Synopsis of thesis titled
“Thermal Fluctuation Spectroscopy and its application in the study of Biomolecules”
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The aim of this thesis is to study the energy fluctuations (leading to thermal fluctuations) during thermal and enzymatic denaturation of biological molecules and to study the variation in fluctuations between simple molecules like the DNA (which have only a secondary structure) to molecules with higher order structures and packaging. We have developed a new technique - Thermal Fluctuation Spectroscopy (TFS) to study these fluctuations. This thesis explores whether this new technique can indeed be made a viable tool to obtain basic physical information on important biological systems. The technique is a new way of doing calorimetry and has been developed during the course of this investigation.

Chromatin is the basic building block of chromosome and this thesis focusses on the constituents this fundamental building block - DNA, histones and nucleosomes.

MOTIVATION
Denaturation studies of biomolecules have always been done using bulk measurement techniques like differential scanning calorimetry (DSC) and UV absorption. These techniques, though extremely useful for knowing the melting temperature and the enthalpy changes involved, cannot give any information on the fluctuations. Also most of the studies during denaturation have been done at a constant rate of change of temperature (or force, in the case of force induced denaturation experiments). This gives rise to a constantly changing energy landscape which blurs out its finer details. To measure fluctuations of a sample in a particular energy landscape, the technique has to be an isothermal one. To detect small fluctuations, the measurement system has to have high precision and has to be extremely sensitive. Thermal Fluctuation Spectroscopy is an isothermal technique where the fluctuations exhibited by a sample can be studied as a function of time under different conditions. The measurement system has a sensitivity of a few parts per billion, which makes it capable of detecting small fluctuations in the sample.

In our system of interest, the questions we have tried to answer using TFS are the following:
- What is the nature of thermal fluctuations in biological systems like DNA, histones, nucleosomes and chromatin?
- How do the fluctuations behave as a function of temperature and as the system undergoes denaturation? How do the fluctuations change as a function of time?
- How exactly does the system undergo denaturation? - Is the process kinetically controlled or does the energy barrier itself change? Does the melting occur in different stages or is it a single step process? Is the process cooperative or is it completely random? If cooperative, what is the degree of cooperativity?
- What are the timescales exhibited by the system and how do they change with temperature and as the system undergoes denaturation?
- In the case of DNA, how do the fluctuations change with different lengths of DNA and with disorder?
- In the case of proteins, what is the structure of the energy landscape? Is the rugged funnel fractal like?
EXPERIMENTAL TECHNIQUE OF TFS

The technique of Thermal Fluctuation Spectroscopy (TFS) was developed by us for the study of biomolecules dynamics with a view to answer the above questions. TFS is a combination of microcalorimetry and noise measurement techniques. The combination of these two powerful techniques has never been exploited before. A very important advantage of this technique is that it is an isothermal technique. Unlike conventional calorimetry, there is no external energy source which is used to heat the calorimeter. The energy source and sink are internal to the system being studied.

In TFS, the sample is mounted in a liquid cell on a substrate which is connected by a weak link to a thermal reservoir. A schematic of the experimental principle is shown in figure 1. When the sample absorbs (or releases) energy, the temperature of the sample substrate system (SSS) decreases (or increases). The SSS also has an internal time constant $\tau_{\text{int}}$ for energy exchange between the sample and the substrate. The condition $\tau_{\text{int}} \ll \tau_{\text{th}}$, where $\tau_{\text{th}}$ is the thermal time constant of the calorimeter (given by the product of the heat capacity of the SSS and the thermal resistance of the link) will ensure that over a timescale $t$ such that $\tau_{\text{int}} \ll t \ll \tau_{\text{th}}$, the temperature change of the substrate $\Delta T$ will give us a faithful measure of the energy release from the sample.

In the other extreme where $\tau_{\text{int}} \gg \tau_{\text{th}}$, the SSS will be in good thermal contact with the reservoir which has a very large heat capacity and $\Delta T$ will be severely suppressed. The trick of the experiment is thus to create a quasadiabatic condition so that in the timescale of the measurement, the SSS is “decoupled” from the reservoir and $\Delta T$ is finite and measurable. Eventually, at longer timescales, $t \gg \tau_{\text{th}}$, the substrate temperature equilibrates to the reservoir temperature.

Thus, any energy exchange between the sample and the substrate is reflected as a thermal fluctuation of the substrate. The substrate has a thin film of platinum (Pt) deposited on it. This Pt film acts as a thermometer for measuring the temperature of the SSS.

![Figure 1: A schematic of the experimental principle.](image)

The experiment involves measuring the temperature of the SSS as a function of time at different temperatures. The timeseries thus obtained has information on the energy exchanges between the sample and the substrate. From this timeseries, we obtain the actual energy released/absorbed by the sample and the time scales of the energy exchanges. The changes in the SSS temperature are very small ~ $\mu$K (which is equivalent to a few nV) and require extremely sensitive noise measurement techniques for detection.

We find that TFS gives us information on the energy landscape of the system (like DNA and protein) as it undergoes denaturation. It can give quantitatively the energy barrier/barriers the system has to cross to reach its denatured state, the difference in energies between the native and denatured states and also the attempt frequency. The system resolution is few parts per billion.
(ppb) and fluctuations in energy ~ 100nJ (which correspond to temperature fluctuations ~ μK) can be measured.

TFS of DNA
We observe that heteropolymeric dsDNA shows extremely large non-Gaussian fluctuation around its melting temperature. For homopolymeric DNA the fluctuations during denaturation are smaller. The thermal fluctuation during denaturation of a heteropolymer in buffer is several orders larger than when the DNA is on a substrate while that for a homopolymer is comparable in both cases.

In heteropolymeric DNA, the thermal fluctuation, \( \frac{<\Delta T^2>}{T^2} \) shows two peaks. In figure 2 we show the \( \frac{<\Delta T^2>}{T^2} \) as a function of temperature for a 100bp heteropolymeric dsDNA. The first peak coincides with the melting temperature of a homopolymeric ATAT dsDNA while the second peak occurs at temperatures below the melting temperature of a homopolymeric GCGC dsDNA.

![Figure 2: Relative variance in temperature fluctuation for a 100bp heteropolymeric dsDNA.](image)

The timeseries of temperature fluctuations shows cooling jumps ~ 334 K as can be seen from figure 3. This is the temperature at which we see the first peak in \( \frac{<\Delta T^2>}{T^2} \). At temperatures between 345 K and 365 K (depending on the DNA), the timeseries shows a few cooling jumps, followed by a very large cooling jump. The timeseries of temperature fluctuation at 361K (the temperature at which we see the second peak) is shown in figure 3, (b) and (c). This large cooling jump, indicative of a major cooperative transition, has superposed on it several small heating and cooling jumps. This is where we see the second peak in \( \frac{<\Delta T^2>}{T^2} \). After this large jump, the SSS temperature returns to the reservoir temperature, beyond which there is no additional fluctuation present. From the data of timeseries of temperature fluctuation, the energies involved in the denaturation process were calculated. The energy absorbed at the first peak corresponds to that of AT bond breaking energy and that at the second peak corresponds to the GC bond energy.

![Figure 3: Time series of temperature fluctuations for the 100bp heteropolymer.](image)
This establishes the important fact that heteropolymeric dsDNA denaturation occurs in two stages. Initially, at around 330 K, bubbles are formed in the AT rich regions. At higher temperatures, the GC rich regions binding them denature in a cooperative transition causing extremely large fluctuations.

TFS OF HISTONES (MONOMERS, DIMERS AND OLIGOMERS) AND NUCLEOSOMES

Histones are proteins, which along with DNA form the nucleosome. There are five kinds of histones – H1, which is the linker histone and H2A, H2B, H3 and H4, which are the core histones. The core histones have a helix-turn-helix structure, while the linker has, in addition to the helix-turn-helix, β-sheets which form the winged helix structure. The denaturation of histones involves unfolding of their structural elements like α-helix and β-sheets, leading to a random coil structure.

TFS on histone monomers showed that only H1 monomer shows an increase in thermal fluctuation in the temperature range studied. This can be observed from figure 4 which shows the \( \frac{\langle (\Delta T)^2 \rangle}{T^2} \) for the histone monomers H1, H2A, H2B and H3. We infer that this is due to the fact that the core histones may not be properly folded when they exist as monomers. It was seen that H1 crosses an energy barrier of 17 kcal/mol to go from its native to denatured state. The transition was kinetically driven with a fixed barrier till 352 K. At 352K, the barrier softened by ~ 1 kcal/mol leading to faster denaturation. A schematic of the energy landscape for H1 denaturation is shown in figure 5.

![Figure 4: Relative variance of temperature fluctuations for histone monomers.](image)

![Figure 5: Schematic of the energy landscape for denaturation of H1.](image)
The core histones when assembled as dimers/oligomers showed an increase in fluctuation at temperatures below 350 K. The H2A-H2B dimer showed a sharp peak at 316.1 K indicating that its dissociation and denaturation were coupled and occurred together. The H3, H4 oligomers show broad peaks centered around 330 K. It appears from the analysis of energy jumps that their transitions are not governed by a simple Arrhenius processes with fixed energy barriers. The transitions could not even be described by processes where the barrier softens during denaturation. We believe that the coupling of the denaturation and dissociation complicates the way the energy landscape changes with temperature.

Nucleosomes consist of two each of the core histones assembled into an octamer which is wrapped around by ~ 147bp of dsDNA. The linker histone H1 binds to the entry and exit sites of the DNA and holds the nucleosome structure in place.

The assembling of these histones and DNA into a mononucleosome causes a very large increase in fluctuation over the entire temperature range studied. TFS showed that the fluctuation during mononucleosome denaturation was much larger than a simple sum of the fluctuations of its constituents (shown in figure 6). From the data we were able to identify that the denaturation starts with dissociation and unfolding of the core histones and the denaturation of AT rich regions of the DNA which leads to the breaking of some of the histone-DNA contacts. At higher temperatures the linker histone H1 and the GC rich regions of the DNA denature, leading to a collapse of the entire nucleosome structure. The broadness of the transition region (the fact that the fluctuation is large over the entire temperature range) was attributed to the presence of different types of contacts and interactions (with different energies) stabilizing the nucleosome structure. The nucleosome was found to favour large energy jumps over smaller ones indicating that the denaturation has an element of cooperativity involved.

![Figure 6: Relative variance of thermal fluctuations of mononucleosomes and the sum of contributions from histones and DNA in their correct proportions.](image)

Thus, using TFS we have studied the denaturation of histones (monomers, dimers and oligomers) and nucleosomes. We have been able to deduce the energy barriers to denaturation involved in the nucleosomes and its constituents. We have also found the exact way in which a nucleosome - the fundamental building block of chromatin, undergoes denaturation.
CONCLUSIONS
Using TFS we have been able to determine the fluctuations involved in the denaturation of biomolecules like DNA, histones and nucleosomes. The energy barriers to denaturation have been determined. We have also been able to give models for the denaturation of these biomolecules. We have also shown that it is possible to study enzymatic digestion using TFS. Thus, the technique of TFS is a viable tool for the study of fluctuations in reactions, in biomolecules, during transitions and in any process where there is an energy exchange involved.