amino acids [15]. This domain has been implicated in transcription initiation since its deletion abolishes transcription [16]. However, the mechanism underlying this observation has not been described. While it is known where TFAM binds in LSP, it has not been studied in great detail which base pairs are responsible for this interaction and whether mutations in this region in human mtDNA can affect transcription initiation. Here we report the use of fluorescence polarization to study TFAM and DNA interaction. By tagging the DNA we can quantitatively probe the necessary TFAM structures for binding. Then, by tagging TFAM, we should be able to find the target DNA sequences for TFAM binding.

Materials and Methods

Materials: Purified TFAM, TFAMdCT, TFAMd10CT, and TC-TFAM were previously prepared in the Cameron laboratory. DNA oligonucleotides were obtained from Integrated DNA Technologies Inc. and purified by PAGE gel electrophoresis. BSA was obtained from New England Biolabs. The Biarsenical Fluorescein derivative FlAsH was obtained from Invitrogen. All other reagents were received from VWR and Fisher.

Annealing: The non-template strand, 5’-ATGTGTAGTTGGGGGGT-GACTGTAAAA-Fl-3’, where Fl means fluorescein, and template strand (reverse complimentary strand) were annealed at 25 μM in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 50 mM NaCl by using a Progene Thermocycler (Techne). The annealing solution was then heated at 90°C for 1 minute, then cooled at a rate of 5oC per minute until it reached 10°C.

TFAM Titration for TFAM-DNA Binding: The reaction mixture for anisotropy for measurement was prepared as follows. TFAM was serially diluted in Enzyme Dilution Buffer (10 mM HEPES, pH 7.5, 20% Glycerol, 100 mM NaCl and 1mM DTT) to acquire the appropriate concentrations. Fluorescein-labeled DNA was diluted in annealing buffer. The DNA binding reaction contained 10 mM HEPES, pH 7.5, 10 mM MgCl2, 1 mM DTT, 0.1 μg/μL BSA, 100 mM NaCl, 0.1 nM DNA, and various concentrations of protein typically in 100 μL. The samples were then transferred to glass tubes and incubated at 25°C for approximately 30 sec before obtaining the mini polarization (mP) values.

Plots of the change in mP as a function of TFAM concentration were used to determine the equilibrium dissociation constant (Kd) for the interaction between TFAM and LSP binding site. The data was fit to a hyperbola (Eq. 1) using the program KaleidaGraph (Synergy Software, Reading, PA). This procedure was used for wild-type TFAM, TFAMdCT and TFAM-d10CT, two other TFAM variants.

Equation 1 \[ mP = \frac{(mP_{max} \times [TFAM])}{(Kd + [TFAM])} \]

Results

TFAM-DNA Binding Characterization using fluorescence polarization (FP): To study TFAM target site we used fluorescence polarization (FP). The advantages of fluorescence polarization include that it is fast, reproducible, reports on the equilibrium in solution and does not require radioactivity materials. FP permits the study of molecular interactions by tracking size changes of fluorescent molecules. The polarization depends on