than ~50 nM due to high background signals. The zero-mode waveguide (ZMW) is a nanophotonic structure consisting of a hole in a metal film on a transparent substrate. In conjunction with laser-excited fluorescence, they provide observation volumes on the order of zeptoliters, three to four orders of magnitude smaller than far-field excitation volumes, allowing fluorescence detection in the μM range. Here, we apply ZMWs and novel detection instrumentation developed by Pacific Biosciences and demonstrate direct observation of multiple rounds of binding and release of tRNAs at high concentrations on various mRNA templates during protein synthesis.

The applicability of ZMWs to translation was confirmed by recapitulation of prior single molecule experiments. We delivered ternary complex of EF-Tu(GTP) with Phe-Cy5-tRNA^{Phe} into the A site of ribosomal initiation complexes with fMet-tRNA^{fMet} with Cy3 in P site immobilized on ZMW surface. FRET observation and tRNA arrival time measured in ZMW at any states was consistent with the prior results obtained by TIRF. Next we monitored the elongation cycle through multiple arrival events of dye-labeled tRNAs onto mRNA-programmed ribosomes. Using a fMet-Phe-Phe-Phe mRNA template, we observed multiple arrivals (up to 3) of Cy5-labeled phe-tRNA^{Phe} in the presence of EF-G; event number and duration depend as expected on EF-G concentration. We demonstrated multicolor observation of three tRNAs using Cy2-labeled Lys-tRNA^{Lys} with Cy2 enables to observe three color elongation cycles for fMet-Phe-Lys-Phe or fMet-(Phe-Lys)x6 mRNA template with green, red and blue excitations. These experiments show that we can observe translation in real time from the perspective of tRNA ligands, and will reveal aspects of translation such as time-dependent tRNA occupancy of the ribosome.

1361-Pos

Following Movement of the L1 Stalk between Three Functional States in Single Ribosomes

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The L1 stalk is a mobile domain of the large ribosomal subunit E site that interacts with the elbow of deacylated tRNA during protein synthesis. Using single-molecule FRET, we follow the real-time dynamics of the L1 stalk and observe its movement relative to the body of the large subunit between at least three distinct conformational states: open, half-closed and fully-closed. Pre-translocation ribosomes undergo spontaneous fluctuations between the open and fully closed states. In contrast, post-translocation ribosomes containing peptidyl-tRNA and deacylated tRNA in the classical P/P and E/E states, respectively, are fixed in the half-closed conformation. In vacant ribosomes, the L1 stalk is observed either in the fully closed or fully open conformation. Several lines of evidence show that the L1 stalk can move independently of intersubunit rotation. Our findings support a model in which the mobility of the L1 stalk facilitates binding, movement and release of deacylated tRNA by remodeling the structure of the 50S subunit E site between three distinct conformations, corresponding to the E/E classical, P/E hybrid and vacant states.

1362-Pos

Side-Chain Reactivity of a Nascent Peptide in the Ribosomal Exit Tunnel

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Nascent peptides begin to fold in the ribosomal exit tunnel. This is not a unilateral act by the peptide. The tunnel, ~100Å in length and 10-20 Å in width, collaborates and is an active participant in translation. The precise mechanisms for this teamwork are unknown. To probe these peptide-tunnel interactions, we have engineered different side-chains adjacent to a cysteine in a molecular tape measure positioned at various locations inside the tunnel. In each case, we measured the kinetics of cysteine modification with a series of reagents of increasing size: trimethyl-, triethyl-, tripropyl-, and tributyl-ammonium maleimides. Three conclusions may be drawn. First, for a given side-chain and a given reagent, the modification rates decrease in going deeper into the tunnel from the exit port to the peptidyltransferase center (PTC). Second, the ratio of modification rate constants for trimethyl to tributyl reagent for an identical reporter cysteine increases monotonically with increasing distance into the tunnel from the exit port. Third, the tunnel near the exit port is relatively insensitive to the choice of adjacent side-chain, whereas a site deeper in the tunnel exhibits side-chain dependent reactivities. As a given amino acid moves along the tunnel during peptide elongation, its interactions with the tunnel are both site-specific and tuned to the unique primary sequence of each nascent peptide. [Supported by NIH grant GM 52302].

1363-Pos

A Universal Zone in the Ribosomal Exit Tunnel for Helix Formation in Kv1.3

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Crystal structures of Kv channels have given us a reasonably complete view of the structure of a mature Kv channel. However, details of its structure acquisition are missing. We now report on the biogenesis of secondary structure of the transmembrane segments and intervening linkers of Kv1.3. Using a combination of accessibility assays, both cysteine pegylation (Tu et al., 2007; Lu and Deutsch, 2005) and N-linked glycosylation (Mingarro et al., 2000), we derive the following principles of folding for nascent sequences within their native contexts. First, native helical transmembrane sequences initially form helices only within the distal 20Å of the ribosomal tunnel near the exit port. We refer to this region as the α-zone. Helix formation in transmembrane segments thus occurs vectorially from N- to C-terminus as each segment moves sequentially into the α-zone. Second, linker sequences also form compact structures inside the α-zone even in the absence of helix formation of their C-terminal flanking transmembrane segments. Third, helical structures, whether transmembrane or linker segments, formed in the tunnel retain their helicity in the translocon. These principles emerge from a diversity of native transmembrane and linker sequences that comprise Kv1.3 and may therefore be applicable to protein biogenesis in general.

[Supported by NIH grant GM 52302].

References: Tu et al., Biochemistry 46: 8075, 2007; Lu and Deutsch, Biochemistry 44: 8230, 2005; Mingarro et al., BMC Cell Biol. 1: 3, 2000.

1364-Pos

Single- Molecule Force Measurement for 30S- mRNA Interaction in Translation Initiation

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Bacterial ribosome is a molecular machine composed of 30S and 50S subunits that translates the genetic code in mRNA into an amino acid sequence through repetitive cycles of tRNA selection, peptide bond formation and translocation. Translation initiation is one of the essential processes in protein synthesis that involves the assembly of initiator fMet-tRNA^{fMet} and three initiation factors (IF1, IF2 and IF3) to 30S subunit with GTP hydrolysis by IF2 to form 70S-mRNA-fMet-tRNA^{fMet} complex efficiently and correctly. These processes are occurred with dynamic intersubunit rotation and repositioning of IF2 and initiator tRNA. We expect that GTP hydrolysis by IF2 plays a key role in 30S-mRNA interactions as well. To understand the dynamics of 30S-mRNA interactions, we performed rupture force measurement between 30S subunit and mRNA by optical tweezer assay.

The rupture force for 30S-mRNA complex in the absence of tRNAs and any IFs showed a single distribution with a peak at 5.7 pN. The addition of tRNA^{fMet} to the complex increased the rupture force to 15.2 pN, while the rupture force for 70S-mRNA-tRNA^{fMet} complex showed 16.5 pN. These results show that the binding of tRNA^{fMet} to the 30S-mRNA complex contributes to the initiation stability, which is greater than 50S binding.

Intriguing results were obtained in the presence of IF2. The rupture force for 30S-mRNA-fMet-tRNA^{fMet}-IF2(GTP) showed 16.1 pN, which is not significantly different from 15.2 pN for 30S-mRNA- tRNA^{fMet}. However, the binding of 50S subunit to this complex led to significant changes, the rupture force for 70S-mRNA-fMet-tRNA^{fMet}-IF2(GDPNP) and 70S-mRNA-fMet-tRNA^{fMet}-IF2(GDP) showing 22.1 pN and 20.5 pN, respectively. These results indicate that IF2 with GTP hydrolysis contributes the initiation stability in 30S-mRNA interactions, which enables efficient initiation of protein synthesis.

1365-Pos

Structural Analysis of Bound Molecules to Ribosome by EM-Fitting

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In the previous study, we systematically analyzed many three-dimensional electron microscopy (EM) density maps of 70S ribosome at various functional states available in the EM DataBank to reveal the global conformational differences between the 70S ribosome structures by our new flexible-fitting approach, in which the best-fitting atomic model for each EM map was built by deforming the PDB structure of the 70S ribosome using normal mode analysis of the elastic network model.

In this study, we analyze the structures of smaller molecules bound to ribosome, such as tRNA and elongation factors, which are included in the EM density maps of the 70S ribosome, by building atomic models of these molecules in the bound state. The structures of these bound molecules may be different from those in the isolated state or the X-ray crystal structures. In this analysis, we need to spot these molecules in the EM density map, which is occupied mostly by the 70S ribosome. By fitting the atomic model of the 70S ribosome into the EM density map and eliminating the density of the regions overlapped with the atomic model, we are able to extract the regions for the bound molecules. Our results show that the best-fitting atomic model of the 70S ribosome built in our previous sturdy can extract the regions for the bound molecules more clearly than the original PDB structure.

1366-Pos

Spontaneous Vs. Allosteric Dissociation of E-Site tRNA During Polypeptide Elongation

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During ribosome-catalyzed polypeptide chain elongation, dissociation of the deacylated tRNA from the E-site has been proposed to be either spontaneous or triggered allosterically by binding of the next cognate ternary complex to the A-site. Using fluorescent labeled tRNAs, we have measured single molecule fluorescence intensities and single molecule FRET between adjacent tRNAs in the ribosome. From these measurements we have been able to determine tRNA occupancy in the ribosome, and thus whether E-site tRNA dissociates before or after A-site occupancy. In the former case there are a maximum of 2 simultaneously bound tRNAs, while the latter case results in transient binding of 3 tRNAs simultaneously. In a total internal reflection florescence microscope, ribosomes were attached to glass microscopes slides via a biotinylated mRNA coding for MRFFRFY.... (single letter amino acid sequence). When synthesis was initiated with $tRNA^{fMet}$ fully charged with formylated-Met, 60-70% of the ribosomes lost their E-site tRNA prior to ternary complex binding (2-tRNA pathway) at the 2nd and 3rd elongation cycles (R and F respectively). In contrast, for synthesis initiated with uncharged initiator $tRNA^{fMet}$, >90% of the ribosomes followed the 2-tRNA pathway in the 2nd cycle, but only ~15% in the 3rd cycle (~75% following the 3-tRNA route, 10% not categorized). In cycles 4 and 5, almost all ribosomes followed the 2-tRNA pathway. Thus, the length of the peptide chain and/or the specific amino acids bound to the P-site tRNA strongly influence the allostery of E-site tRNA dissociation. Such allostery is also sensitive to the presence or absence of polyamines, Mg^{2+} concentration, and the specific codons in positions 2 and 3. Supported by NIH R01grant GM080376 and NIST ATP grant 70NANB7H7011 through Anima Cell Metrology, Inc.

1367-Pos

Enhancement of Single Molecule Fluorescence Signals by Colloidal Silver Nanoparticles in Studies of Ribosome Dynamics

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Metal enhanced fluorescence (MEF), in which a surface plasmon near a noble metal alters the spectral properties of an organic fluorophore, increases fluorescence intensity without a concomitant increase in photobleaching rate. To improve recordings of single molecule fluorescence signals from individual ribosomes, we studied the emission of Cy3- and Cy5-labeled ribosomes and tRNAs attached near 50-80 nm silver colloidal particles on a glass microscope coverslip. The fluorescence of Cy3 and Cy5 labeled initiation complexes (ICs) near 50 nm silver particles was increased 4- and 5-fold, respectively, compared to labeled ICs in the absence of silver colloids. Photobleaching lifetime was not significantly accelerated, resulting in 4-5 fold enhancement of total photon emission before photobleaching. Fluorophores showing enhanced fluorescence were colocalized with the colloidal particles, as detected by light scattering. Other ribosomes or tRNAs that were farther away had intensities similar to those on plain glass. Aggregates of silver colloidal particles themselves produced wavelength-shifted luminescence similar to fluorescence, presumably due to resonance between nearby metal particles. With ribosomes bound to the glass substrate near the silver particles via a short mRNA, interaction between $tRNA^{Arg}$-Cy3 in the ribosomal P-site and fMet-Arg-Phe-$tRNA^{Phe}$-Cy5 in the A-site had FRET efficiency and dynamics similar to ribosomes farther away and on plain glass. Binding of Cy5-Arg-$tRNA^{Arg}$ to ICs labeled with Cy3 on the large subunit protein L11, in the absence of the translocase EF-G, produced FRET efficiency and dynamics characteristic of specific codon-dependent A-site binding. Addition of EF-G reduced FRET efficiency, as expected. These tests demonstrate that the colloidal silver nanoparticles increase fluorescence and total photon emission without compromising the biophysical activity of ribosomes.
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1368-Pos

Establishing a Fluorescence-Based Helicase Assay for Monitoring Eukaryotic Protein Synthesis Initiation

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Efficient mRNA recruitment to the human ribosome requires a region of single stranded RNA close to the cap structure. The eukaryotic initiation factor 4A (eIF4A) is a DEAD box helicase that is essential for unwinding any stable secondary structure that would inhibit this step. Although studied for many years, the majority of helicase assays involving eIF4A and its associated proteins have failed to rigorously analyze the kinetic events that occur during mRNA unwinding. To provide this kinetic understanding we have developed a continuous fluorescence-based assay to measure RNA duplex unwinding events. This assay utilizes an RNA oligonucleotide modified with cyanine 3 (Cy3) annealed to a complimentary strand modified with black hole quencher (BHQ). Separation of the RNA duplex region significantly enhances the Cy3 fluorescence, enabling us to measure RNA helicase activity in real time by fluorescence spectroscopy. Data will be presented to show how we are using this assay to determine the kinetic role of accessory factors on the helicase activity by eIF4A. Moreover, we show how this data combined with a continuous coupled ATPase assay is enabling us to determine the relationship of ATP hydrolysis to the unwinding of duplex RNA by this DEAD box helicase.
We will also present data employing RNA duplexes of different lengths, allowing us to understand how the processivity of eIF4A is influenced by other initiation components.
This study will provide us with the foundation to begin understanding the kinetic framework of mRNA recruitment to the human ribosome.

DNA, RNA Structure & Conformation I

1369-Pos

Structure and Mechanism of the glmS Ribozyme

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The self-cleaving glmS ribozyme is a mechanistically unique functional RNA among both riboswitches and RNA catalysts as its catalytic activity provides the basis of genetic regulation and depends upon glucosamine-6-phosphate (GlcN6P) as a coenzyme. A substantial body of biochemical and biophysical data relating the structure and function of the glmS ribozyme has been amassed, in our laboratory and others, in a relatively short period of time since its discovery. However, a precise and comprehensive mechanistic understanding of coenzyme function in glmS ribozyme self-cleavage has not been elaborated. Careful consideration of the available biochemical and biophysical data relating the structure and function of the glmS ribozyme necessitates that general acid and general base catalysis in a coenzyme-dependent active site mechanism of RNA cleavage are inherently interdependent. We propose a comprehensive mechanistic model wherein the coenzyme, GlcN6P, functions both as the initial general base catalyst and consequent general acid catalyst within a proton-relay thus fulfilling the apparent biochemical requirements for activity. This analysis in combination with other considerations regarding the effects of coenzyme binding on riboswitch structure and function suggests that the development of glmS ribozyme agonists as prospective antibiotic compounds must satisfy strict chemical requirements for binding and activity.

1370-Pos

Antibiotic Development by Investigation of the glmS Riboswitch

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Although bacterial infections have always been of significant interest to researchers and physicians, the drug-resistant bacterial strains that have recently developed are causing new concerns and are much more difficult to combat. Our current methods for treating bacterial infections include broad-spectrum