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その現状と将来展望

肉 魚 野菜 惣菜料理に安心感を!!

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Ⅱ

ゲノム（遺伝子）が作り出す産物

21世紀は蛋白質の時代になる

—BSE（牛海綿状脳症）の感染因子“プリオン”の衝撃—

キーワード

①ＢＳＥ　②プリオン　③ゲノム（遺伝子）
④３体問題　⑤αヘリックス　⑥βシート
⑦プラスミノーゲン活性化因子インヒビター（ＰＡＩ）１
⑧βラクトグロブリン　⑨アルツハイマーβ線維
⑩ヒトプリオン　⑪スクレイピー型

ポイントは何か

—21世紀の新しい総合的生命科学—

ウシのBSEやヒトのヤコブ病の病原体が，ウイルスと異なり，遺伝子を持たない「蛋白質」であったという事実は，生物学・医学にとって非常に大きな衝撃であった。21世紀の生物学には，この「蛋白質の異常構造」という問題の解明とともに，疾患に対する分子立体構造に基づく新概念の構築が求められる。本節では1838年，ドイツの科学者Müdlerの「Protein」（ギリシャ語で“もっとも原初的の”という意味）以来の蛋白質の基本コンセプト，αヘリックスからβシートへの転移を中心に，BSEの原因物質プリオンの異常構造の解明及びその制御を検討する。

（1）蛋白質とは？—基本的な考え方—

①　生命を司る蛋白質の複雑さ

1）遺伝子生物学の基礎—ヒト・ゲノムの配列決定—

20世紀初頭，アインシュタインやハイゼンベルグは量子力学を創出し，20世紀後半，人類は遺伝子生物学の基礎を確立した。21世紀初頭，ヒト・ゲノムの配列決定がほぼ終了した。しかし，ゲノムの並び方がわかっても，その意味を解読することは容易ではない。

2）BSE の本態が分る

ゲノム（遺伝子）が作り出す産物，それは蛋白質である。蛋白質はアミノ酸が鎖状に連なったものであり，BSE の感染因子であるプリオンの場合，約3,000個の原子からなっている。ここまで来れば，BSE の本態はもう分ったといえるのではないだろうか？

しかし，ここで新たな問題が生ずる。3,000個の原子が連なった紐（ひも）が，どのように振舞うのか，我々はこれをどうすれば理解できるのだろうか？

②　多体問題の困難さ

1）多体問題は解析的には解けない

古来より３体問題，すなわち，三つの物体が互いに引力で引き合った結果，どのように動くか？という問題は，解析的な一般解が求められず，計算機で数値的に逐一シミュレーションしなければ解けないことが知られている。さらに一回の計算で得られる解

は，一つの初期条件の下での一例であり，初期条件が異なると解も異なり，一般的な振舞いは分らない。これは古典力学の例であるが，量子力学においても基本的に同じである。多体問題は解析的には，解けないのである。

2）個々の粒子が個性を持って特別な振舞いをする

しかし，多数の粒子が，ばらばらに運動していれば，このような問題を統計的に扱うことが出来，ここに統計力学が成立する。しかし，粒子同士が開いた一本の紐で結ばれているときは，簡単に平均してしまうことは出来ない。すなわち，一個一個の粒子が個性を持って，特別な振舞いをするようになる。

③　蛋白体仮説

1）蛋白質は脳を形成する

この世の中で一番難しいパズルは何だろうか？フェルマー予想は解けた。リーマン予想は未だ解けない。しかし，いずれは解決されるだろう。このような問題を考え出し，解くといった行為を行うのは，我々の脳である。ところが，この脳は，実は蛋白質で出来ている。レセプターもチャンネルも，細胞内情報伝達素子も全て蛋白質で出来ている。では一体，蛋白質が，蛋白質より複雑なものを理解できるだろうか？ここで，一つの仮説が成り立つかもしれない。

2）蛋白体仮説とは？

「脳が考えうる問題の複雑さの濃度（適当に定義すれば）は，蛋白質（蛋白体）の複雑さの濃度を超えることは出来ない。」

すなわち，蛋白質を解き明かすことは，最も複雑な問題を解く

ことと同等であり，かつ，それは生命そのものを解明することに等しい，といえるのではないだろうか？

（2）αヘリックス→βシート転移とは？

①　蛋白質内部の自己組織化は，αからβへと進む

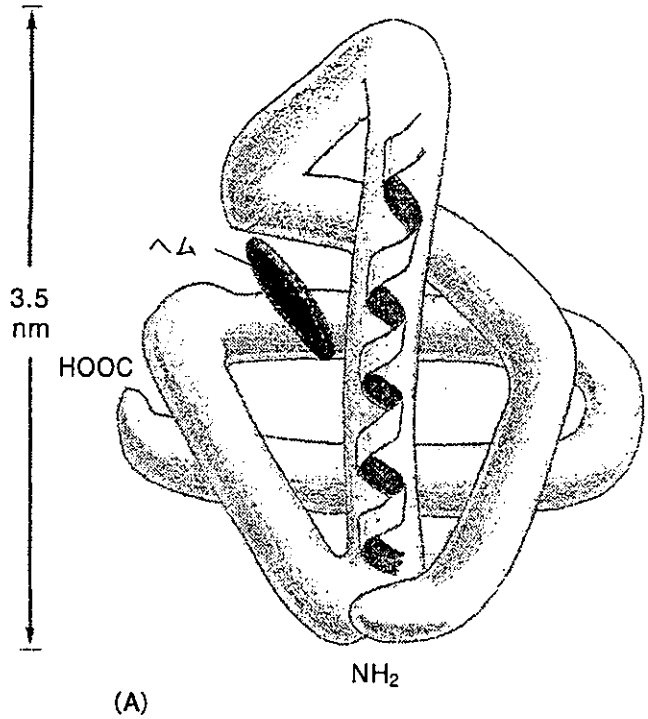
1）蛋白質の基本構造αヘリックスとβシート

蛋白質は，細胞内で合成された直後は，伸びた一本の紐である。この紐は，やがて毛糸の玉のように，自分自身で丸まってゆく。この丸まり方には，大雑把に言って2種類ある。一つは，鎖が，らせん状に巻く，αヘリックスという構造（図1）で，もう一つは，手編みのように交互に並ぶ構造でβシート（図2）と呼んでいる。このようなβシート構造は，たんぱく質が生理的機能を行うために必要なものである。

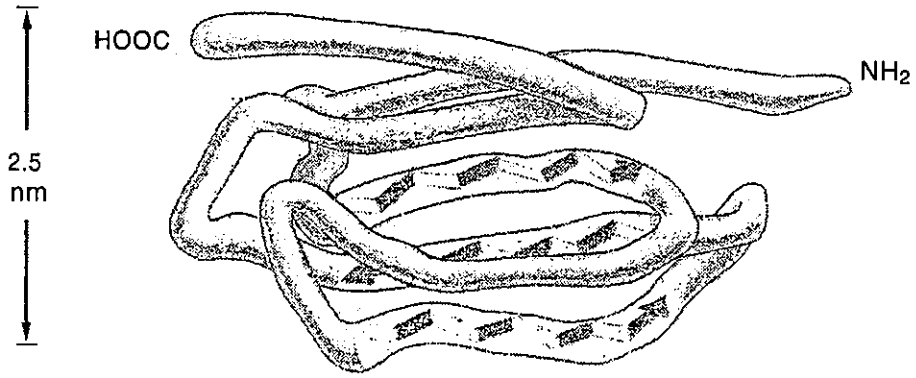
不思議なのは，このαヘリックスからβシートへの変換が存在することである。（図3）はプラスミノーゲン活性化因子インヒビター（PAI）1の構造転移を示している。

（PAI）1は，初期は（図3の左）に示すような，3回巻きのαヘリックスを持っているが，時間が経つと，（図3の右）に示すような構造に変化する。このとき，前のαヘリックスは，βシートへと変化している。このような構造変化をα→β転移と呼んでいる。左の構造のみが生理活性を有する。

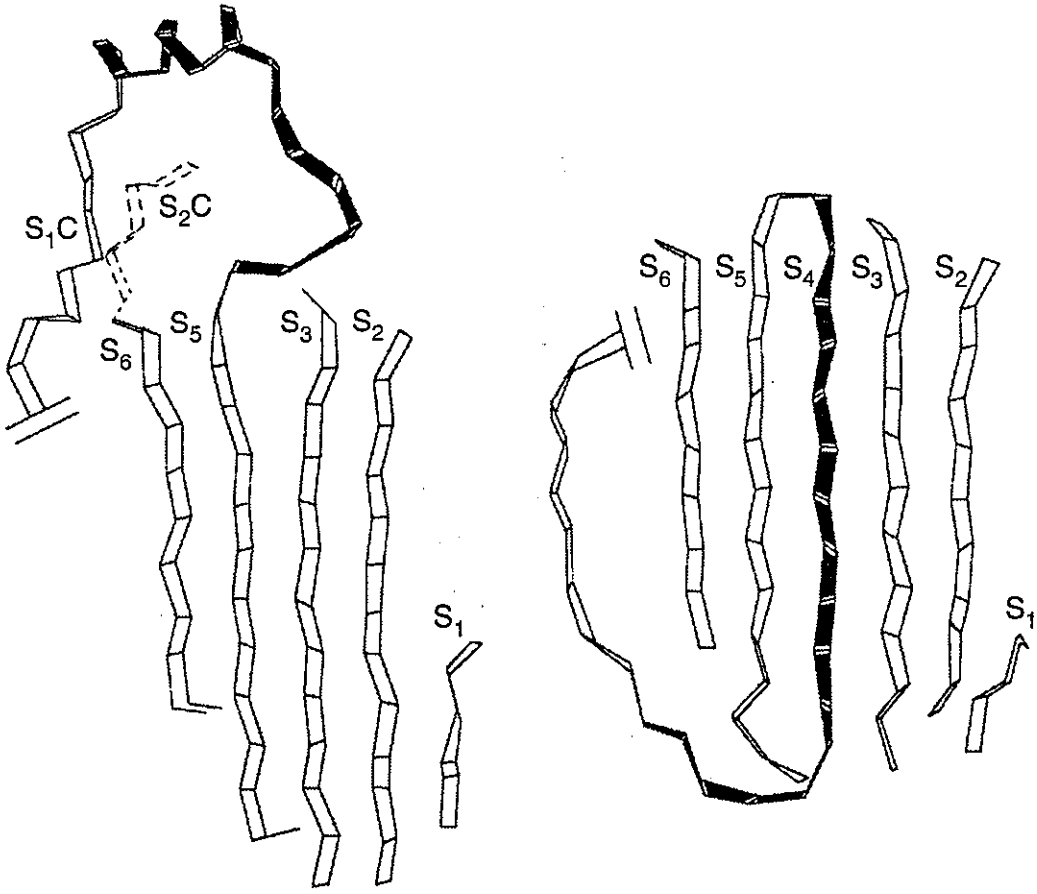
このような現象は他の蛋白質，たとえば，βラクトグロブリンでも観測されている(注3)（図4）。βラクトグロブリンはA～Hの8本のβシートからなるが，構造形成の初期に，ヘリックスが



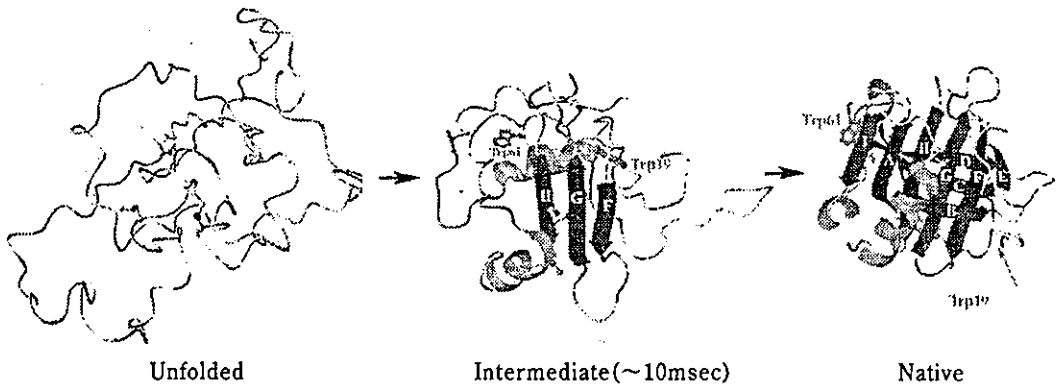
（図１）筋肉に存在するミオグロビンの立体構造。らせん状のαヘリックスからなる典型的な構造を有する（注１）。



（図２）βシートを有する免疫グロブリン分子の一部。３本の鎖が逆平行に並んでいる（注１）。



（図３）プラスミノーゲン活性化因子インヒビター（PAI）１の構造転移（注２）



（図４）βラクトグロブリンの立体構造形成過程

一時的に形成され，このヘリックスが最終的には，βシートＡとなる。

2）柔い構造から固い構造へ

蛋白質の構造には様々なものがあるが，上にみられるように，ポピュレーションとしては，αヘリックスからβシートへと変化する。生理的条件下では，この逆は起こらない。

それは何故だろうか？αヘリックスと，βシートとの違いは，αヘリックスでは，水素結合が4残基離れたアミノ酸同士の間に形成されるのに対し，βシートでは，何十残基も離れたアミノ酸同士の間に形成される。そのため，βシートは板状となり，構造揺らぎの少ない，強固な構造を形成する。つまり，蛋白質の構造形成は，アミノ酸同士のつながりが，弱い構造（αヘリックス）から強い構造（βシート）へと進んでゆくようである。

これは恰も，人間同士の繋がりが近隣の地域社会から，遠く離れた国際社会へとITを介して進んで来たのとどこかよく似ているようにも思われる。

②　βシート構造と難治性疾患

1）蛋白質の構造異常により引き起こされる約1,000種類の疾患カテゴリー

蛋白質の構造異常により，引き起こされる疾患カテゴリーとして，約1,000種類が知られている。この中には，プリオン病，癌，神経変性疾患（アルツハイマー病等）などの重要な難治性疾患が含まれている。その中でもアルツハイマー病等のような神経変性疾患は，多くの場合βシート構造が関与することが知られている。このような場合，分子内ではなく，分子間のβシートが問題となる。例えば，アルツハイマー病の原因となる，アルツハイマーβ線維はβシートからなる線維状物質である（図5参照）。これは，

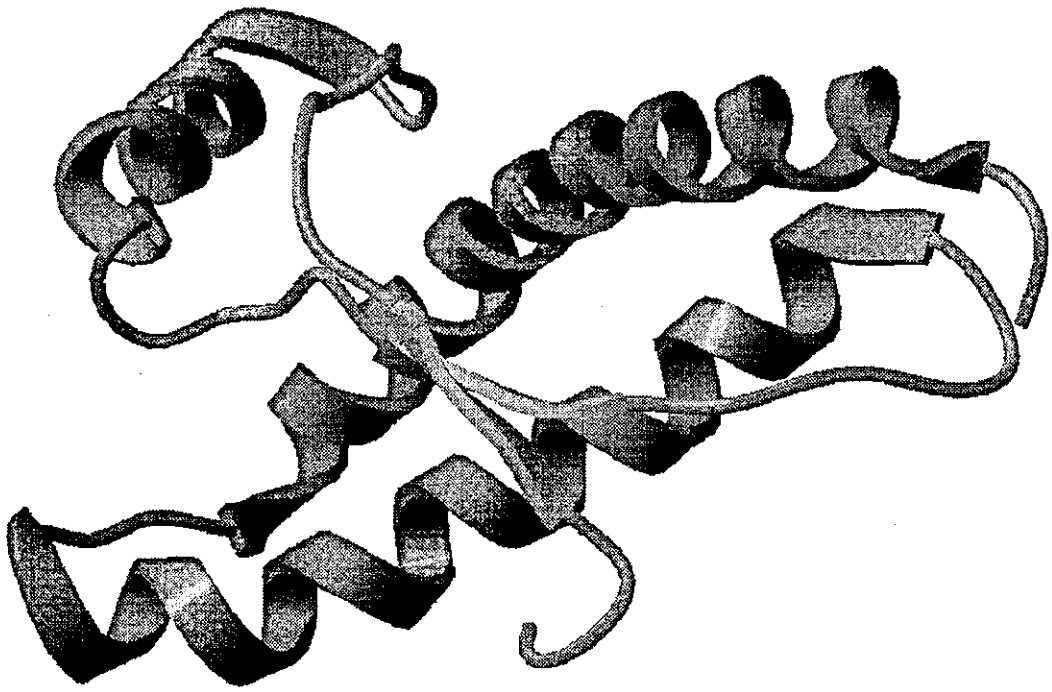
約40個のアミノ酸からなるペプチドが，分子間でβシートを作り，次々に会合して線維を形成したものである。

（図５）アルツハイマー病の原因となるアルツハイマーβ線維の電子顕微鏡写真。約20万倍　（岐阜大学医学部電子顕微鏡室，奥村年彦氏との共同実験）

（３）プリオンとは？—BSE等のプリオン病メカニズムを解明し，新薬を開発する—

①　BSEの原因物質“プリオン”におけるα→β転移の実像

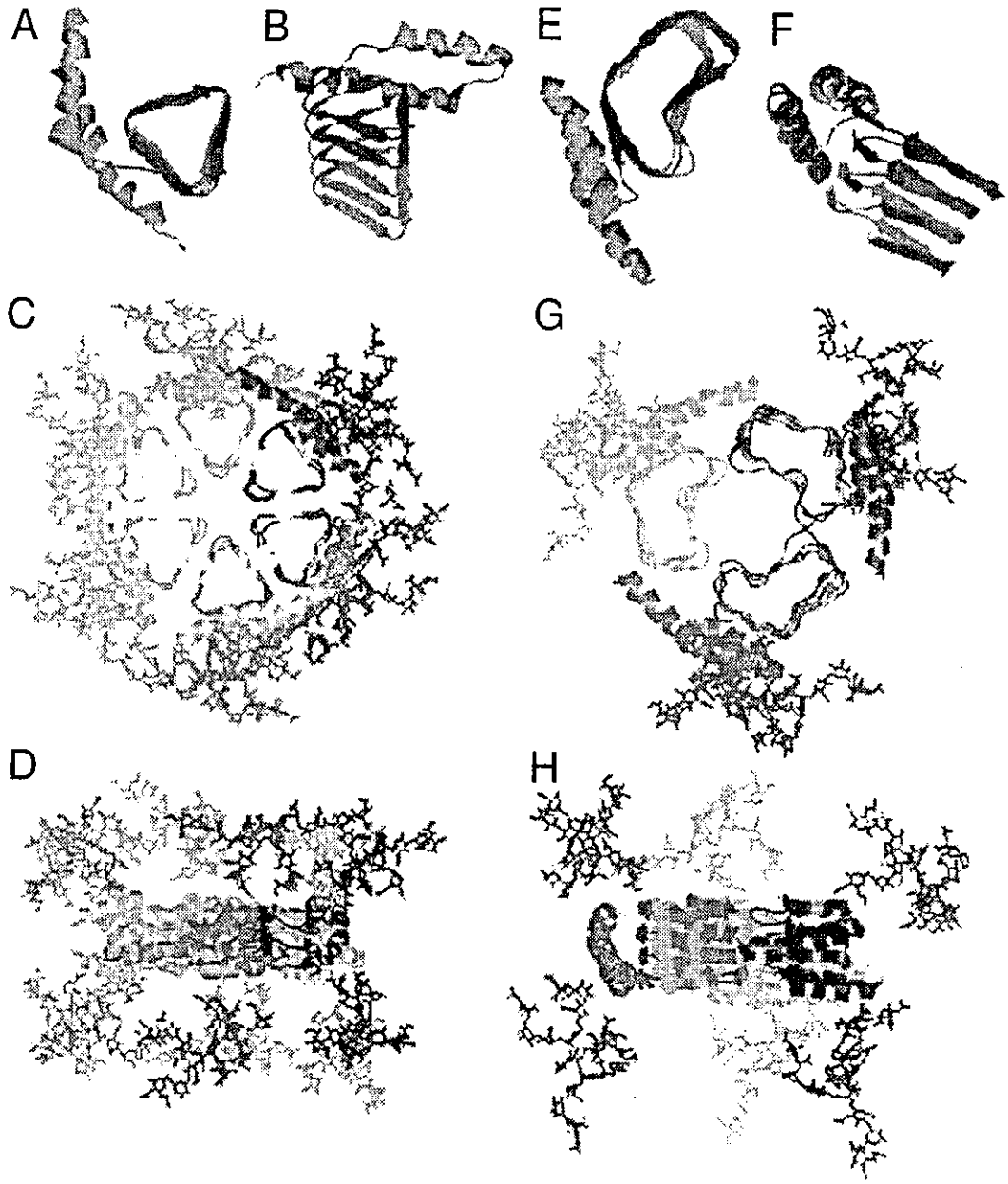
正常ヒトプリオンの三次元立体構造を，（図６）に示す。



（図６）ヒトプリオンの立体構造

正常型プリオンは，進化の上でよく保存されてきており，現在未だ詳細は不明ではあるが，重要な生理機能を有すると考えられている。正常型が異常型に変化するとき，正常型のβシート周辺の構造は，細胞毒性に関係し，αヘリックス周辺の構造は，感染性に関与する(注４)と考えられている。また，感染性を有する異常型（スクレイピー型）では，このβシート周辺の構造が次第に伸びてゆき，やがて蛋白全体の半分程度がβシートになるものと予想されている。

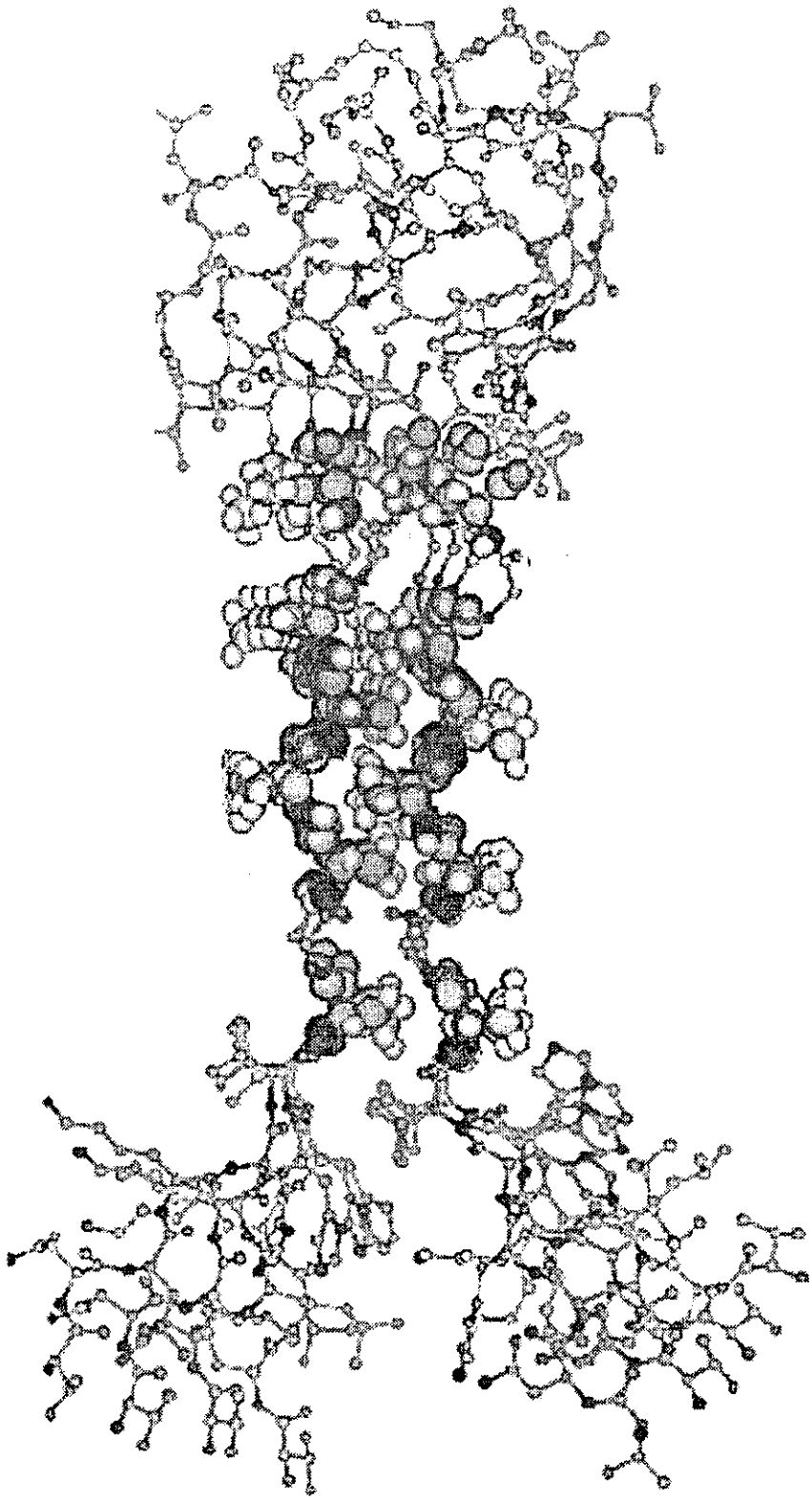
次の（図７）に感染性を有する異常型（スクレイピー型）のモデル構造を示す(注５)。



（図７）スクレイピー型のモデル構造

（図７）において，複数のモデル構造があるが，いずれも電子顕微鏡写真に基づく類推であって，構造生物学的な根拠に基づく立体構造は未だ提出されていない。

これに対し，近年，プリオン蛋白の毒性部分のオリゴマー構造の一端が，明らかにされた(注６)。その構造を（図８）に示す。正常構造（図６）のβシート周辺の構造は，オリゴマー（２～10個



（図８）プリオン毒性部分のオリゴマー立体構造。平行βシート構造をとる。

程度の分子が集合した形態）を形成し，細胞毒性を発揮すると考えられる。

（図８）の構造は，感染性を有するスクレイピー型そのものではないが，感染型がオリゴマーであるとすれば，やはりこのような構造を部分的に有すると考えられ，（図７）の異常構造モデルのなかでβシート部分に対応している。

②　治療法開発—新薬の開発へ—

以上，見てきたように，BSE等のプリオン病のメカニズムは，その異常構造にある，と考えられる。従って，その異常構造形成を制御することにより，治療が可能となる。上述のように立体構造を決める目的は，治療薬の開発にある。分子立体構造が正確に求めることが出来れば，その構造にフィットするような分子をデザイン・合成することが可能となる。このような技術を「構造に基づく創薬」(Structure Based Drug Design, SBDD) と呼んでいる。最近では「構造ダイナミクスに基づく創薬」(Dynamics Based Drug Design, DBDD) も開発され(注7)，これは，上述のα→β転移を制御するための新技術である。これら構造生物学的新創薬方法を駆使して，多くの難治性疾患の治療薬が，今後開発されてゆくだろう。

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A Pitfall in Diagnosis of Human Prion Diseases Using Detection of Protease-resistant Prion Protein in Urine

CONTAMINATION WITH BACTERIAL OUTER MEMBRANE PROTEINS*

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Because a definite diagnosis of prion diseases relies on the detection of the abnormal isoform of prion protein (PrP^{Sc}), it has been urgently necessary to establish a non-invasive diagnostic test to detect PrP^{Sc} in human prion diseases. To evaluate diagnostic usefulness and reliability of the detection of protease-resistant prion protein in urine, we extensively analyzed proteinase K (PK)-resistant proteins in patients affected with prion diseases and control subjects by Western blot, a coupled liquid chromatography and mass spectrometry analysis, and N-terminal sequence analysis. The PK-resistant signal migrating around 32 kDa previously reported by Shaked *et al.* (Shaked, G. M., Shaked, Y., Kariv-Inbal, Z., Halimi, M., Avraham, I., and Gabizon, R. (2001) *J. Biol. Chem.* 276, 31479–31482) was not observed in this study. Instead, discrete protein bands with an apparent molecular mass of ~37 kDa were detected in the urine of many patients affected with prion diseases and two diseased controls. Although these proteins also gave strong signals in the Western blot using a variety of anti-PrP antibodies as a primary antibody, we found that the signals were still detectable by incubation of secondary antibodies alone, *i.e.* in the absence of the primary anti-PrP antibodies. Mass spectrometry and N-terminal protein sequencing analysis revealed that the majority of the PK-resistant 37-kDa proteins in the urine of patients were outer membrane proteins (OMPs) of the *Enterobacterial* species. OMPs isolated from these bacteria were resistant to PK and the PK-resistant OMPs from the *Enterobacterial* species migrated around 37 kDa on SDS-PAGE. Furthermore, nonspecific binding of OMPs to antibodies could be mistaken for PrP^{Sc}. These findings caution that bacterial contamination can affect the immunological detection of prion protein. Therefore, the presence of *Enterobacterial* species should be excluded in the immunological tests for PrP^{Sc} in clinical samples, in particular, urine.

Prion diseases are a group of neurodegenerative disorders pathologically characterized by accumulation of an abnormal isoform of prion protein (PrP^{Sc}) in the central nervous system. A definite diagnosis of prion diseases relies on the detection of PrP^{Sc} (1). Concerning the link between bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease (CJD),[1] the iatrogenic occurrence of prion diseases after dural transplantation, and the recent remarkable progress in therapeutic approaches have made it urgently necessary to establish a non-invasive *in vivo* test to enable a definite diagnosis of human prion diseases in the early or preclinical stage of the disease.

Diffusion-weighted magnetic resonance imaging of the brain is currently one of the most helpful techniques to detect abnormal high intensity lesions in the cerebral cortices and basal ganglia in the early stage of the disease (2). The detection of 14-3-3 proteins and measurement of phosphorylated tau protein in the cerebrospinal fluid has been found to be useful in supporting the clinical diagnosis of CJD (3,4). Although these tests are clinically useful, they are surrogate markers and therefore cannot provide direct evidence of the presence of PrP^{Sc}. Moreover, although a brain biopsy can reveal the deposition of PrP^{Sc} in the brain (5), it is highly invasive and is not suitable for preclinical screening or early diagnosis. Detection of PrP^{Sc} in body fluids such as blood and cerebrospinal fluid has been extensively investigated, but these tests still need a new technological device to increase the sensitivity (6).

As a potentially non-invasive diagnostic test, Shaked *et al.* (7) reported the presence of protease-resistant PrP in the urine ($UPrP^{Sc}$) of humans and animals affected with prion diseases. Their data suggests that $UPrP^{Sc}$ will reflect the presence of PrP^{Sc} in the central nervous system and will also be a useful preclinical diagnostic test for prion diseases. In the present study, we have examined the urine protein of humans affected with prion diseases and controls using Western blot analysis to evaluate diagnostic usefulness and reliability of the $UPrP^{Sc}$ assay in human prion diseases. A detailed analysis using coupled liquid chromatography and mass spectrometry (LC/MS) and N-terminal protein sequencing revealed that bacterial contamination might account for the misinterpretation in the interpretation of protease-resistant protein in urine.

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[1] The abbreviations used are: CJD, Creutzfeldt-Jakob disease; PBS, phosphate-buffered saline; PK, proteinase K; OMP, outer membrane proteins.

TABLE I

Protease-resistant protein in urine and characteristics of patients and controls

The abbreviations used are: GSS; Garstmann-Sträussler-Scheinker syndrome; HDS-R; revised Hasegawa Dementia Rating Scale; MMSE; Mini-Mental State Examination; and MELAS; mitochondrial myopathy, lactic acidosis, and stroke-like episodes.

Clinical diagnosis[a]	No. of cases	Mean age[b]	Mean clinical duration at examination	CSF 14-3-3 protein positive ratio	Brain DWI MRI[c] positive ratio	Protease-resistant protein in urine[d] positive ratio
		years	*months*		%	
Prion diseases						
Sporadic CJD	45	65.9 (42-83)	5.5 (1.5-18)	93.5 (29/31)	75.0 (15/20)	66.7 (30/45)
Dural graft-associated CJD	4	53.8 (15-69)	20.5 (6-48)	100 (3/3)	25.0 (1/4)	100 (4/4)
Familial CJD (E200K)	2	58.5 (53-64)	3.8 (3.5-4)	50 (1/2)	100 (2/2)	100 (2/2)
GSS (P102L)	3	57.3 (47-72)	45.3 (28-72)	NE[e]	NE	66.7 (2/3)
	54	58.9	9.58	91.7 (33/36)	69.2 (18/26)	70.4 (38/54)
				Mean scores		
				HDS-R	MMSE	
Diseased controls with dimentia						
Alzheimer's disease	19	74.2 (56-87)	NA[f]	13.5 (0-23)	18.8 (0-30)	0 (0/19)
Cerebrovascular dementia	1	76	NA	19	24	0 (0/1)
Diseased controls without dimentia						
Diabetes mellitus	7[g]	67.5 (53-75)	NA	NE	NE	14.3 (1/7)[h]
Cerebral infarction	6[g]	73.3 (62-77)	NA	NE	NE	16.7 (1/7)[h]
Multiple sclerosis	4	49.3 (24-62)	NA	NE	NE	25.0 (1/4)
Pneumonia	2	72.0 (70-74)	NA	NE	NE	0 (0/2)
Epilepsy	2	46.0 (20-72)	NA	NE	NE	0 (0/2)
Myasthenia gravis	1	35	NA	NE	NE	0 (0/1)
Encephalitis	1	57	NA	NE	NE	0 (0/1)
Chronic renal failure	1	79	NA	NE	NE	0 (0/1)
MELAS	1	21	NA	NE	NE	0 (0/1)
	23	27.5 (19-59)		NE	NE	0 (0/23)
Healthy controls		56.5				3 (2/66)

[a] Clinical diagnosis of prion diseases was performed in accordance with the criteria proposed by WHO.
[b] Mean age at onset in groups of prion diseases, at examination in controls.
[c] Abnormal high intensity signals in cerebral cortices or basal ganglia on diffusion-weighted (DWI) MRI of the brain.
[d] Determined by the presence of PK-resistant signal around 37 kDa.
[e] NE, not examined.
[f] NA, data not available.
[g] Two patients were included in both groups.
[h] Identical patient.

EXPERIMENTAL PROCEDURES

Analysis of Human Urine—Urine samples were collected from patients affected with prion diseases and control subjects under informed consent (Table I). A clinical diagnosis of prion disease was made by neurologists in order to follow the diagnostic criteria proposed by the World Health Organization (1). First morning urine samples were used whenever possible.

Protein was isolated from urine as previously described by Shaked *et al.* (7) with minor modifications. After dialysis and sedimentation by ultracentrifugation, the pellets obtained from 15 ml of urine were re-suspended in 30 μl of PBS (pH 7.4) containing 0.5% Nonidet P-40 and 0.5% sodium deoxycholate, instead of STE buffer containing 2% Sarkosyl, and digested with 40 μg/ml proteinase K (PK) (Roche Diagnostics) at 37 °C for 1 h. In several samples, urine protein was re-suspended in 2% Sarkosyl STE buffer prior to PK digestion as described by Shaked *et al.* (7). Western blot analysis was performed using monoclonal antibodies 3F4 at 1:10,000 (Signet Laboratories), 6H4 at 1:5,000 (Prionics, Switzerland), or 3O8 at 1:1,000 (Cayman Chemical) followed by incubation with donkey-derived anti-mouse IgG (AP192A, Chemicon), goat-derived anti-mouse IgG (H + L) (S372B, Promega), or the $F(ab')_2$ fragment of rabbit-derived anti-mouse IgG (710–4520, Rockland) and development in a chemiluminescent substrate (CDP Star or ECL-Plus, Amersham Biosciences). Some blots were labeled with PrP2B, rabbit-derived polyclonal antibody raised against PrP89-103, followed by incubation with donkey-derived anti-rabbit IgG (AP182A, Chemicon). In some blots, incubation with primary antibody was omitted for the experimental purpose.

Coupled Liquid Chromatography and Mass Spectrometry (LC/MS) Analysis of Protease-resistant Proteins—A PK-resistant signal of 37 kDa on a SDS-polyacrylamide gel was cut out and transferred to a clean, siliconized Eppendorf tube. In-gel digestion was performed as previously described (8). After an overnight incubation of gels with trypsin at 37 °C, the digested protein was extracted twice with 50% acetonitrile, 50% trifluoroacetic acid and concentrated by vacuum centrifugation. An LC/MS analysis was performed using the QSTAR XL system (Applied Biosystems) and MAGIC 2002 liquid chromatography (Michrom BioResourse). The obtained protein masses were queried against entries for all species in the SwissProt data base using the Mascot Search program offered by Matrix Science.[2]

N-terminal Protein Sequencing—PK-resistant protein was obtained from the urine of patients sCJD 4, 5, and 7 and dural graft-associated CJD-1 as described above, or from urine of other patients as described by Shaked *et al.* (7). After separation of the protein samples by 12% mini SDS-PAGE gels (Bio-Rad), proteins were transferred onto Immobilon-P^{SQ} transfer membrane (Millipore). PK-resistant bands visualized by Coomassie Brilliant Blue staining were cut out and stored at 4 °C until the sequencing procedure. N-terminal protein sequencing by automated Edman degradation was performed using the Procise 491cLC protein sequencer (Applied Biosystems), as previously described (9). N-terminal sequencing proceeded for 13 to 23 cycles. The obtained amino acid sequences were queried against entries for all species in the SwissProt data base using the FASTA search program offered by GenomeNet.[3]

Assays of Protease Resistance of Outer Membrane Proteins (OMP)—OMPs were isolated from *Klebsiella pneumoniae* and *Salmonella typhimurium* as previously described (10) with minor modifications. In brief, cells harvested from overnight cultures in Super Broth medium were recovered by centrifugation. After washing with 10 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, cells were broken by sonication. Unbroken cells were eliminated and cell envelopes were recovered at 100,000 × *g* for 1 h. After solubilization in 10 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 0.5% Nonidet P-40, 0.5% deoxycholate for 30 min at 25 °C, insoluble OMPs were recovered by ultracentrifugation at 100,000 × *g* for 1 h. OMPs were resuspended in 0.5% Nonidet P-40, 0.5% deoxycholate in PBS (pH 7.4), or 2% Sarkosyl STE buffer and digested with 40 μg/ml of PK at 37 °C for 1 h, under the same conditions as urine proteins. In parallel, 15 μg of ovalbumin was digested with PK as a control.

Binding of OMPs to $F(ab')_2$ Fragment of Immunoglobulin—Isolated OMPs were separated by 12% SDS-PAGE and transferred onto the

[2] Available at www.matrixscience.com.
[3] fasta.genome.ad.jp.

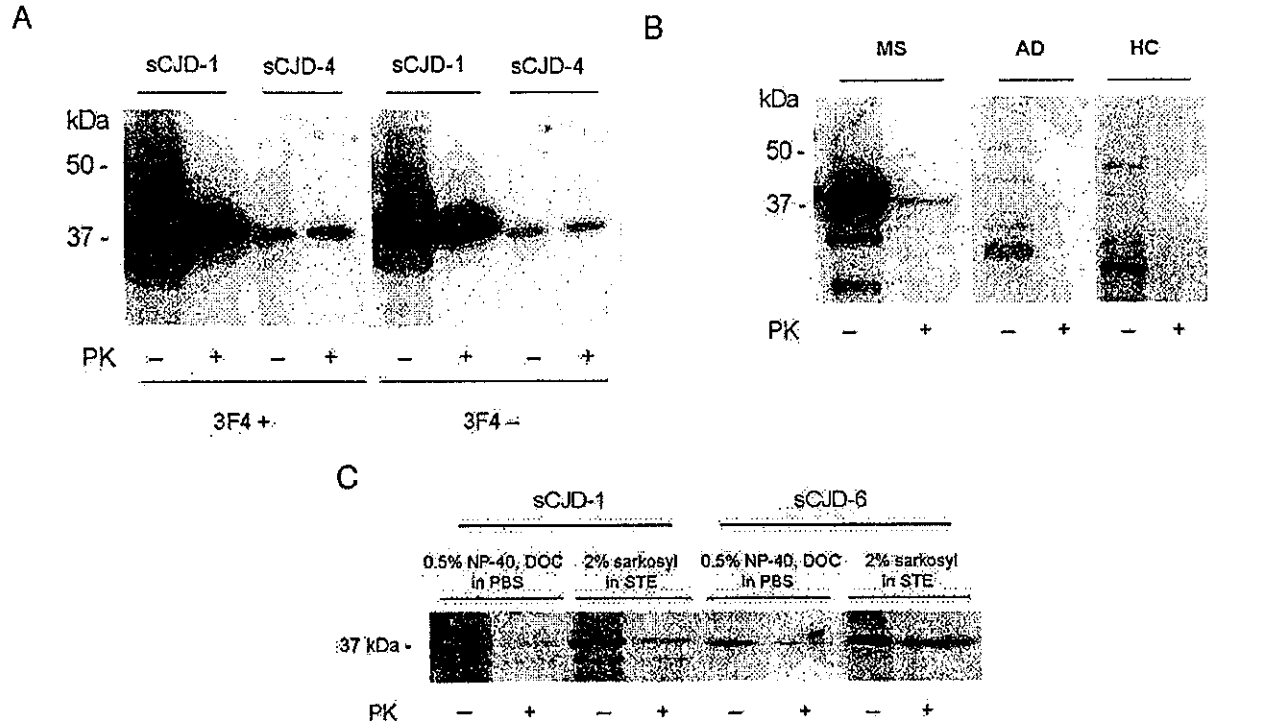

FIG. 1. **Western blot analysis of urine proteins.** Protein obtained from 15 ml (*A* and *B*) or 7.5 ml (*C*) of urine was applied in each lane. *PK*, digestion with proteinase K. *A*, urine proteins were drawn from sCJD-1 and sCJD-4 patients. Patient ID number corresponds to that in Table III. Proteins were re-suspended in PBS (pH 7.4) containing 0.5% Nonidet P-40 and 0.5% deoxycholate (*DOC*). The blot was probed with 3F4 monoclonal antibody followed by incubation with secondary antibody AP192A (*left panel*) or incubated with secondary antibody omitting the probing with 3F4 (*right panel*). *B*, urine proteins were obtained from patients with multiple sclerosis (*MS*) bearing a urethral catheter, with Alzheimer's disease (*AD*), and from a healthy control subject (*HC*). Proteins were re-suspended in PBS (pH 7.4) containing 0.5% Nonidet P-40 and 0.5% deoxycholate. A PK-resistant signal was detected in one patient affected with MS. The membranes were probed with 3F4 monoclonal antibody followed by incubation with AP192A. *C*, urine proteins were drawn from sCJD-1 and sCJD-6 patients, and re-suspended in PBS (pH 7.4) containing 0.5% Nonidet P-40 and 0.5% deoxycholate or in STE buffer containing 2% Sarkosyl as described by Shaked *et al.* (7). The blot was incubated with a rabbit-derived $F(ab')_2$ fragment of anti-mouse IgG.

polyvinylidene difluoride membrane. To determine whether OMPs bind to antibodies via the Fc region of immunoglobulin, the membrane was incubated with the $F(ab')_2$ fragment of anti-mouse IgG conjugated with alkaline phosphatase (710–4520, Rockland) after blocking with 5% nonfat milk. The membrane was then developed in a chemiluminescent substrate (CDP Star, Amersham Biosciences).

RESULTS

PK-resistant Protein in the Urine of Humans Affected with Prion Diseases Directly Reacted with Secondary Antibodies—The results of urine protein examination and patient and control characteristics are summarized in Table I. We examined the PK sensitivity of urine proteins of patients affected with prion diseases (n = 54), healthy controls (n = 23), and disease control patients with (n = 20) and without dementia (n = 23). Clinical durations between disease onset and urine collection were 1.5 to 72 months in prion diseases. 14-3-3 protein in cerebrospinal fluid was frequently positive in sporadic CJD (93.5%, 29/31) and dural graft-associated CJD (100%, 3/3). Abnormal high intensity signals in the cerebral cortices or basal ganglia were observed in the majority of patients with sporadic CJD (75.0%, 15/20). Most patients carried methionine homozygosity at codon 129 in the prion protein gene (PRNP), except for one case that was affected with Gerstmann-Sträussler-Scheinker syndrome. No patients carried lysine polymorphisms at codon 219 in the PRNP.

Three kinds of signals migrating around 37, 28, and 22 kDa were observed after PK treatment of the urine. PK-resistant signals of 37 kDa were prominent and observed in all positive cases, whereas the other two signals were usually faint and not always observed in all the positive cases. The signals of 28 kDa were also observed in controls after digestion with PK, suggesting that it represented a nonspecific signal because of PK itself. PK-resistant signals around 32 kDa, detected by Shaked *et al.* (7) in CJD patients, were not observed in the present study. Therefore, we decided to utilize the 37-kDa signal as a PK-resistant protein in urine in this study. PK-resistant protein signals of 37 kDa were detectable in 70.4% (38/54) of the patients affected with prion diseases, whereas 3% (2/66) of the control subjects were positive for PK-resistant signals. The PK-resistant signal was not detectable in healthy controls or diseased controls with dementia (Table I and Fig. 1*B*).

Although PK-sensitive and -resistant signals were detectable by labeling with 3F4 (Fig. 1*A*, *left panel*), 6H4, 3O8, or PrP2B (data not shown), these signals were also detectable with anti-mouse IgG antibody alone, omitting the incubation with 3F4 (Fig. 1*A*, *right panel*). This phenomenon was observed in all cases (11 cases; sporadic CJD, one case; dura-associated CJD) tested and reproducible using three kinds of anti-mouse IgG antibodies (AP192A, Chemicon; S372B, Promega; and 710–4520, Rockland) and an anti-rabbit IgG antibody (AP182A, Chemicon) (data not shown).

To examine the possible influence of assay conditions on the detection of PK-sensitive or -resistant signals, urine proteins were re-suspended prior to PK digestion in 2% Sarkosyl STE buffer as described previously by Shaked *et al.* (7) or in 0.5% Nonidet P-40, 0.5% deoxycholate, PBS buffer. As shown in Fig. 1*C*, 37-kDa signals were similarly detectable in both assay conditions, indicating that the difference of the assay conditions did not influence the detection of these signals.

Contamination of Urine with Bacterial Outer Membrane Proteins—To characterize the PK-resistant protein of 37 kDa on Western blot analysis, the bands from the urine of three pa-

TABLE II
List of proteins detected by LC/MS analysis
Protein masses were queried against entries for all species in the SwissProt database. Patient's ID correspond to that in Table III.

Patient ID	Significant hits		Peptides matched
	Protein identification	Species	
sCJD-1[a]	Outer membrane protein C precursor	*K. pneumoniae*	11
	Outer membrane protein C, chain A	*K. pneumoniae*	8
	Outer membrane protein C precursor	*S. typhimurium*	3
sCJD-2	Outer membrane protein C precursor	*E. coli*	13
	Outer membrane protein C precursor	*E. coli* O157:H7	13
	Outer membrane protein C precursor	*S. typhimurium*	4
	Translation initiation factor IF-2	*Geobacillus stearothermophilus*	3
	Outer membrane protein S2 precursor	*S. typhimurium*	2
	Outer membrane protein F precursor	*S. typhimurium*	2
sCJD-3	Outer membrane protein C precursor	*E. coli* O157:H7	16
	Outer membrane protein C precursor	*E. coli* O6	11
	Outer membrane protein C precursor	*S. typhimurium*	5
	Outer membrane protein C precursor	*K. pneumoniae*	4
	Outer membrane protein (fragment)	*Sodalis glossinidius*	2
	Outer membrane protein S1 precursor	*S. typhimurium*	2
	Outer membrane protein S2 precursor	*S. typhimurium*	2
	Outer membrane protein F precursor	*S. typhimurium*	2
	Glial fibrillary acidic protein homolog	*Carassius auratus*	2
	Elongation factor P-like protein	*S. typhimurium*	1

[a] sCJD, sporadic CJD.

tients were purified from the SDS-polyacrylamide gel and prepared for LC/MS as previously described (8). One of these patients was diagnosed as probable sporadic CJD and the others were pathologically definite sporadic CJD. Protein mass analysis using LC/MS demonstrated that the major component of the PK-resistant signal was the OMPs of bacteria such as *Escherichia coli, K. pneumoniae,* and *S. typhimurium* (Table II). No molecules of human origin, including PrP and immunoglobulin, were detected with any significance. Fig. 2 demonstrated the results of LC/MS analysis of a patient (sCJD-2).

We performed an N-terminal sequencing analysis to confirm the results of the LC/MS more quantitatively. It revealed that prominent PK-resistant signals in the urine of these three patients and all other patients examined (10/10) consisted of a mature chain of OMPs (Table III). Neither PrPs nor immunoglobulins were detected.

As described above, two other minor signals migrating around 28 and 22 kDa were observed on SDS-PAGE after PK treatment in the urine of some patients and controls. The N-terminal protein sequence analysis revealed that the signal at 28 kDa corresponded to the fragment of PK used for the assay and another signal at 22 kDa corresponded to the fragment of OMPs.

OMPs Are Resistant to PK—To evaluate PK sensitivity, OMPs were isolated from overnight cultured *K. pneumoniae* or *S. typhimurium* in Super Broth medium. After digestion with PK, a considerable amount of OMPs remained undigested and migrated around 37 kDa on SDS-PAGE (Fig. 3*A*, *fourth* and *sixth lanes*), whereas ovalbumin was completely digested under the same conditions (Fig. 3*A*, *second lane*). The electrophoretic mobility of PK-resistant OMPs was similar to that of the PK-resistant urine protein isolated from a patient affected with sporadic CJD (Fig. 3*A*, *seventh lane*).

OMPs Reacted with the F(ab') Portion of Immunoglobulins—To evaluate if OMPs bind to the Fc region of immunoglobulins like protein A, a cell wall component of *Staphylococcus aureus*, one of the gels was blotted onto a polyvinylidene difluoride membrane to perform Western blot analysis. As shown in Fig. 3*B*, OMPs bound to the $F(ab')_2$ fragment of anti-mouse IgG, indicating that OMPs bind to immunoglobulins in a manner that is different from that of protein A with immunoglobulins. This observation as well as PK resistance of OMPs was not influenced by the difference in assay conditions (Fig. 3*C*).

DISCUSSION

In the present study, we found that the PK-resistant protein was frequently detected in the urine of patients affected with prion diseases. However, the LC/MS and N-terminal protein sequencing analysis revealed that the majority of PK-resistant proteins in the urine of patients, which migrated around 37 kDa on SDS-PAGE and reacted non-specifically with several secondary antibodies, comprised OMPs of bacteria such as *E. coli, K. pneumoniae,* and *S. typhimurium*, the popular causative agents for urinary tract infections. This finding indicated that bacterial contamination of urine might cause false-positive results in the assay for detecting $UPrP^{Sc}$.

It is known that urinary tract infections associated with urethral catheterization is the most common nosocomial infection and is often asymptomatic. In this study, the majority of patients affected with prion diseases were already bearing catheters because of severe deterioration of intellectual and motor functions as they were suspected to be suffering from prion diseases. Furthermore, two of the diseased control patients with positive PK-resistant urine protein were also bearing persistent or intermittent urethral catheters. One patient suffered from neurogenic bladder because of multiple sclerosis and another was long bedridden because of cerebral infarction. The signal intensity decreased after PK digestion in a patient affected with multiple sclerosis (Fig. 1*B*), whereas it was not significant in patients affected with sporadic CJD (Fig. 1*A*). Differences in bacterial species or growth conditions in urine between the cases might cause such a variation in PK sensitivity of OMPs. On the other hand, all diseased controls affected with Alzheimer's disease or cerebrovascular dementia were outpatients; therefore, they were thought to be at lower risk of urinary tract infections. These circumstances strongly supported that possible bacterial contamination resulted in the detection of confusing PK-resistant protein in urine.

OMPs are 36- to 39-kDa membrane spanning proteins that form channels in the outer membranes of Gram-negative bacteria. The primary and secondary structures of OMPs are well conserved in *Enterobacterial* species containing 16-stranded antiparallel β barrels to form channels (11). Biochemically,