Doctoral Thesis:

Physico-Chemical Studies on the Regulatory Factors of Folded Structure of Single Giant Duplex-DNA Chains

巨大二本鎖DNAの単分子凝縮構造の
調節要因に関する物理化学的研究

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CONTENTS

GENERAL INTRODUCTION

Correlation Between the Function and Structure of DNA

In general, the function of a biopolymer is closely associated with the characteristics of its higher-order structure. To better understand the actual physiological functions of various types of biopolymers, it is essential to study the manner and mechanism of the formation of the higher-order structure in solution from a physico-chemical viewpoint.

For example, in the case of protein molecules, it is well known that a specific folded structure (native structure) is required for the execution of their original functions. If the native structure is destroyed (denatured structure), the protein loses its original functions. Since the function of protein is so closely related to the characteristics of its folded structure, and can not be clarified without a detailed knowledge of the structure, there have been many studies on protein folding.

On the other hand, in the case of another primary cellular biopolymer, DNA, the relationship between function and the higher-order structure is less understood compared to that in proteins. The "native structure" of genomic DNA *in vivo* is called "nucleoid" in prokaryotic cells, and "chromatin " in eukaryotic cells, respectively. The manner of the folding of genomic DNA in eukaryotes, *i.e.*, chromatin, is known to decisively affect the regulation of reactions such as transcription and replication *in vivo*. When these reactions proceed, chromatin should locally unfold to provide appropriate steric conditions for the interaction between DNA and other enzymes. This has been confirmed by the following experimental findings.

First, several studies have examined the structure of the transcriptionally active region of chromatin, which is called "active chromatin". Active chromatin has been found by an analysis of the DNase I hypersensitive site (DHS: see reviews, Elgin, 1988; van Holde, 1988b; Nacheva et al., 1989; Groston and Kadonaga, 1993). For

almost all of the regions on chromatin where active transcription occurs, DNase I can approach and cut the DNA strand. Since DNase I is a relatively large enzyme (approx. 30kDa), it usually cannot attack regions where chromatin exhibits its regular tightly-compact structure, and this regular chromatin structure is therefore considered to generally have a suppressive effect on transcription (see reviews, Grunstein, 1992; Workman and Buchman, 1993; Paranjape et al., 1994). In addition, the patterns of the distribution of highly expressed genes in chromatin of various types of differentiated cells correspond to DHS patterns, which depend on the cell type (Weintraub and Groudine, 1976; Garel et al., 1977; Yaniv and Gereghini, 1986; Evans et al., 1990; Dillon and Grosveld, 1993). Thus, DHS can be accessed not only by DNase I but also by other transcription factors. Based on the above results, it is believed that the expression of many genes can be activated by the relaxed structure of the nucleosome, as seen in the active chromatin region.

Second, the tightly compacted structure of chromatin is known to generally have a strong suppressive effect on gene expression. The chromatin region where the compacted structure is retained throughout the cell cycle is called "heterochromatin". Heterochromatin was first morphologically found by light microscopic observation (Heitz, 1928), and was biochemically noted to have a strong suppressive effect on gene action (e.g., Fig. 8-26 in the textbook, Albert et al., 1994). A similar effect has been observed for the chromatin structure in the cell mitotic phase (so-called "chromosome"), which is highly compacted and exhibits little transcriptional activity (Creanor and Mitchison, 1982; Pardee et al., 1986). One of the most remarkable relationships between transcriptional activity and the chromatin structure can be observed in "facultative heterochromatin". A representative example of facultative heterochromatin can be seen in X-chromosome inactivation in mammalian cells (Brown, 1966). One of the two X-chromosomes in mammalian females (so-called "sex chromatin") is always completely heterochromatized and transcriptionally inactivated. Interestingly, although the two X-chromosomes have the same primary structure, only the other X-chromosome remains euchromatic and transcriptionally active. The

existence of such facultative heterochromatin suggests that the regulation of gene expression depends not only on the information in the base sequence of the DNA molecule but also on the manner of folding in the higher-order structure.

Third, replication can also be influenced by the higher-order structure. This can be observed as differences in the timing of replication in different parts of the same chromatin. The highly compacted heterochromatin region is replicated at the terminal stage in S phase (Lima-de-Faria and Jaworska, 1968; Stubblefield, 1975), while the loosely compacted active chromatin region is replicated in the beginning of S phase (Brown, 1987). Here again, the degree of compaction of chromatin directly affects the kinetics of replication.

Fourth, the active chromatin which is replicated early in S phase has a G-C-rich base sequence, while the heterochromatin that is replicated later is A-T-rich (Bickmore and Sumner, 1989). Interestingly, most housekeeping genes (approx. 40,000) are particularly located in G-C-rich regions (Antequera and Bird, 1993). On the other hand, A-T-rich regions tend to include many tissue-specific genes (Holmquist, 1989; 1992). Since the G-C base pair has three hydrogen bonds while the A-T pair has only two, G-C-rich regions of double-stranded DNA should exhibit stiffer characteristics upon DNA-folding than A-T-rich regions. Recently, it was verified experimentally that the stiffer G-C-rich regions of DNA strands show a higher probability of nucleation & growth upon strand folding (Yoshikawa and Matsuzawa, 1996). Thus, the manner of folding of a giant DNA chain significantly depends on the strand's stiffness. The expression of various kinds of housekeeping or tissue-specific genes that are distributed in chromatin is expected to be regulated through the manner of the folding of giant DNA due to variation in the strand's stiffness, such as that caused by differences in % G-C and % A-T.

Although the above biological studies emphasize the contribution of the higher-order structure of DNA to the regulation of its functionality, the actual manner and mechanism of the structural regulation of compacted DNA are not yet clear. This is due to the complexity of the system. Since many biological studies of the native

structure of DNA inevitably include various factors for the compaction of DNA to maintain its higher-order structure, it is difficult to quantitatively analyze the properties of DNA itself in solution. *In vitro* physico-chemical studies are needed to examine this point.

Current Studies on the Higher-Order Structure of DNA *in vitro*

To physico-chemically investigate the higher-order structure of DNA, *in vitro* condensation of DNA has been actively studied as a model of DNA compaction *in vivo* (see review, Bloomfield, 1996). There have been two main approaches in the study on DNA condensation; polymer- and salt-induced condensation (so-called "Ψ-condensation") and cation-induced condensation. In the former, condensation is induced by a concentrated aqueous solution of a hydrophilic neutral polymer such as polyethylene glycol (Lerman, 1971; 1973; Laemmli, 1975). In the latter, condensation is induced by a low molecular-weight multivalent cation such as polyamine or hexaamine cobalt (III) (Gosule and Schellman, 1976; Widom and Baldwin, 1980; Arscott et al., 1990; Plum et al., 1990; Bloomfield, 1991). From extensive studies of DNA condensation *in vitro*, a rich variety of chemical species which exist in the living cellular environment in addition to these two kinds of condensation agents have been shown to potentially condense DNA molecules. In addition, the specific morphology of the condensate, such as a toroid- or rod-like structure, has received close attention with regard to DNA compaction into the phage head (see Chapter 2). Although these studies have successfully identified the chemical conditions necessary to condense DNA and to generate a specific morphology of the condensate, three problems remain unresolved: (1) the nature of the folding of a **single** molecule, (2) the nature of the folding of a **giant** molecule, and (3) the actual behavior **in solution**. Clarification of these three problems is required for an understanding of the behavior of single genomic DNA in the cellular environment.

With regard to the first point, it was difficult to determine whether a single or multi-chain is involved in a DNA condensate. In living cells, since individual genomic DNA chains are compacted independently without knotting to each other, accurate discrimination between single-chain compaction and the condensation of multiple DNA chains is essential for obtaining clear insight into the genetic function of DNA. Although DNA condensation *in vitro* has been actively studied over the past several decades as mentioned above, most researchers have examined the intermolecular condensation of multiple DNA chains (Arscott et al., 1990; Plum et al., 1990; Bloomfield, 1991; 1996). In addition, various traditional experimental techniques, such as light scattering, sedimentation, circular dichroism, and so on, have a serious drawback, in that they can essentially only provide information on an ensemble average of many DNA molecules.

The second point of ambiguity involves the size of the DNA examined. Genomic DNA is generally extremely large and consists of more than several kilo- or mega-base pairs. Since the preparation and treatment of such giant DNA is rather troublesome, and it is difficult to obtain clear results by traditional experimental methods, short DNA on the order of several thousand base pairs has been successfully used in many studies to avoid this disadvantage. While this approach may be adequate for studying the condensation of short and multiple DNA chains, it cannot clarify the conformational behavior of genomic DNA chains.

The third problem is also related to the limitations of experimental techniques. The actual morphology or conformation of a DNA condensate is often examined by electron microscopy. However, this method does not allow us to observe the structure of DNA chains in solution due to the methods used to pretreat samples, such as vapor deposition, staining by heavy metals, freezing & etching, etc. (excluding the cryo-technique).

Thus, due to the lack of an suitable methodology which simultaneously satisfies the above three requirements, there is currently little information available regarding the behavior of single molecules of giant DNA in solution.

Aim and Composition of the Present Thesis

For the physico-chemical elucidation of the properties of the folding of single giant DNA molecules in solution, observation with fluorescence microscopy has recently been shown to be quite useful (see Chapter 1). This method provides direct images of the conformation of single giant DNAs and quantitative data on the conformational distribution of DNA chains. Using this technique, it becomes possible to characterize the properties of single giant DNA in solution, which is expected to be useful for examining the functional aspects of the structural regulation of DNA chains. Thus, in the present thesis, I sought to obtain a basic idea of the relationship between the function and higher-order structure of DNA, with particular focus on the case of single giant DNA chain in solution.

Chapter 1 describes the technique of fluorescence microscopic observation of single giant DNA chains. In addition, I also discuss the intrinsic nature of the data obtained by this method; *i.e.*, the characteristics of an ensemble from the perspective of statistical physics.

Chapter 2 presents the properties of the collapse transition of single giant DNA chains in solution induced by a low molecular-weight multivalent cation. In addition, a theoretical prediction of the stability of the collapsed toroidal state of a single giant DNA chain is experimentally verified by electron microscopy. The state varies with both the contour length of DNA chain and the nature of the solvent. A toroidal globule appeared as an intermediate state between the elongated coil state and the spherical globule state. The conditions for such structural regulation are discussed.

In Chapter 3, to gain insight into how information on the temporal axis regulates the folded structure of DNA chains, the effect of the time-course of sample preparation on the regulation of DNA conformation was examined. Single giant DNA complexed with polypeptide (poly-Arg) in NaCl solution was investigated using fluorescence

microscopy. The degree of folding of DNA chains depended not only on the concentration of poly-Arg, but also on the time-course of the addition of poly-Arg and NaCl. This hysteresis effect is discussed in relation to the possible mechanism of the self-regulation of gene expression in living cells.

In Chapter 4, to understand the conformational behavior of a single giant DNA in a mixed system with various kinds of proteins, such as in the cytoplasmic or intranuclear space, folded DNA in the presence of a polycation (poly-arginine), a polyanion (poly-glutamic acid), and a highly concentrated neutral polymer (polyethylene glycol) was analyzed. In a crowded medium, the folding and unfolding of giant DNA is sensitively regulated by the interplay between polycations and polyanions. The effect of crowding by the inert polymer on the folding transition of giant DNA is discussed.

In Chapter 5, as a simple example of the correlation between function and higher-order structure in an actual biological system, the medical application of DNA condensation was investigated. In this case, the condensed structure of DNA chains was closely related to efficient gene transfection.

CHAPTER 1

Application of Fluorescence Microscopy for the Characterization of DNA-Folding

"Both Staudinger and Flory were certainly right!" This is a comment by a biophysical scientist, J.Hearst, at the Cold Spring Harbor Symposium on Quantitative Biology in 1983 (Yanagida and Hiraoka, 1984), when the vido-images of giant DNA molecules in solution were shown by M. Yanagida and his collaborators (Yanagida, et. al., 1983). They have succeeded first in visualization of the DNA molecules by means of fluorescence microscopy (FM) (Matsumoto, et al., 1981). That was the first moment when the actual behavior of a single chain-molecule fluctuating in solution was visually shown. It can be said that at the very moment, the appearance of chain-molecules predicted by both H.Staudinger and P.J.Flory have clearly been substantiated with the naked eye. This fact has potentially implied the excellent applicability of FM for a characterization of physico-chemical properties of the single chain-molecule in solution. In fact, high availability of FM for such purpose has been shown in case of giant DNA molecule in recent years (Minagawa et. al., 1991;1994; Matsuzawa and Yoshikawa, 1994; Yoshikawa and Matsuzawa, 1995; 1996; Mel'nikov et. al., 1995a;1995b; Yoshikawa et. al., 1996a; 1996b; Kidoaki and Yoshikawa, 1996; Ueda and Yoshikawa, 1996; Mel'nikov and Yoshikawa, 1997; see review, Bloomfield, 1996).

In this chapter, I would like to discuss the general merit of FM and the innovative meaning of FM data. The essential introduction on experimental idea for the characterization of DNA-folding is given, which is used throughout the present thesis.

1-1. Application for Quantitative Analysis

F. M. is an ideal tool for the characterization of physico-chemical properties of single chain-molecules, because following quantitative data can simply be obtained (Matsumoto et al., 1992); *i.e.*, 1) contour length, 2) average extent or gyration radius, 3) persistence length, 4) conformational distribution, 5) translational diffusion constant, 6) rotational relaxation time, 7) diffusion constant of segment.

It is noted that almost any item of measurement which is usually carried out by light scattering, centrifugation, viscometry, and so on is covered, except molecular weight. It should also be noticed that the items 1), 4), 6) can never be measured by other experimental techniques. Especially, item 4) has a important key to better understand the conformational behavior of single chain-molecules, as will be discussed in next section.

To obtain highly reliable data on the conformational behavior of DNA chains by FM, some basic experimental settings are required about optimization of optical system, choice of appropriate fluorescence dye, avoidance of DNA cleavage, and so on (Matsumoto et al., 1981, Bustamante, 1991). However, in addition to such settings, there need more special cares concerning the following two points; A) concentration of DNA chains should be set to be quite low, B) depth of sample solution which is mounted on microscope-stage should be thick enough. These two requirements are especially indispensable in case that the folding phenomenon of DNA chains is investigated.

Condition A) is necessary to observe an isolated **single** molecules with avoiding an intermolecular interaction between many chains of DNA. To establish such a situation, I have made the DNA concentration ultra-dilute (less than 0.3μM in phosphate) throughout the present studies. If the higher concentration than this dilute condition is used, aggregation between many DNA chains may happen, depending on sample composition, then the analysis of the single molecular behavior

will be failed.

Condition B) is indispensable in order to observe the DNA chains **in solution**. Namely, the observation avoiding an effect from glass surfaces that are used to enclose a sample solution. To obtain the conformational distribution of DNA chains, it needs to collect many images of the DNA chain observed in the bulk of the solution. When the sample solution was enclosed as in the usual manner of the fluorescence microscopy that uses only two cover-glasses (5~10μm), the influence of glass surface for the DNA conformation cannot be ignored. Because there is some cases that the mean size of DNA molecules in pure water reaches *ca.* 3~5μm. Then, the analysis of the conformational behavior in solution will be failed. Concerning this problem, I deviced the proper manner of sample-enclosure inside the glass plates. If sample solution is situated between two glass plates at a depth of *ca.* 150 μm using spacer glass plates (Fig. 1-1), many DNA chains in the bulk of the solution become to selectively be observed. Though the device is quite simple, it is essential one in order to ensure the quantitative analysis with high reliability on the physico-chemical properties of a **single** DNA chain **in solution**. Experimental procedure is quite simple, but this is also one of the remarkable merits of FM. Because the simple procedure enable one to perform a quick measurement and analysis.

As was discussed in the above, FM has first two marked merits; to be possible to obtain a highly reliable quantitative data, and the quick procedure. However, a true essence of the merit of FM is not the very these two things. FM data contains a crucial character concerned with the statistical physics, which is never acquired by other usual experimental techniques.

1-2. Analysis of Hierarchical Ensemble

Let me clarify the conclusion first. The true essence of FM is to be possible to characterize the ensemble both on DNA chains and on segments within a single

DNA chain. FM data reflects such a hierarchical information on the ensembles.

First hierarchy is a system of the ensemble consisted of DNA chains. By collecting many observed images of DNA chain, this kind of ensemble can be obtained (Fig.1-2 A). In addition, this ensemble of DNA images also contains informations on the statistical ensemble on a single DNA chain. Because, the chains composing the ensemble have almost the same chain-length in case that we use natural DNA molecules (high mono-dispersity). Though the DNA chains have the same primary structure, each chain microscopically exhibits different conformational states in solution due to thermal fluctuation. Therefore, if we collected infinite number of the DNA images, we could expect that within the ensemble, all possible microscopic states of the single DNA chain is realized under the thermal equilibrium condition. This is no less than a definition of statistical ensemble. Although it's of course impossible to observe the infinite number of DNA chains in reality, if we collect enough large number of images of DNA chain, we can know the statistical properties of a single DNA chain with good precision. In this sence, by applying FM, we can also obtain the information on the distribution of the statistical ensemble through a distributional analysis of the images of DNA chain.

Second hierarchy is a system of the ensemble consisted of segments within a single DNA chain. For example, when a single giant DNA chain is divided with Kuhn length, the chain appears as an ensemble of a number of segments. Though the segments have almost uniform structure, each segment exhibits a different microscopic state due to thermal fluctuation. In this time, the whole higher-order structure of the single DNA chain is determined with accumulation of many different microscopic states of the segments. Thus, observed image itself of a single DNA chain becomes to reflect distributional information on the segment-ensemble.

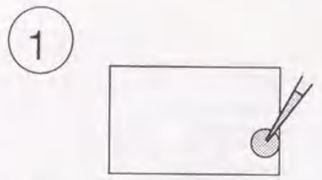
What kind of actual measurement is carried out in FM in order to analyze the segment-ensemble? And, what kind of information can be obtained from the analysis? Let us consider about a typical images of DNA chain (Fig.1-2D, E). For example, Fig.1-2 D is a image of a random coiled state of bacteriophage T4 DNA chain. Since a

local fluorescence intensity on the chain reflects its segment density, spatial distribution of the segment density can be estimated from the 3-dimensional plot of the fluorescence intensity. The 3-D profile of Fig. 1-2D indicates that the segments of a random coiled DNA chain are almost uniformly distributed within the observed image, as shown in Fig.1-2B. On the other hand, in case of Fig.1-2E, the 3-D profile of fluorescence intensity shows steep single peek, though the observed DNA chain is the same one as the case of Fig.1-2D. Interpretation for this unusual profile is that all segments of the chain are concentrated within a narrow space, because integrations of the fluorescence intensity of both Fig.1-2 D and E becomes to be almost equal each other. Since the globular image of Fig.1-2E can be considered to contain almost the same number of segments with random coiled DNA chain, the state of Fig.1-2 E is identified as a globule state of a single DNA chain.

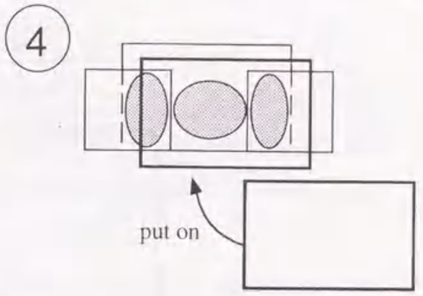
As was discussed in the above, FM can measure both the image of the DNA conformation and 3-D profile of the fluorescence intensity. This is the very key to enable one to analyze distributional information on the segment-ensemble. In conclusion, FM provide the information both on "chain-ensemble" and on "segment-ensemble". Indeed, this character is the essence of merit of FM

To better understand physico-chemical regulatory factors of folded structure of the **single giant** DNA chain **in solution**, the use of FM is essential. Because, other usual methods, such as light scattering, sedimentation, circular dichroism, etc., give only the information on the average of the chain-ensemble. Besides, it was found by Yoshikawa and his collaborators recently that the behavior of the folding transition of single DNA chain exhibits marked difference depending on the level of the analyzed ensemble (Yoshikawa et al., 1996b; Yoshikawa, 1997). To precisely characterize the behavior of the folded DNA chain, it is necessary to observe not only the average information on the chain-ensemble but also the distributional information both on the chain-ensemble and on the segment-ensemble. FM can meet this requirement. This is the reason that FM has been employed in the present studies.

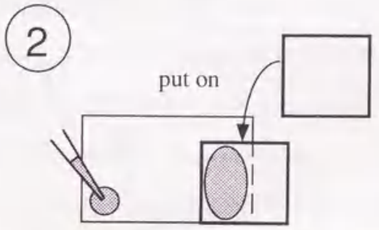
Use a couple of large (30×40mm) and small (20×20mm) cover glasses



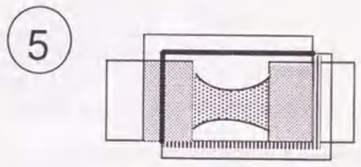
To fix the spacer glasses, drop the sample solution 1.2μl at the shadow position of the first large cover glass.



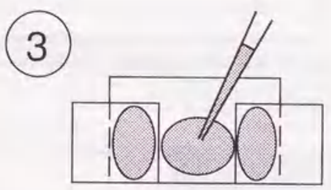
Put on the second large cover glass.



Fix two small glasses as the spacer.



Seal the gap between the large glasses by some sealing agent (e.g., hand nail) in order to avoid efflux of sample solution.

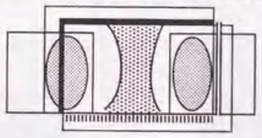


Drop the sample solution 40~45μl on the area between the spacer glasses. Here, the sample depth c.a. 120μm is prepared.

Front side part where should be sealed

Back side part where should be sealed

Undesirable manner of enclosure



Sample solution has contacted with the sealing agent. This situation will occur due to the insufficient amount of sample solution In protocol 3.

Fig.1-1:Method of sample enclosure

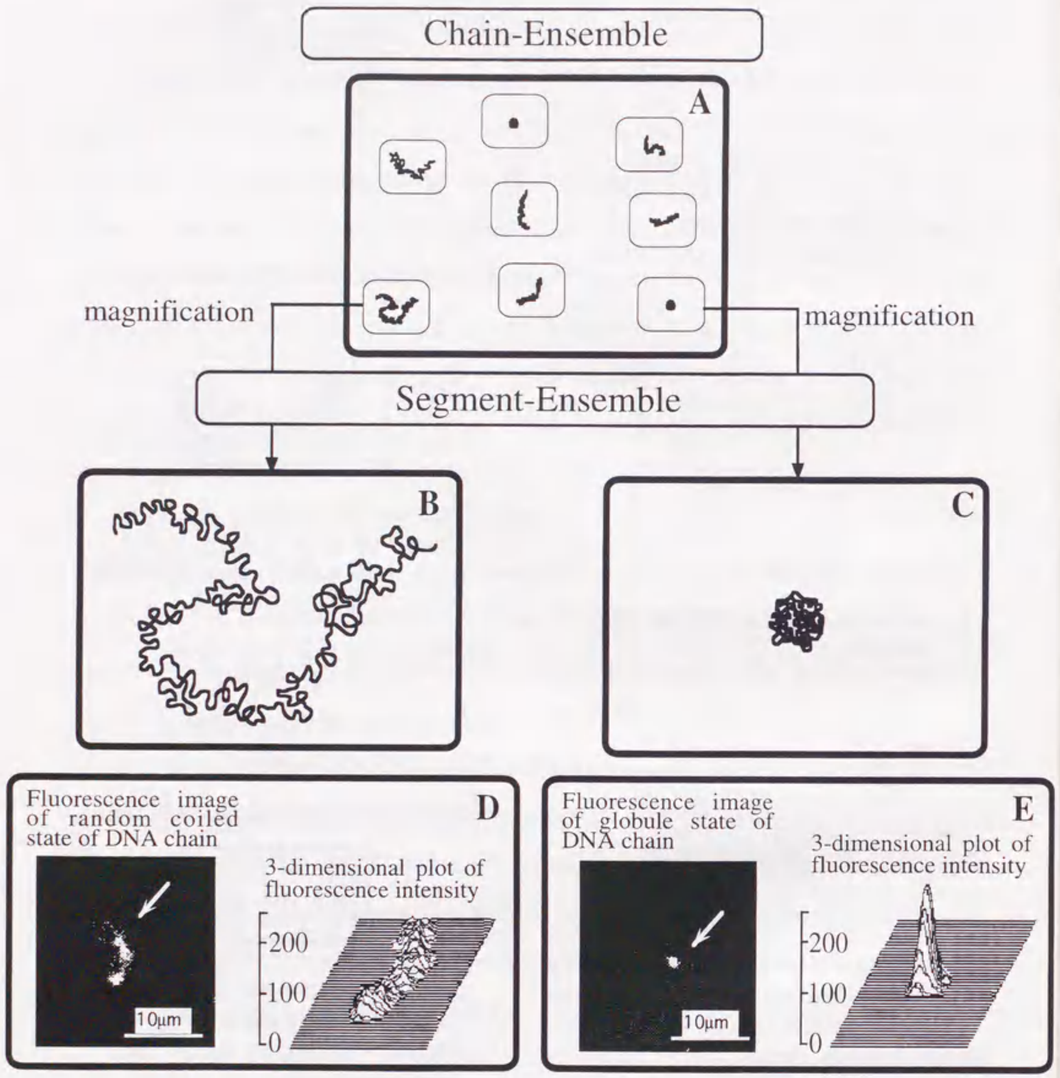


Fig.1-2:Analysis of hierarchical ensemble

CHAPTER 2

Collapse Transition of Giant DNA Induced by Low Molecular Weight Multivalent Cation

This chapter deals with the problem of the morphological regulation on the single molecular condensate of giant DNA. For the first of the investigation for giant DNA-folding phenomenon, the behavior of a collapsed persistent macromolecule in poor solvent is studied theoretically as a model of collapse transition of single double-stranded DNA chain, and the diagram of states in the variables with contour length of a macromolecule and quality of the solvent is constructed. It is found that the state of toroidal globule exists as an intermediate state between the states of elongated coil state and the spherical globule. Theoretical result suggests that a single linear macromolecule with a high degree of polymerization can form a toroidal globule. However, the range in which the toroidal structure is stable decreases as the macromolecule length increases. Experimental observation with transmission electron microscopy has been performed to study the globular structure of single DNA chain (bacteriophage T4 DNA, λ DNA) collapsed by hexaamine cobalt (III) at different concentrations. I found that an extremely long chain of T4 DNA (166 kbps), with a contour length of 56 μm, actually forms a toroidal globule, and that isotropic spherical globule appears at higher hexaamine cobalt concentration.

2-1. Introduction

Collapse of a single giant DNA chain: Coil-globule transition

As was discussed in chapter 1, it has been clarified by an application of FM that a single giant DNA chain can be folded from an elongated coiled state into compacted globular state by a compositional change of solution. This phenomenon is known as the coil-globule transition in a field of statistical polymer physics, which has been the focus of many theoreticians and experimentalists for these thirty years (for example, see, Park et al., 1987; Grosberg and Kuznetsov, 1992a; b; c; d). The reason is that the coil-globule transition significantly represents a simple basic model for a lot of phenomena in various kinds of polymer systems, such as protein folding, polymer network collapse, interpolymer complex, native DNA packing, and so on. Since the investigation of coil-globule transition is also quite helpful for the fundamental understanding of interrelations between segment-segment interaction and segment-solvent interaction of polymer chain (*i.e.*, problem of solvent quality, and of excluded-volume interaction), this phenomenon has been special theoretical interest from the early stage of polymer physics (Flory, 1952). Although many theoretical studies on the problem have been carried out (see review, Lifshitz et al., 1978), experimental substantiation for coil-globule transition was rather behind the theoretical predictions. Actually, the first observation of coil-globule transition of synthetic polymer was reported in 1979 (Nishio et al., 1979). This delay was due to the extreme difficulty to observe the transition without macroscopic phase-separation (or precipitation) at finite certain polymer concentration. Though coil-globule transition should be observed under ultra-dilute polymer concentration, appropriate experimental technique for the characterization has been lacked for a long time. Hence, the experimental characterization for the transition has been regarded as one of the most difficult experimental challenges among the many unsolved polymer physics problems.

Experimental studies on coil-globule transition have dealt with mainly two kind of polymer system; *i.e.*, flexible synthetic polymer system such as polystyrene in cyclohexane solution, and stiff polymer system such as the double-stranded DNA in aqueous solution. In the former system, since the temperature-induced coil-globule transition was early found (Swislow et al., 1980; Sun et al., 1980), refined theoretical treatment has become to be possible (Grosberg and Kuznetsov, 1992a; b; c; d). As the solvent composition within polymer coil may very well differ from that in the bulk of the solvent, it is more difficult to find out general fundamentals in case of the solvent compositional change induced coil-globule transition involving several kinds of component. Concerning this issue, in the latter case of the DNA system, the temperature-dependent coil-globule transition has not been observed yet until present stage. DNA-globule formation has been mainly investigated from the view of the transition induced by the solvent compositional change, which is so-called DNA condensation phenomenon.

Here, it is noted that the term "condensation" has been used in many studies in order to refer to the formation of a polymer-rich state of high segment density; therefore, condensation applies both to aggregation of many molecules, and to compaction of a single molecule. On the other hand, the term "collapse" is usually reserved for single-molecular event with the following changes of polymer conformation; a large reduction in gyration radius and formation of tightly folded globule state (These terminology is in conformity to the definition used by Post and Zimm, 1982a;b.); *i.e.*, "collapse transition" of a giant DNA chain indicates the coil-globule transition of the DNA chain, which is mainly induced by the changes of the solvent-quality.

Concerning the coil-globule transition of DNA chain induced by the compositional changes, it has been special issue for many years whether the transition is discrete or diffuse. For example, Post and Zimm theoretically predicted that the transition can exhibit the discrete character similar to first-order phase transition (Post and Zimm, 1979). However, Widom and Baldwin have experimentally concluded

that the transition is diffuse in case of monomolecular condensation of λ-DNA induced by hexaamine cobalt (III) (Widom and Baldwin, 1983). For this issue, in recent year, the clear evidence for discrete character of the coil-globule transition of DNA chain has been reported by Yoshikawa and his collaborators (Minagawa et al., 1994). In addition, interesting findings was reported that the transition of T4-DNA chain induced by hexaamine cobalt (III) not only exhibits the first-order phase transition but also generate the toroidal globule that can be considered as a kind of single molecular crystal (Yoshikawa et al., 1996a).

Structure of collapsed DNA chain in solution

DNA condensation induced by various kinds of condensation agents has been intensively studied so far (Lerman, 1971; 1973; Arscott et al., 1990; Plum et al., 1990; Bloomfield, 1991; 1996). However, the problem of how the compacted structure of a single giant DNA is partly unfolded in solution by a compositional change of the solution environment remains completely unclear, which is seen for example in the process of transcription or replication in nuclear region of cells. In other words, the problem of structural regulation of collapsed DNA chain. As for this problem, FM recently contributes to provide a crucial picture of the collapsed DNA structure, from which one can detect the character of the collapsed morphology of DNA chain. According to the obtained fluorescence images, it has been found in solution that the collapsed structure of DNA chain depends on the kind of the condensation agents. Its typical appearances are classified as following three cases: a) the coexistence of random coil state and collapsed tight globule (not observed intermediate structure), b) the appearance of intermediately folded state; *i.e.*, loose globule, c) the coexistence of several tight collapsed parts and not condensed parts within one DNA chain.

In a) case, structural distribution of the collapsed DNA in solution exhibits bimodality. This type of collapse transition is induced by the addition of hydrophilic neutral polymer (e.g., PEG (Minagawa, 1994)), low molecular-weight multivalent cation (e.g., hexaamine cobalt (III) (Yoshikawa et al., 1996a), polyamine (Yoshikawa

and Yoshikawa, 1995; Yoshikawa et al., 1996b)), cationic surfactant (e.g., cetyltrimetylamonium bromide (Mel'nikov et al., 1995a; 1995b)) and even neutral surfactant (e.g., Triton-X (Mel'nikov and Yoshikawa, 1997)). The character of the transition has been recognized as the first-order phase transition (Minagawa et al., 1994; Vasilevskaya et al., 1995). On the other hand, b) type of collapse is characterized as a diffuse transition between coil and "tight" globule, due to the appearance of intermediate state of the "loose" globule (the exact definition for "tight" and "loose" globule is given in Chapter 4.) Polycation-induced collapse of single giant DNA seems to be classified into this category, as far as I examined on basic polypeptide (e.g., poly-Arg (Kidoaki and Yoshikawa, 1996), polylysine (data not shown), histone H2B (data not shown)). Finally, c) case is characterized as intrachain phase separation, which is induced by some kind of neutral polymer (e.g., poly-vinylpirrolidone (Starodoubtsev and Yoshikawa, 1997)) and alcohol (e.g., ethanol (Ueda and Yoshikawa, 1996)).

Stability of the toroidal globule

It is known that the character of coil-globule transition depends essentially on the stiffness of macromolecules (Lifshitz et al., 1979; Post and Zimm, 1979; Grosberg and Khokhlov, 1994). The size of a flexible macromolecule smoothly decreases as solvent quality becomes poorer. Whereas the width of coil-globule transition for stiff macromolecules is very narrow and this transition can be treated as first order phase transition. The structure of arising globule from a flexible and a stiff macromolecule differs as well (Grosberg, 1979; Grosberg and Khokhlov, 1981; Boehm and Martire, 1986).

Electron microscopy has shown that a double-stranded DNA macromolecule, as well as plural number of smaller DNAs, can form a toroidal structure as the globule state (Haynes et al., 1970; Olins and Olins, 1971; Evdokimov et al., 1972; Gosule and Schellmann, 1976; Chattoraj et al., 1978; Marx and Ruben, 1984; 1986; Plum et al., 1990; Bloomfield, 1991; Reich et al., 1991). A qualitative explanation on

the stability of the toroidal structure has been given more than 15 years ago (Grosberg, 1979; Chattoraj et al., 1978). It can be summarized as follows: due to the rigidity of DNA chain, DNA has usually no points for keen bending. To minimize the loss of energy associated with bending, DNA wraps itself circumferentially (Fig. 2-1). As a result, a hole is formed in the center of the DNA globule. The density of the DNA strands compacted into this toroidal form can be relatively high.

The study on the structure of condensed DNA was followed by the experimental investigations on the so-called Ψ–condensation of DNA. Ψ–condensation was discovered by Lerman in a study on the sedimentation coefficient of DNA immersed in an aqueous solution of linear polymer (such as polyethylene glycol) and salt (Lerman, 1971). Lerman found that, at certain threshold concentrations of polymer and salt, the size of DNAs decreased sharply. Later it was found that condensation of DNAs can also be induced by the addition of other species, such as organic solvents or polyvalent cations (Gosule and Schellmann, 1976; Laemmli, 1975; Wilson and Bloomfield, 1979; Post and Zimm, 1982b). Recently, interest on the higher order structure of collapsed DNA was renewed, since it was recognized that such highly compacted structures are typical for DNA packing in viral bacteriophage pathogens (Hud, 1995; Hud, et al., 1995).

Theoretical studies on Ψ-condensation of DNA and toroidal structure generated through the condensations were performed in refs (Grosberg, 1979; Bloomfield, 1991; Riemer, and Bloomfield, 1978; Frisch and Fesciyan, 1979; Manning, 1981; Grosberg et al., 1981; Grosberg et al., 1982; Grosberg and Zhestkov, 1985; 1986; Marquet and Houssier, 1991; Vasilevskaya et al., 1995; Ubbink and Odijk, 1995). Generally speaking, there are two different approaches in the theoretical investigations on the toroidal globule of DNA. The first way involves the estimation of the relevant importance from several different contributions to the free energy of collapsed DNA (Bloomfield, 1991; Riemer and Bloomfield, 1978; Manning, 1981; Marquet and Houssier, 1991). One of the most important factors is the electrostatic contribution to the total free energy of DNA as it has been stressed in an early report (Riemer and Bloomfield,

1978; for recent publication on this subject, see Ubbink and Odijk, 1995). Here it should be mentioned that, in order to evaluate the electrostatic contribution depending on the properties, such as DNA chain length and salt concentrations, several approximations are adapted unavoidably and that the reliability of these approximation is usually not so clear.

On the other hand, the formation of toroidal structure seems to be a common property for compact globule state formed from a macromolecule with enough stiffness. Theoretical investigation on the collapse of electroneutral polymer with certain stiffness, including the construction of full diagram of the states is, thus, important. Such second way of the theoretical investigation has been carried out in refs (Grosberg, 1979; Grosberg and Zhestkov, 1985; 1986). The theoretical treatment in these articles was limited to the case of globules with almost closest packing of strands within the globule. In addition to this, they concluded that the toroidal globule can be observed only for rather short macromolecules; and that spherical isotropic globule is firstly formed from coiled state and then transformed to toroidal globule upon further worsening of the solvent quality. In contrast to these theoretical expectations, actual experimental results (Laemmli, 1975) indicated that a very long DNA chain actually forms the toroidal shape, and that the density of DNA packing in the toroid can be lower than the corresponding close packing density (Maniatis et al., 1974). It is also noted that compacted globule from a single long DNA loses almost all of the negative charge owe to the ion-pair formation with the counter ion, as has been revealed from the measurement of light scattering under external electric field (unpublished result by Yoshikawa et al.). Actually the net charge for a toroid formed from a giant T4 DNA (166kbp) in the presence of spermidine is found to be only of the order of 10. Thus, theoretical treatment on the stability of toroidal globule for a persistent chain is expected to serve as a good model for the compacted DNA, even for the level of approximation without the electrostatic term.

The purpose in this chapter is to investigate, as the first theoretical trial, the conditions necessary for the stability of toroidal structure with different densities of

segment packing. A simple situation is considered: single persistent macromolecule present in a low-molecular weight solvent with different quality; *i.e.*, the general trend on the structure of the globule is investigated without keeping in the complicated situation connected with the electrostatic contribution to the free energy which is not exactly known. The diagram of states of the persistent macromolecule in low-molecular weight solvent is constructed in terms of contour length vs. a parameter of interaction between segments belonging to a single molecular chain. To confirm the theoretical analysis, by use of electron microscopy I have studied the structure of collapsed DNA (bacteriophage T4 DNA, λ DNA) that has been condensed in the presence of hexaamine cobalt (III) with different concentrations. Here, the increase of the concentration of hexaamine cobalt (III) corresponds to the change of the interaction parameter between the segments of DNA to be more attractive, or to the change of the solvent quality to be poorer. One of the significant reasons, why DNA molecules have chosen in order to check the present theoretical consideration, is the fact that bacteriophage DNAs are the best material with respect to "mono-dispersity" among giant macromolecules regardless natural or synthetic.

In Section 2-2, the free energy of the toroidal globule is derived. Section 2-3 contains the results of the mathematical analysis, which are summarized in the diagram of states. The experimental results from electron microscopy and the discussion are presented in Section 2-4, and the conclusions are given in Section 2-5.

2-2. Free Energy of the Toroidal Globule

Let us consider a giant persistent macromolecule with the total contour length L. Let l be the length of the Kuhn segment; and d be its thickness. The stiffness of a macromolecule is characterized by the ratio p=l /d, which is very high for DNA (p~20-50). Let χ denote the "effective" interaction energy between monomer links. It was assumed that persistent macromolecule takes the form of a toroid whose geometry

is characterized by the external radius R of the toroid and the radius r of its cross section (Fig. 2-1).

The free energy of the toroidal globule, F^{gl}, can be written as the sum of three terms:

$$F^{gl} = F_{el} + F_{sur} + F_{int} \qquad (2\text{-}1)$$

where F_{el} is the elastic component of the free energy of the globule; F_{sur} describes the surface free energy; and F_{int} is contribution describing the mixing free energy.

The surface free energy F_{sur} can be written as:

$$F_{sur} = S\sigma \qquad (2\text{-}2)$$

where S is the area of the globule surface (S~Rr) and σ is the surface tension. The surface tension depends on the manner of compaction of the polymer globule (Grosberg and Pakhomov, 1989). If the globule is isotropic and the volume fraction of the polymer is not close to unity, σ can be estimated as (Lifshitz et al., 1979):

$$\sigma \sim T\frac{lB^2}{C^{3/2}} \qquad (2\text{-}3)$$

where B and C are the second and third virial coefficients of the interaction of DNA links and T is the temperature in energy units.

The virial coefficients B and C for the persistent macromolecule can be approximately represented as (Grosberg and Khokhlov, 1994):

$$B \sim l^2 d(0.5 - \chi)$$
$$C \sim l^3 d^3 \qquad (2\text{-}4)$$

Here, in relation to the coil-globule transition of DNA, it is possible to regard that χ parameter partly contains the contribution from short-range electrostatic attraction

between segments (Plum et al., 1990; Bloomfield, 1991).

By substituting these approximations into (2-3), next equation can be obtained:

$$F_{sur} = T\frac{Rrp^{1/2}}{d^2}(0.5-\chi)^2 \qquad (2\text{-}5)$$

Equation (2-5) is similar (except for the proportional constant) to the theoretical expression used in refs (Grosberg, 1979; Grosberg and Zhestkov, 1985; 1986), where the stability of toroidal globule with close packing has been discussed. To focus only on the essential factors determining the stability of toroid, any dependence of the proportionality constant on the density of the segment packing in the toroid was ignored.

For the elastic free energy F_{el},

$$F_{el} = T\frac{Ll}{R^2} \qquad (2\text{-}6)$$

which describes the entropy loss of the macromolecule due to the collapse.

To write the free energy F_{int} of mixing, next expression was employed, which was proposed in (Khokhlov and Semenov, 1985) for the description of concentrated solution of rigid macromolecules:

$$F_{int} = -\,T\frac{L}{d}\bullet\frac{Ld^2}{Rr^2}\chi - \frac{1}{2}T\frac{L}{d}\bullet\ln\left(1-\frac{Ld^2}{Rr^2}\right) \qquad (2\text{-}7)$$

here the first contribution is free energy of the energetic interactions between monomer links; second one describes the steric interaction.

Thus, the total free energy F^{gl}, of the toroidal globule can be written as:

$$\frac{F^{gl}}{T} = \frac{Ll}{R^2} + \frac{Rrp^{1/2}}{d^2}(0.5-\chi)^2 - \frac{L}{d}\bullet\frac{Ld^2}{Rr^2}\chi - \frac{1}{2}\bullet\frac{L}{d}\bullet\ln\left(1-\frac{Ld^2}{Rr^2}\right)$$

(2-8)

To obtain the equilibrium parameters of the toroidal globule, the total free energy F^{gl} (2-8) is minimized with respect to R and r:

$$\frac{\partial F^{gl}}{\partial R} = 0 \quad ; \quad \frac{\partial F^{gl}}{\partial r} = 0 \qquad (2\text{-}9)$$

The estimations for optimal values of radii R and r were determined by the analysis of relative importance of different terms in system (2-9).

2-3. Diagram of State

The analysis of the system (2-9) is summarized in Fig. 2-2: I is the region of the toroidal globule; II is the region where the globule is spherical; and III corresponds to the elongated coil state. Two distinct regimes exist within region I for the toroidal globule. In the first regime, the volume fraction of DNA links within the bulk of the toroid ($\phi_t \sim (Ld^2/Rr^2)$) is much less than unity ($\phi_t < 1$; I_A). The second regime represents the close packing of DNA segments ($\phi_t \sim 1$; I_B). The radii R and r of the toroid in region I_A are estimated as:

$$R \sim \frac{L^{1/6}d^{7/12}l^{1/4}}{(\chi-0.5)^{2/3}}$$
$$r \sim L^{1/2}\frac{d^{3/4}}{l^{1/4}} \qquad (2\text{-}10)$$

The density of monomer packing within the bulk of toroid can be characterized by the average distance ε between different strands of the DNA macromolecule. This value can be estimated as follows. The total number of DNA strands which form a toroid with radius R is approximately L/R; and the density within one cross section

of the toroid is approximately $(L/R)/r^2$. Thus,

$$\varepsilon \sim r\sqrt{\frac{R}{L}} \qquad (2\text{-}11)$$

For a toroidal structure of I_A type, the following relationship using equations (2-10) and (2-11) was obtained:

$$\varepsilon \sim \frac{1}{(\chi-0.5)^{1/3}} \bullet \frac{d^{25/24} L^{1/12}}{l^{1/8}} \qquad (2\text{-}12)$$

The average distance ε between the DNA strands decreases with an increase in the attraction parameter χ. If $(L/d) < (l/d)^{3/2}$, ε decreases until ε ~ d at the crossover to the region of toroidal structure with close packing of DNA strands (line *d*, Fig. 2-2). Scaling estimates for the radii R and r of the toroidal globule with close packing of strands gives:

$$R \sim \frac{L^{1/5} d^{3/5} l^{1/5}}{(\chi-0.5)^{4/5}}$$

$$r \sim \frac{(\chi-0.5)^{2/5} L^{2/5} d^{7/10}}{l^{1/10}} \qquad (2\text{-}13)$$

The estimations for R and r obtained in refs (Grosberg and Zhestkov, 1985; Ubbink and Odijk, 1995) gave the same scaling dependence on contour length L.

As χ increases (the quality of the solvent decreases), the external radius R of the toroid decreases, while the radius r of its cross section increases; the transition from toroidal globule to spherical globule occurs when these two radii become approximately equal: R ~ r. The crossover lines from the region of toroidal globule to the region of spherical globule are shown as lines *b* and *c* in Fig. 2-2. The density of links within the spherical globule near the transition point depends on the degree of polymerization of the macromolecule: the greater the total number of Kuhn segments

in the DNA molecule, the lower the density of the spherical globule near crossover line *b*.

At low values of χ (*i.e.*, in the case of weak attraction between DNA links), the globular state becomes unstable and DNA undergoes a transition to the coil state. To find the transition line, the free energy between the globule and coil states was compared.

The free energy, F^{coil}, of the coil state of a persistent macromolecule can be written as:

$$\frac{F^{coil}}{T} = \frac{R^2}{Ll} + \frac{Ll}{R^2} + \frac{L^2 d}{R^3}(0.5 - \chi) \qquad (2\text{-}14)$$

where the first two terms describe the elastic free energy: the first term reflects the entropy loss of the macromolecule as it expands from its ideal size, while the second term describes the entropy change under contraction. The third term in equation (2-14) is the free energy of the excluded volume interaction at low values of the volume fraction of the polymer, and represents the contribution from the pair interaction between monomer links, since all other contributions are negligibly small for the macromolecule in its coil state.

The transition from coil to globule occurs when the free energy of the coil state, F^{coil}, becomes equal to the free energy, F^{gl}, of the globule state (lines *a* and *f* on Fig. 2-2). Near the coil-globule transition, the globule expands gradually, so that the scaling estimate of the radius R is the same as that for an ideal coil: $R \sim L^{1/2} l^{1/2}$. To the left of line *a*, the macromolecule has the conformation of an ideal chain: $R \sim L^{1/2} l^{1/2}$. However, if the contour length of the macromolecule L is shorter than the Kuhn segment, the size R of the macromolecule depends on L linearly: (line *f*).

Thus, the toroidal globule state is generated as an intermediate state between the coil and spherical globule states. This result seems reasonable. In fact, short macromolecules ($L << l$) are easier to bend by kinking than by forming spherical globules. The range of χ, where the toroidal globule of the long macromolecule (

$(L/d) > (l/d)^{3/2}$) can exist, is rather narrow; its width $\Delta\chi$ decreases as the total number L/l of Kuhn segments increases in the macromolecule:

$$\Delta\chi \sim \frac{1}{(L/l)^{1/2}} \qquad (2\text{-}15)$$

The size R of the toroidal structure formed by the long persistent macromolecule can be smaller than its persistent length ($R > l$). The region of a small globule ($R < l$) is shown by lines g and h on Fig. 2-2.

On the other hand, if the macromolecule is very short ($L << l$), it is necessary to apply large forces to induce its bending. Theoretical analysis shows that if the degree of macromolecule polymerization is small, *i.e.*: $(L/d) < (l/d)^{1/4}$, the macromolecule remains in a stretched state ($R \sim L$) even for very poor solvent (line *e*, Fig. 2-2).

Here, let's briefly discuss on the nature of the transition given in Fig. 2-2. As for the transition between elongated coil and compacted globule, recently Yoshikawa and his collaborators have obtained the clear evidence on the discreteness in the transition for individual DNAs, where the transition is continuous in the ensemble of DNA chains (Vasilevskaya et al., 1995; Yoshikawa and Matsuzawa, 1996). The discrete character of the transition indicates that the transition is first order and that the free energy profile exhibits double minimum near the transition points. The important aspect of the transition deduced from such free energy profile is that there exists finite region of the coexistence between coil and globule and that the relative population is determined from the Boltzmann distribution. Thus, the coexistence between different states is generally expected near the lines in Fig. 2-2.

In conclusion, the theoretical analysis has shown the following important new features for the collapse transition of persistent macromolecules: (i) Toroidal globule can be formed from a very long chain. (ii) For a stiff enough chain toroidal globule is transformed from coiled state at first. (iii) The size of the collapsed macromolecule

diminishes upon the transformation from toroid to spherical globule. In the next section some of these conclusions are discussed in connection with the results of our experimental observation.

2-4. Experiment

Bacteriophage T4 DNA (166 kbps) and λ DNA (48 kbps) were purchased from Nippon Gene. hexaamine cobalt (III) chloride was obtained from Sigma Chemicals and recrystallized from hot H_2O-HCl. DNA and hexaamine cobalt (III) were dissolved in distilled water that had been purified using a 0.22-μm Millipore filter. The sample solution is consisted with DNA in nucleotide, 1.2 μM, and hexaamine cobalt (III), 150 μM.

Transmission electron microscopic observation of the complex between DNA and hexaamine cobalt (III) was carried out using the negative staining method. Carbon-coated 400 Cu mesh grids (EM Shizai) were rendered hydrophilic by glow discharge. A grid was floated on a droplet of sample solution (20μl); after 2 min. it was drained on filter paper, and then stained with an aqueous solution of 1% uranyl acetate for 30s. The sample was examined by a JEM1200EX electron microscope (JEOL) operated at an acceleration voltage of 100 kV.

In this chapter, the structural stability of the toroidal DNA globule was focused on. To my knowledge there has been no previous report of the formation of a toroid from a giant DNA macromolecule with more than 100 kbps induced by a small ion or molecule. The only report on toroid formation from giant DNA uses poly-cations as the condensation reagent (Laemmli, 1975). Thus, in this study, I investigated whether extremely giant T4 DNA (166 kbp) can form a toroidal globule using a small condensation agent such as a cobalt complex. It have been confirmed that actually it can. The morphology of arising globule dependent on the concentration of hexaamine cobalt (III) is also interesting .

Electron micrographs of compacted T4 DNAs are shown in Fig. 2-3. The morphology of the resulting condensates is variable even under fixed experimental conditions: DNA can form toroidal, rod or even more complicated shapes, such as a bent rod or a deformed toroid (Fig. 2-3A). For comparison, a toroidal globule of λ DNA is shown in Fig. 2-4.

The varied morphology of the DNA condensate has been reported and discussed previously (*e.g.*, Evdokimov et al., 1972; Chattoraj et al., 1978). However, in most of previous studies on the toroidal structure of DNA, the observed toroidal condensates were estimated to be formed from plural number of DNA chains. To investigate whether I observed toroidal globule formed from single DNA macromolecule, I make a simple estimate. The self-volume V_o of the DNA macromolecule was assumed, *i.e.*, the total volume occupied by DNA links, and compare this to volume V_t of the observed toroid structure. The self-volume V_o of a DNA chain can be estimated as:

$$V_0 = \pi L r_h^2 \qquad (2\text{-}16)$$

where L is the contour length of DNA and r_h is the radius of the double-helix of DNA (r_h ~10Å).

The contour length L of DNA depends on the number N of base pairs:

$$L \sim 3.4 \cdot N \quad (\text{Å}) \qquad (2\text{-}17)$$

From equations (2-16) and (2-17), it was found that the self-volume V_o of the macromolecule is about $0.46 \cdot 10^8 Å^3$ for λ DNA and $1.7 \cdot 10^8 Å^3$ for T4 DNA. The volume V_t of bulk part in the toroid can be estimated as $V_t \sim 2\pi^2 R r^2$ (see Fig. 2-1). I measured the external radius R of the toroid and the radius r of its cross sections in the electron micrographs in Figs. 2-3 and 2-4. From the calculations, the volume V_t of the toroid bulk was approximately found to be equal to $0.54 \cdot 10^8 Å^3$ for λ DNA (Fig. 2-4), and $2.6 \cdot 10^8 Å^3$ (Fig. 2-3 B) for T4 DNA. If we take into account the possible

errors in our evaluation of volume V_o (for example, a few percent of error in equation (2-17), which is about 10% of the diameter of the double-helix r_h), I conclude that the toroidal globules shown in Fig. 3-4 are derived from single λ and T4 DNA. It is also to be noticed that the toroids are not necessarily the constituent of most dense packing with the DNA segments, as has been discussed in the preceding part in this paper. A comparison of V_o and V_t suggests that the density of packing of the DNA strands can be significantly less than that of hexagonal most-dense packing.

Based on Fig. 2-3 and on the results of the measurement for similar systems, the toroidal globule of T4 DNA seems to be characterized by a large hole in the center: *i.e.*, a relatively large external radius R and a relatively small radius r, in contrast to the toroidal globule of λ DNA and the toroidal aggregates from short DNA fragments.

Concerning the size of the toroidal globule, it has been reported (Marx and Ruben, 1986; Bloomfield, 1991) that the external radius R of the DNA toroid increases slightly with the molecular weight of DNA. This observation corresponds to the theoretical conclusion (see formulas (2-10) and (2-13)) that the external radius R weakly depends on the contour length L. It is worth mentioning that, based on previous experiments, short DNA macromolecules with less than 150 bps never form the toroidal state (Bloomfield, 1991; Widom and Baldwin, 1980). This observation also corresponds to the theoretical result in Section 2-3.

Concerning the stability of the toroid, from the measurement of electron microscopy, I have obtained the preliminary result that the shape of toroid tends to be deformed and also to be transformed to spherical structure accompanied with the increase of the concentration of added hexaamine cobalt (III). Fig. 2-5 shows the actual example on somewhat deformed spherical globule formed from single T4 DNA molecule. Such an experimental trend corresponds well to the present theory as is indicated in Table 2-I, where the relative population is given between toroidal and spherical obstacles in the electron microscopic observation. The toroidal globule becomes unstable with the increase of concentration of hexaamine cobalt (III) in the

both cases of T4 and λ DNAs. Further detailed and careful study is awaited to make clear the full detail on the morphological change including the "rod" obstacle.

2-5. Conclusion

The stability in the collapse state of a persistent semiflexible macromolecule was studied as a simple model for the compaction of a single double-stranded DNA. The theoretical results suggest that even giant macromolecules can form toroidal structure. This structure exists an intermediate state between the elongated coil and the spherical globule. Such theoretical prediction has been ascertained by the electron microscopic study of giant λ and T4 DNAs condensed by hexaamine cobalt (III). Indeed, the toroidal structure of such DNA was observed and it was found that it transforms to the spherical globule at high concentration of hexaamine cobalt (III).

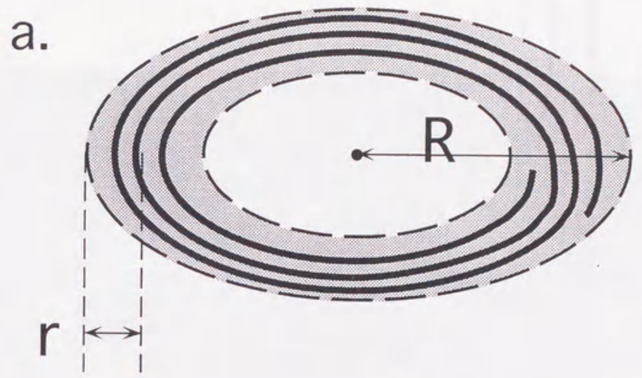


Fig.2-1: Schematic representation of a toroidal globule. a) overall view. R is the external radius of the toroid; r is the radius of the toroid cross section. b) cross section of a toroidal globule. ε is the distance between different segments of a DNA macromolecule.

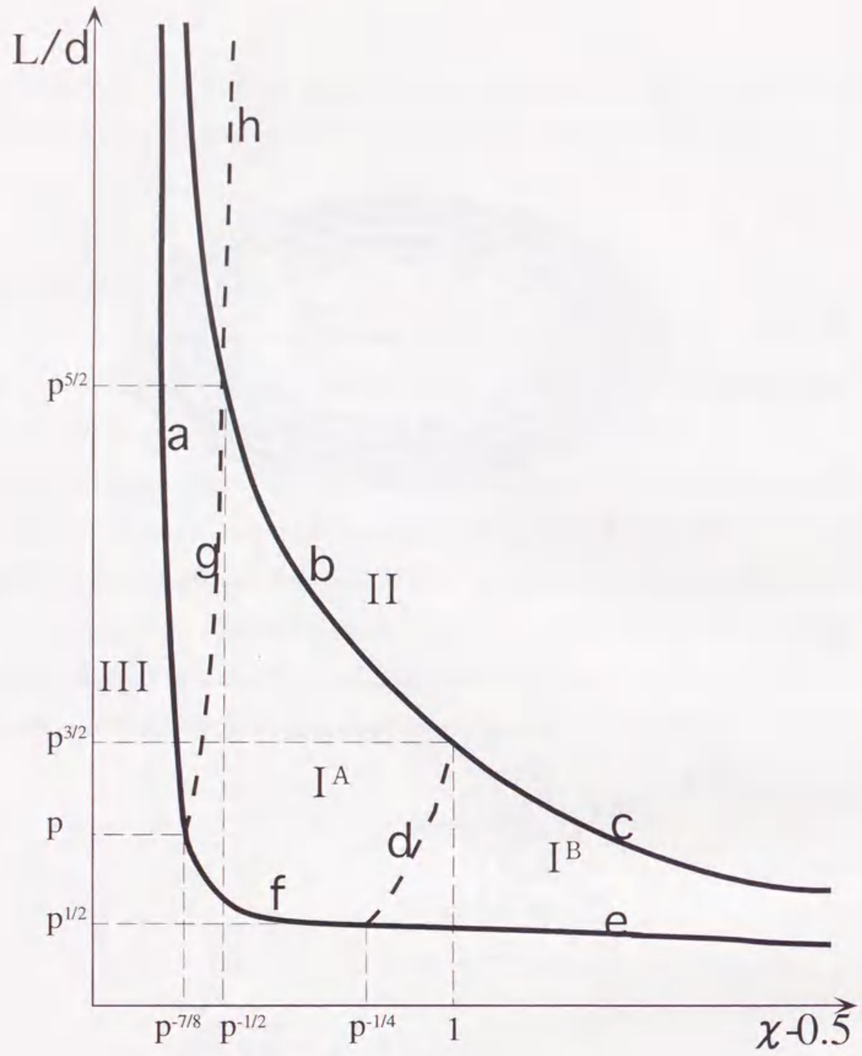


Fig.2-2: Diagram of state in a persistent macromolecule with the total number L/d of monomers vs. solvent quality c. The solid line distinguishes regions I; toroidal globule, II; spherical globule and III; coil. The equations for the boundaries are:

a) $\frac{L}{d} \sim p^{-3/4} \frac{1}{(\chi-0.5)^2}$ b) $\frac{L}{d} \sim p^{3/2} \frac{1}{(\chi-0.5)^2}$ c) $\frac{L}{d} \sim p^{3/2} \frac{1}{(\chi-0.5)^6}$

d) $\frac{L}{d} \sim p^{3/2}(\chi-0.5)^4$ e) $\frac{L}{d} \sim p^{1/4} \frac{1}{(\chi-0.5)}$ f) $\frac{L}{d} \sim p^{3/10} \frac{1}{(\chi-0.5)^{4/5}}$

g) $\frac{L}{d} \sim p^{9/2}(\chi-0.5)^4$ h) $\frac{L}{d} \sim p^3(\chi-0.5)$

All other comments and notations are given in the text.

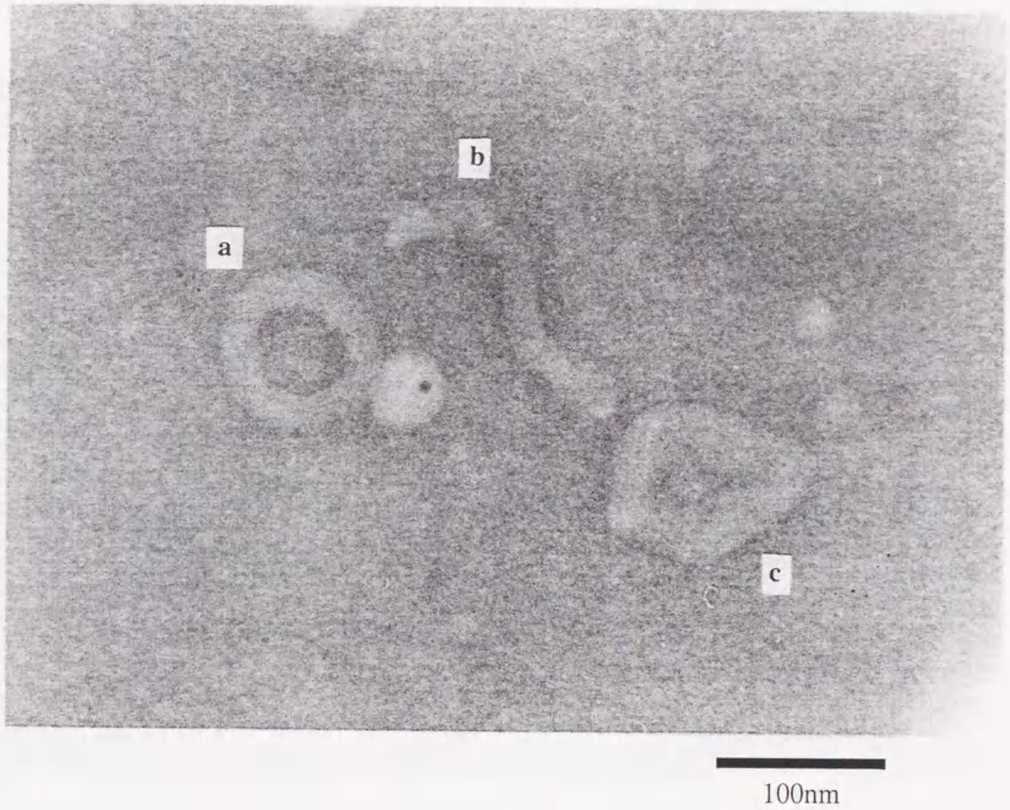
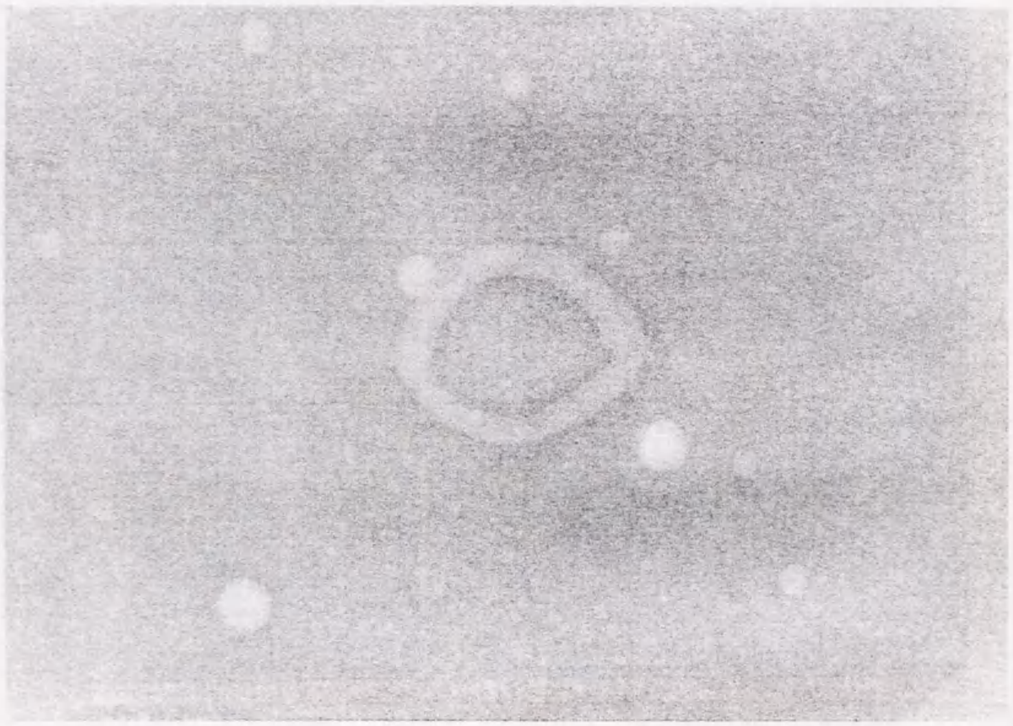
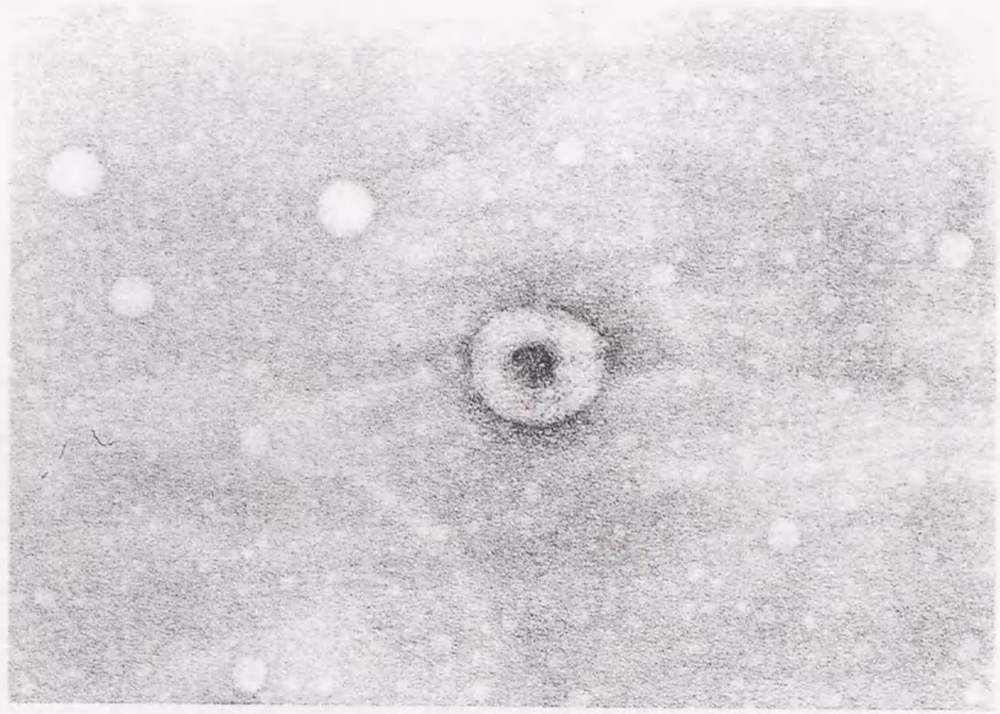


Fig.2-3 A: Electron micrographs of a globule structure formed from a single T4 DNA: (a) toroid (b) bending rod (c) deformed toroid.



100nm

Fig.2-3 B: Electron micrographs of a globule structure formed from a single T4 DNA: toroid.



100nm

Fig.2-4: Electron micrograph of a toroidal globule from a single λ DNA.

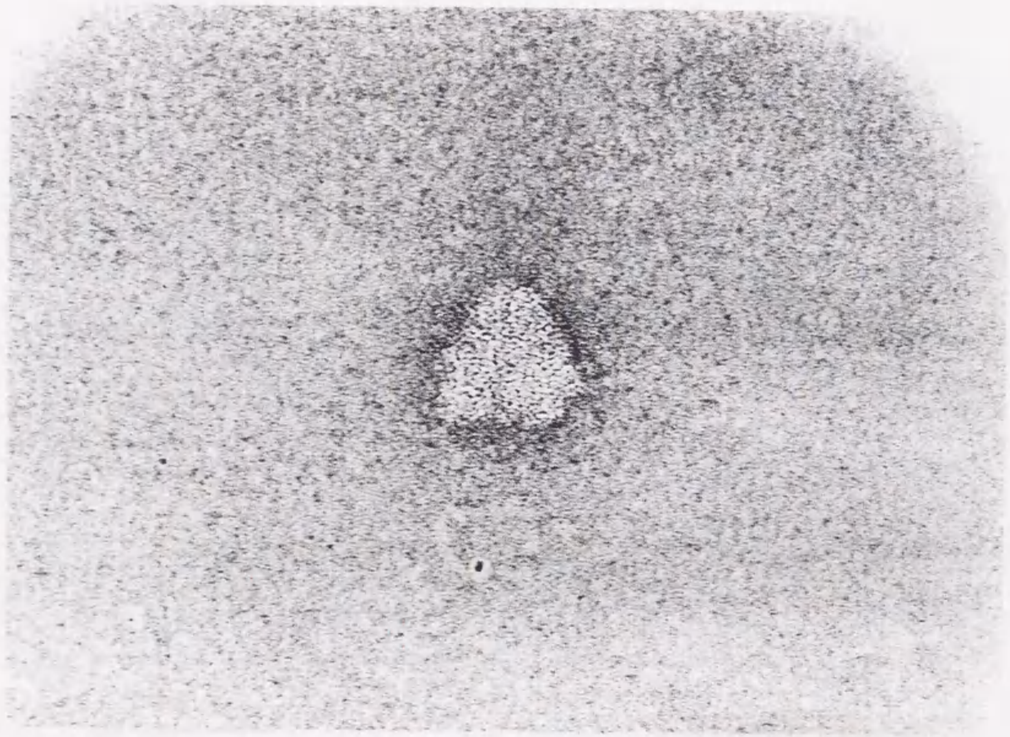


Fig.2-5: Electron micrograph of a quasi-spherical globule from a single T4 DNA.

Table 2-I: The population*1 of toroidal and spherical structures in the collapsed DNA as is observed by electron microscopy.

Concentration of Hexammine cobalt (III) / μM		50	100
T4 DNA	Toroidal obstacles	86.0%	53.8%
	Spherical obstacles*2	14.0%	46.2%
λ DNA	Toroidal obstacles	74.5%	11.8%
	Spherical obstacles*2	25.5%	88.2%

*1 Number of counted obstacles is *ca.* 50 for the individual conditions.
*2 We have counted deformed spherical globules as the "spherical obstacles" in addition to the genuine spherical ones.

CHAPTER 3

Hysteresis Effect on the Folding of Single Giant DNA

In this chapter, the effect of past history of solution on a regulation of the higher-order structure of a single giant DNA is studied in order to consider a possible mechanism for a novel informational processing of genomic DNA molecules excepting informations stored on its primary structure. As was discussed in the General Introduction, many evidences has been reported concerning that the higher-order structure or the manner of folding of genomic DNAs deeply affect functional regulations of the DNA. To explore the possible physico-chemical mechanism on such a correlation between function and structure is quite important.

The higher-order structure of compacted single giant DNA induced by complexation with polypeptide (poly-Arg) in NaCl solution is investigated using fluorescence microscopy. As the poly-Arg concentration increased, the mean size of extended DNA chains gradually decreased. In the presence of excess poly-Arg, individual DNA chains collapsed into compact globules, and the degree of collapse of the DNA chains depended not only on the concentration of poly-Arg, but also on the time-course of the addition of poly-Arg and NaCl, indicating that the structure of the collapsed DNA is not determined simply according to the minimum free energy. I discuss theoretically the presence of multiple-stationary states based on a consideration of simple kinetics in the process of binding. Depending on the past history, the number of poly-Arg and Na^+ which bind to each DNA changes markedly. This interesting characteristic of long DNA is discussed in relation to the possible mechanism of self-regulation of gene expression in living cells.

3-1. Introduction

In living prokaryote and eukaryote cells, individual double-stranded DNA chains usually exist in a highly compacted state. For example, in mammalian cells, individual DNA chains have a contour length or full-stretch length, L, on the order of cm (Watson et al., 1987). Since the persistence length λ of a DNA chain is usually on the order of 500Å (Hargerman, 1988), a DNA chain with L=1cm corresponds to a linear freely-jointed polymer chain with $N \sim 10^5$ segments, where $N=L/(2\lambda)$. If DNA chains act as ideal linear polymers, the average end-to-end distance is $\langle R \rangle \sim 2\lambda N^{1/2}$ = ca.30μm. In fact, however, in actual living cells, a long DNA chain is compacted into a small space on the order of 1 μm, indicating that DNA chains are more tightly compacted than an ideal chain. In this chapter, I refer to a DNA chain that is significantly compacted compared to the ideal chain as "globule" DNA. On the other hand, if a purified DNA chain is dissolved in a typical aqueous solution, it swells more than the ideal polymer chain, due to the excluded volume effect between the segments in the DNA chain. I refer to this elongated form as "coil" DNA. It should be noted that our use of the term "coil" does not refer to the denatured random structure in single-stranded DNA, and that both the coil and globule DNAs preserve their double-stranded structure. Upon coarse-graining of the double-stranded structures, individual long DNA chains appear as either elongated coils or compacted globules.

Among globule DNAs *in vivo*, various types of higher-order structure have been reported. In some viruses, giant DNA chains are highly packed and exhibit a liquid crystalline-like structure (Earnshaw and Casjens, 1980). In eukaryote cells, DNA chains are compacted to form a chromatin structure. It is well known that the morphology of the compacted DNA molecule changes drastically depending on the stage in the cell-cycle. Thus, a detailed understanding of the manner of packing in long DNA chains is important in biological science. However, despite this importance, our understanding of the "globule" or compacted DNA structure in an aqueous

environment remains at a primitive level. This may be due to the lack of a suitable experimental methodology for investigating the higher-order structure of single giant DNA chains in an aqueous environment.

Most previous studies on the higher-order structure of giant DNA chains *in vitro* have been carried out for an ensemble of DNA chains, using experimental tools such as light-scattering, sedimentation and viscosity. For example, studies on so-called Ψ-condensation (Lerman, 1971; 1973), polymer- and salt-induced condensation, have frequently been carried out for the aggregate, or the condensate, of DNAs. Condensation of DNAs induced by various kinds of cationic species have been studied extensively (Arscott et al., 1990; Plum et al., 1990; Bloomfield, 1991). In living cells, since individual DNA chains are compacted independently without knotting to each other, accurate discrimination between single-chain compaction and condensation of multiple DNA chains is essential for obtaining clear insight into the genetic function of long DNA.

Recently, it has been found that individual long DNA chains undergo a discrete transition between coil and globule states with the addition of various kinds of condensation reagents, such as multivalent cation (Yoshikawa, Y. and Yoshikawa, K. 1995; Yoshikawa et al.,1996a; 1996b), neutral flexible polymer (Minagawa et al.,1994; Vasilevskaya et al.,1995), alcohol (Ueda and Yoshikawa, submitted for publication), and cationic surfactant (Mel'nikov et al.,1995). These findings were obtained by single-chain observation using fluorescence microscopy. The significance of our recent finding is that the transition is first order on the level of single DNA chain, whereas the transition is steep but continuous in the level of ensemble of DNAs (Yoshikawa et al.,1996b). This clarifies why there has been essentially no experimental report on the discrete transition in the higher-order structure of DNA. With single-chain observation by fluorescence microscopy, single DNA chains have been shown to undergo a process of "nucleation and growth" (Yoshikawa and Matsuzawa, 1995), which confirms that the coil-globule transition follows first-order phase transition. The products of DNA compaction induced through the process of nucleation and growth exhibit

various shapes, such as toroid and rod, which are considered "single molecular crystals" (Yoshikawa et al.,1996a). However, the relationship between the steric structure of compacted DNA and the time-course of compaction has not yet been clarified, despite its importance in biological science. Therefore, I have extended the studies of higher-order structure of DNA to address the following problems: What is the fundamental principle which controls the steric structure of compacted DNA in an aqueous environment *in vitro* and *in vivo* ? Does a giant DNA chain reflect its past history in its compacted structure?

To investigate the effect of the order dependence of a multi-step procedure on the collapse process of long duplex-DNA, the solution structure of DNA/poly-arginine complex is analyzed in this chapter. It has become clear that the folding of single DNA can reflect its past history. I discuss such hysteresis effect with the aid of time-dependent nonlinear differential equation.

3-2. Materials and Methods

Sample solutions

Bacteriophage T4 DNA and poly-L-Arginine (poly-Arg) with an average degree of polymerization of 236 were purchased from Nippon Gene and Sigma Chemicals, respectively. T4 DNA is composed of 166 kilo base pairs, and has a contour length of 56μm in aqueous solution (Yoshikawa and Matsuzawa, 1995). I used Tris-Borate buffer (45mM Tris and 45mM Borate) throughout the experiments. The buffer solution was purified by Millipore filter before measurement. The fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) was obtained from Wako Pure Chemicals.

Fluorescence microscopy

The complex of a single T4DNA with poly-Arg was observed using fluorescence microscopy. I added 0.3μM DAPI to the DNA solution (0.3μM in phosphate), to stain

the DNA molecules. Under this condition, the ratio of DAPI molecules bound to DNA is estimated to be one per twenty base pairs (Matsuzawa and Yoshikawa, 1994). It has been previously confirmed that the presence of DAPI at this concentration has no significant effect on the persistence length or the contour length of DNA. In addition, under this ultra-dilute condition of DNA, there is almost no chance for the DNA molecules to collide with each other during the observation period of several minutes.

Sample solution was situated between two thin glass plates (Matsunami No.1, thickness:120μm~170μm) at a rather width depth of *ca.* 150μm using spacer glass plates. Since a sample depth was set with greater than that in usual fluorescence measurement, many DNA molecules remained in the bulk solution during the observation period. It is noted that the mean size of T4 DNA molecules are less that 3μm. Thus, it has become possible to observe the higher-order structure just of the complex in bulk solution which hardly has interaction with glass surface, discounting the images of DNAs absorbed onto the glass surface.

Fluorescence images of the complex were recorded on videotape and processed using an image-processor Argus 50 (Hamamatsu Photonics). Long-axis length was measured on 50~100 randomly chosen complexes under fixed conditions, where the long-axis length is defined as the longest distance in the fluorescence image (Fig. 3-1).

Electron microscopy

Electron microscopic observation of the complex was carried out by the negative-staining method with an electron microscope JEM1200EX (JEOL). Carbon-coated copper grids were treated with glow discharge. The grid was floated on a droplet of sample solution (20μl) for 2 min, drained on filter paper, and finally stained for 30 s with an aqueous solution of 1 % uranyl acetate.

3-3. Results

Fig. 3-2 shows the average long-axis length of fluorescent images as a function of the poly-Arg concentration. The length was measured with two different time-dependent procedures.

Route A: Poly-Arg and then sodium chloride were added to the DNA solution.
Route B: Sodium chloride and then poly-Arg were added to the DNA solution.

There was no difference in the final concentrations of poly-Arg and sodium chloride (40mM) between Route A and Route B. For both routes, the individual DNA chains tend to shrink with an increase in the poly-Arg concentration. This gradual change is in contrast to the discrete behavior in the coil-globule transition using other condensation agents, such as polyamine (Yoshikawa et al.,1996b), hexaamine cobalt(III) (Yoshikawa et al., 1996a), polyethylene glycol (Minagawa et al., 1994) and cationic surfactant (Mel'nikov et al., 1995). Fig. 3-2 indicates that there is a definite difference in the conformation of the complex depending on the time-course of the preparation. The system in Fig. 3-2 is expected to attain a stationary state, since it remains essentially the same even 1 week after the sample preparation.

Fig. 3-3a) shows the experimental distribution of the long-axis length for DNA chains obtained through routes A and B, in which individual samples were measured after being allowed to stand for 3 days at 4°C. It have been confirmed that the profile of this distribution remains almost constant even after 7days (data not shown), indicating that the results in Fig. 3-3 reflect a stationary state. As shown in the difference between the distributions, the complex formed through route A is folded more tightly than that formed via route B.

To verify the presence of differences in the distributions with the two routes, I performed a statistical analysis using a rank sum test. Note that the distribution of the long-axis length of the complex should deviate widely from a normal distribution,

due to the heterogeneity of the number of poly-Arg bound to DNA. Therefore, I used the Mann-Whitney-Wilcoxon rank sum test (Mann and Whitney, 1947) as a static nonparametric analysis, in which the null hypothesis to be examined is that the samples to be compared are randomly chosen from the same population distribution. The approximate normal deviate Z was 2.93, suggesting that the null hypothesis is rejected at the 5% level; *i.e.*, the two distributions have a statistically significant difference in the shaded region in Fig. 3-2. On the other hand, for poly-Arg concentrations between 0.6 and 1.0 and between 0 and 0.2, no meaningful difference was recognized between the two different routes.

These results indicate that the shape of the DNA complex is dependent on the sequence of sample preparation. The most plausible explanation for this phenomenon involves the difference in the number of poly-Arg bound to DNA between routes A and B.

Next, an analysis has been carried out by classifying the profile of the fluorescence intensity of the images of individual DNAs (Fig. 3-4). Class I includes fluorescence images with a compact bright light spot. This class has one sharp peak of fluorescence intensity within the image of a DNA chain. Class II corresponds to the images with slight loosening from the tightly compacted state having a single maximum and a small tail on the light-intensity distribution. Class III corresponds to the images with several maxima in the fluorescence-intensity distribution. Class IV includes images revealing the shape of thick filaments with a rather broad intensity distribution without intense maximum on the image. Finally, Class V corresponds to the elongated coil state. This class has no fluorescence peak within the image. The distribution of DNA images in each class is shown in Fig. 3-3 b), indicating that route A tends to enhance the collapse of the DNA chain more than route B.

To obtain information regarding structural details from the fluorescence microscopic shapes of compacted DNA, the complexes were observed by transmission electron microscopy (TEM). Fig. 3-5 shows the TEM image of a globular complex formed in the presence of excess poly-Arg: [Arg]/[nucleotide] = 3.0. It was found

that DNA globules induced by complexation with poly-Arg exhibited an amorphous-like state. Unfortunately, from the TEM measurement, it was very difficult to study the structural change of DNA chain in relation to the poly-Arg concentration and also to the time-dependent procedure on the sample preparation (hysteresis effect).

3-4. Discussion

The results in this chapter indicate that the manner of packing of giant DNA chains is determined by kinetics, rather than by the principle of the minimum free energy. To shed light on the mechanism how the observed difference depends on the time-dependent procedure (Fig. 3-3), a simple theoretical discussion based on a simple mathematical model as seen in the following has been done.

Let us discuss the effects of kinetics on the higher-order structure in the competitive binding of two cationic species, poly-Arg and Na^+, to the DNA molecule. A double-stranded DNA is regarded as an uniformly charged, stiff rod-cylinder. In addition, parameters θ_1 and θ_2 is introduced, which represent the degree of binding of cationic species, poly-Arg and sodium ion, to the negatively charged DNA. For simplicity, differences in the manner of binding of individual poly-Arg molecules to DNA are not taken into account. Here, I would like to use the term "binding" as the state where the cationic species is located near the negatively charged phosphate groups in DNA. In other words, the term "binding" of cations is used without distinguishing tight binding and delocalized binding (Oosawa, 1971; Manning, 1978). With this scheme of the binding, the kinetics of the binding of poly-Arg and Na^+ ion can be given as:

$$\begin{cases} \dfrac{d\theta_1}{dt} = k_1(c_1 - a\theta_1)(1-\theta_1-\theta_2) \\ \dfrac{d\theta_2}{dt} = k_2(c_2 - b\theta_2)(1-\theta_1-\theta_2) \end{cases} \qquad (3\text{-}1)$$

where the suffixes 1 and 2 correspond to poly-Arg and sodium ion respectively. θ_i is the variable corresponding to the fraction occupied by the ith chemical species on sites with a negative charge along the DNA chain. The symbol k is the rate constant. Parameters c_1 and c_2 are the initial concentrations of poly-Arg and sodium ion in the bulk solution, respectively. Parameters a and b are numerical coefficients representing the saturated amount of binding of the respective species, poly-Arg and Na^+, per one DNA molecular chain.

To draw a phase diagram of Eq.3-1 in relation to the actual experiments, the following parameters was established. First, it is noted that the condition $c_1/a \geq 1$ should be satisfied because the concentration of free poly-Arg in bulk solution, $(c_1 - a\theta_1)$, must not be smaller than zero for all θ_1 in $0 \leq \theta_1 \leq 1$. Similarly, the relationship $c_2/b \geq 1$ is expected in the case of Na^+. Considering that a small amount of free poly-Arg molecules remains in bulk solution at the experimental condition of globule formation, we have adapted the value of $c_1/a = 1.2$. On the other hand, Na^+ ions remain in the bulk under the condition of the excess addition of Na^+. Thus, the value $c_2/b=1.5$ was chosen. Supposing that the rate of adsorption of poly-Arg is faster than that of Na^+, the ratio of the rate constants were set as $k_2/k_1=0.2$. As for the parameters a and b, the ratio were set as $b/a=1$ for simplicity.

Fig. 3-6 shows an example of the phase diagram of the time-trajectory based on Eq.3-1, indicating that steady states are located along the line, $\theta_1+\theta_2=1$.That is, numerous numbers of final steady states exist along the line of stationary states $(\theta_1+\theta_2=1)$, depending on the initial conditions of θ_1, θ_2 $(0<\theta_1, \theta_2<1$ at $t=0)$. Thus, it is concluded that the final, or the stationary, state is not determined solely from the final concentrations of the individual chemicals (DNA, poly-Arg and Na^+) in the system; the state of the system depends on its past history. For example, let us consider route A. If we consider that the initial addition of poly-Arg corresponds to a change along the line $\theta_2=0$ and the system will stop at a certain point as shown in Fig. 3-6 a). Thereafter, with the addition of Na^+, the system will reach state A according to the dynamics of Eq.3-1. On the other hand, route B leads the complex into state B

(Fig. 3-6 b). At first, the system will move and stop at a point on the line, $\theta_1=0$, and then reach state B. Since DNA in state A binds much more poly-Arg than that in state B, the DNA chain is expected to be more compacted through route A than through route B. This theoretical expectation corresponds well to the present experimental findings.

The most significant point in the above analysis is that the final higher-order structure of single long duplex-DNA chain is not determined solely by the principle of minimum free energy but highly depends on the time-course in the steps of sample preparation. Although almost all of the nonlinear effect on the dynamics have been neglected in the above theoretical treatment, including the rather complex interactions between the higher-order structure and the number of the bound ions, the essential aspect on the effect of the time-dependent procedure has been interpreted.

As a next step, let us discuss the relationship between the higher-order structure in a DNA chain and the degree of binding with poly-Arg. Even without such a theoretical consideration, it may be easy to expect that enhanced binding of poly-Arg will cause the higher-order structure of DNA to be more compact. However, in order to gain insight into the effect of the size distribution in a semi-quantitative manner, here I would like to develop the following theoretical treatment.

A DNA chain is considered to be a stiff string which contains N segments with persistence length λ. The free energy can be written as (Grosberg and Khokhlov, 1994):

$$\frac{F(\alpha)}{T}=\frac{3}{2}\left(\frac{1}{\alpha^2}+\alpha^2\right)+\frac{BN^{\frac{1}{2}}}{\alpha^3\lambda^3}+\frac{C}{\alpha^6\lambda^6} \qquad (3\text{-}2)$$

where the first two terms describe the contribution from the elastic energy, and the third and fourth terms are the contribution from the interaction energy. The variables α is the swelling coefficient, $\alpha=S/S_0$; S is the radius of gyration of DNA chain, while S_0 is the gyration radius for a Gaussian chain with the same contour length. With a

good approximation, S is expected to be proportional to the long-axis length. B and C are the second and third virial coefficients for the effective interaction among the segments, including solvent effects. In Eq. 3-2, the effect of counter ion binding on free energy has been ignored for simplicity.

Here it is supposed that B and C change depending on the degree of binding of poly-Arg, θ_1. Besides, it is also supposed that B has a negative value because of the bridging effect of poly-Arg between the DNA segments. With these assumptions, the third and fourth terms can be rewritten as:

$$\frac{BN^{\frac{1}{2}}}{\lambda} \equiv \beta = -m\theta_1 \qquad (3\text{-}3)$$

$$\frac{C}{\lambda^6} \equiv y = n\theta_1 \qquad (3\text{-}4)$$

where β and γare the reduced 2nd and 3rd virial coefficients, respectively. The effect of binding of Na^+ on these coefficients was ignored, because the effect of Na^+ is expected to be much smaller than that of poly-Arg. Eqs. 3-3 and 3-4 show that B becomes more negative and C becomes more positive as the amount of bound poly-Arg increases. For simplicity, it was assumed that both B and C are zero when θ=0 (ideal chain).

The distribution function of the DNA chain is calculated from the following relationship:

$$P(\alpha) = \frac{\alpha^2 \exp(-F(\alpha)/T)}{\int \alpha^2 \exp(-F(\alpha)/T) d\alpha} \qquad (3\text{-}5)$$

Fig. 3-7 a) exemplifies the theoretical results of the change in the distribution function with m=2.0 and n=0.05. The resulting size distributions deduced from the theory (Fig. 3-7 a) correspond rather well to those in the experiments (Fig. 3-7 b; reconstructed from Fig.3-2, route B). The values for m and n in the calculation has been chosen

because of the following reason.

It is noted that large m value corresponds to large attractive interaction between DNA segments. It is also to be indicated that, in order to take into account the effect of self-volume of DNA chain in a proper manner, n should be larger/smaller accompanied with the increase/decrease of m in general. When the parameter m becomes smaller, the bimodal character in the density profile becomes to be enhanced. In other words, there exists distinguishable two-peaks in the theoretical distribution with small m value. For example at the conditions that m=0.44 and n=0.001, clear bimodal distribution is obtained with the above theoretical equation (data are not shown). The distribution profile with bimodality corresponds rather well to the experimental distributions for the coil-globule transition of giant DNA induced by small cationic species, such as tri-cationic amine (Yoshikawa et al.,1996b) and tri-valent cobalt complex (Yoshikawa et al.,1996a). It is expected that poly-Arg with a lot of positively charged monomers mediates larger attractive interaction between negatively charged DNA segments than small cationic species do. Thus, in the calculation of Fig. 3-7 a), somewhat large value of m has been chosen to include the large attractive interaction between DNA segments mediated by poly-Arg. As for the parameter n, this should be positive. If this is negative, the globule state becomes infinitely small, which is prohibited in the collapse of actual DNA chain. In addition to this, it is to be mentioned that, when n=0.05 and m=2.0, the free energy minimum on the side of globule is found to be located around α=0.3~0.5, corresponding to the experimental trend in the present study.

Thus, these analysis clarify how the amount of bound poly-Arg is related to the conformation of the DNA chain.

3-5. Conclusion

It has become clear that the higher-order structure of DNA in solution is dependent

on the time-course of sample preparation. Such a time-dependent effect has been discussed in relation to multi-stability in the higher-order structure of DNA. The multi-stability is attributable to the competition between different binding species. Since there exist various species which bind to DNA in the actual environment of living cells, it is expected that the competitive effect, together with the hysteresis effect, contribute significantly on the determination of the higher-order structure of DNA *in vivo*. In relation to the manner of compaction, or folding, in a DNA chain, it should be noted that DNA chains *in vivo* must unfold from compacted states to transfer the information in the base sequence into RNA, and then into proteins. In other words, change in the manner of compaction for a DNA chain is expected to be neatly concerned with the efficiency in gene expression. Actually, in the eucaryotic cell nucleus the degree of condensation in chromatin structure is considered to have a close relation on the transcriptional activity (Alberts et al, 1994). It has become clear that, though the active chromatin region with loosely condensed nucleosomal arrangement is often expressed, heterochromatin region has no transcriptional activity. In this way, the control of transcriptional activity is subject to the degree of condensation by changing the higher order structure of long DNA chain. Thus, the manner of packing DNA chain depending on the past history of its environment is highly expected to concern with the mechanism of self-regulation on gene expression.

The present finding that the higher-order structure of giant DNA chains reflects their past history suggests another possible informational source in living cells, in addition to the information stored in the primary structure of DNA. Using an analogy to computer architecture, it may be worthwhile to consider that the primary structure of DNA acts as a kind of read-only memory (ROM) whereas the higher-order structure, which reflects the time-course of the interaction with coexisting chemical species, acts as a kind of random-access memory (RAM). Studies on controlling the higher-order structure of DNA by the binding of various chemical species may be important in solving the long-standing problem in life science of how living cells can regulate gene expression using only the one-dimensional information stored as the base

sequence along the DNA chain. The textbook explanation is that gene expression is controlled by various kinds of regulatory proteins. However, it should be noted that regulatory proteins are also produced according to the information stored in the primary structure of DNA. Thus, to solve this chicken-or-the-egg problem, other sources of information besides the base sequence should be considered. The new finding in the present study, that the higher-order structure of a collapsed single chain of long DNA can reflect its past history, is expected to shed light on the unsolved problem: How is DNA compaction programmed together with temporal and spatial DNA events inside living cells?

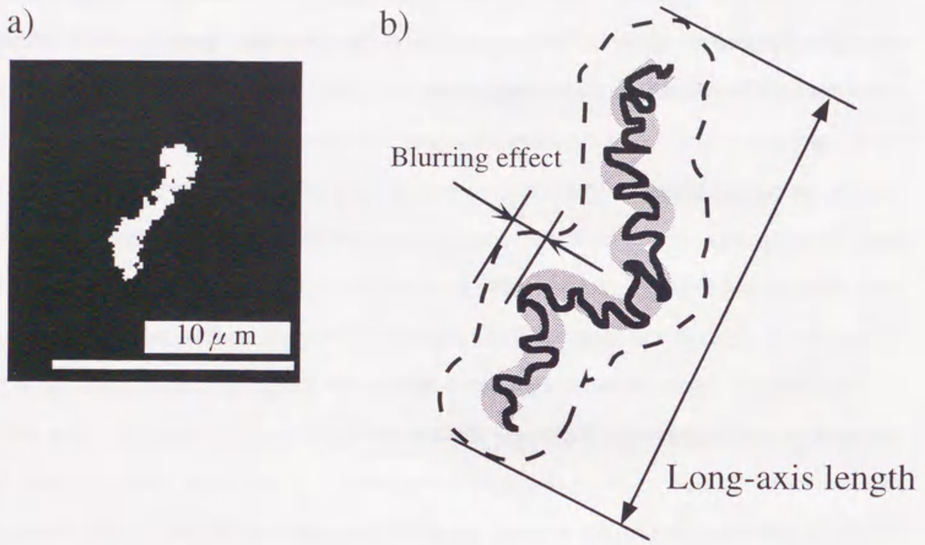


Fig. 3-1:
a) Fluorescence image of a random coil T4 DNA molecule in aqueous solution.
b) Schematic diagram of the blurring effect on the fluorescence image. Long-axis length is defined as the longest distance in the image.

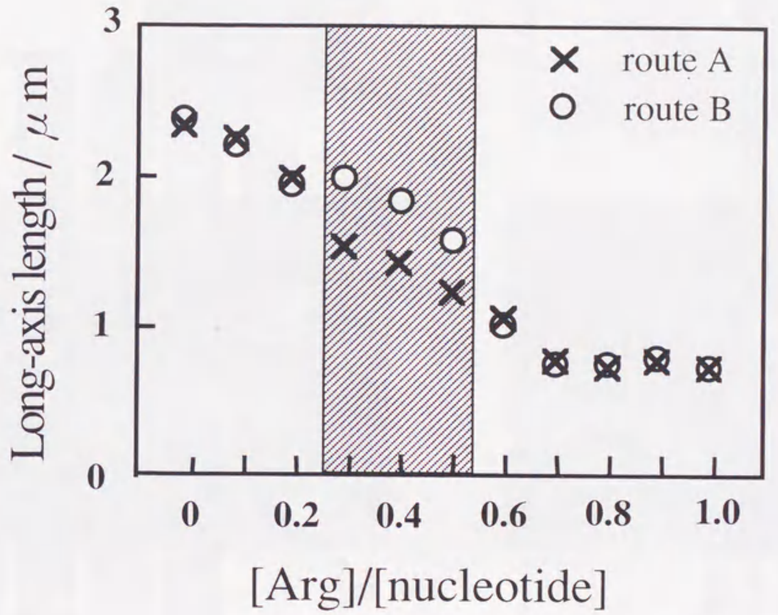


Fig. 3-2: Average long-axis length of T4 DNA as a function of the poly-Arg concentration in stationary states. The shaded area indicates the region with a distinct difference between routes A and B. The concentrations, [Arg] and [nucleotide], are the monomer concentrations of poly-Arg and DNA. [nucleotide]=0.3μM, [NaCl]=40mM.

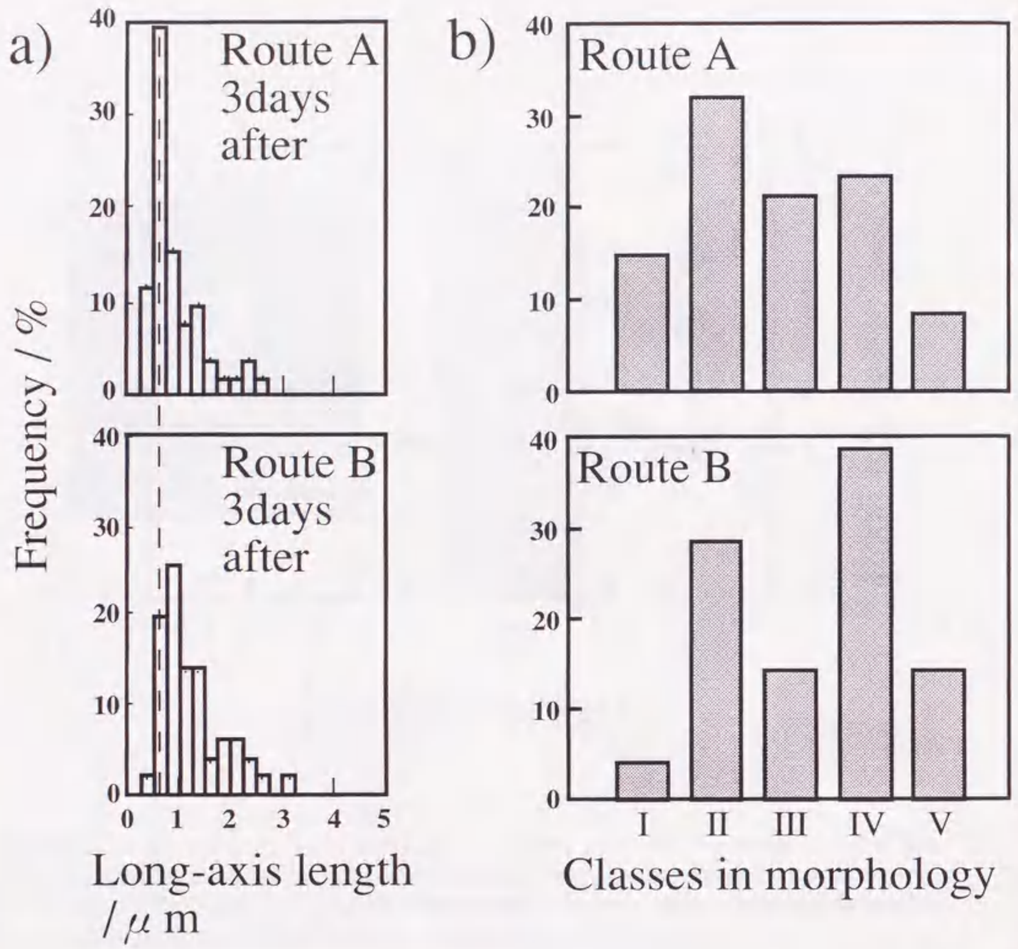


Fig. 3-3:
a) Difference in the distribution of the long-axis length of the complex at [Arg]/[nucleotide] = 0.3 in routes A and B.
b) Difference in the morphology in routes A and B according to the classification given in Fig. 3-4 .

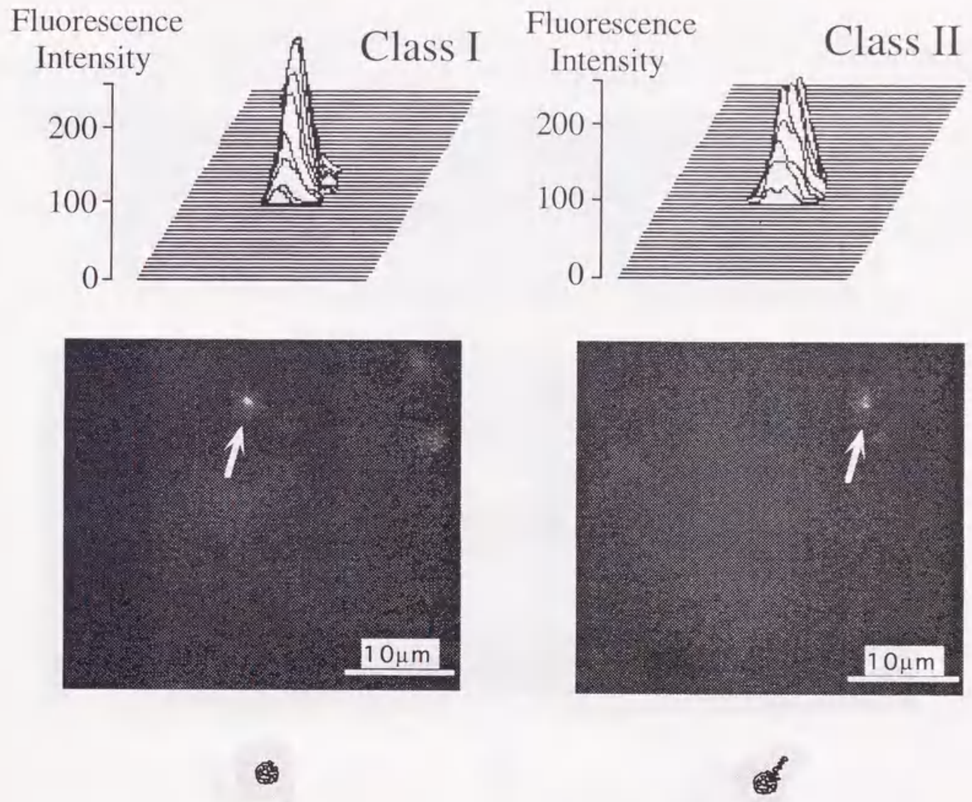


Fig. 3-4-1: Classification of the complex morphology for fluorescent images at [Arg]/[nucleotide] = 0.3. The top panel shows a quasi 3-dimensional profile of the fluorescence intensity of the complex. The middle panel is the actual fluorescence image. The bottom panel is a schematic representation of each fluorescence image together with a depiction of the blurring effect. Class I: tightly compacted complex. Class II: slightly distorted from the class I structure.

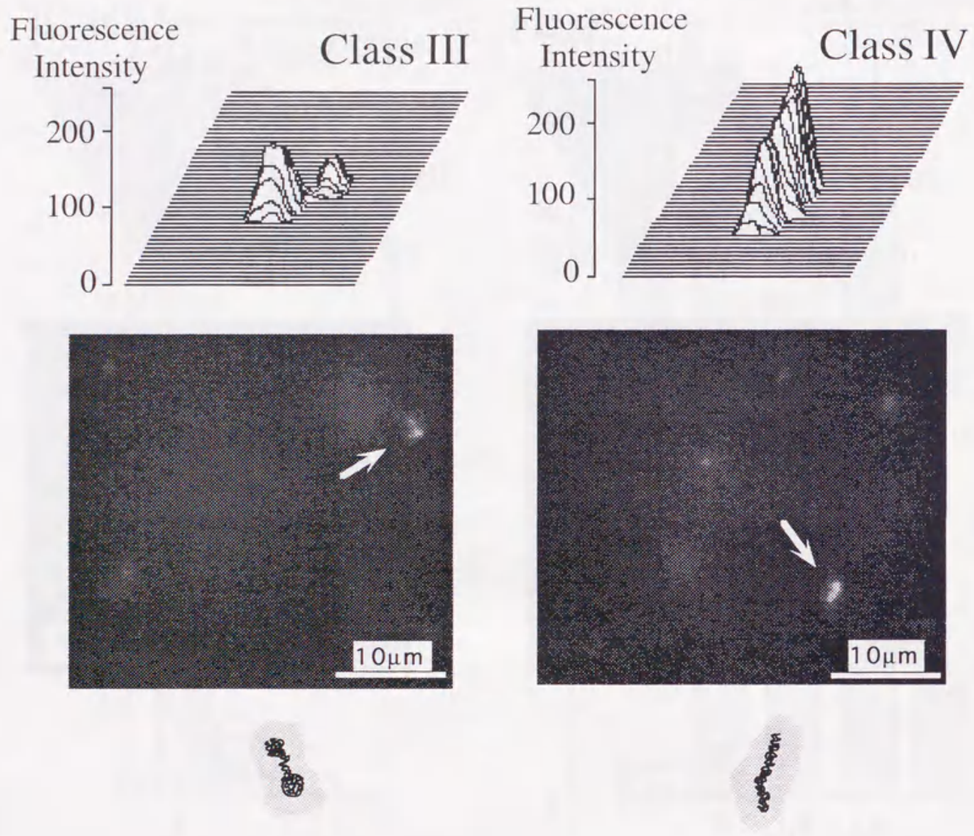


Fig. 3-4-2: Class III: Image with several maxima in the fluorescence intensity distribution due to the existence of condensed areas within the complex. Class IV: thick filament with a broad intensity distribution.

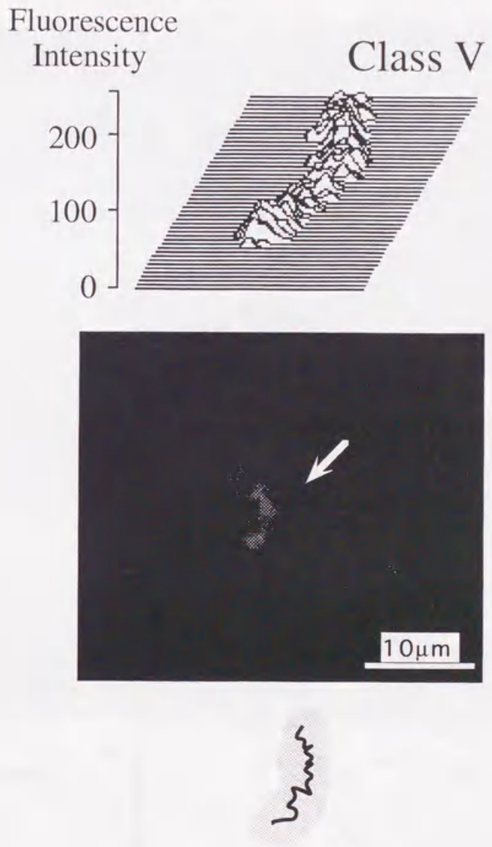
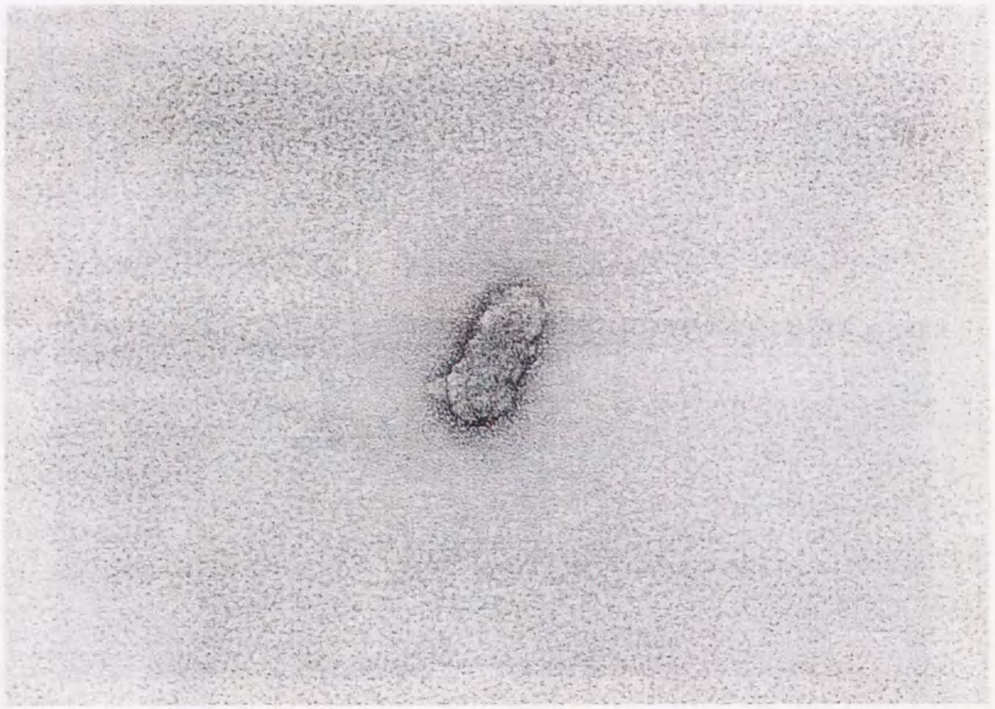
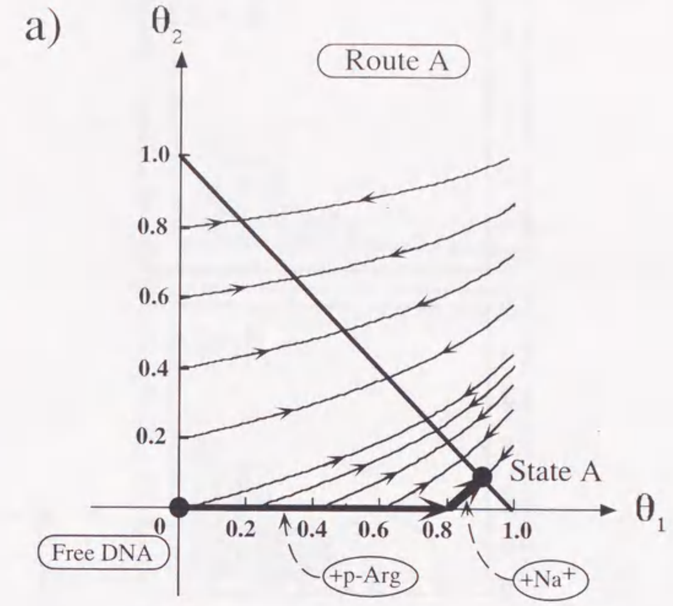


Fig. 3-4-3: Class V: random coil state.



100nm

Fig. 3-5: Electron microscopic photograph of DNA/p-Arg complex corresponding to a Class I fluorescent image of Fig. 3-4 at [Arg]/[nucleotide] = 3.0.



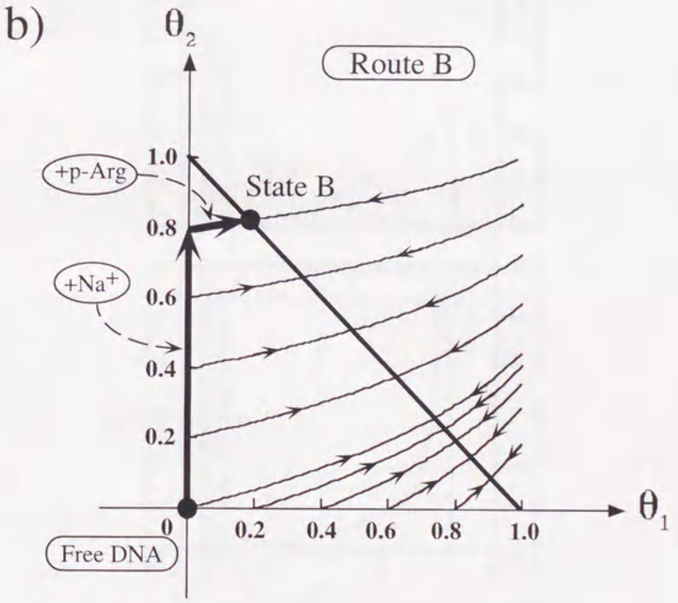


Fig. 3-6: Phase diagrams of the trajectory in Eq. 3-1, indicating that different stationary states are attained depending on the time-procedure, even when the final concentrations of the chemical species in the system are the same.

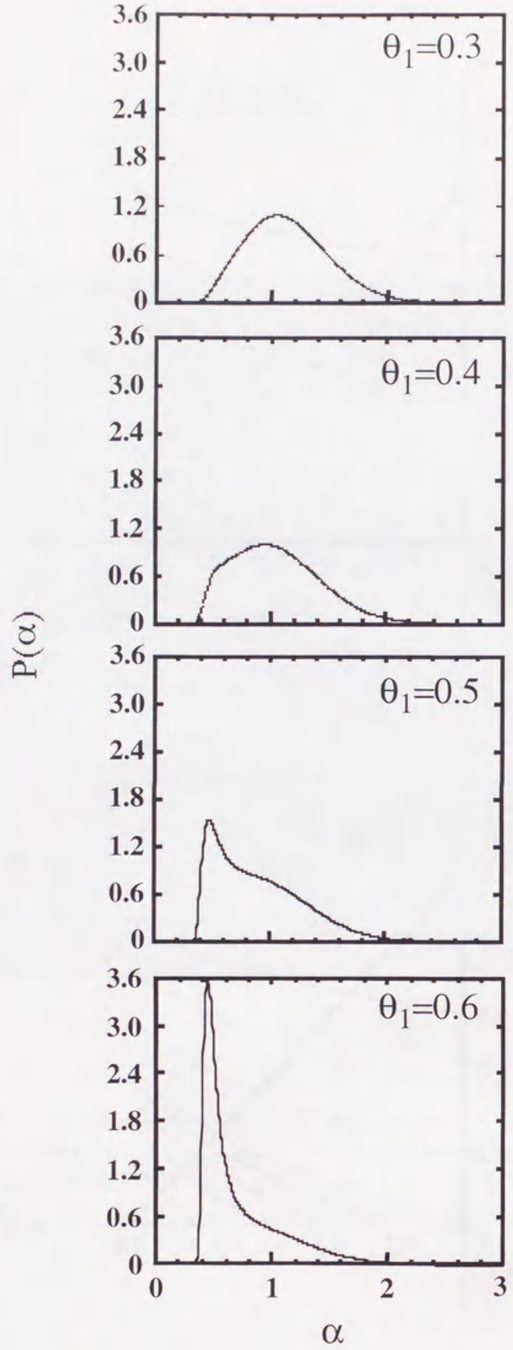


Fig. 3-7: a) Changes in the distribution of a (swelling coefficient) depending on the degree of binding of cationic species obtained from Eq. 3-5, with m=2.0 and n=0.05.

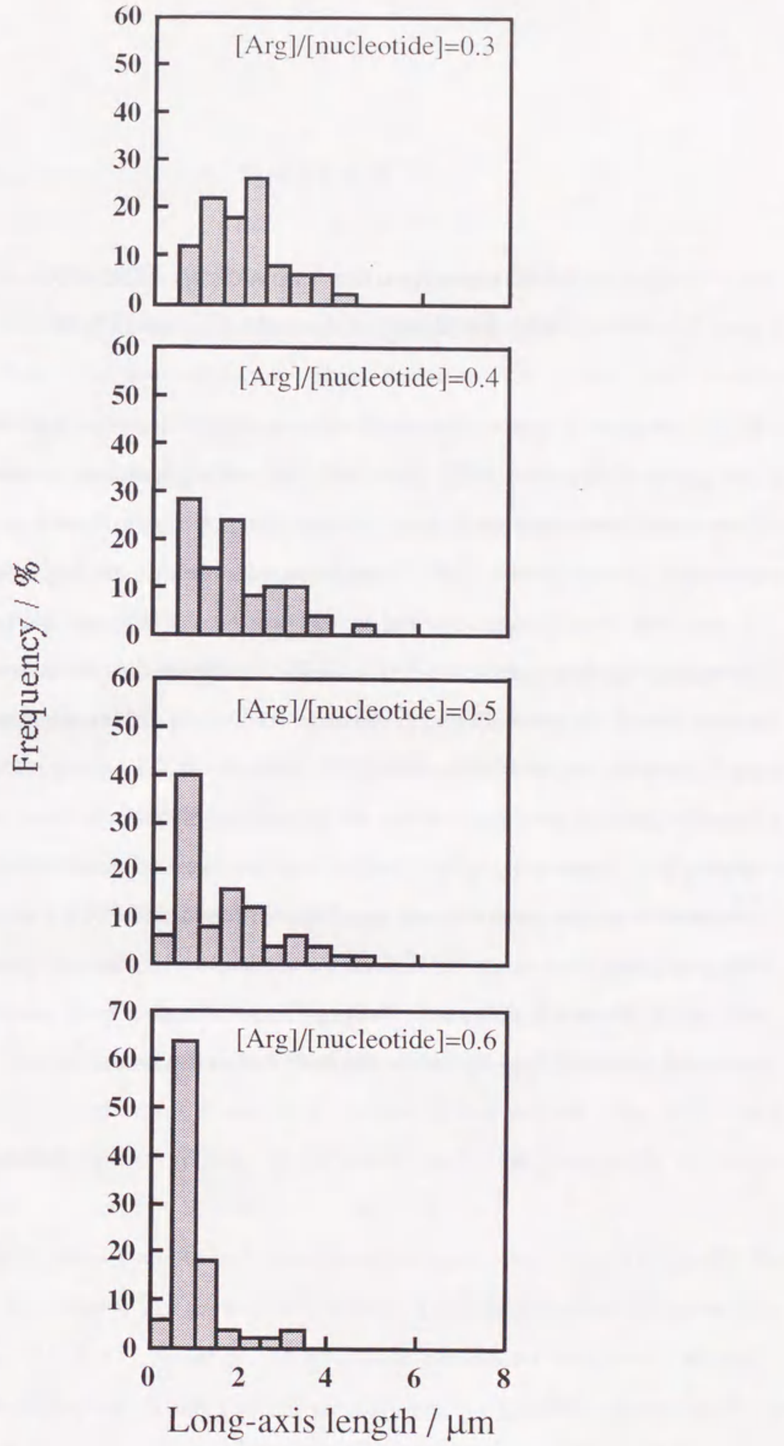


Fig. 3-7: b) Changes in the distribution of long-axis length obtained from the experiment with fluorescence microscopy, based on the result for route B in Fig. 3-2.

CHAPTER 4

Macromolecular Crowding Effect on the Folding of Single Giant DNA

In this chapter, to physico-chemically understand the effect of surroundings of concentrated macromolecules on a regulation of higher-order structure of a single giant duplex-DNA, the conformational behavior of the DNA chain in a mixed solution with various biopolymers with different state of ionization, the higher-order structure of the DNA is analyzed with FM in the presence of polycations (poly-arginine), polyanions (poly-glutamic acid), and neutral polymers (polyethylene glycol) as a model for cellular environment. Concentrated medium with neutral polymer induced the discrete folding transition of the DNA. At the threshold condition for the transition, addition of small amount of either the polycation or the polyanion caused marked structural changes in the folded DNAs. Based on thermodynamic considerations, the potential to induce the structural regulation of the folded DNA was experimentally found to be represented as the summation of the individual contributions of cationic, anionic, and neutral polymers. Physico-chemical aspect on the neural polymer's potential on the folding-regulation of giant DNA is discussed.

4-1. Introduction

In general, giant DNA molecules *in vivo* such as genomic DNAs are highly folded, and packed into a very narrow region inside cells. For example, while the full-stretch length of an individual genomic DNA with on the order of mega-base pairs reaches on the order of centimeter contour length, the actual size of the folded DNA is around several micrometers (Cairns, 1963; Holliday, 1970). Although the giant DNAs in cellular environment retain the compact structure as a whole, selective structural changes such as the local unfolding in a particular region would be induced at the proper moment of the expression of the desired genes.

Concerning the folded state of DNA, DNA condensation *in vitro* has been actively studied as a model for DNA compaction *in vivo* (Lerman, 1971; 1973; Arscott et al., 1990; Plum et al., 1990; Bloomfield, 1991; 1996). DNA condensation is induced *in vitro* by the various kinds of condensation agents together with the presence of adequate concentration of salt; e.g., a hydrophilic neutral polymer such as polyethylene glycol (Lerman, 1971; 1973; Laemmli, 1975), a low molecular-weight multivalent cation such as polyamine and hexaamine cobalt (III) (Gosule and Schellman, 1976; Widom and Baldwin, 1980), a basic polypeptide (Laemmli, 1975; Olins and Olins, 1971), a cationic and neutral surfactant (Mel'nikov et al., 1995a; 1995b; Mel'nikov and Yoshikawa, 1997), and so on. From these studies, it has become clear that a rich variety of chemical species in the cellular environment can potentially condense DNA molecule.

Among the above-mentioned condensation agents, main contribution for the DNA compaction *in vivo* are attributed not only to the binding basic-proteins such as histone, but also to the presence of highly concentrated environment with other proteins. For examples, in the case of prokaryote, the typical concentration of cytoplasmic proteins is considerably high with on the order of hundreds of milligrams per milliliter (Fulton, 1982; Zimmerman and Trach, 1991; Cayley et al., 1991). Although

the cytoplasmic proteins have no direct interaction with the genomic DNAs (in this sense, the proteins is often referred to as "inert" polymers for genomic DNAs), such a concentrated medium with cytoplasmic proteins has been shown to play an important role in functions and structures of the DNAs (referred to as the macromolecular crowding effect (Minton, 1981; 1983; Zimmerman, 1993; Zimmerman and Minton, 1993; Minton, 1997)). In addition, it should be noted that there also exists polyanions such as transcribed RNAs and newly replicated DNAs in both nuclear region of prokaryote and eukaryote. The chemical surroundings of genomic DNAs is thus generally characterized by the presence of polycations and polyanions in the nuclear region and of highly concentrated medium with various cytoplasmic proteins. It is therefore important to understand how the conformational behavior of a single giant duplex-DNA is affected by environmental changes in a mixed solution with various polymers with different state of ionization.

To better understand the conformational behavior of a single giant duplex-DNA in such a mixed solution, I adapted the model system composed of polyethylene glycol, poly-arginine, and poly-glutamic acid, and observed the manner of folding of single giant DNAs by means of fluorescence microscopy. It is to be noted here that I have investigated the characteristics of chain-folding in isolated giant DNAs. Although DNA condensation has been actively studied during the past several decades, most of the researches has examined on the condensation of a plural number of DNA chains (Lerman, 1971; 1973; Arscott et al., 1990; Plum et al., 1990; Bloomfield, 1991; 1996). The traditional experimental techniques such as light scattering, sedimentation, circular dichroism, etc., usually afford information only on an ensemble average of many DNA molecules. Observation with fluorescence microscopy has been shown to be useful for investigating the characteristics of the chain-folding of single giant DNAs. Using this method, the hidden properties of the chain-folding of single giant DNA induced by various condensation agents have been unveiled in recent years (Minagawa, 1994; Yoshikawa and Yoshikawa, 1995; Ueda and Yoshikawa, 1996; Yoshikawa et al., 1996a; 1996b; Starodoubtsev and Yoshikawa, 1997).

In this chapter, I applied the technique of fluorescence microscopy to analyze the structural behavior of folded single giant DNAs in the above-mentioned mixed solution, by avoiding the interaction between different DNA molecules in a very dilute condition. It was found that the manner of chain-folding in giant DNA sensitively depends on the compositional balance of coexisting polycations and polyanions in the crowded medium with the inert polymers.

4-2. Materials and Methods

Sample preparation

Bacteriophage T4 DNA (166 kilo base pairs) was purchased from Nippon Gene (Tokyo, Japan). Poly-L-arginine (poly-Arg; MW; 10800, DP; 56), and poly-L-glutamic acid (poly-Glu; MW; 14300, DP; 95) was purchased from Sigma Chemicals (St. Louis, MO). Polyethylene glycol (PEG; MW; 10000, DP; 227) was available from MERCK-Schuchardt (Schuchardt, German).

Sample solutions were prepared according to the following mixing procedure. First, stock solutions of DNA, poly-Arg, poly-Glu, and PEG were made at the following concentrations in distilled and sterilized pure water; [DNA]=30μM in phosphate, corresponding to 9.75μg/ml; [poly-Arg] and [poly-Glu]=3μM in residue; [PEG]=10M in monomer unit. A stock solution of Tris-Borate buffer (10xTB; 900mM Tris and 900mM Borate, pH=8.16) and a fluorescent dye solution (4',6-diamidino-2-phenylindole, DAPI, obtained from Wako Chemicals, Osaka, Japan; 30μM in pure water) were also prepared. To obtain the desired composition of each substance in the fixed volume of sample solutions (500μl), an appropriate volume of each stock solution was added to a suitable volume of pure water by the following procedure. 1) addition of 10xTB, 50μl (final concentration, 90mM), 2) addition of PEG, 3) addition of poly-Arg, 4) addition of DAPI, 5μl (final concentration, 0.3μM), 5) addition of 2-mercaptoethanol (2-ME; purchased from Wako Chemicals), 20μl (final concentration,

4% (v/v)), 6) vortexing, 7) addition of DNA, 5µl (final concentration, 0.3µM in phosphate), 8) standing still at 55°C for 15 minutes, 9) incubation at 20°C for 30 minutes, 10) addition of poly-Glu, 11) standing still at 55°C for 15 minutes, 12) incubation at 20°C for 30 minutes before observation. Finally, observation was done at 20°C.

Here, the ascending heat to 55°C was carried out to complete mixing DNAs with concentrated PEG solution, instead of mixing by strong vortexing with avoiding a cleavage of the giant T4 DNA. It has already been confirmed that the presence of DAPI at this concentration has no significant effect on the persistence length and on the contour length of DNA (Matsuzawa and Yoshikawa, 1994). 2-ME was added as an antioxidant reagent (Matsumoto et al., 1981).

Single molecular observation of giant DNAs in solution

Fluorescence images of the DNA/poly-Arg complex in PEG and poly-Glu solution were obtained using a fluorescence microscope Axiovert 135TV (Carl Zeiss, German), recorded on videotape, and processed using the image-processor Argus 50 (Hamamatsu Photonics, Japan).

Sample solutions were situated between two thin glass plates (Matsunami No.1, thickness:120µm~170µm) at a depth of *ca.* 150 µm using spacer glass plates. When the sample depth was set as in the usual observation with the fluorescence microscope (~5µm), almost all of the complex became attached to the glass surface. Related to this, it is noted that the mean size of T4 DNA molecules in pure water is *ca.* 3~5µm. By avoiding the surface effect with the relatively large sample depth, we succeeded in conformational observation of the DNAs in the bulk solution. Since the DNA concentration (0.3µM in phosphate, or 0.1µg/ml) was chosen to be very low so as to minimize the interchain interaction between DNAs, it has become possible in the present study to analyze the properties of a **single giant** duplex-DNA chain **in solution**.

4-3. Results

Evaluation of the manner of folding in a giant DNA

Figure 4-1 shows typical fluorescence images of single duplex-T4 DNAs with the corresponding profiles of fluorescence intensity and schematic representations of the conformation of the DNA chains.

As shown in Figure 4-1a), T4 DNAs in TB buffer solution exhibit an elongated random coil state with a long-axis length of *ca.* 3~5µm. While the contour length, or the full-stretch length, is 57µm in T4 DNA (Matsuzawa and Yoshikawa, 1994), the random coil state of the DNA chain in solution is naturally shrunken to the small size due to its entropic nature. The coil state is defined by the character of a marked inter-chain thermal fluctuation (Grosberg and Khokhlov, 1994). When the condensation agents such as PEG and poly-Arg are added to the solution, the DNA molecule is folded as shown in Figure 4-1 b)~d).

Figure 4-1b) shows tightly collapsed globular state of a single T4 DNAs in 8M PEG solution with the single steep maximum in fluorescence intensity, which exhibits an enhanced translational Brownian motion in keeping the tight conformation.

Figure 4-1 c) shows a loosely folded state of the DNAs in 0.12µM poly-Arg solution (in residue; [Arg]/[phosphate]=0.4) with a lower peak-height than those observed in the tightly collapsed state as seen in Fig. 4-1 b). The fluorescence intensity distribution is somewhat diffuse. Here, I refer to the b-type of folded DNA as a "tight globule" and to the c-type as a "loose globule". Especially in this chapter, the term "globule" is used with the following definition; *i.e.*, the state of the chain that is compressed to smaller size than that in its "θ-state" conformation, in which the correlation radius of link concentration fluctuations is much smaller than the size of the DNA (Grosberg and Khokhlov, 1994), (definition A; tight globule), or the state that has a markedly condensed core and a fluctuating short tail within a single chain (definition B; loose globule). The size of θ-state in T4 DNA chain is deduced to

approximately be 1.2μm from Khun length; 120nm and the contour length; 57μm (Doi and Edwards, 1986).

Figure 4-1 d) shows a coexistence of the tight globule and the loose globule in the mixed aqueous system of 4M PEG, 0.12μM poly-Arg, and 0.6μM poly-Glu (in residue; [Glu]/[phosphate]=2.0). The tight globule and the loose globule were discriminated by their fluctuating character; *i.e.*, tight globule exhibits no significant time-dependent fluctuation of the folded morphology within a period of observation more than several minutes, and retain the compact bead-like structure. On the other hand, loose globule simultaneously has both the fluctuating tail and the condensed core within a single chain as seen in Fig. 4-1 c), d). It is noted that since the coil state does not have such condensed part, the discrimination between coil and loose globule is also well-defined.

As will be shown later, the relative population of such tight and loose globules changes sensitively depending on the chemical composition of the solution. In this chapter, to quickly evaluate the population changes of folded state of giant DNAs under various conditions of mixed solution, I especially measured fraction of the tight globules that were observed in ensemble containing more than 50 DNA molecules at each fixed condition.

PEG-induced chain-folding in a single giant DNA

Inert polymer such as PEG has been found to induce large discrete transitions in the higher-order structures of individual single giant DNA chains (Minagawa et al., 1994). With the addition of the inert polymer, the conformation of the DNA chain is altered from an elongated random coiled state to the collapsed globule state; *i.e.*, the coil-globule transition. The critical concentration and width of the coexistence region depends on the ionic strength of the solution (Vasilevskaya et al, 1995). To characterize the conditions for the folding transition in a single giant DNA in the 90mM TB buffer solution used throughout the present study, the dependence of the long-axis length of the DNAs on the PEG concentration was examined.

Figure 4-2a) shows the distributions of long-axis length in single T4 DNAs induced at different PEG concentrations. Here, the long-axis length is defined as the longest distance within the fluorescence image (see the schematic representation in Fig. 4-1). At PEG concentrations of less than 4M, all DNA chains were found to be in the elongated coil state with an average size of *ca.* 3 μm. When the PEG concentration became higher than 6M, however, all the DNAs were folded into the globule state with a size less than 1 μm. It is to be noted that all the globules were tightly collapsed without the appearance of loose globule. In other words, the transition is all-or-none. In the region of [PEG]= 4~6M, the elongated coil and tight globule coexisted at thermal equilibrium, as is shown in the bimodal distribution in Figure 4-2a). As previously reported (Minagawa et al., 1994; Yoshikawa et al., 1996a; 1996b; Vasilevskaya et al, 1995), this bimodality characterizes the PEG-induced folding of a single giant DNA as a first-order phase transition between a random coil and a tight globule. The fraction of tight globules increased along with an increase in the PEG concentration as shown in Figure 4-2b).

DNA/poly-Arg complex in PEG-crowded medium

Figure 4-3 shows the dependence of the fraction of tight globules on the poly-Arg concentration at different concentrations of PEG. Lower, middle, and upper lines indicate results with PEG concentrations of 0, 2, and 4M, respectively. Under these conditions, the fraction of tight globules increased with an increase in poly-Arg concentration. It is apparent from the lower line that in the absence of PEG, all DNAs were collapsed into tight globules when the ratio of [Arg]/[phosphate] reached 1.0. This implies that the collapse is induced at conditions where the poly-Arg concentration is nearly equimolar to DNA (Kidoaki and Yoshikawa, 1996). On the other hand, in the presence of PEG, DNA is collapsed into tight globules even in conditions where [Arg]/[phosphate]<1, indicating the presence of a cooperative effect between poly-Arg and PEG on the folding transitions.

It should be noted that at the 4M PEG conditions, more than half of the DNA

chains transformed into tight globules, even when [Arg] is only 30% of [phosphate]. 4M of PEG is the minimum concentration to generate the tight globule in the coexistence region. Only a small amount of polycation was found to induce the folding transition in the crowded medium.

Interaction between the DNA/poly-Arg complex and poly-Glu in PEG- crowded medium

Next, the effect of the polyanion, poly-Glu, was investigated on the folding transition of DNA in a PEG-crowded medium containing the polycation, poly-Arg. As the system is complicated, only the essential portion of the results is shown in Fig. 4-4, which indicate the particular effect of poly-Glu on the complex in the PEG medium.

When [Arg]/[phosphate]=0.7 in 4M PEG, there is almost no effect from the addition of poly-Glu up to the ratio of [Glu]/[phosphate]=5.0. In other words, essentially all of the DNAs remain in the tight globule, even with a large excess of the polyanion. In contrast, when [Arg]/[phosphate] =0.4, the addition of the poly-Glu induces a rather remarkable effect, with tight globules becoming unfolded. According to control results on giant-DNA/poly-Glu interaction appearing in (Ichiba and Yoshikawa, 1998, BBRC, in press), poly-Glu does not affect the conformation of giant T4 DNA in solution until the concentration reaches 0.8 M in residues; *i.e.*, [Glu]/[phosphate]=2.7x10^6. Thus, it is noted that poly-Glu is completely inert for the giant DNAs in the present range of the concentration of [Glu]/[phosphate]=0~5.0.

Such a result indicates that the manner of folding of a single DNA chain present in a crowded medium with inert neutral polymer is affected by a delicate compositional balance between the existing polycations and polyanions.

4-4. Discussion

Energetical character of the macromolecular crowding effect on the structural regulation of the folded single giant DNAs

In this study, isolated single giant T4 DNA molecules (test macromolecule) were situated in a concentrated solution environment of hydrophilic neutral polymer, PEG. The complex formation between the single DNA and other polycations, poly-Arg, has been examined in the concentrated PEG medium. Particular effects on the properties of the test macromolecules which are created by such concentrated situations have often been referred to as the macromolecular crowding effect, indicating (a) a favoring effect for the formation of a compact conformation of the test macromolecule and (b) an enhancement of the binding between the test macromolecule and other macromolecules, etc. (Minton, 1981). Both effects (a) and (b) can be seen in Figure 4-3; effect (a) can be seen in the vertical section with the fixed concentration of poly-Arg, and effect (b) from comparison among the slopes of each line of PEG concentration in the region of low poly-Arg concentration. The present findings visually confirm that the macromolecular crowding effect affects on the regulation of higher-order structure of the single giant DNA through both of the folding of the DNA chain and of the enhancement of complex-formation with other polycations.

Here, I would like to discuss the physico-chemical meaning of the crowding potential to the structural regulation of giant DNA affected by the addition of poly-Arg and poly-Glu from a thermodynamic viewpoint. Tight globules and loose globules were found to coexist under equilibrium conditions in the present system, as shown in Figure 4-1 c), d). From the relative population, the free energy gap between the tight and loose globules was deduced (see Fig. 4-5).

The plotted points lay roughly in straight lines fitted with the least squares method, indicating that the potential for poly-Arg to stabilize the tight globule is almost constant. From the slopes in Figure 4-5, the potential of poly-Arg is calculated to be approximately 66~95x10^6kJ/mol per 1M Arg-residues. Since the linearities of the fitting results are retained in a nearly parallel manner with each other regardless of the concentration of PEG, it is found that the potentialities to induce the folding of DNA can roughly be represented as the linear summation of the two contributions

from PEG and poly-Arg. From the vertical section with the fixed concentration of poly-Arg, the average potential of the PEG-crowded condition is calculated to be approximately 1.8kJ/mol per 1M PEG-monomers.

Figure 4-6 shows the dependence of the free energy gap between tight and loose globules on the added poly-Glu concentration under the condition of [PEG]=4M and [Arg]=0.4, calculated from Figure 4-4. The linearity again holds for the effect of DNA-unfolding by poly-Glu. From the slope in the graph, the destabilization energy of the tight globule state is calculated to be approximately 3.2×10^6kJ/mol per 1M Glu-residues. As the potential of poly-Glu for the unfolding transition of DNA also exhibits linearity in the PEG-crowded medium, it can be concluded that the change in the total free energy in the folding transition of DNA can be represented as the summation of the contributions from the crowding inert polymers, polycations, and polyanions:

$$\Delta G_{total} = \Delta G_{PEG} + \Delta G_{p\text{-}Arg} - \Delta G_{p\text{-}Glu} \qquad (4\text{-}1)$$

In the recent publications of Yoshikawa and his collaborators, it has been made clear that ΔG_{PEG} shows the double-minimum profile as seen in the first-order phase transition (Minagawa et al., 1994; Yoshikawa et al., 1996a; 1996b; Vasilevskaya et al, 1995). This can also be seen from Figure 4-2a), where the distribution clearly exhibits the bimodality. Therefore, the folding and unfolding of the DNA chain induced by the polycation and polyanion are interpreted to be caused by the change in the relative energy difference in the double-minimum of the crowding potential, ΔG_{PEG}.

Biological implications of the structural regulation of the folded giant DNAs observed in the present mixed medium

The biological significance of the macromolecular crowding effect has been investigated in many studies (see reviews, Zimmerman, 1993; Zimmerman and Minton,

1993; Minton, 1997), because the cellular environment is highly concentrated with various kinds of biopolymers in general, and the biopolymers originally function under the physiologically crowded situation. It has well been characterized that the macromolecular crowding causes *in vitro* DNA condensation, which has been known as Ψ-condensation (Lerman, 1971; 1973). Recently, it is advocated that the macromolecular crowding yields a mandatory condensation of DNA *in vivo* (Zimmerman and Murphy, 1996). The effect is becoming one of the essential issues in the biological research on the DNA-condensation phenomena (Bloomfield, 1996; Minton, 1997).

The results in this chapter indicate that the structural changes in the folded state of single giant duplex-DNA sensitively depends on the compositional balance of coexisting polycations and polyanions, especially in the crowded medium with inert polymers. Whether retaining or local unfolding the tightly collapsed structure of the DNA-polycation complex is determined by the concentration both of the binding polycations and of the surrounding polyanions in the crowded medium.

The higher-order structure of genomic DNAs *in vivo* is known to be regulated by the interplay between cationic and anionic proteins. An example *in vivo* system is seen in an interaction between sperm chromatin and nucleoplasmin in fertilized eggs. Sperm chromatin is tightly compacted by binding of sperm-specific protein, protamine, that is highly basic with arginine residues over 80% in its amino acid sequences (Wouters-Tyrou et al., 1991). On the other hand, nucleoplasmin is a thermostable acidic protein (Earnshaw et al., 1980) and has clusters of charged residues including a long poly-glutamic acid tracts (Dingwall et al., 1987; Bürglin et al., 1987). Nucleoplasmin decondenses sperm nuclei of *Xenopus*, *Mytilus*, salmon, human, etc. due to the removal of protamines from the sperm chromatin (Itoh et al., 1993; Rice et al., 1995; Iwata et al., 1997). In sperm nuclei, the interplay between polycations (protamines) and polyanions (nucleoplasmins) plays a essential role for the structural regulation of folded state of genomic DNAs. It is noted here that the interplay should naturally be affected by the presence of other proteins inside nucleus such as nuclear

matrix proteins, and by the compressive potential imposed by the crowded medium with inert proteins in cytoplasm. The present results suggest physico-chemical aspect of how the interplay affecting for the structural regulation of genomic DNAs is influenced by the cellular crowded surroundings.

4-5. Conclusion

In this chapter, it has been demonstrated that the potential of folding of DNA, or the free energy difference between the tight globule and the unfolded state, can be represented as the simple summation of the individual contributions of neutral, cationic, and anionic polymers. Due to the double minimum profile of the crowding potential, especially at the threshold conditions in the folding transition of DNA induced by the crowding polymer, a small change in the concentration of either of the polymers can induce a large change in the higher-order structure of giant DNA. Since the solution environment in the living cell physiologically establishes such a polymer situation, the present findings can be expected to provide insight into the regulation of the folded structure of genomic DNA *in vivo*.

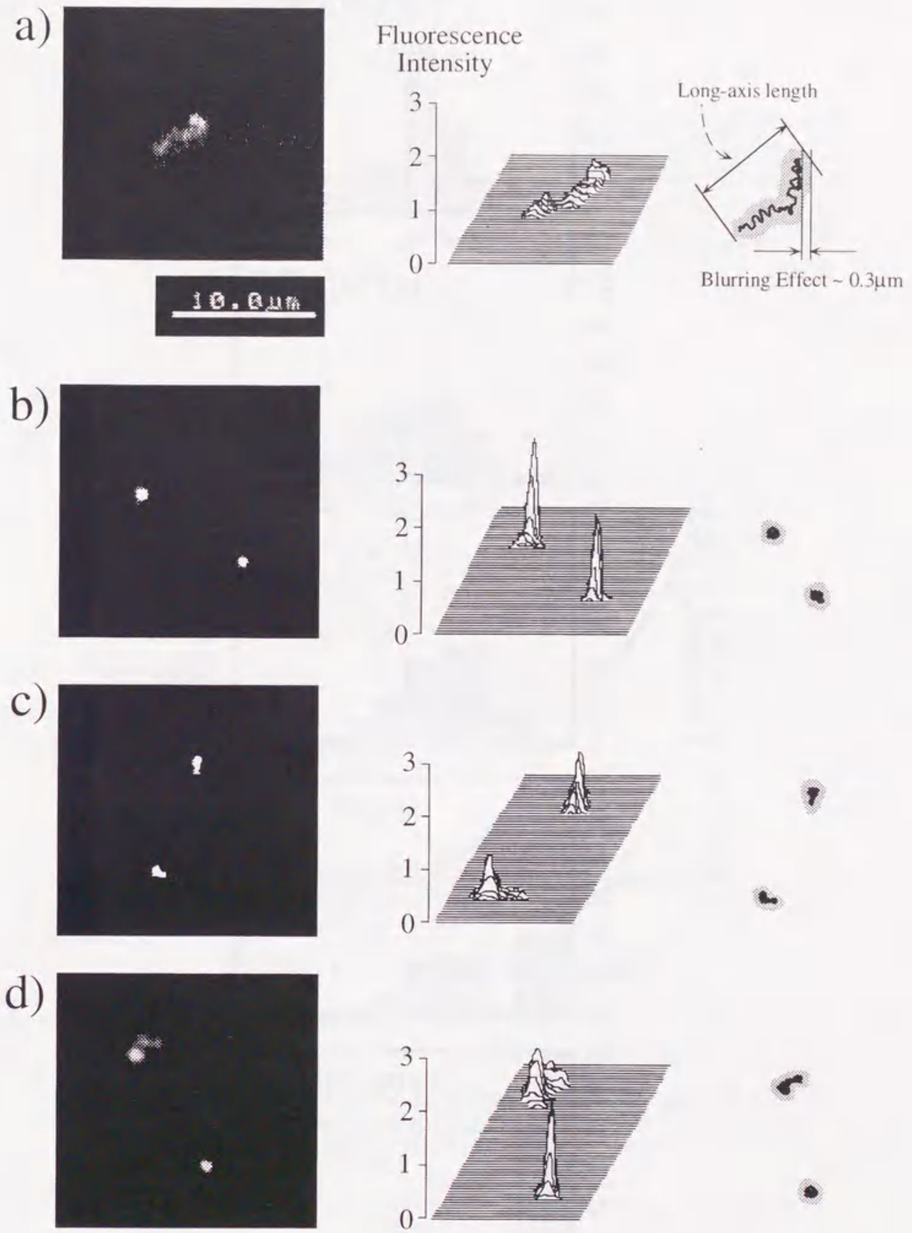


Fig. 4-1: The left: fluorescence images of the single T4 DNA molecules. (a) random coiled state in 90mM TB buffer, (b) tightly collapsed globule state in [PEG]=8M solution, (c) loosely condensed globule state in [Arg]/[nucleotide]=0.4 solution, (d) the tight globule and the loose globule in the mixed system of [PEG]=4M, [Arg]/[nucleotide]=0.4 and [Glu]/[nucleotide]=2.0. The middle: quasi three-dimensional profiles of fluorescence intensity distribution for the corresponding left-hand-side images. The noise in the fluorescence images has been smoothed out with image processing. The right: schematic representation of the correspondence between the conformation of DNAs and the fluorescence images.

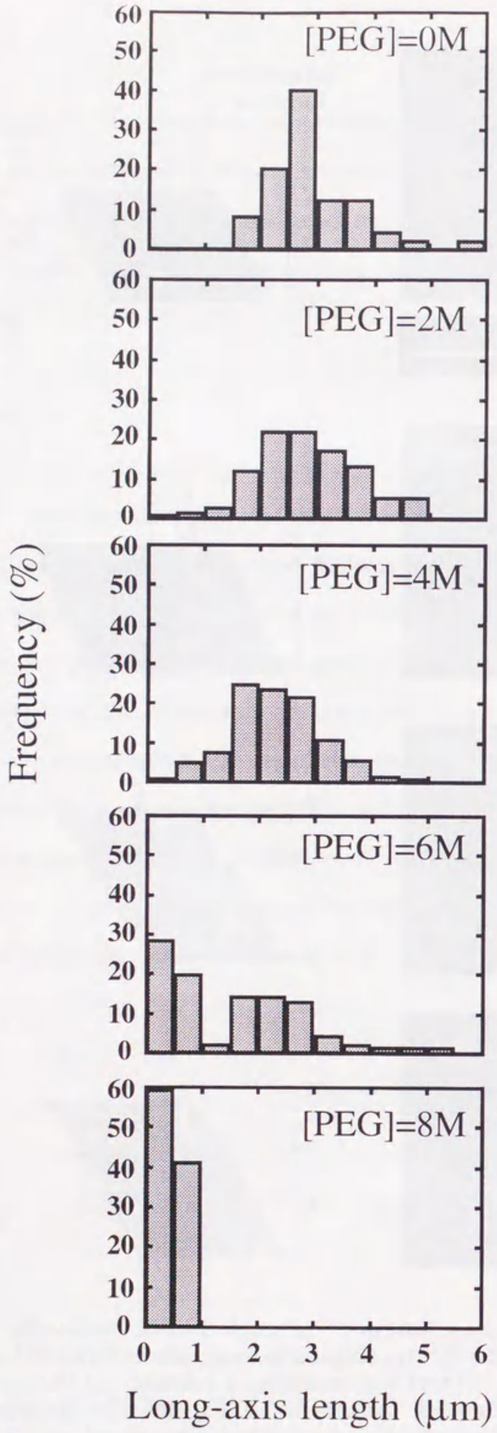


Fig. 4-2. (a) Distribution of the long axis length of the T4 DNA molecules at various PEG concentrations. Fifty DNAs have been analyzed at each concentration.

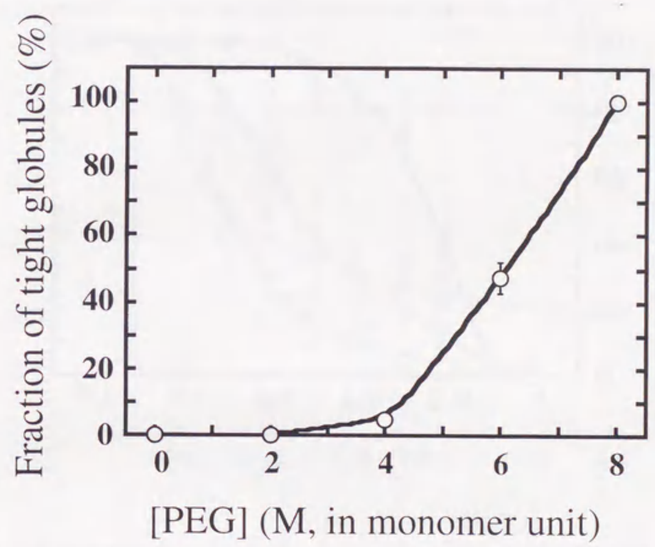


Fig. 4-2 (b) The fraction of tight globules dependent on the PEG concentration. Error bars show the standard error in 3 experimental runs, each with 50 images.

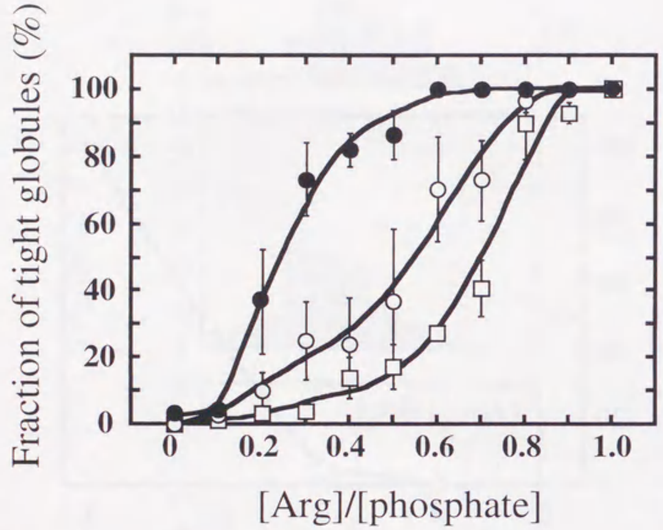


Fig. 4-3. Dependence of the fraction of T4 DNA tight globules on the poly-Arg concentration in the PEG medium with different concentrations. "Tight globule" is defined in the text. Lower (open square), middle (open circle), and upper lines (closed circle) correspond to [PEG]=0, 2, 4M in monomer units, respectively. Error bars show the standard error in 4~6 experimental runs, each with 50 images.

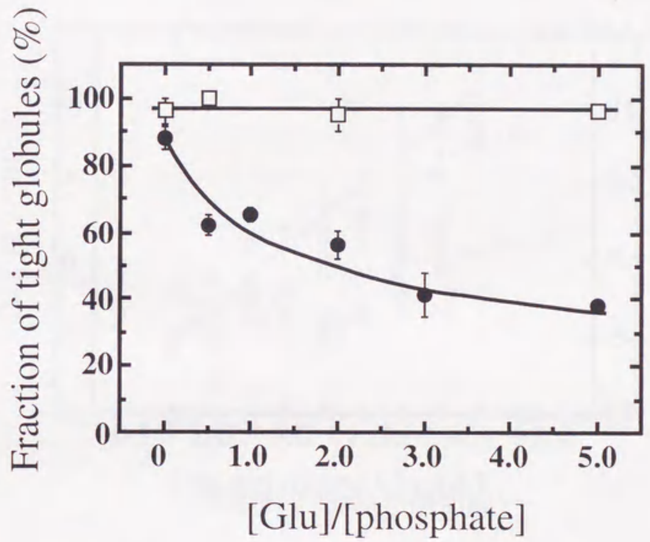


Fig. 4-4. Change of the fraction in T4 DNA tight globules with poly-Glu concentration in the solution containing 4M PEG and poly-Arg. [Arg]/[phosphate]=0.7 for the open squares and [Arg]/[phosphate]=0.4 for the solid circles. Error bars show the standard error in 4~6 experimental runs, each with 50 images.

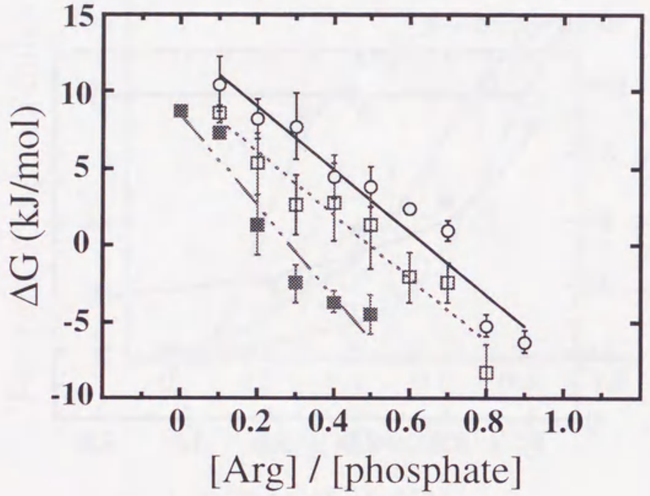


Fig. 4-5. Free energy differences between tight and loose globules vs. the poly-Arg concentration when coexisting PEG is 0M in monomer unit (open circle), 2M (open square) and 4M (closed square). Error bars show the standard error in 4~6 experimental runs.

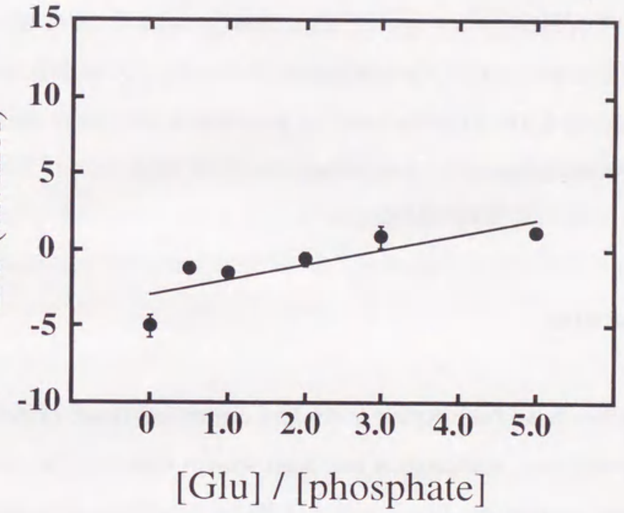


Fig. 4-6. Dependence of free energy differences between tight and loose globules on the added poly-Glu concentration in the presence of [Arg]/[phosphate]=0.4 and 4M PEG. Error bars show the standard error in 4~6 experimental runs.

CHAPTER 5

Appropriate Steric Conditions of Condensed DNA Chains for the Efficient Gene Transfection

This chapter is devoted to show a simple example of a correlation between the function and the condensed structure of DNA chains. To extract a physico-chemically fundamental viewpoint as for specific functions generated from the formation of condensed state of DNA chain, the interaction between DNA and cell is investigated; the gene transfection. The importance of an surface-effect of DNA condensate on the execution of DNA's original function is discussed.

5-1. Introduction

Many researchers have been trying to find an optimized DNA carrier-complex for efficient gene-delivery. Although it has been shown that complex formation with substances such as cationic lipids and polylysine is effective (Ledley, 1995), our understanding of the optimal structure for a DNA carrier-complex remains at a preliminary level, especially with regard to non-receptor-mediated transfection.

On the other hand, it is well known that DNA molecules condense to form aggregates with the addition of various cationic and neutral chemical species: e.g., basic polypeptide (polylysine (Laemmli, 1975), poly-arginine (Minagawa et al., 1991; Kidoaki and Yoshikawa, 1996)), etc. These condensing reagents drastically change the higher-order structure of the DNA molecule. In some cases, DNA even undergoes a large discrete transition, or first-order phase transition (Minagawa et al., 1994). According to these findings, a carrier-complex of DNA is also expected to have a characteristic higher-order structure, depending on the properties of the interaction.

To analyze the higher-order structure of a DNA-carrier complex, in the present study we used a fluorescence microscope technique. This technique is useful for the direct observation of DNA molecules in solution. Using this method, the structure of the complex of a reporter gene and condensation agents was observed in aqueous solution, and was compared with its transfection efficiency.

Before beginning the experiments, the literature for various natural DNA-condensing materials was searched. Sperm protamine is known to compact sperm DNA into a highly condensed stable complex in spermiogenesis. The peptide sequence of cuttlefish protamine is highly basic: RRRRRSRR RRRRSRRRSR SPYRRRYRRR RRRRRRRSRR RRYRRRRSYS RRRYRRRR (Wouters-Tyrou et al., 1991). Over 80% of the amino acids are arginine residues. Thus, it was expected that poly-L-arginine (poly-Arg) may be useful for condensing genomic DNA and transferring DNA into the cell. In this chapter, poly-Arg peptide as a DNA carrier molecule was focused on and it was found that the complex of DNA / poly-Arg is effective in gene transfer.

5-2. Materials and Methods

Preparation of DNA/ poly-Arg complex.

β-galactoshidase gene as the reporter was ligated into the Bam HI site of retroviral vector pLRNL downstream from the Molony murine leukemia virus (MoMLV) LTR in the same transcriptional orientation as the viral transcriptional unit to form construct pLZRN (Li et al., 1989). The MoMLV-based retroviral vector pLRNL contains the Neo-resistance (Neo^R) gene under control of the promotor of the Rous sarcoma virus (RSV) between LTRs. pLZRN was added to buffer solution (PBS(-)) containing poly-L-Arginine (poly-Arg; Mr = 139200; Sigma Chemicals). The final concentrations were DNA 30μM (in phosphate) and poly-Arg 0 ~ 100 μM (in residue). The solution of the DNA/poly-Arg complex was incubated for 30 minutes at 20°C.

For fluorescence microscopic observation, the fluorescent dye 4',6-diamidino-

2-phenylindole (DAPI; Wako Pure Chemicals; 30μM) and the anti-oxidant 2-mercaptoethanol (2-ME; Wako Pure Chemicals; 4% (v/v)) were added.

Fluorescence microscopic observation

A fluorescence microscope (Axiovert 135V, Carl Zeiss) with a 100×oil-immersion objective lens was used. Sample solution was situated between two thin glass plates (Matsunami No. 1, thickness: 120μm~170μm) separated by *ca.* 50μm using spacer glass. Fluorescence images of the complex were recorded on videotape and processed using an image processor (Argus 50, Hamamatsu Photonics).

To analyze a distribution of the higher-order structure of the complex, long-axis length was measured on 50~100 randomly chosen complexes on a fixed conditions, where the long-axis length is defined as the longest distance in the fluorescence image.

Cell and gene transfer protocol

The adherent cell line 208F (rat fibroblast) was cultured at a density of about 5×10^4 cells per well in 24-well culture dishes in Iscove's modified Dulbecco's medium (IMDM) with 10% (v/v) fetal bovine serum (FBS) in a 5% CO_2 atmosphere at 37°C. Poly-Arg complexes with plasmid DNA were prepared in microfuge tubes by mixing 2μg pLZRN with serially diluted peptides (10μM to 160μM) in plain medium in a final volume of 200μl. In a typical experiment, cells were transfected in PBS with DNA/poly-Arg complex at 37°C. After 2 h of incubation, solution was removed and replaced by medium containing 10% FBS with 100μM of chloroquine. Three hours later, the cells were washed and replaced by medium. As a positive control, 10μl of Lipofectin (GIBCO BRL) was used instead of poly-Arg.

β-galactosidase assay

Expression of β-galactosidase genes was measured after 24 h of incubation at 37°C in 5% CO_2 with the β-galactosidase Enzyme Assay System (Promega: Madison WI)

according to the manufacturer's instructions. The transfected cells were rinsed once with PBS and then suspended in 200μl of the reaction buffer in each 24 well. 100μl of 5mg/ml o-nitrophenyl β-D-galactopyraboside solution was added to the cell extract and incubated at 37°C for 30min, the absorbance at 420nm was measured.

For *in situ* β-galactoshidase activity, cell were washed with PBS and fixed with 1.25% glutaraldehyde solution for 15min at room temperature. After fixation, cell were washed three times with PBS and icubated with X-Gal solution (0.2% X-Gal, 1mM $MgCl_2$, 150mM NaCl, 3.3mM $K_4Fe(CN)_6$) for 4hr at 37°C. After incubation, the X-Gal solution was removed and cells were washed with PBS.

5-3. Results and Discussion

The phase diagram in Figure 5-1 is based on observation of the carrier-complex in solution using fluorescence microscopy. The vertical axis shows the average size of the carrier-complex, and the horizontal axis shows the concentration of poly-Arg. The size distribution was obtained by measuring the long-axis length. The standard deviation in the size distribution of the complex is also shown for each sample. As shown, the complex drastically increases in size when the ratio of nucleotide to Arg residue is 1:1.

Actual fluorescence images of the complex are shown in Figure 5-2. At low Arg concentrations before the transition, the complexes appear as bright spots, suggesting the formation of a compact globular structure (Fig. 5-2A). On the other hand, the complexes become very big (bigger than 10μm) at Arg concentrations above the transition (Fig. 5-2B). Even with fluorescence microscopy, it was impossible to recognize individual DNAs because the size of pLZRN DNA is not large enough for monitoring by fluorescent light (wave length: ~0.4μm); the contour length and gyration radius are expected to be around 3μm and 0.5μm, respectively. Therefore, it is likely that both small and big complexes (Fig. 5-2A and 5-2B, respectively) are

composed of many DNA chains. On average, large complexes (Fig. 5-2B) are about ten times greater in volume than small complexes (Fig. 5-2A). As will be discussed later, the experimental conditions for generating big complexes were successful for transfection.

To assess the efficiency of gene transfer with the DNA/poly-Arg complex, a transfection experiment was carried out with 208F cells. The DNA/ poly-Arg ratio for maximal transfection was determined according to the β-galactosidase activity of lac-Z expression vector-transfected cell lysate (Fig. 5-3). β-gal activity was detected at a poly-Arg concentration of greater than 40μM. Maximal transfection was found at a poly-Arg concentration of 80μM in plain Dulbecco's medium. At a poly-Arg concentration of around 160μM, the transfected cells were round and peeled from the plate. This may be due to the cytotoxic effect of poly-Arg peptides at higher concentrations. Using poly-Arg at 80μM , β-gal expression in 208F cells was about 5-fold greater than that obtained with lipofection. Since 0.01μg /ml DNA is equivalent to a phosphate concentration of about 30μM, the mole ratio of the amino acid residues in poly-Arg and the phosphate group of DNA was Arg / DNA : 0.3-5.0. The minimum ratio for gene transduction was 1.

Comparing the data in Figures 5-1 and 5-3, it is clear that a big aggregate structure is relatively more efficient for the delivery of plasmid DNA. Here, it should be noticed that the number of the complex in solution becomes smaller when the big complexes are formed than when only the small complexes are found, because the total amount of DNAs in sample solution is taken to be the same in the present experiments. It is, thus, obvious that the probability for the big complex to interact with cells is lower than that for the small complex. In spite of this low probability of the big complex on the contact with cell, the transfection efficiency was found to be higher under the condition where the big complexes are formed. This result suggests that the ability of the big complex to transfect DNA into cell is much higher than the small complex.

This result is attributable to the stabilization effect for the big complex adsorbed

on cell surface, because of the enlargement of contacting area between carrier-complex and cell surface. In fact, estimating the area ratio roughly from Fig. 5-2 based on the sizes of big and small complexes, the ratio of small complex to big one is about 1:100. In the case of small complex, it seems reasonable to suppose that desorption rate of small complex from cell surface is rather fast due to its small contacting area and the complex cannot stay on cell surface over enough time to be transformed into cytoplasmic space. It is, therefore, concluded that gene transfer mediated by cationic polyarginine requires a formation of big carrier-complex of DNA aggregate.

The efficiency of some types of gene transfer can be augmented by the use of pharmacological agents that disrupt the endocytic transfer of DNA-ligand complexes. Chloroquine has been used in this capacity to increase the expression of transgenes. Chloroquine acts by increasing the pH inside lysosomes, which inhibits the activity of hydrolyzed enzymes. The effect of chloroquine on transfection efficiency was studied by performing transfection in medium containing 100μM of this lysomotrophic agent. The β-gal activity after transfection was increased several-fold when chloroquine treatment was performed. The stability of endocytosed DNA-ligand complexes and their resistance to degradation are critical for the effective expression of the transgene, since many ligands are transferred to lysosomes. The association of polypeptides with DNA permits the DNA-ligand complex to escape endosomal degradation pathways. Poly-Arg may contribute to preventing the degradation of DNA-ligand complex by making DNA inaccessible to lysosomal nucleases.

The formation of the DNA/poly-lysine complex was also assessed by the same method. The threshold concentration for large aggregates was almost the same as that in the experiment with poly-Arg. On the other hand, the efficiency of gene transfer was several-fold lower than that with the poly-Arg complex. This may be due to the difference in the steric structure of the residues in the amino acids. Lysine residue contains in its structure longer by a hydrophobic group, CH_2, than arginine residue does. Since the contact area between adsorbed big complex and cell surface become large, it is considered that even a slight difference on chemical structure of

residue is accumulated over all the Lys residues included in the contact area and the hydrophobic effect is amplified to an extent which cannot be ignored. This effect should bring about the reduction of transfection efficiency in case of poly-lysine mediated gene transfer.

5-4. Conclusion

The cationic polypeptide poly-arginine promotes transfection efficiency at certain concentration. Peptide-mediated gene transfer, peptidefection, has also been shown to be successful with a lysomotrophic agent. It was found that the formation of the big carrier-complex of DNA aggregate is essential in the poly-Arg peptidefection. The results in this chapter suggests not only possible steric conditions of the carrier-complex for the process of gene transfection mediated by cationic polypeptide, but also the simple physico-chemical aspect of correlation between structures and functions on DNA condensate or folded DNA.

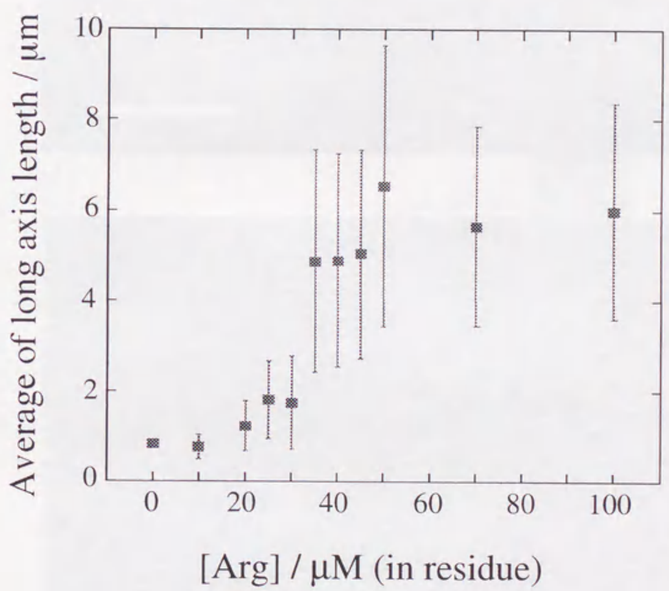


Fig. 5-1. Formation of aggregates of DNA/poly-Arg complex

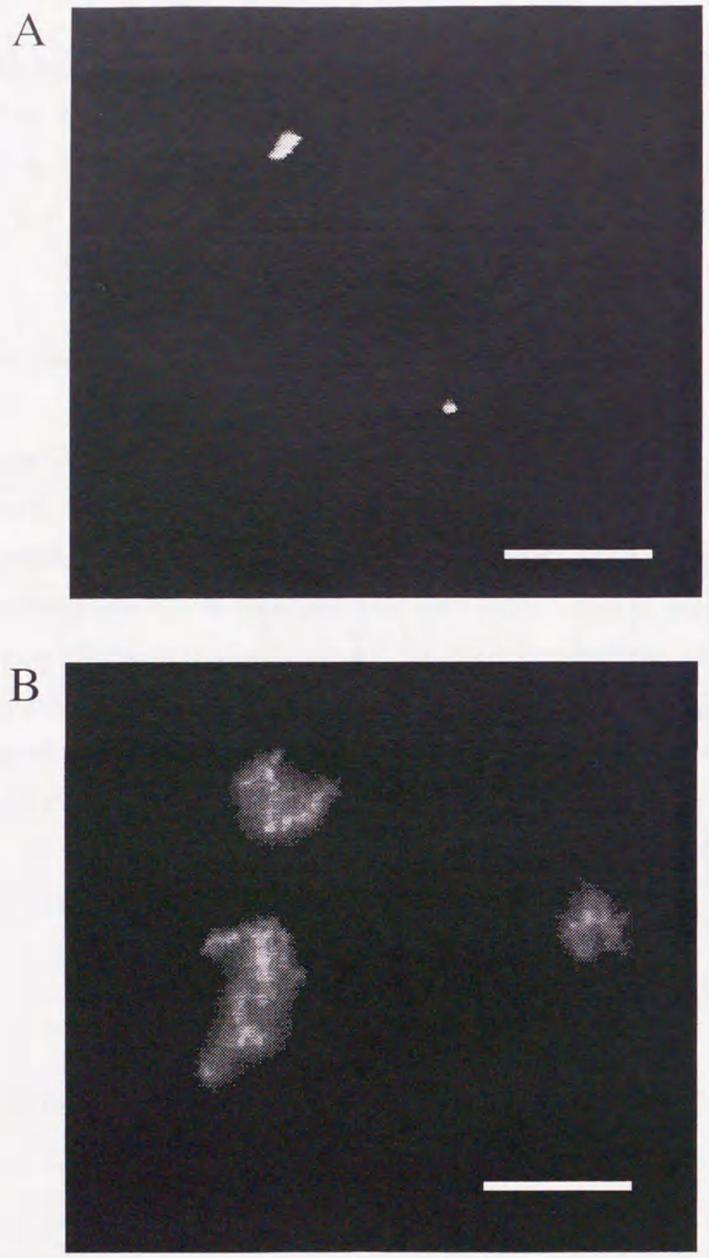


Fig. 5-2 Fluorescence microscopic observation of the DNA/poly-Arg complex. (A)Small aggregates formed with 30μM poly-Arg. (B)Large aggregates formed with 50μM poly-Arg. The scale bar shows 10μm.

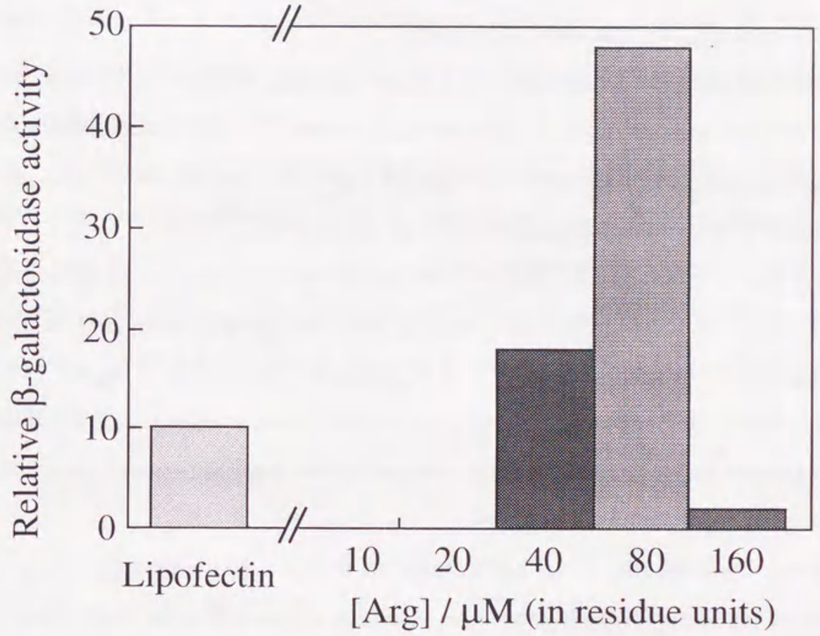


Fig. 5-3 Relative β-galactosidase activity after gene transfer with poly-Arg peptides.

GENERAL CONCLUSION

In the present thesis, to better understand the actual manner and mechanism of the formation of the higher-order structure of genomic DNA in solution from a physico-chemical perspective, the nature of the folding of giant duplex-DNA chains in solution was analyzed by applying fluorescence microscopy and electron microscopy. As a result, the following findings were obtained:

(1) A folded giant DNA induced by a low molecular-weight multivalent cation has two different phases depending on the concentration of the condensation agent; an orderly compacted phase with a toroidal morphology and an amorphous phase with a disorderly twined globular morphology (Chapter 2).

(2) The folded state of a giant DNA chain retains memory of its solution history. This is due to the multistable nature of the free-energy of the system resulting from competitive interaction among the coexisting chemical species (Chapter 3).

(3) The folding and unfolding of giant DNA present in medium crowded with an inert neutral polymer are sensitively affected by the balance between the existing polycations and polyanions (Chapter 4).

From these results, it has been extracted that the following five factors are essential for regulation of the higher-order structure of giant DNA; (a) elastic property of DNA chain itself, (b) energetical contribution from surface area of folded structure, (c) volume interactions between DNA-segments, which are sensitively affected by the change of the concentration of condensation agents, (d) competitive interaction among coexisting chemical species, and (e) the potential for DNA-folding imposed by an environment crowded with inert polymers. These five fundamental phenomena are also expected to deeply affect the structural behavior of genomic DNA *in vivo*, since the chemical environment surrounding genomic DNAs naturally establishes conditions in which these five factors have an effect.

In addition, the size of the DNA used in the present study should be noted. A

DNA chain with on the order of one hundred base pairs, such as T4 DNA, forms a specific higher-order structure in the compacted state of genomic DNA chains *in vivo*; *i.e.*, the so-called "radial loop domain" (Cook and Brazell, 1975; Gasser and Laemmli, 1986; Boy de la Tour and Laemmli, 1988; Jackson et al., 1990). The radial loop domain has been confirmed to exist in both prokaryotes (Worcel and Burgi, 1972) and eukaryotes (Benyajati, C. and A. Worcel, 1976; Callan et al, 1987), and has recently been considered to be one of the basic units of the chromatin structure (van Holde, 1988a). The folding and unfolding of DNA strand with on the order of one hundred base pairs essentially reflect the structural regulation which occurs on the loop domains of chromatin.

In conclusion, the results in the present study, especially with the above-mentioned five fundamental factors, are highly expected to provide groundwork for future research on the correlation between the structural regulation of chromatin by the surrounding chemical environment and its functional regulation, with especially suggesting the fundamental mechanism on the structural regulation of the loop domain of chromatin.

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Note

In the present doctoral thesis, each chapter has appeared in the above publications with minor modifications as follows.

Chapter 1: No. 7, 8;
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