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論文内容の要旨
Summary of thesis contents

The thesis consists of four chapters, **Chapter 1.** "General introduction," **Chapter 2.** "The use of spin desalting columns in DMSO-quenched H/D-exchange NMR experiments," **Chapter 3.** "The H/D-exchange kinetics of the *Escherichia coli* co-chaperonin GroES studied by 2D NMR and DMSO-quenched exchange methods," and **Chapter 4.** "Summary and future perspectives."

In Chapter 1, I describe general introduction about protein folding, molecular chaperones and hydrogen-exchange techniques. For proper biological functions of proteins, they have to fold into the native three-dimensional (3D) structures. How a protein folds from its primary structure into the native 3D structure has been a major research interest in the field of biophysical, biochemical and biomedical sciences. As the interior of biological cell is very much crowded, the cytoplasm does not serve as an ideal place for protein folding, and there exist machineries which assist protein to fold into the native state *in vivo*, and such machineries are called "molecular chaperones." Among all chaperones, the GroEL/GroES complex, found in *Escherichia coli* (*E. coli*), is one of the extensively studied molecular chaperones. The GroEL/GroES chaperonin complex is very huge with the co-chaperonin G roES of a molecular weight of 73 kDa (7 subunits) and GroEL of a molecular weight of 800 kDa (14 subunits), resulting in the megadalton chaperonin machinery. There are a number of studies on the GroEL/GroES complex, but the structural flexibility and fluctuations of the chaperonin complex remains to be understood. For free heptameric GroES and the GroES portion of the GroEL/GroES complex, however, it is quite feasible to characterize the structural fluctuations by the hydrogen/deuterium (H/D)-exchange methods combined with two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy, although we still need sophisticated new techniques to overcome low quality of the GroES spectra taken by the conventional NMR techniques. The use of the H/D-exchange methods can shed light on the structural flexibilities and dynamic behavior of the GroEL/GroES complex. In the H/D-exchange methods, the peptide amide protons are exchanged with solvent deuterons at every amino-acid residue, and the H/D-exchange reactions can be monitored as time-dependant changes in the amide-proton signals by NMR spectroscopy. With the help of observed H/D-exchange rate constants and predicted theoretical H/D-exchange rate constants, we can easily evaluate protection factors for the individual amino-acid residues, which ultimately gives us crucial information about the structural fluctuations and dynamics of the chaperonin complex. Hence, the general introduction of Chapter 1 gives brief descriptions of these aspects.

In Chapter 2, I describe a new method, which I developed for measurement of H/D-exchange kinetics, i.e., the use of spin desalting columns in dimethylsulfoxide (DMSO)-quenched H/D-exchange NMR experiments. The DMSO-quenched H/D-exchange (DMSO-QHX) method is a powerful method to characterize the

H/D-exchange behaviors of proteins and protein assemblies, and it is potentially useful for investigating non-protected fast-exchanging amide protons in the unfolded state. However, this method has not been used for studies on fully unfolded proteins in a concentrated denaturant or protein solutions at high salt concentrations. In all of the current DMSO-QHX studies of proteins so far reported, lyophilization was used to remove D_2O from the protein solution, and the lyophilized protein was dissolved in the DMSO solution to quench the H/D-exchange reactions and to measure the amide proton signals by 2D NMR spectra. The denaturants or salts remaining after lyophilization;thus, prevent the measurement of good NMR spectra. In this new method, I found that the use of spin desalting columns is a very effective alternative to lyophilization for the medium exchange from the D_2O buffer to the DMSO solution. In this method, the medium exchange by a spin desalting column took only about 10 min in contrast to an overnight length of time required for lyophilization, and the use of spin desalting columns has made it possible to monitor the H/D-exchange behavior of a fully unfolded protein in a concentrated denaturant. I analyzed H/D-exchange kinetics of the unfolded ubiquitin in 6.0 M guanidinium chloride and related results are discussed in Chapter 2.

In Chapter 3, I studied H/D-exchange reactions of peptide amide protons of free heptameric GroES complex. To map H/D-exchange kinetics, I used two different techniques: (1) 2D $^{1}H–^{15}N$ transverse-optimized NMR spectroscopy and (2) the DMSO-QHX method combined with conventional $^{1}H–^{15}N$ heteronuclear single quantum coherence (HSQC) spectroscopy. By using these techniques together with direct HSQC experiments, I quantitatively evaluated the exchange rates for 33 out of the 94 peptide amide protons of GroES and their protection factors, and for the remaining 61 residues, I obtained the upper and lower limits of the exchange rates. The protection factors of the most highly protected amide protons were on the order of $10^{6}–10^{7}$, and the values were comparable in magnitude to those observed in typical small globular proteins, but the number of the highly protected amide protons with a protection factor larger than 10^{6} were only 10, significantly smaller than the numbers reported for small globular proteins, indicating that the significant portions of free heptameric GroES are flexible and natively unfolded. The highly protected amino-acid residues with a protection factor larger than 10^{5} were mainly located in three β-strands that form the hydrophobic core of GroES, while the residues in a mobile loop (residues 17–34) were not highly protected. The protection factors of the most highly protected amide protons were orders of magnitude larger than the value expected from the equilibrium unfolding parameters previously reported, strongly suggesting that the equilibrium unfolding of GroES is more complicated than a simple two-state or three-state mechanism and may involve more than a single intermediate.

In Chapter 4, I summarize the present study and discuss future perspectives. Development of the spin-column technique was very useful to follow the H/D-exchange kinetics of free GroES and the GroEL/GroES complex, whose solutions

contained certain amounts of salts, as well as to study fully unfolded ubiquitin in 6.0 M guanidinium chloride. The H/D-exchange protections were mostly observed in strands B, C and D of free heptameric GroES, but the mobile loop region formed by residues 17–34 and the reverse turn formed by residues 49–52 were not highly protected against the H/D-exchange. Hence, these results of Chapters 2 and 3 are summarized in this chapter. In addition, I also describe preliminary results of the H/D-exchange kinetics of the GroES complexed with a single ring mutant (SR1) of GroEL in the presence of ADP. In this preliminary study of the SR1/GroES complex, I observed significant protections (protection factors of 10^5–10^6) for the amide protons in the mobile loop region (residues 17–34), indicating that the mobile loop is highly organized and plays a significant role when the co-chaperonin GroES forms a complex with SR1. Thus, the study in my thesis has overall clarified detailed changes in the dynamic behaviors of the GroES portion of the GroEL/GroES chaperonin complex.

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博士論文の審査結果の要旨

Summary of the results of the doctoral thesis screening

蛋白質の生物機能発現にその構造揺らぎは重要であり，蛋白質の構造揺らぎを調べる実験手法として水素/重水素（H/D）交換二次元NMR法がよく知られている。しかし，GroESのような蛋白質超分子複合体では良好なNMRスペクトルが得られないため，横緩和最適化スペクトル（TROSY）法やジメチルスルホキシド（DMSO）停止H/D交換法を用いるなどの工夫が必要とされる。本論文では，これらの手法を用いて大腸菌コシャペロニンGroESの構造揺らぎを調べ，構造揺らぎと機能発現との関係を明らかにするとともに，GroESの実効熱力学的安定性の評価からアンフォールディング転移の分子機構についても議論している。

本論文は全4章より成る。第1章は全体の緒言であり，蛋白質のフォールディング問題，大腸菌シャペロニン複合体GroEL/GroESを始めとする分子シャペロンの細胞内フォールディングにおける役割，H/D交換反応と蛋白質構造揺らぎとの関係，二次元NMRスペクトル法などについて述べられている。

第2章では，出願者自身によって開発された，スピン脱塩カラムを利用した新規なDMSO停止H/D交換二次元NMR法について述べられている。この手法を用い，6 M塩酸グアニジン中で完全変性したユビキチンのアミドプロトンの水素交換反応測定に成功し，その結果がまとめられている。

第3章では，第2章で記述されたH/D交換二次元NMR法，および，H/D交換TROSY法を用いて，天然条件下における遊離GroES 7量体のペプチド・アミドプロトンのH/D交換反応を追跡した結果についてまとめられている。GroESの94個のアミドプロトンの内33個のプロトンの交換速度を定量的に求め，これらの保護因子（protection factor (P_f)）の決定に成功した。他の61個のアミドプロトンについてはP_f の上限値が求められた。これらの結果から，以下が結論されている。(1) 最も強く保護されたアミドプロトンのP_f は10^6–10^7であり，これは通常の球状蛋白質のP_f 値と同程度であるが，P_f 値10^6 以上の残基数は僅か10であり，7量体GroESのかなりの部分が大きな構造揺らぎを持ち天然変性状態にある。(2) P_f 値10^5以上の強く保護されたアミノ酸残基は三本のβストランドにより形成される疎水性コアに局在しているのに対し，残基17–34の可動性ループは保護されていない。(3) 最も強く保護された残基のP_f から求められた，アンフォールディング転移の実効自由エネルギー変化（ΔG_{eff}）は，GroESの変性に関する実験的な先行研究結果から期待されるΔG_{eff} 値よりも顕著に大きく，GroESのアンフォールディング転移が二つ以上の中間体を伴う複雑な過程であることを示している。

第4章では，全体のまとめと今後の展望について述べられているが，SR1/GroES/ADP複合体のGroES部分のH/D交換反応を追跡した予備的な結果についても記述されている（SR1はGroELの単一リング変異体を示す）。複合体形成によって可動性ループが強く保護され，このループがGroESのGroELによる認識にとって重要であることが明らかとなった。

以上のように，本研究は，蛋白質超分子複合体である GroES の構造揺らぎを H/D 交換二次元 NMR 法によって初めて解き明かしたものであり，生体分子科学の基盤的な研究として高く評価される。本研究の実験，解析，結果の取り纏めから論文作成の一連の過程において，出願者が主体的に行っていることが認められる。また，本論文の内容の一部は，

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出願者が筆頭著者の英文学術論文二報として国際的な学術雑誌に公表済みである。したがって，本審査委員会は，出願論文が博士(理学）の授与に値すると全員一致で判断した。