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論　文　要　旨(博士)

論文題目	1分子DNA操作技術を用いたDNA-タンパク質間相互作用の解析

(要旨 1,200字程度)

近年の生化学・分子生物学・遺伝学・細胞生物学の進展により、DNA-タンパク質間相互作用について徐々に判明してきている。しかしこれまでに得られている知見は多数の分子を解析対象とした生化学的・分子生物学的・遺伝学的解析がほとんどであり、詳細が解明できていない部分も数多く残されている。一方で1分子観察・1分子計測技術の進展も著しく、生体高分子の1分子観察・1分子操作が可能になり、生化学反応が起きているその場で、しかもリアルタイムで観察できるようになってきた。電気泳動など従来行われてきた解析手法では、100万分子以上を解析の対象とせざるを得ないため、得られた結果は多数の分子の挙動を平均したものになる。従って、このようにして得られた結果から個々の分子の挙動やその分布を知ることはできない。それに対し1分子計測による解析では、多分子解析では隠れてしまう個々の分子の挙動やその分布を知ることができるので、従来法では得られない生体高分子の生化学的機能に関する新たな知見を得ることができると考えられる。そこで本論文では、1分子DNA操作技術・1分子観察技術に基づくDNA-タンパク質間相互作用の1分子動態解析を行った。まずDNA消化酵素exonucleaseIIIが1分子DNAを消化していく様子を1分子直接観察した。ここではシリコーンラバーを用いて微細流路を作製し、光ピンセットを用いた1分子DNAの操作と反応開始制御を可能とする1分子観察系を構築した。exonucleaseIIIのように比較的消化速度が遅く、またdistributiveな酵素に対しても1分子DNA蛍光観察によるDNA-タンパク質間相互作用の解析が可能であることを示した。続いてexonucleaseIIIによるDNAの消化反応において、DNAを伸張させた場合と弛緩させた場合とで1分子観察を行った。ここではDNAの形態を溶液の流れのON/OFFによって制御しながらDNA消化反応の1分子観察を行い、DNAの形態が反応に影響を及ぼすかどうか実験的検討を行った。その結果、DNAを伸張させた場合と弛緩させた場合で消化速度に2倍以上の違いが生じることが示された。さらにDNAだけでなくタンパク質も蛍光標識してDNA-タンパク質間相互作用の1分子解析を行った。真核細胞の1本鎖DNA結合タンパク質であるReplication Protein A (RPA)の発現・精製および蛍光標識を行い、さらにssDNA-RPA複合体の1分子観察を試みた。DNA結合タンパク質を蛍光標識するためにDNA結合タンパク質アフィニティーカラムを用い、DNA結合活性を保持しつつ蛍光標識する方法を確立した。また溶液中でssDNA-RPA複合体を蛍光観察することに成功した。このように、本研究では1分子DNA操作技術・観察技術をDNA-タンパク質間相互作用の解析を1分子レベルで行う実験系を確立した。さらに1分子DNAを操作しながら生化学反応させることができるという1分子解析の利点を生かして、DNAの形態と酵素反応の関係を直接証明することに成功した。本研究の成果は今後各種DNA-タンパク質間相互作用の解析に適用されることが期待され、新たな知見の獲得に貢献できると考えられる。

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論　文　要　旨(博士)

論文題目	Analysis of DNA-protein interactions by single-molecule DNA observation and manipulation

(要旨 1,200字程度)

This thesis entitled as "Analysis of DNA-protein interactions by single-molecule DNA observation and manipulation" described mainly two subjects. One is single-molecule analysis of DNA hydrolysis reaction by *E.coli* exonuclease III. The other is development of fluorescent labeling technique for single-stranded DNA-binding protein and single-molecule imaging of DNA-protein complex. In a conventional assay for DNA-protein interaction based on biochemistry and molecular biology, it is impossible to analyze the dynamics of an individual DNA or enzyme because it can only observe the average behavior of a number of molecules. On the other hand, single-molecule imaging and single-molecule detection techniques have been investigated to analyze various biochemical reactions. Real-time fluorescent observations are particularly helpful for understanding single-molecule phenomena since the positions of individual protein or DNA molecules can be directly detected by fluorescent labeling. In this thesis, I applied single-molecule techniques to analyze DNA-protein interactions.

In chapter 3, at first, I observed single-molecule DNA digestion by exonuclease III (ExoIII), which has 3' to 5' exonuclease activity. Prior to this single-molecule experiment, I evaluated the effect of YOYO-1, a fluorescent dye, as well as anti-fade reagents on the ExoIII reaction. It was found that YOYO-1 and the anti-fade reagents did not inhibit hydrolysis, and the digestion rate of ExoIII was estimated. For the single-molecule experiments, I designed a single-molecule experiment based on an optical trap and two-layer laminar flow, in which mixing between the two layers was negligible. First, a DNA-bead complex was optically trapped in one layer in the absence of ExoIII permitted the DNA to be stretched by the laminar flow. The ExoIII reaction was initiated by moving the trapped DNA-bead complex to another layer of flow which contained ExoIII. As the reaction proceeded, the fluorescently-stained DNA was observed to shorten. The process was photographed; examination of the photographs showed that the DNA molecule shortened in a linear fashion with respect to the reaction time. The digestion rate obtained from the single-molecule experiment was compared to that measured from a bulk experiment and was found to be ca. 28 times higher than the bulk digestion rate. I concluded that the disagreement between the result of bulk experiments and single-molecule experiments might be the cause of the difference of DNA/enzyme ratio and physical form of DNA in both experiments. In the present study, single-molecule DNA fluorescence experiments were conducted using a distributive enzyme with low digestion rate and its effectiveness as an exonuclease was confirmed.

In chapter 4, I studied the effect of physical form of DNA on ExoIII reaction. In chapter 3, the observed digestion rate from the single-molecule experiment was much faster than bulk experiments. I supposed that the physical form of DNA molecules affected digestion by ExoIII. In bulk experiments, all DNA molecules are in random coil structure and always change their shape randomly. On the other hand, individual DNA molecules were stretched by buffer flow. The effect of DNA tension on ExoIII activity can be demonstrated only by single-molecule experiments that can control tension of DNA. In this chapter, I immobilized one-ends of fluorescently stained DNA on a glass surface, and then they were digested by ExoIII under both conditions: stretched state by flow and relaxed state. The sequentially captured photographs demonstrated that the digested DNA molecules were linearly shortened with the reaction time. The digestion rate obtained from the stretched condition was two times higher than the relaxed condition. I clearly demonstrated that the physical form of DNA affects ExoIII activity. I think that it is very important for understanding the mechanism of DNA-protein interactions to compare ExoIII digestions both by bulk assay and the single-molecule approach. Single-molecule measurement that can control the physical form of individual DNA molecules is a powerful method for obtaining new knowledge about the correlation between DNA-tension and enzyme activity.

In chapter 5, I developed a fluorescent labeling technique for Replication Protein A (RPA), which is a eukaryotic single-stranded DNA (ssDNA)-binding protein. RPA binds to and stabilizes single-strand DNA generated during DNA replication and repair. Fluorescent labeling sometimes damage the proteins seriously resulting in very low activity. This problem may be caused by the binding of fluorescent dye to functional site of the protein. In my method, RPA bound to single-stranded DNA immobilized on cellulose was chemically conjugated with amine-reactive fluorescent dye Alexa succinimidyl ester. In the next step, fluorescent dye was washed out from the chromatographic column and RPA were released from single-stranded DNA with the buffer containing high concentration of sodium chloride. DNA-binding activity of fluorescently labeled RPA was not affected. I observed single-molecule ssDNA-RPA complexes. Fluorescent-labeled RPA bound to ssDNA generated by ExoIII in fluorescent microscopic field. As a result of fluorescent observation, ssDNA-RPA complexes were stretched or aggregated in flow of solution.

I conclude that single-molecule techniques are effective in analyzing DNA-protein interactions. Especially I think single-molecule DNA manipulation technique is necessary to demonstrate the effect of physical form on DNA-protein interactions. Further single-molecule experiments should elucidate the mechanism of DNA-protein interactions and provide additional insight into these interactions in living cells.