In classical folding studies folded proteins are initially denatured into an unfolded state before the (re-) folding process can be studied. It has been demonstrated that protein folding takes place already during the elongation of the nascent chain (co-translational folding).
Here we present an approach employing a two color single molecule sensitive fluorescence wide-field microscope in order to visualize surface tethered fluorescently labeled ribosomes and *de novo* synthesized GFP molecules in real time [1]. Suppression of protein release after synthesis keeps the synthesized GFP bound to the ribosome and allows to image GFP fluorescence for extended observation times.
We demonstrate that the characteristic time for the production of the mature GFP mutant Emerald (GFPem) is five minutes, which is one of the fastest maturation times for a GFP mutant observed so far. Early GFPem molecules appear even faster, within one minute. Processes precedent to chromophore formation, such as polypeptide synthesis and protein folding, are fast and last less than one minute. Thus cellular processes within a time range of a few minutes can be followed by GFPem.
[1] A. Katranidis et al. (2009)Angewandte Chemie Int. Edit., **48**, 1758-1761

1355-Pos
Fluctuating tRNA Guided by Induced Fit as a Basis of High Fidelity Translation by the Ribosome
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Positional fluctuations and structural flexibility of molecules play important roles in various cellular processes. We implemented a single molecule method that can simultaneously measure fluorescence resonance energy transfer (FRET) and the anisotropy of FRET acceptor emission. Based on the method, we monitored the process of aa-tRNA selection by the ribosome and evaluated the positional fluctuations of a cognate and a near-cognate tRNA at the GTPase activated state. The FRET measurements revealed that a cognate tRNA at the GTPase activated state samples the ''A'' site of the ribosome more frequently than a near-cognate. A cognate tRNA is found fluctuating more slowly within a more restricted space as compared to a near-cognate. These results suggest that induced fit steers a fluctuating cognate tRNA more accurately toward the ''A'' site of the ribosome. Based on our findings, we propose that fluctuating tRNA guided by induced fit may be the basis of high efficiency tRNA selection by the ribosome.

1356-Pos
Simulations of the Bacterial Ribosomal Decoding Switch
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Gentamicin is a potent antibiotic often used in therapy for methicillin-resistant Staphylococcusaureus. Gentamicin works by flipping a conformational switch on the ribosome, disrupting the reading head (i.e., 16S ribosomal decoding bases A1492-A1493 used for decoding messenger RNA. We use explicit solvent all-atom molecular simulation to study the thermodynamics of the ribosomal decoding site and its interaction with gentamicin. The replica exchange molecular dynamics simulations allow enhanced sampling of the unbinding free-energy landscape, including a rigorous treatment of enthalpic and entropic effects. The decoding bases flip on a timescale faster than that of gentamicin binding, supporting a stochastic gating mechanism for antibiotic binding, rather than an induced-fit model where the bases only flip in the presence of a ligand. The study also allows us to explore the nonspecific binding landscape near the binding site and reveals that, rather than a two-state bound/unbound scenario, drug dissociation entails shuttling between many metastable local minima in the free-energy landscape. Additional simulations address the effect of mutation/modification of the A-site on the free energy landscapes. In particular, mutations of base A1408 known to confer high-level resistance in ''superbug'' bacteria to common antibiotic therapies are investigated. Special care is dedicated to validation of the obtained results, both by direct comparison to experiment and by estimation of simulation convergence.

1357-Pos
Single-Molecule Study of Programmed Ribosomal Frameshifting
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Programmed ribosomal frameshifting is involved in regulation of gene expression at the translation level in bacteria, and the frameshifting efficiency has to be well controlled. For example, the dnaX gene of *E. coli* encodes two subunits (gamma and tau) of the DNA polymerase III, and the ratio of these two subunits is determined by the frameshifting efficiency. Factors that affect frameshifting have been extensively studied in vitro and in vivo, but the dynamic features of this process are still not well understood. Here we use optical tweezers to follow stepwise translation in real time at the single ribosome level to understand how frameshifting is controlled. Our preliminary data show that the ribosome pauses for various times at, or even one to two codons prior to, the slippery sequence where frameshifting occurs. The pause may be caused, at least in part, by an internal Shine-Dalgarno sequence, which is located upstream to the slippery sequence and is known to induce frameshifting. Correlation between pause duration and occurrence of frameshifting is under investigation.

1358-Pos
Single-Molecule Optical-Tweezers Studies of Ribosome Translation and Unwinding of Messenger RNA
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Ribosomes translate the genetic code in an mRNA into a protein; three nucleotides-one codon-code for one amino acid. Because natural mRNAs contain base-paired regions, the ribosome needs to unwind these structures into single-strands before the structured regions can be translated. Furthermore, mRNA secondary and tertiary structures are involved in translation regulation mechanisms, such as frame-shifting. Bulk studies have shown that mRNA structures slow down the translation rate and that the unwinding is inherent to the ribosome. But the unwinding mechanism is not well understood. We used optical tweezers to apply force to the ends of an mRNA hairpin being translated by a single ribosome. At constant force, the mRNA end-to-end distance increases as the ribosome translates the message and converts double-stranded RNA into single strand. This technique allows observation of codon-by-codon translation. The translation rate dependence on force provides information on the ribosome unwinding mechanism. We found that the translation rate increases quickly as force is increased within a narrow force range, but the rate plateaus below and above this force range. We also found that the translation rate on duplex mRNA (low force plateau) is ~50% of the rate on single-stranded mRNA (high force plateau). The observed force dependence is inconsistent with a passive unwinding model, or the active unwinding models that have been applied to T7 and NS3 helicases. Instead, the data call for a different type of active unwinding mechanism. This result suggests that there might be fundamental differences in the unwinding mechanism of ribosomes and of other nucleic acid helicases. Our results show a tight coupling of the ribosome translation and unwinding activities, and should facilitate understanding of translation regulation mechanisms, such as frame-shifting.

1359-Pos
Insights into Translational Termination from Crystal Structures of the 70S Ribosome Bound to Release Factor
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Here we report high-resolution crystal structures of release factor 2 (RF2) bound to 70S ribosome. These structures emulate the translational states directly before and after hydrolysis of the ester bond in peptidyl-tRNA on the ribosome during translational termination. Our structures show detailed molecular interactions between the ribosome decoding center and RF2 upon stop-codon recognition. After a stop-codon is recognized, the universally conserved GGQ motif extends directly into the peptidyl transferase center forming a tightly packed catalytic core. Nucleotide A2602 in the 23S rRNA forms a favourable stack with the GGQ motif in RF2. The ribose of the A76 in the peptidyl-tRNA adopts C2'-endo conformation. Our structures help to rationalize a decade of biochemical and computational data on translational termination. Based on the structures, a mechanistic model on how the ester bond in the peptidyl tRNA is hydrolyzed is proposed.

1360-Pos
Realtime Observation of tRNA Dynamics at High Concentrations in Single Molecule Translation
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Conventional TIRF methods can probe the dynamics of complex biological systems, but only at concentrations of fluorescent components that are less

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.