

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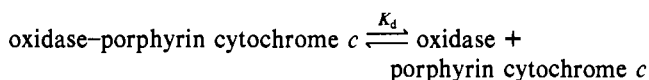
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## Volume Changes Associated with Cytochrome *c* Oxidase-Porphyrin Cytochrome *c* Equilibrium<sup>†</sup>

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**ABSTRACT:** The binding of a fluorescent derivative of cytochrome *c* to cytochrome *c* oxidase has been studied by use of pressure to perturb the equilibrium.  $\Delta V^\circ$  for the reaction



was small and favored dissociation of the complex. Pres-

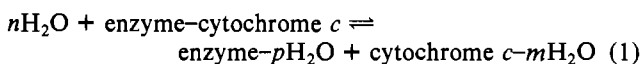
sure-induced dissociation is to be expected if the major forces governing the equilibrium are electrostatic in nature. The dependence of  $\log K_d$  on pressure is not linear but biphasic; high pressures lead to a decrease in  $K_d$  and association of the reactants. The latter fact indicates that the net compressibility of the complexes is greater than that of the free reactants, an unexpected result.

**C**ytochrome *c* oxidase (EC 1.9.3.1) catalyzes electron transfer from cytochrome *c* to oxygen. The oxidase is a mitochondrial protein, the role of which is to couple electron transport to the production of ATP. This is accomplished through the intermediate establishment of an electrochemical gradient across the mitochondrial membrane. There appears little doubt that the gradient is set up, at least in part, by the active pumping by the oxidase of protons from the mitochondrial matrix space to the cytosolic space (Wikstrom, 1977). The oxidase is an intrinsic membrane protein; it is large ( $M_r$  200 000) and contains at least seven subunits, two hemes, and two coppers. The structure of the oxidase has been admirably reviewed recently by Capaldi et al. (1983).

The interaction of cytochrome *c* oxidase with cytochrome *c* is similar to the interactions that cytochrome *c* shows with its other protein acceptors. A summary of the available information indicates that the interacting face of cytochrome *c* viewed from any of its acceptor molecules (cytochrome *c* reductase, mammalian sulfite oxidase, and cytochrome *c* peroxidase, as well as cytochrome *c* oxidase) consists of a positively charged surface with the center of the charge located near phenylalanine-82; Koppenol & Margoliash (1982) have postulated that the driving force for the orientation of the cytochrome *c* with respect to its partners is the large dipole moment that is associated with the sum total of positive and negative charge on the cytochrome *c*. The acceptor molecules have been postulated to have a complementary surface as part

of their interaction domains. In support of this statement, all of the acceptor molecules show similar ionic strength dependencies (Wainio et al., 1960; Davies et al., 1964; Nicholls, 1964), similar interactions with modified cytochromes *c* or the cytochromes *c* of different species (Errede & Kamen, 1978; Davis et al., 1972; Brautigan et al., 1978; Ferguson-Miller et al., 1978; Smith et al., 1980, 1981), similar reaction rate constants (Errede & Kamen, 1978; Smith et al., 1981), and similar tendencies to form tight binding complexes (Yonetani & Ray, 1965; Nicholls, 1964; Mochan & Nicholls, 1972; Yu et al., 1975; Ferguson-Miller et al., 1976). In the case of the peroxidase, where a high-resolution three-dimensional structure is known, a negatively charged surface (Poulos & Kraut, 1980), complimentary to the positively charged surface of the cytochrome *c* (Swanson et al., 1977), has been located. In the case of the oxidase, it is likely that some of the negative charge on the oxidase comes from the cardiolipin that is normally associated with the protein (Erecinska et al., 1980).

One consequence of the binding of cytochrome *c* by the oxidase is that spaces between them are eliminated:



The total volume occupied by the reactants<sup>1</sup> of eq 1 may be greater or less than that of the products;  $\Delta V^\circ$  for the reaction may be either positive or negative but is unlikely to be zero. As such, the equilibrium of (1) can be perturbed by pressure. From a study of the pressure dependence, one can deduce how

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<sup>1</sup> Reactants or products, as used here, must be taken to mean the sum of all the interacting species on either side of eq 1. This includes interactions of the oxidase and porphyrin cytochrome *c* with solvent, detergent, and other components of the buffer.