

P3 施設におけるプリオン感染サルへの治療薬静脈内投与・採血 及び脳脊髄採取

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研究要旨 クロイツフェルト・ヤコブ病などを含むプリオン病は、多くの治療研究がなされているが、未だ有効な治療法が確立されていない。低分子シャペロン P092 塩をプリオン感染させたマウスに投与したところ、有意な寿命の延命効果が見られたことから、抗プリオン治療薬として有望されている。そこで、ヒトに近いサルを用いたプリオン病モデルによる P092 の評価をおこなうこととした。プリオン病のひとつである牛海綿状脳症（BSE）プリオンに感染したサルを 3 群、1) 感染 10 ヶ月目の発症前から投与を開始した群（発症前投薬群）、2) 感染 16 ヶ月目の発症後から投薬を開始した群（発症後投薬群）、2) 非投薬群各 2 頭に分け、臨床経過の比較を行った。その結果、非投薬群と比較して、発症前投薬群の 2 頭及び発症後投薬群の 1 頭において、臨床症状の明らかな遅延が認められた。P092 は、動物種やプリオン株に関係なく、抗プリオン効果を有することが示唆された。

A. 研究目的

低分子シャペロン P092 塩をプリオン（福岡 1 株）感染マウスに腹腔内投与すると、有意な延命効果が示されている。本研究では、BSE プリオン

（C-BSE）に感染したサルにおいても P092 が、抗プリオン効果を有するのか、その有効性と安全性を評価するために、C-BSE 感染ウシ脳乳剤を接種、発症したカニクイザルの脳乳剤（サル馴化 BSE プリオン）を脳内接種した C-BSE 感染ザルを 3 群に分け、発症前投薬群、発症後投薬群、非投薬群間で臨床経過の比較を行った。

B. 研究方法

1) 供試動物

新日本科学で繁殖された雄カニクイザル 6 頭（1.3～2.3 歳）を用いた供試動物は、医薬基盤研究所霊長類医科学研究センターの P3 実験動物施設のアイソレーター内ステンレス製ケージにおいて馴化、飼養後、感染実験を開始した。アイソレーター内環境は室温 23-27°C、相対湿度 50-60%、12 時間照明（7 時～19 時）に設定し、アイソレーターの全面及び側面は透明になっており、他のアイソレーター内で飼養されているサルの体動、声を感じることでできる環境とした。また、ストレス軽減のためにケージ内にコングトイ等を入れた。飼料は固形飼料（CMK-2; CLEA Japan, Inc.）70 g とリンゴ 100 g を 1 日 1 回給餌した。

2) 接種方法

塩酸ケタミンとキシラジンの混合麻酔下のカニクイザルの頭部を剃毛し、イソジンで消毒後、頭皮膚切開し、左側頭部頭蓋骨に直径 2 mm の穿孔部を作製し、C-BSE（定型 BSE JP/8 和歌山）感染ウシ脳乳剤を脳内接種・発症したカニクイザル（#7）の 10% 脳乳剤 0.2 mL を線条体に注入した。注入後、皮膚を縫合し、手術日より 3 日間抗生物質及び鎮痛剤を筋肉内投与をした。

3) 材料採材方法

接種後、定期的に血液、尿、唾液及び脳脊髄液（CSF）を採取した。採血は塩酸ケタミン麻酔下で大腿静脈より行った。CSF は塩酸ケタミン麻酔下で背部剃毛し、イソジンで消毒後、第 3～5 腰椎椎間より採取した。

安楽死は塩酸ケタミン筋肉内投与による鎮静後、ペントバルビタール静脈内投与による深麻酔下において、放血後過剰のペントバルビタール投与を行った。安楽死後、脳及び主要臓器の組織の一部を摘出し、凍結保存及びホルマリン浸漬を行った。

4) P092 投薬方法

C-BSE 接種サルを 3 群に分けた。I) 発症前投薬群：2 頭（#3, #4）は、発症前の接種 10 ヶ月目より P092 溶液を毎週 10 mg/kg で伏在静脈または撓側皮静脈より点滴静注した（100 mL/h）。接種

13ヶ月目より隔週 10 mg/kg に、接種 14ヶ月目より隔週 2 mg/kg に、接種 16ヶ月目より毎週 2 mg/kg に投薬を変更した。II)発症後投薬群：2頭 (#1, #5) は、発症前投薬群と同様の投薬スケジュールで接種 10ヶ月目より生理食塩水の点滴静注を行い、発症後の接種 16ヶ月目より P092 溶液を毎週 2 mg/kg で点滴静注を開始した。III) 非投薬群：2頭 (#2, #6) は、発症前投薬群と同様の投与スケジュールで接種 10ヶ月目より生理食塩水の点滴静注を開始した。

4) MRI 撮像

P3 動物実験区域内で管理されている動物は生存したまま管理区域外への搬出ができないため、MRI 撮像は安楽死直後に行った。安楽死したサルは密封コンテナに封じ込めて MRI 室に搬送した。発症したカニクイザルは塩酸ケタミンにより鎮静後、ヘパリンを静脈内注射し全身血液の凝固防止を行った後、ペントバルビタールナトリウム過剰投与により安楽死を行った。アクリル製密封型コンテナに動物を保定して MRI 撮像室への移動を行った。MRI 撮像は 3T MRI 装置 (MAGNETOM Allegra [Siemens 社]) を用いた。カニクイザルの脳撮像に際し、空間分解能・解像度を上げるために撮像視野を小さくし、高い S/N を持つ撮像条件を設定し、ヒト用ヘッドコイルの CP 型コイルを用いて T1-3D、T2、プロトン強調画像、Flair 画像を撮像した。

(倫理面への配慮)

C-BSE 接種サルはすべて改良型の P3 アイソレータケージ内に収容した。本アイソレータはサル類が社会的動物であることを考慮して、アイソレータ内で視覚、聴覚による相互の社会的コミュニケーションを可能とするよう改良した。また、材料採取においては麻酔下において実施した。臨床症状発現後は症状に応じて、健康状態を維持すべく給餌方法の対応及び輸液療法等による維持管理を行った。

安楽死は塩酸ケタミンによる鎮静後、過剰量のペントバルビタールナトリウム静脈内投与により行った。

動物実験の実施に当たっては、「動物の愛護及び管理に関する法律」、「実験動物の飼養及び保管に関する基準」、「厚生労働省の所管する実施機関における動物実験棟の実施に関する基本指針」を遵守し、医薬基盤研究所動物実験委員会の承認を得て行った。病原体の取扱については、医薬基盤研究所バイオセーフティー委員会の承認を得て行った。

C. 研究結果

1) 投薬方法の変更

発症前投薬群では、感染 10ヶ月目より P092 溶液を毎週 10 mg/kg で点滴静注を始めたが、投薬 3ヶ月目より、2頭とも点滴ルートの静脈及びその血管周辺組織の硬化に伴い、点滴ルートの確保が難しくなったため、投薬 4ヶ月目より投与を隔週に変更した。また、手の運動機能障害も観られ、投薬 4ヶ月目より投薬量を 2 mg/kg に変更した。投薬量を減らした結果、静脈及びその周辺の硬化は回復し、静脈の点滴ルートの確保が出来るようになった。その後、投薬に伴う有害事象がなかったため、投薬 6ヶ月目より投薬量は変えず毎週投薬に変更した。発症後投薬群の毎週 2 mg/kg 及び非投薬群の生理食塩水の点滴静注では、有害事象は起こらなかった。

2) 臨床症状

感染 15~16ヶ月目より、非投薬群及び発症後投薬群の計 4頭が発症し始めた。その時点で発症前投薬群の 2頭 (#3, #4) は、臨床症状はほとんど呈していなかった。その後、非投薬群 2頭 (#2, #6) 及び発症後投薬群の 1頭 (#1) の臨床症状は悪化し、予後不良により、3頭とも感染 19ヶ月目に安楽死を行った。発症の遅延が認められた発症前投薬群及び発症後投薬群の 1頭に関しては、その後、臨床発現が緩徐に進行した。

3) 脳 MRI 画像解析

安楽死直後の MRI 撮像において、発症した 3頭とも脳室拡張による脳の委縮の所見と、Flair 画像では大脳皮質辺縁、海馬、小脳で高信号域が認められた (図 1)。

D. 考察

C-BSE 感染カニクイザルを用い、P092 の有効性と安全性を評価した。感染 15~16ヶ月目より、非投薬群 (#2, #6) 及び発症後投薬群 (#1, #5) で発症し始めたが、その時点で発症前投薬群 (#3, #4) はほとんど発症しておらず、少なくとも発症半年前からの投薬により、発症の遅延効果が示唆された。発症後投薬群の内、1頭 (#5) は発症直後から投薬を開始したが、発症前投薬群と同様に発症の遅延効果が示唆された。しかしながら、発症後投薬群のもう 1頭 (#1) は投薬によるに効果があまり観られず、非投薬群と同じ臨床経過を呈した。これは投薬を開始した時期が発症直後ではなく、発症後 1ヶ月ほど過ぎ、抗プリオン効果を示せなかったものと推察された。従って、投薬時期が重要と考えられる。発症の遅延が認められた発症前投薬群 (#3, #4) 及び発症後投薬群の 1頭 (#5)

に関しては、その後、臨床発現が緩徐に進行したことから、P092 は完全に発症を抑制することは難しいことが示唆された。

今後、病理解析や経時的に採取した体液類サンプルの解析等を通じ、P092 投薬による発症遅延を裏付けるデータを出していく必要がある。

当初、P092 の毎週 10 mg/kg 点滴静注では、点滴ルートである静脈の血管壁及びその血管周辺の組織が硬化する事象が生じたが、その後、投薬量を 2 mg/kg に減らした結果、回復したことから、投薬量による一過性の有害事象と推察された。なお、別のサルへの P092 単回投与実験により、2 mg/kg 隔週投与でも十分な脳内濃度を維持されることが示唆されている。

P092 をプリオン感染マウスに腹腔内投与すると、有意な延命効果が示されていたが、C-BSE 感染カニクイザルにおいても、投薬群と非投薬群の臨床経過の比較より P092 が抗プリオン効果の有効性が示唆された。

E. 結論

C-BSE 発症カニクイザルへの発症前 P092 投薬により、発症を遅らせる効果が示唆された。従って、P092 は、動物種やプリオン株に関係なく、抗プリオン効果を有することが示唆された。P092 の抗プリオン製剤としての臨床応用が期待される。

F. 健康危険情報

特になし。

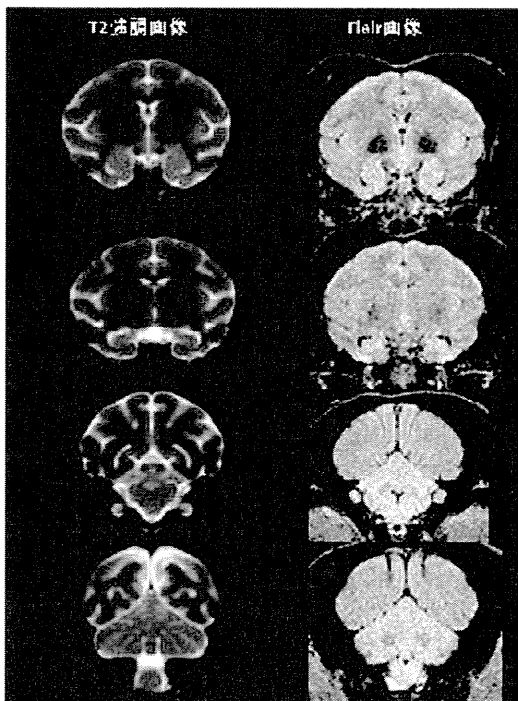
G. 研究発表

特になし。

H. 知的財産の出願・登録状況

1. 特許取得
なし
2. 実用新案登録
なし
3. その他
なし

A



B

C
D

図1、プリオン病発症カニクイザルの安楽殺直後のMRI画像
 各パネルの左側はT2強調画像、右側はFlair画像。(A) 非感染正常カニクイザル脳 (B) 発症後投薬群 (#1) 発症カニクイザル脳。(C) 非投薬群 (#2) 発症カニクイザル脳。(D) 発症後投薬群 (#6) 発症カニクイザル脳。

[III] 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌 (英文原著)

1	発表者氏名	Honda RP, Kei-ichi Yamaguchi, Kuwata K						
	論文タイトル名	Acid-induced Molten Globule State of a Prion Protein: Crucial Role of Strand 1-Helix 1-Strand 2 Segment						
	発表誌名	The Journal of biological chemistry	巻号	289(44)	ページ	30355-30363	出版年	2014
2	発表者氏名	Hattori T, Orimo S, Hallett M, Wu T, Inaba A, Azuma R, <u>Mizusawa H</u>						
	論文タイトル名	Relationship and factor structure in multisystem neurodegeneration in Parkinson's disease.						
	発表誌名	Acta Neurol Scand	巻号	130(6)	ページ	347-53	出版年	2014
3	発表者氏名	Nishina T, Numata J, Nishina K, Yoshida-Tanaka K, Nitta K, Piao W, Iwata R, Ito S, Kuwahara H, Wada T, <u>Mizusawa H</u> , Yokota T						
	論文タイトル名	Chimeric antisense oligonucleotide conjugated to α -Tocopherol						
	発表誌名	Mol Ther Nucleic Acids.	巻号	4	ページ	220	出版年	2015
4	発表者氏名	Nakamura Y, Ae R, Takumi I, Sanjo N, Kitamoto T, Yamada M, <u>Mizusawa H</u>						
	論文タイトル名	Descriptive Epidemiology of Prion Disease in Japan: 1999-2012.						
	発表誌名	J Epidemiol.	巻号	25(1)	ページ	8-14	出版年	2015
5	発表者氏名	Qina T, Sanjo N, Hizume M, Higuma M, Tomita M, Atarashi R, Satoh K, Nozaki I, Hamaguchi T, Nakamura Y, Kobayashi A, Kitamoto T, Murayama S, Murai H, Yamada M, Mizusawa H						
	論文タイトル名	Clinical features of genetic Creutzfeldt-Jakob disease with V180I mutation in the prion protein gene.						
	発表誌名	BMJ Open	巻号	4(5)	ページ	e004968	出版年	2014
6	発表者氏名	Akasaka K, Maeno A, Murayama T, Tachibana H, Fujita Y, Yamanaka H, Nishida N, Atarashi R:						
	論文タイトル名	Pressure-assisted dissociation and degradation of "proteinase K-resistant" fibrils prepared by seeding with scrapie-infected hamster prion protein.						
	発表誌名	Prion	巻号	8(4)	ページ	314-318	出版年	2014
7	発表者氏名	Sano K, Atarashi R, Ishibashi D, Nakagaki T, Satoh K, Nishida N						
	論文タイトル名	Conformational properties of prion strains can be transmitted to recombinant prion protein fibrils in real-time quaking-induced conversion.						
	発表誌名	J Virol	巻号	88(20)	ページ	11791-11801	出版年	2014
8	発表者氏名	Yu Mizuno, Yasuhisa Sakurai, Izumi Sugimoto, Keiko Ichinose, Shoichiro Ishihara, Nobuo Sanjo, Hidehiro Mizusawa, Toru Mannen						
	論文タイトル名	Delayed leukoencephalopathy after carbon monoxide poisoning presenting as subacute dementia						
	発表誌名	Internal Medicine	巻号	53	ページ	1441-1445	出版年	2014
9	発表者氏名	Fumiko Furukawa, Satoru Ishibashi, Nobuo Sanjo, Hiroshi Yamashita, Hidehiro Mizusawa						
	論文タイトル名	Serial magnetic resonance imaging changes in sporadic Creutzfeldt-Jakob disease with valine homozygosity at codon 129 of the prion protein gene						
	発表誌名	JAMA Neurology	巻号	71	ページ	1186-1187	出版年	2014

10	発表者氏名	Temu Qina, Nobuo Sanjo, Masaki Hizume, Maya Higuma, Makoto Tomita, Ryuichiro Atarashi, Katsuya Satoh, Ichiro No						
	論文タイトル名	Clinical features of genetic Creutzfeldt-Jakob disease with V180I mutation in the prion protein gene						
	発表誌名	BMJ Open	巻号	4	ページ	e004968	出版年	2014
11	発表者氏名	Yosikazu Nakamura, Ryusuke Ae, Ichiro Takumi, Nobuo Sanjo, Tetsuyuki Kitamoto, Masahito Yamada, Hidehiro Mizu						
	論文タイトル名	Descriptive epidemiology of prion disease in Japan: 1999-2012						
	発表誌名	Journal of Epidemiology	巻号	25	ページ	8月14日	出版年	2015
12	発表者氏名	Kokoro Ozaki, Nobuo Sanjo, Kinya Ishikawa, Miwa Higashi, Takaaki Hattori, Naoyuki Tanuma, Rie Miyata, Masaharu Hayashi, Takanori Yokota, Atsushi Okawa, Hidehiro Mizusawa						
	論文タイトル名	Elevation of 8-hydroxy-2'-deoxyguanosine in the cerebrospinal fluid of three patients with superficial siderosis						
	発表誌名	Neurology and Clinical Neuroscience	巻号		ページ	in press	出版年	2014
13	発表者氏名	Kimura N, Okabayashi S, Ono F.						
	論文タイトル名	Dynein dysfunction disrupts β -amyloid clearance in astrocytes through endocytic disturbances.						
	発表誌名	Neuroreport.	巻号	7;25(7)	ページ	514-20	出版年	2014
14	発表者氏名	Murayama Y, Masujin K, Imamura M, Ono F, Shibata H, Tobiume M, Yamamura T, Shimozaki N, Terao K, Yamakawa Y,						
	論文タイトル名	Ultrasensitive detection of PrP(Sc) in the cerebrospinal fluid and blood of macaques infected with bovine spongiform encephalopathy prion.						
	発表誌名	J Gen Virol.	巻号	95(Pt 11)	ページ	2576-88.	出版年	2014

雑誌 (和文総説)

1	発表者氏名	能勢裕里江、水澤英洋						
	論文タイトル名	プリオン病						
	発表誌名	感染症診療-生涯教育シリーズ-87-	巻号	第143巻特別号	ページ	415-417	出版年	2014
2	発表者氏名	三條伸夫、水澤英洋						
	論文タイトル名	VII. プリオン病.						
	発表誌名	神経感染症を極める アクチュアル脳・神経疾患の臨床	巻号		ページ	278-285	出版年	2014
3	発表者氏名	三條伸夫、水澤英洋						
	論文タイトル名	感染症関連ガイドラインと使用法の注意 プリオン病.						
	発表誌名	神経感染症を極める アクチュアル脳・神経疾患の臨床	巻号		ページ	352-354	出版年	2014
4	発表者氏名	佐藤克也, 新 竜一郎, 西田教行						
	論文タイトル名	プリオン病の新しい診断法						
	発表誌名	Dementia Japan	巻号	28(2)	ページ	189-196	出版年	2014
	発表者氏名	三條伸夫						

5	論文タイトル名	プリオン病						
	発表誌名	神経内科研修ノート	巻号		ページ	372-375	出版年	2014

書籍

なし

[IV] 研究成果の刊行物・別刷

Acid-induced Molten Globule State of a Prion Protein

CRUCIAL ROLE OF STRAND 1-HELIX 1-STRAND 2 SEGMENT*

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Background: The oligomerization mechanism of a prion protein is not fully understood.

Results: We found an acid-induced molten globule state (A-state) as a pre-oligomer state, in which the Strand 1-Helix 1-Strand 2 segment was unfolded.

Conclusion: The A-state formation is the initial step of the oligomerization.

Significance: This work may offer a clue for understanding the prion's pathogenic conversion mechanism.

The conversion of a cellular prion protein (PrP^C) to its pathogenic isoform (PrP^{Sc}) is a critical event in the pathogenesis of prion diseases. Pathogenic conversion is usually associated with the oligomerization process; therefore, the conformational characteristics of the pre-oligomer state may provide insights into the conversion process. Previous studies indicate that PrP^C is prone to oligomer formation at low pH, but the conformation of the pre-oligomer state remains unknown. In this study, we systematically analyzed the acid-induced conformational changes of PrP^C and discovered a unique acid-induced molten globule state at pH 2.0 termed the "A-state." We characterized the structure of the A-state using far/near-UV CD, 1-anilino-8-naphthalene sulfonate fluorescence, size exclusion chromatography, and NMR. Deuterium exchange experiments with NMR detection revealed its first unique structure ever reported thus far; *i.e.* the Strand 1-Helix 1-Strand 2 segment at the N terminus was preferentially unfolded, whereas the Helix 2-Helix 3 segment at the C terminus remained marginally stable. This conformational change could be triggered by the protonation of Asp¹⁴⁴, Asp¹⁴⁷, and Glu¹⁹⁶, followed by disruption of key salt bridges in PrP^C. Moreover, the initial population of the A-state at low pH (pH 2.0–5.0) was well correlated with the rate of the β -rich oligomer formation, suggesting that the A-state is the pre-oligomer state. Thus, the specific conformation of the A-state would provide crucial insights into the mechanisms of oligomerization and further pathogenic conversion as well as facilitating the design of novel medical chaperones for treating prion diseases.

Prion diseases (1, 2) are a group of fatal neurodegenerative diseases that includes Creutzfeldt-Jakob disease and kuru in humans, as well as scrapie and bovine spongiform encephalopathy in animals. The conversion of the cellular prion protein (PrP^C)² to its pathogenic conformational isoform (PrP^{Sc}) is a crucial event in the pathogenesis of prion diseases (1, 2). PrP^C contains three α -helices (Helix 1 (H1), Helix 2 (H2), and Helix 3 (H3)), where H2 and H3 are connected by a disulfide bridge between Cys¹⁷⁹ and Cys²¹⁴, and a small β -sheet (Strand 1 (S1) and Strand 2 (S2)) (3). In contrast, PrP^{Sc} is mainly composed of a β -strand structure, as revealed by Fourier transform infrared spectroscopy (4), and it was reported that the infectious particles in prion diseases (PrP^{Sc}) include oligomers (5, 6). Thus, the β -sheet formation and oligomerization process may be a crucial step during the formation and/or stabilization of PrP^{Sc}. To understand and further regulate these complex processes, it would be beneficial to clarify the conformational characteristics of the pre-oligomer state that may exist immediately before the β -sheet and oligomer formation.

Initially, β -rich oligomers were reported to be formed at a mildly acidic pH (pH 2.5–5.0) in the presence of denaturant (7–10) or at a higher temperature (11), and recently it was also reported that they were generated at a highly acidic pH (pH 2.0) without denaturant (12, 13). Unfortunately, the detailed physicochemical studies at pH 2.0 were mainly on the salt-induced aggregates (13–16), and the monomer state was only observed at the pH range from 5.2 to 3.0, in which a large chemical shift perturbation was observed in the C-terminal region of H2 and the H2-H3 loop (residues 185–195) (17). Thus, overall conformational characteristics of PrP^C at a highly acidic pH within the monomer ensemble essentially remain unknown, especially at the residue level resolution.

In the present study, we systematically analyzed the acid-induced conformational change in mouse prion protein fragment 90–231 (mPrP(90–231)) by reducing the pH down to 2.0 within the monomeric state and detected a previously unre-

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² The abbreviations used are: PrP^C, cellular prion protein; PrP^{Sc}, prion protein pathogenic isoform; mPrP, mouse prion protein; H1–H3, Helix 1–3, respectively; S1 and S2, Strand 1 and 2, respectively; ANS, 1-anilino-8-naphthalene sulfonate; HSQC, heteronuclear single quantum coherence.

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ported thermodynamically stable state of the PrP^C. Far-UV CD, near-UV CD, size exclusion chromatography, 1-anilino-8-naphthalene sulfonate (ANS) fluorescence, and NMR revealed that the conformational properties of this state were similar to those of the acid-induced molten globule state ("A-state") (18). We subsequently performed a conformational analysis of the A-state using deuterium exchange coupled with NMR and found that the S1-H1-S2 segments were almost completely unfolded, whereas the H2-H3 segment was still weakly protected. The A-state in this report is the only monomeric intermediate ever reported thus far, which is characterized by its markedly high tendency to β -rich formation. The A-state is easily aggregated into β -rich oligomer at pH 2.0 by increasing the ionic strength. Moreover, the initial population of the A-state at low pH (2.0–5.0) was well correlated with the rate of the β -rich oligomer formation, suggesting that the A-state is the pre-oligomer state. We further discuss the detailed conformational properties of the A-state based on pH titration analysis, which provides insights into the detailed mechanism of the oligomerization process.

EXPERIMENTAL PROCEDURES

Acid Titration—A recombinant mPrP(90–231) with an N-terminal linker containing His₆ was prepared according to a protocol described previously (19, 20).

In the acid titration experiments, the pH was adjusted by slowly adding drops of 0.1–12 M HCl to the native mPrP(90–231) solution, which contained 20 mM sodium acetate at pH 4.6 at 20 or 37 °C. 10 and 70 μ M of mPrP(90–231) were used for the far- and near-UV CD measurements, respectively. 10 μ M mPrP(90–231) solution and 100 μ M ANS were used for ANS fluorescence analysis. Far-/near-UV CD was monitored by a quartz cell with a light path of 1 or 10 mm using an AVIV model 215s spectropolarimeter (AVIV Biomedical, Lakewood, NJ) and by ANS fluorescence using a Hitachi F-7000 fluorescence spectrophotometer (Hitachi High-Tech, Tokyo, Japan) with excitation at 385 nm and emission at 400–600 nm.

The acid titration data obtained from the far-/near-UV CD observation were fitted globally using the theoretical curve generated by assuming a two-state model (Equations 1 and 2) (21) with the nonlinear least squares methods using the software Igor Pro (Wave Metrics Inc., Lake Oswego, OR),

$$\alpha(\text{pH}) = \frac{(\alpha_N + m_N \times \text{pH})}{1 + K_{NA}} + \frac{(\alpha_A + m_A \times \text{pH}) K_{NA}}{1 + K_{NA}} \quad (\text{Eq. 1})$$

$$K_{NA} = \frac{[A]}{[N]} = 10^{\Delta\nu_{H^+} \times (\text{pH}_{\text{mid}} - \text{pH})} \quad (\text{Eq. 2})$$

where α_N , m_N , α_A , and m_A are the intercepts and the slopes of the native state and A-state baselines, respectively; pH_{mid} and $\Delta\nu_{H^+}$ represent the midpoint of the transition and the steepness of the transition (or the number of bound or released H⁺), respectively; and K_{NA} represents the equilibrium constant of the N-to-U transition.

The secondary structure contents of PrP^C were estimated from far-UV CD spectra using SELCON3 (22). The pK_a values for charged residues were predicted theoretically from the

three-dimensional structure of native PrP^C (Protein Data Bank entry 1AG2) based on the continuum electrostatics model using H⁺ (23).

Urea Titration at Variable pH Values Monitored by Far-UV CD—A solution of mPrP(90–231) with a protein concentration of 10 μ M was titrated with 10 M urea solution containing the same protein and buffer at 20 °C and monitored by far-UV CD. The buffers utilized were 20 mM glycine (pH 2.0–3.1), 20 mM sodium citrate (pH 3.2–3.9), or 20 mM sodium acetate (pH 4.0–4.6). The urea concentration was measured using the refractive index values. The far-UV CD spectra were measured according to the same protocol described above. The mean residue ellipticities $[\theta]$ obtained as a function of the urea concentrations were fitted using the theoretical curve generated by the following two-state model (Equations 3 and 4),

$$[\theta] = \frac{[\theta]_N}{1 + K_{NU}} + \frac{[\theta]_U \times K_{NU}}{1 + K_{NU}} \quad (\text{Eq. 3})$$

$$K_{NU} = \frac{[U]}{[N]} = \frac{\ln(-\Delta G_{NU} + m_{NU} \times \text{Urea})}{RT} \quad (\text{Eq. 4})$$

where $[\theta]_N$ and $[\theta]_U$ represent the mean residue ellipticity of the native (N) and unfolded state (U) at 0 M urea, respectively; K_{NU} represents the equilibrium constant of the N-to-U transition; m_{NU} represents the m value (or cooperativity) of the transition; and ΔG_{NU} represents the Gibbs free energy difference between the N and U states in the absence of urea.

Size Exclusion Chromatography—The hydrostatic properties of mPrP(90–231) were studied using a gel filtration column, TSKgel G3000SW, which was connected to an ÄKTA chromatography system (GE Healthcare). Further, pH 4.6 buffer (pH 4.6, 20 mM sodium acetate buffer, and variable NaCl concentrations) and pH 2.0 buffer (pH 2.0, 20 mM glycine, and variable NaCl concentrations) were used for the native and A-state conditions, respectively. The column was equilibrated using the buffer with 4 column volumes before the experiment. To protect the column, the injected solutions were passed through 0.2- μ m filters before their application. All of the experiments were performed at a flow rate of 0.5–1.0 ml/min at 25 °C. The molecular weight was estimated from a standard curve obtained using standard proteins.

NMR Measurements and Analysis—To acquire both the ¹H one-dimensional NMR and ¹⁵N-¹H HSQC data, an ¹⁵N-uniformly labeled mPrP(90–231) solution was prepared at a concentration of 0.5 mM at pH 4.6 or 2.0 in 5 mM acetate-d₄ buffer with 95% H₂O, 5% D₂O. The NMR spectra were recorded at 20 °C using an Avance 600 spectrometer at Gifu University.

For the line shape analysis, the peaks in HSQC spectra were assigned based on the chemical shifts for mPrP(23–231) and mPrP(121–231) (3, 24). An exponential window function with 8-Hz line broadening was used for this analysis. The ¹H one-dimensional slices were extracted at the maximum of each peak from the ¹⁵N-¹H HSQC spectrum, and the intensity as a function of the proton frequency was fitted to a Lorentzian function (Equation 5),

$$I(\nu) = \frac{C_0}{T_2^* \times \left(\left(\frac{1}{T_2^*} \right)^2 + (\nu - w_0)^2 \right)} \quad (\text{Eq. 5})$$

where T_2^* , w_0 , and C_0 represent the apparent T_2 relaxation time, the frequency of resonance, and the normalization constant, respectively.

Deuterium Exchange Coupled with NMR—We performed a deuterium exchange experiment according to a procedure described previously (25, 26). Briefly, for the hydrogen exchange study of the native state, lyophilized mPrP(90–231) was dissolved in 20 mM sodium acetate at pD 4.6 with 100% D_2O (27) at a final protein concentration of 5.0×1.0^{-1} mM. This solution was incubated at 20 °C for up to 4 weeks, and the HSQC spectra were acquired at the desired incubation times. For the hydrogen exchange study of the A-state, lyophilized mPrP(90–231) was prepared in 20 mM glycine at pD 2.0 with 100% D_2O solution at a concentration of 50 μM and stored at 20 °C. Samples were collected after incubation periods (0 min, 7.5 min, 15 min, 1 h, 2 h, 4 h, 8 h, and 16 h) and washed three times with 20 mM sodium acetate at pD 4.6 and with 100% D_2O buffer prepared using Millipore Ultra 3K concentrators at 5 °C. Further, the samples were concentrated until the final concentrations of mPrP(90–231) and pD were $5.0 \pm 1.0 \times 1.0^{-1}$ mM and 4.6 ± 0.2 , respectively, for acquiring the HSQC spectra.

We assigned 51 peaks on the HSQC spectra at pD 4.6 (*i.e.* 3 of 3 for S1 (residues 129–131), 6 of 7 for H1 (residues 147–153), 3 of 3 for S2 (residues 161–163), 7 of 20 for H2 (residues 174–193), 20 of 24 for H3 (residues 203–226), and 12 of 36 for the hydrophobic core (residues 132–146, 154–160, 194–202, and 227–231)). The volume of the assigned peaks was obtained using the software TOPSPIN3 (Bruker), and values were normalized as per the protein concentration. The volumes of the normalized peaks as a function of time were fitted to a single exponential curve using the nonlinear least squares methods to obtain the rate constants of deuterium exchange (k_{ex}). The protection factors were obtained based on the ratio of the intrinsic rate constants of random coil (k_{int}) to those observed experimentally (k_{ex}). The k_{int} value was predicted using SPHERE, which was developed by Prof. Heinrich Roder's laboratory at Fox Chase Cancer Center.

Kinetics of β -Rich Oligomer Formation—The mPrP(90–231) was incubated with a protein concentration of 70 μM , 10 mM glycine, 10 mM sodium acetate at various pH values (2.0–5.0) in an ionic strength of 160 mM (~ 150 mM NaCl) at 37 °C, and the β -rich oligomer formation was monitored using size exclusion chromatography. The fraction of monomer was calculated by dividing the area of the monomeric peak (9.5–11.5 ml) by the total area in the elution profile. The fraction of the monomer as a function of time was fitted using the theoretical curve generated by assuming a second-order reaction (Equation 6),

$$[M](t) = \frac{1}{1/([M]_0 - [M]_\infty) + k_{\text{app}} \times t} + [M]_\infty \quad (\text{Eq. 6})$$

where $[M]_0$ and $[M]_\infty$ represent the fraction of monomer at time zero and infinity, respectively, and k_{app} represents the apparent rate constant.

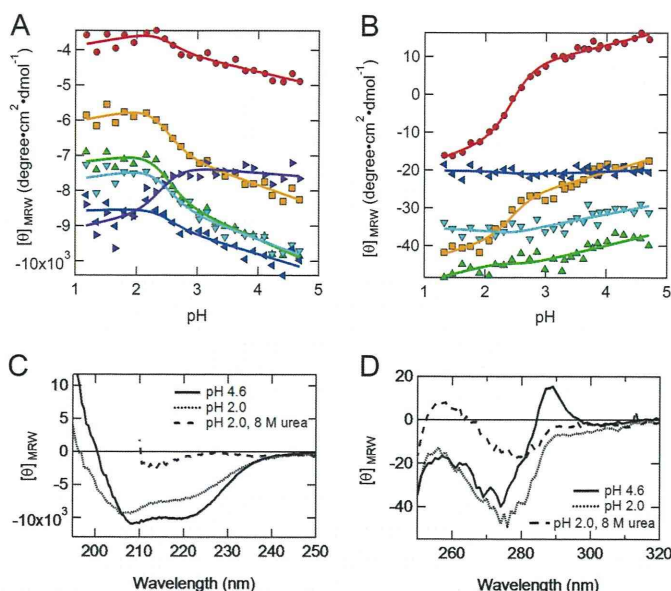


FIGURE 1. Acid titration monitored using far-/near-UV CD. *A*, acid titration monitored as the mean residue ellipticity at 230 (red circles), 225 (orange squares), 220 (green triangles), 215 (light blue inverted triangles), 210 (blue left-pointing triangles), and 205 nm (purple right-pointing triangles). *B*, acid titration monitored as the mean residue ellipticity at 288 (red circles), 281 (orange squares), 274 (green triangles), 267 (light blue inverted triangles), and 260 nm (blue left-pointing triangles). The solid curves represent the global fits to the two-state unfolding model (Equations 1 and 2). The pH_{mid} and $\Delta\nu_{\text{H}^+}$ values obtained were 2.44 ± 0.06 and 2.28 ± 0.47 , respectively. *C*, far-UV CD spectra of the native state at pH 4.6 (solid curve), non-native state at pH 2.0 (dotted curve), and unfolded state at pH 2.0 with 8 M urea (dashed curve). *D*, near-UV CD spectra of the native state at pH 4.6 (solid curve), non-native state at pH 2.0 (dotted curve), and unfolded state at pH 2.0 with 8 M urea (dashed curve).

RESULTS

Molten Globule-like Partially Unfolded State at pH 2.0—We performed acid titrations to examine the conformational changes of PrP^C when the pH ranged from 4.6 to 1.2 at 20 °C. The mean residue ellipticities as a function of the pH in both the far-UV (Fig. 1A) and near-UV CD region (Fig. 1B) were fitted well by the theoretically generated curves according to Equations 1 and 2, which assumed a two-state transition with a pK_{mid} of 2.44 ± 0.06 and a $\Delta\nu_{\text{H}^+}$ of 2.28 ± 0.47 . All of the curves shown in Fig. 1, *A* and *B*, were fitted simultaneously using the common variables, pK_{mid} and $\Delta\nu_{\text{H}^+}$ in Equations 1 and 2 except for α_{N} , m_{N} , α_{A} , and m_{A} . Overall, the transition of PrP^C obtained by reducing the pH from 4.6 to 1.2 can be described by a two-state model, which indicated the formation of a non-native state at pH 2.0. The far-UV CD spectrum indicated that the secondary structure content (α -helix (%), β -sheet (%), and turn (%)) estimated using SELCON3 for the non-native state at pH 2.0 is 26, 19, and 20%, whereas that of the native state at pH 4.6 is 39, 13, and 15% (Fig. 1C). Thus, the non-native state largely retains α -helical native-like secondary structure. In addition, the near-UV CD spectrum of the non-native state was distinct from that of both the native and unfolded state (Fig. 1D). For example, the peak at 288 nm in the non-native state was relatively close to that of the unfolded state, whereas the peak at 275 nm was relatively close to the native state (Fig. 1D), suggesting that the tertiary structures of the non-native state retained the characteristics of both the native and unfolded states.

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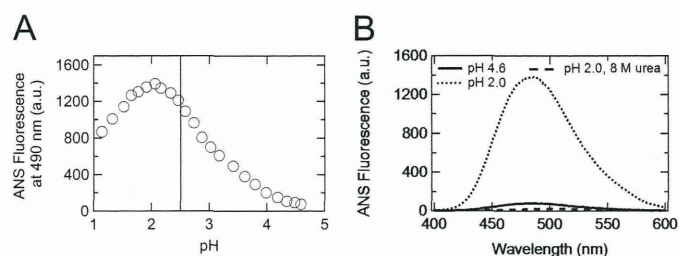


FIGURE 2. Acid titration monitored with ANS fluorescence. *A*, acid titration monitored with ANS fluorescence at 490 nm. The *perpendicular solid line* represents the midpoint of the two-state transition observed during the near- or far-UV CD monitoring. *B*, ANS fluorescence spectra of the native state at pH 4.6 (*solid curve*), non-native state at pH 2.0 (*dotted curve*), and unfolded state at pH 2.0, with 8 M urea (*dashed curve*). *a.u.*, arbitrary units.

The acid titrations were also monitored with ANS fluorescence (Fig. 2*A*). In contrast to the far-/near-UV CD, ANS fluorescence as a function of pH did not exhibit a two-state transition, partially because of the acid quenching of the fluorescence below pH 2.0 (Fig. 2*A*). Even above pH 2.0, it was poorly fitted to the two-state transition equation (data not shown), but it was clear that the ANS fluorescence at pH 2.0 was much higher than that of both the native and unfolded states (Fig. 2*B*), indicating the increased hydrophobic surface area after the formation of the non-native state at pH 2.0.

Overall, the non-native state at pH 2.0 was characterized by its “molten globule-like” properties (*i.e.* a native-like secondary structure (Fig. 1*C*) and increased hydrophobic surface area (Fig. 2*B*)) (18). In addition, the native-like tertiary structure was found to be retained relatively well, even in the non-native state at pH 2.0 (Fig. 1*D*), which was similar to the molten globule state of equine lysozyme (28). Thus, we conclude that the non-native state at pH 2.0 can be considered as an acid-induced molten globule state (A-state).

Furthermore, the NMR data supported the molten globule characteristics of the non-native state at pH 2.0. The ^1H one-dimensional NMR spectra at pH 2.0 indicated the loss of peaks in both the methyl and aromatic regions (Fig. 3, *A* and *B*), which are characteristic for the molten globule state (18). In ^{15}N - ^1H HSQC spectra of the prion protein at pH 2.0 (Fig. 3*D*), peaks at ~ 7.5 and ~ 9.0 ppm were almost absent but number of peaks at ~ 8.5 ppm were increased compared with the HSQC spectra at pH 4.6 (Fig. 3*C*). The varied peak intensities and shapes in HSQC at pH 2.0 indicate that the non-native state is not fully unfolded (Fig. 3*D*). Intriguingly, the apparent T_2 relaxation times of peaks in the N-terminal unfolded domain (residues 90–119) at pH 2.0 were approximately the same as those at pH 4.6 (Fig. 3*E*), suggesting that the flexibility in this domain is conserved in the A-state. Thus, the A-state probably maintains a monomer conformation, satisfying one of the prerequisites for molten globule states.

Low m Value and Stability of PrP^C in Highly Acidic pH Conditions—To investigate the stability of the PrP^C in acidic pH conditions, we next performed urea titration at various pH values by monitoring mean residue ellipticity at 220 nm ($[\theta]_{220}$). At the pH values tested, $[\theta]_{220}$ as a function of urea was well described by the two-state model (Fig. 4*A*). At mildly acidic pH levels (3.5–4.5), the m values were relatively independent of

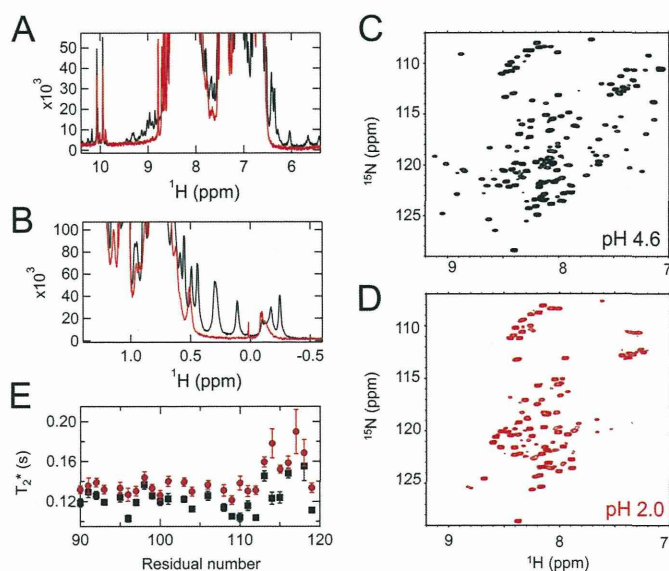


FIGURE 3. *A* and *B*, ^1H one-dimensional NMR spectra of the native state at pH 4.6 (*black*) and the A-state at pH 2.0 (*red*) in the methyl (*A*) and aromatic regions (*B*). *C* and *D*, ^{15}N - ^1H HSQC spectra of the native state (*C*) and the A-state (*D*). Most peaks from the C-terminal domain (7.0–7.8 or 8.6–9.0 ppm of ^1H) were lost in the A-state. *E*, apparent T_2 relaxation time of the amide protons in the N-terminal unfolded region (amino acids 90–119) in the native state (*black*) and A-state (*red*).

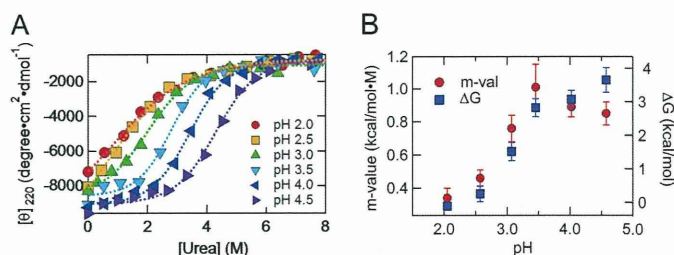


FIGURE 4. Urea titration monitored as the mean residue ellipticity at 220 nm. *A*, urea titrations at pH 4.6 (*purple right-pointing triangles*), 4.0 (*blue left-pointing triangles*), 3.5 (*light blue inverted triangles*), 3.1 (*green triangles*), 2.5 (*orange squares*), and 2.0 (*red circles*) were monitored as the mean residue ellipticity at 220 nm. The *dotted lines* represent the mean least squares fits to the two-state unfolding model (Equations 3 and 4). *B*, m value (*red circles*, left axis) and ΔG (*blue squares*, right axis) at various pH values obtained from Fig. 4*A*. Error bars, S.D.

pH, and they almost corresponded to the theoretical m values or 0.96 ± 0.15 , which were calculated based on the ΔSASA (where ΔSASA indicates changes in solvent-accessible surface area) between the native and unfolded states of PrP^C (29), implying that PrP^C forms a native structure under these conditions. However, at highly acidic pH (2.0–3.0), the m values were substantially low, indicating a significant decrease in cooperativity of unfolding. ΔG also decreased significantly in highly acidic pH conditions (Fig. 4*B*) and reached ~ 0.3 kcal/mol at pH 2.0. This change in m values and ΔG under highly acidic pH is well explained by the formation of the A-state (Fig. 1, *A* and *B*), which is generally characterized by low cooperativity of unfolding as well as low stability (18).

Measurement of the Molecular Size of the A-state by Size Exclusion Chromatography—Next, We performed size exclusion chromatography to investigate the molecular size of the A-state. We initially measured the elution volumes of native state at pH 4.6 in the presence of various concentrations of

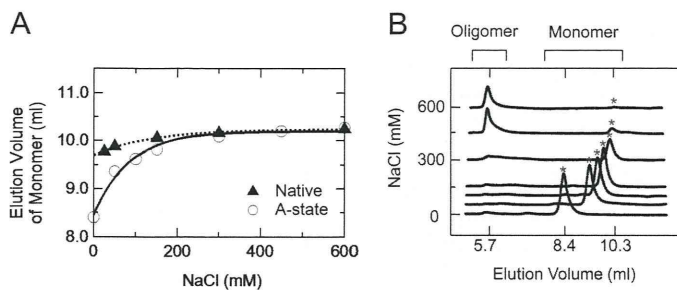


FIGURE 5. Size exclusion chromatography of native state and A-state with varying NaCl concentrations. *A*, elution volumes of native state at pH 4.6 (filled triangles) and the A-state at pH 2.0 (empty circles) as functions of the NaCl concentration. The elution volume as a function of NaCl was fitted to an exponential function, $y_0 + A \times \exp(\text{invTau} \times [\text{NaCl}])$, where y_0 , A , and invTau are $1.0 \pm 0.1 \times 10^1$, $-5.4 \pm 1.4 \times 10^{-1}$, and $7.4 \pm 0.6 \times 10^{-3}$, respectively, for the native state and $1.0 \pm 0.2 \times 10^1$, $-1.8 \pm 0.3 \times 10^0$, and $1.2 \pm 0.5 \times 10^{-3}$ for the A-state. *B*, elution profiles of the A-state at pH 2.0 with different NaCl concentrations. The gray asterisks represent the elution peaks of the A-state. The peak around ~ 5.7 ml represents the elution of the β -rich oligomer irreversibly transformed from the A-state (see also Fig. 7B).

NaCl (Fig. 5A). The elution volume of the native state were increased gradually by increasing the NaCl concentration. We estimated the elution volume of native state without NaCl as 9.7 ml (Fig. 5A), which corresponded to a molecular mass of 28 kDa. This mass is much larger than that predicted from the amide acid sequence in the folded domain (residues 90–231) (19 kDa), assuming a spherical shape because of the N-terminal unfolded domain of PrP^C (residues 90–120). Actually, after the NaCl concentration was increased to >300 mM, the elution volume almost reached a constant value of 10.3 ml, corresponding to a molecular mass of 19 kDa. The decrease in the apparent molecular mass can be explained by the decrease of electrostatic repulsions within the unfolded domain caused by the shielding effect of NaCl.

The elution volume of the A-state at pH 2.0 also increased gradually with increasing NaCl concentration (Fig. 5, *A* and *B*). We estimated the elution volume of the A-state without NaCl as 8.4 ml (Fig. 5A), which corresponded to a molecular mass of 65 kDa. This value is larger than that of the native state or 28 kDa; therefore, the A-state of PrP^C is significantly expanded rather than the native state just like a typical molten globule state (18). In contrast, the elution volume of the A-state at high NaCl concentrations was comparable with that of the native state (Fig. 5A), suggesting that the expansion in the A-state is caused by intramolecular electrostatic repulsions. Note that the secondary structural content was not affected by increasing NaCl concentration (data not shown); therefore, the change in the apparent molecular weight is not attributed to a conformational alternation of the A-state.

Structural Characterization of the A-state Based on Deuterium Exchange—To characterize the conformational properties of the A-state at amino acid resolution, we performed a deuterium exchange coupled with NMR detection at pD 2.0. ¹⁵N-uniformly labeled PrP^C was initially dissolved with pD 2.0 buffer and incubated with variable times and then returned into a native condition (pD 4.6) to assign the NMR peaks. Overall protection factor of the A-state was much less than those of the native state, by 4 orders of magnitude (Fig. 6, *A* and *B*, and Table 1). The protection factor profile (Fig. 6A and Table 1) showed

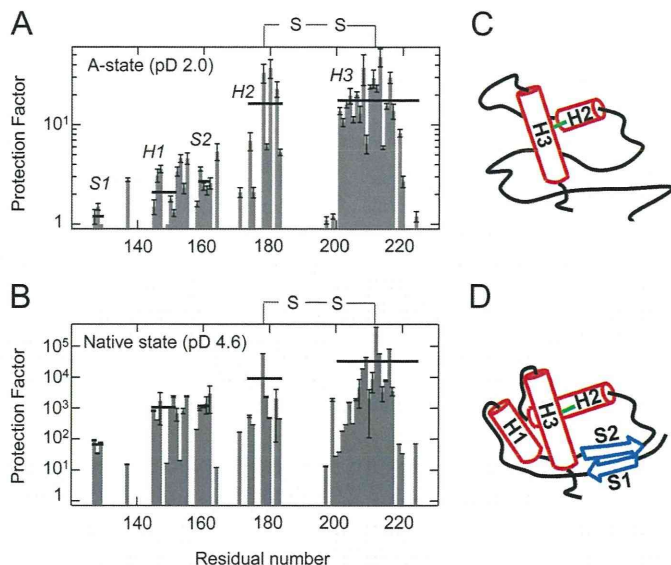


FIGURE 6. *A* and *B*, protection factors of the A-state at pD 2.0 (*A*) and native state at pD 4.6 (*B*). The solid horizontal lines represent the average protection factor within each secondary structure (S1, H1, S2, N-terminal H2, and H3). See also Table 1. *C*, schematic structure of the A-state, where the H2-H3 region forms a relatively stable folded structure (red solid rods), whereas the S1-H1-S2 region is highly unstable (black line). *D*, schematic structure of the native state based on the three-dimensional structure of PrP^C (Protein Data Bank entry 1AG2). H1, H2, and H3 are shown as red solid rods, and the antiparallel β -strands are shown as blue arrows. Error bars, S.D.

TABLE 1

Protection factors of each structure in the A-state and the native state

	A-state (pD 2.0)	Native state (pD 4.6)
Strand 1	1.2	66
Helix 1	2.1	1058
Strand 2	2.7	1171
Helix 2 ^a	16.3	>9056
Helix 3	17.6	$>32,650$
Tertiary hydrogen bond	2.7	561
Overall	10.2	$>14,422$

^a N-terminal H2 (amino acids 174–185).

that the amide protons of H2 (residues 176–185) and H3 (residues 203–226) were strongly protected as compared with those of S1 (residues 129–131), H1 (residues 147–153), and S2 (residues 161–163). We also observed weak protection factors of the protons even in the hydrophobic core regions (residues 139, 154–160, 164, 166, 173, and 199–202), suggesting the loose packing characteristics of the A-state. Therefore, it is considered that the A-state forms a molten globule-like structure, where the H2-H3 region is marginally stable while the S1-H1-S2 region is largely unstable. Notably, even in PrP^C at pH 4.6 (Fig. 6B and Table 1), the protection factors of the S1-H1-S2 region and hydrophobic core were 1 or 2 orders of magnitude less than those of the H2-H3 region. Fig. 6, *C* and *D*, depicts a model of conformational exchange between the A-state and native state structures.

The β -Rich Oligomer Formation at pH 2.0—The β -rich oligomer formation was observed at pH 2.0 with a high salt concentration (150 mM NaCl) at 20 °C. Immediately after the start of incubation, the secondary structure of the A-state was almost the same as that in pH 2.0 solution without salt (Fig. 7A), and most of the PrP^C formed the monomer (Fig. 7B). With a few h of incubation at 20 °C, the oligomerization and α -to- β transition occurred (Fig. 7, *A* and *B*). The secondary struc-

A-state of a Prion Protein

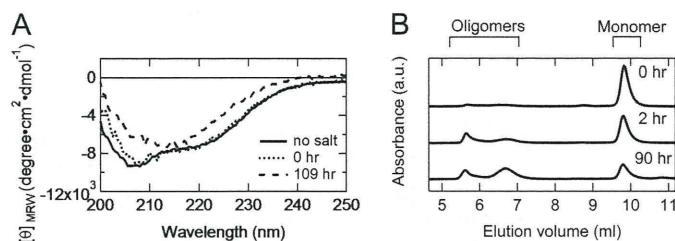


FIGURE 7. β -Rich oligomer formation at pH 2.0. *A*, far-UV CD spectra at pH 2.0 in the absence of 150 mM NaCl (solid curve) in the presence of 150 mM without incubation (dotted line) and in the presence of 150 mM after 4 days of incubation (dashed line). *B*, elution profiles after variable times of incubation at pH 2.0, 150 mM NaCl. The pH 2.0 buffer (pH 2.0, 20 mM glycine, 150 mM NaCl) was utilized for the elution buffer. This experiment was performed at 20 °C with a protein concentration of 10 μ M. *a.u.*, absorbance units.

ture content of the oligomer after 4 days of incubation, which was estimated using SELCON3 (α -helix (%), β -sheet (%), and turn(%)), was 16, 34, and 21% (Fig. 7A) in contrast to the α -rich A-state (26, 19, and 20%) (Fig. 1C). Thus, the A-state is stable and monomeric without added salt but is easily aggregated into the β -rich oligomer by increasing the NaCl concentration to 150 mM.

The Rate of β -Rich Oligomer Formation Was Strongly Correlated with the Initial Fraction of A-state—The oligomerization kinetics at various pH values (pH 2.0–5.0) was monitored with the change of the monomer fraction using size exclusion chromatography. This experiment was performed at 37 °C in the presence of \sim 150 mM NaCl, which mimics the physiological acidic conditions. The oligomerization kinetics at every pH value was approximately described by a second-order reaction (Equation 6) (Fig. 8A). It was clear that the oligomerization was gradually accelerated by lowering the pH from 5.0 to 2.0. This acceleration may be explained by the increase of the initial fraction of the A-state at low pH (Fig. 1, A and B) because the A-state has a tendency to form the β -rich oligomer (Fig. 7, A and B). In fact, the logarithm of the apparent rate constant (k_{app}) was linearly correlated with the logarithm of the initial fraction of the A-state ($R^2 = 0.98$) (Fig. 8B). Although the detailed mechanism underlying the oligomerization process is under investigation, this linear correlation strongly suggests that the A-state is a pre-oligomer state just prior to the oligomerization reaction. Thus, the A-state is considered to be a pre-oligomer state and to play a physiologically important role in the oligomerization process occurring under physiological acidic conditions, such as in the endosome.

DISCUSSION

Characterizations of the A-state Structure—In the present study, we identified the A-state of PrP^C at pH 2.0 and clarified its structure using far-/near-UV CD, ANS fluorescence, and deuterium exchange. The deuterium exchange analysis revealed that the S1-H1-S2 region of the A-state was significantly fragile (Fig. 6A). Previously, a synthetic H1 peptide was shown to retain its native-like structure even at pH 2.0, although its α -helicity was decreased by 41% compared with that at pH 4.5 (30). Thus, the S1-H1-S2 region may undergo rapid exchange between the folded and unfolded structures. In contrast, the H2-H3 region should retain a relatively stable folded structure because its backbone protection factors are

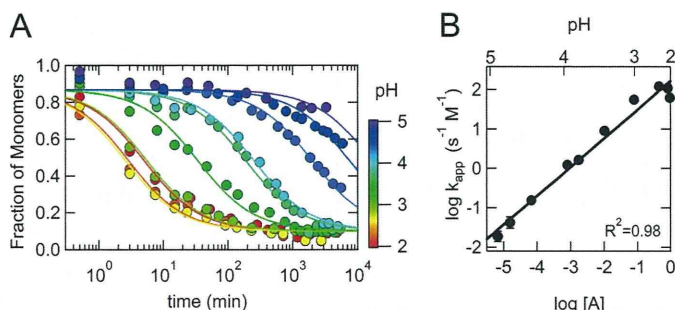


FIGURE 8. pH dependence of the oligomerization process. *A*, oligomerization process at various pH values (pH 2.0–5.0) monitored by the change of the monomer fraction using the size exclusion chromatography. The pH 4.6 buffer (pH 4.6, 20 mM sodium acetate, 150 mM NaCl) was utilized for the elution. The solid lines represent the least squares fits to a second-order reaction (Equation 6). $[M]_0$ and $[M]_{\infty}$ values (0.87 ± 0.01 and 0.10 ± 0.01 , respectively) were commonly used for the fitting. This experiment was performed at 37 °C with a protein concentration of 70 μ M in the presence of \sim 150 mM NaCl. *B*, the logarithms of the apparent rate constant (k_{app}) plotted against pH (top axis) and the logarithm of the initial fraction of the A-state (bottom axis), which was calculated using Equations 1 and 2 based on an acid-titration experiment at 37 °C (2.58 and 2.28 of pH_{mid} and $\Delta\nu_H$, respectively). The solid line represents the least squares fit to a linear function (0.73 ± 0.04 and 2.22 ± 0.11 of the slope and the intercept, respectively).

significantly high (Fig. 6A). The large difference in stability between the two regions may be attributed partly to the disulfide bond that connects H2 and H3 (Cys¹⁷⁹-Cys²¹⁴). Although the protection factors at the fully unfolded state have not been obtained for PrP^C, those for an unfolded β -lactoglobulin (31) around the disulfide bond region (Cys¹⁰⁶-Cys¹¹⁹) were shown in Fig. 5C in Ref. 31. Although the orders of magnitude of the protection factors in the unfolded β -lactoglobulin were similar to those of the A-state, residues with relatively high protection factors span only several residues from the Cys¹⁰⁶ or Cys¹¹⁹. On the other hand, in the A-state, residues with the high protection factors span more than 10 residues from Cys²¹⁴, as shown in Fig. 6A, suggesting that H3 regions are stabilized by interactions other than the disulfide bond, which may also stabilize the overall secondary structure of the A-state.

The A-state of PrP^C was also characterized based on its native-like tertiary structure (Fig. 1D) similar to the molten globule state of equine lysozyme (28). In the molten globule state of equine lysozyme, the native-like tertiary structure was located in the region where the backbone protection factors are relatively high. Thus, the native-like tertiary structure of the A-state may be localized mainly at the H2-H3 region. Moreover, the synthetic H2-H3 peptide retained its native-like tertiary structure (32), implying that this region can maintain its native structure almost independently of the structure of the S1-H1-S2 region.

Effects of the Protonation of Charged Residue(s) on A-state Formation—The acid titration experiments (Fig. 1, A and B) showed that the pH_{mid} and $\Delta\nu_{H+}$ values of the N-to-A transition were 2.44 ± 0.06 and 2.28 ± 0.47 at 20 °C, respectively, indicating that this transition is triggered by the protonation of two or three charged residues with pK_a values of \sim 2.5. We identified 4 carboxyl residues where the pK_a value corresponded to the pK_{mid} value of the N-to-A transition within an error of 1.0 using H⁺⁺ (23) (Asp¹⁴⁴, Asp¹⁴⁷, Glu¹⁵², and Glu¹⁹⁶) (Table 2). These residues are located on the interface

TABLE 2
Predicted pK_a values of the side chain carboxyl groups

Residue	pK_a
Asp ¹⁴⁴	2.37
Glu ¹⁴⁶	3.93
Asp ¹⁴⁷	2.45
Glu ¹⁵²	3.30
Asp ¹⁶⁷	3.61
Asp ¹⁷⁸	0.65
Glu ¹⁹⁶	2.09
Glu ²⁰⁰	4.07
Asp ²⁰²	0.64
Glu ²⁰⁷	3.66
Glu ²¹¹	3.63
Glu ²²¹	4.17

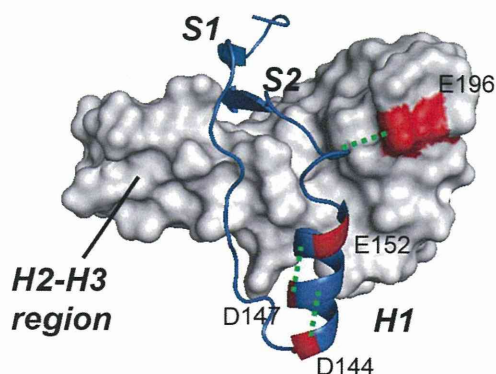


FIGURE 9. Charged residues whose protonations may trigger A-state formation. The charged residues with pK_a values of 2.5 ± 1.0 are shown in red in the three-dimensional structure of native PrP^C (Protein Data Bank entry 1AG2) (Asp¹⁴⁴, Asp¹⁴⁷, Glu¹⁵², and Glu¹⁹⁶). The salt bridges between these residues and neighboring residues are represented as green dotted lines (Asp¹⁴⁴–Arg¹⁴⁸, Asp¹⁴⁷–Arg¹⁵¹, and Arg¹⁵⁶–Glu¹⁹⁶). The S1-H1-S2 region is shown as a blue ribbon diagram, whereas the H2-H3 region is shown as a white surface diagram.

between the S1-H1-S2 and H2-H3 region (Glu¹⁹⁶) or within the S1-H1-S2 region (Asp¹⁴⁴, Asp¹⁴⁷, and Glu¹⁵²) (Fig. 9). Thus, protonation of these residues can destabilize the S1-H1-S2 region and initiate the N-to-A transition. Moreover, three of these residues probably form a salt bridge with the neighboring residues (Asp¹⁴⁴–Arg¹⁴⁸, Asp¹⁴⁷–Arg¹⁵¹, and Arg¹⁵⁶–Glu¹⁹⁶) (Fig. 9) (33), which is expected to contribute significantly to the stability of native PrP^C. Thus, the protonation of charged residue(s), especially Asp¹⁴⁴, Asp¹⁴⁷, and Glu¹⁹⁶, may trigger the N-to-A transition.

Biological Role of the A-state—The A-state is characterized by a high tendency to β -conversion (Fig. 7A) and oligomerization (Fig. 7B). Early studies suggested that PrP^C transformed into a β -rich partially unfolded state at acidic pH values in the presence of salt (9). However, subsequent extensive studies revealed that this state is not monomer but oligomer (8, 10, 13) and found that high ionic strength is required to convert an α -rich monomeric PrP^C into the β -rich oligomer (10, 13, 14). These studies coincide well with our current results, in which PrP^C forms the A-state or a stable α -rich monomer with low ionic strength (Fig. 7B), whereas it is rapidly converted into the β -rich oligomer by adding 150 mM NaCl (Fig. 7, A and B). In addition, we showed that the rate of oligomerization at low pH (pH 2.0–5.0) in the presence of salt was strongly correlated with the initial fraction of the A-state (Fig. 8, A and B). Thus, our current study suggests that the A-state is a pre-oligomer state at low pH and that the destabilization of the S1-H1-S2 region (Fig.

6, A and C), which may be triggered by the disruption of key salt bridges (Fig. 9), is crucial to understand the oligomerization process of PrP^C.

Interestingly, it has been suggested that critical interactions in H1 are involved in the pathogenic conversion mechanism (34), which has been further verified experimentally (35). An R148H mutation that disrupts the Asp¹⁴⁴–Arg¹⁴⁸ hydrogen bond causes symptoms similar to the sporadic Creutzfeldt-Jakob disease MV2 subtype (36). In addition, a E196K mutation is a phenotype of inherited prion disease, which typically presents a rapidly progressive dementia and ataxia (37). Thus, it is possible that the A-state, where these salt bridges are lost, is actively involved in the conversion mechanism. Actually, it has been suggested that the pathogenic conversion occurs at low pH in the endosome pathway (38). It might also be interesting to pursue whether or not the A-state corresponds to an intermediate state toward PrP^{Sc}, such as PrP* (39).

Although conformational change of the A-state into the β -rich oligomer was not clarified in the present study, the following two models were suggested as the initial process based on the A-state conformation (Fig. 6, A and C): (a) refolding of the highly unstable S1-H1-S2 region into a β -sheet structure and their subsequent intermolecular interactions (40) and (b) intermolecular interaction between the H2 and H3 regions, where the hydrophobic core is probably exposed to the solvent in the A-state because of the unstable S1-H1-S2 region (32, 41, 42). Recently, β -sheet conversion of residues 118–122 in the crystal structure of the human prion protein has been observed (43). Thus, mechanism a might be also plausible.

Drug Design—This information about the A-state structure could also facilitate drug design by stabilizing the PrP^C conformation and further regulation of the conversion process (44). The A-state of the PrP^C described in the present study shows that the salt bridge between Arg¹⁵⁶ and Glu¹⁹⁶ is crucial for maintaining the PrP^C conformation and for preventing further oligomerization. It should be noted that Glu¹⁹⁶ undergoes a slow fluctuation of the time scale between submilliseconds and milliseconds (45), which suggests that Glu¹⁹⁶ might be actively involved in a kinetic intermediate formation. In addition, Glu¹⁹⁶ is one of the major binding sites of GN8 (19). The interaction between Glu¹⁹⁶ and GN8 prevents the major structural breakdown of the PrP^C into the A-state. A medical chaperone (44) that stabilizes the PrP^C conformation should include Glu¹⁹⁶ in the binding region. The involvement of Asp¹⁴⁴ and Asp¹⁴⁷ in the binding site would further strengthen the anti-prion efficiency of the medical chaperone in terms of the structural stabilization of PrP^C.

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プリオン病診療ガイドライン 2014

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プリオン病及び遅発性ウイルス感染症に関する調査研究班

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プリオン病のサーベイランスと感染予防に関する調査研究班

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発行にあたって

このたび、厚生労働科学研究費補助金 難治性疾患等克服研究事業（難治性疾患克服研究事業）・『プリオン病及び遅発性ウイルス感染症に関する調査研究班』並びに『プリオン病のサーベイランスと感染予防に関する調査研究班』では、『プリオン病診療ガイドライン2014』を発行する運びとなりました。両研究班は協力してプリオン病の疫学、臨床病態、発症機序・分子病態を解明し、新たな診断法や治療法の開発等を行い、プリオン病を克服することを使命としております。その活動の1つに最新の診療ガイドラインの作成と提供があります。本ガイドラインはプリオン病を専門としない一般医師向けにプリオン病に関する最新の情報を提供し、本症に関する診療水準の向上をはかることを目的としております。

本ガイドラインは、『プリオン病及び遅発性ウイルス感染症に関する調査研究班』のプリオン分科会及び『プリオン病のサーベイランスと感染予防に関する調査研究班』のメンバーによって原案が執筆され、両研究班全体による討議を経て、更に関連学会のご意見やパブリックコメントを得て発行されたものです。関係各位のご尽力に心より感謝いたします。

プリオン病の診療ガイドラインにつきましては、2002年1月に厚生労働省・特定疾患対策研究事業『遅発性ウイルス感染に関する調査研究班』（主任研究者：北本哲之）によって発刊された『クロイツフェルト・ヤコブ病診療マニュアル・改訂版』があり、そこにはプリオン病の診療面ばかりでなく、感染防御についての項目が含まれていました。『プリオン病及び遅発性ウイルス感染症に関する調査研究班』は、感染防御については『プリオン病感染防御ガイドライン（2008年版）』（主任研究者：水澤英洋、編集責任者：黒岩義之）を発刊しており、本ガイドラインは診療面に特化した内容になっております。本ガイドラインは、プリオン病研究の飛躍的な進歩を踏まえて、プリオン病診療に関する最新の情報をなるべく分かりやすくコンパクトに盛り込むという編集方針のもとに作成されました。治療の項目では、近年、臨床試験が行われてきた治療法の推奨グレードを示しました。また、プリオン病患者・家族に対する心理社会的支援、研究班による診療支援の項目を設けました。プリオン病は難病中の難病ですが、有効な治療法が1日も早く確立されることを目指して、本態解明や治療法開発のための研究が急速に進行しています。

本ガイドラインは小冊子として、また研究班ホームページ (<http://prion.umin.jp/index.html>) 上に公表されます。本ガイドラインを第一線で臨床に携わる諸先生方のご診療に役立ていただければ誠に幸いに存じます。

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