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The ribosome is the molecular motor responsible for the protein synthesis within all cells. Ribosome motions along the messenger RNA (mRNA) to read the genetic code are asynchronous and occur along multiple kinetic paths. Consequently, observation and manipulation at the single macromolecule level is desirable to unravel the complex dynamics involved. In this communication, we present the study of translation kinetics of single ribosomes via the direct observation of fluorescent amino-acid incorporations.

In order to study the kinetics of amino-acid incorporation inside the growing protein by the ribosome, we use a home-made total internal reflection single-molecule fluorescence microscope (TIRFM). The mRNA-ribosome complex is attached to a polyethylene glycol modified glass coverslip surface by a streptavidin-biotin linkage. The ribosome is labelled with a quantum dot (QD) in order to localize it on the surface while a specific amino acid (lysine) is marked with Bodipy-FL [1]. This fluorescent dye is small enough to enter the ribosomal channel thus leaving intact ribosomal activity. The protein synthesis is observed in real time as the labelled amino acids are incorporated into the polypeptidic chain by the co-localization of QD and Bodipy-FL fluorescence signals.

We will discuss the future application of this technique to single-molecule observation of the translation process, proof reading or even protein folding.

Reference

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2983-Pos Board B30

How Initiation Factor 2 Regulates the Fidelity of Translation Initiation

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During prokaryotic translation initiation, the small, 30S and large, 50S ribosomal subunits, along with formylmethionyl-transfer RNA (fMet-tRNA^{fMet}), assemble at an authentic AUG messenger RNA (mRNA) start codon. The fidelity of initiation is regulated by three initiation factors (IFs) which kinetically control individual steps along the reaction pathway. One such step is recruitment of the 50S subunit to a 30S initiation complex (30SIC), composed of the 30S subunit, mRNA, tRNA, and IFs 1, 2, and 3. Although the GTPase IF2 has been shown to be principally responsible for selectively accelerating the rate of 50S subunit joining to a correctly initiated 30SIC, the molecular mechanism underlying this catalytic activity remains unknown. In order to elucidate the mechanism through which IF2 selectively catalyzes 50S subunit joining, we have developed single-molecule Förster resonance energy transfer (smFRET) signals between fluorescently-labeled IF2 and two tRNAs, an initiator fMet-tRNA^{fMet} and an elongator Phe-tRNA^{Phe}. Using these IF2-tRNA smFRET signals, we have characterized the dynamics of IF2-tRNA interactions within correctly and incorrectly initiated 30SICs. Our data reveal that the residence lifetime of IF2 on the 30SIC (τ_{30SIC}), a parameter that directly controls IF2's ability to recruit the 50S subunit to the 30SIC, is highly regulated during initiation. We find that τ_{30SIC} is exquisitely sensitive to: (1) the presence of IF1 and IF3, (2) the GTP- *vs.* GDP- or nucleotide-free forms of IF2, (3) the presence of fMet-tRNA^{fMet} *vs.* Phe-tRNA^{Phe}, and (4) the presence of a correct AUG start codon. Thus, only in the presence of IF1, IF3, GTP, and a correctly formed codon-anticodon interaction between an authentic AUG start codon and an initiator fMet-tRNA^{fMet} is IF2 binding to the 30SIC significantly stabilized such that 50S subunit joining is efficiently catalyzed, ensuring the fidelity of this step of translation initiation.

2984-Pos Board B31

Coupling of Ribosomal L1 Stalk and tRNA Dynamics during Translation Elongation

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Translation elongation necessarily requires large-scale movements of transfer RNAs (tRNAs) through the ribosome. While it is likely that these highly-coordinated tRNA movements are directed by conformational changes of the translating ribosome, data directly correlating ribosome and tRNA conformational dynamics are lacking. Using single-molecule ribosome-ribosome and ribosome-tRNA Förster resonance energy transfer (smFRET) signals, we have characterized the intrinsic conformational dynamics of the ribosomal L1 stalk as well as the coupling of L1 stalk and tRNA dynamics during translation elongation. We find that in post-translocation ribosomal complexes, the L1 stalk can occupy either an opened or closed conformation, with the probability and rate of transitions between these two states dependent on the occupancy and identity of the deacylated tRNA in the classical E site. Upon delivery of aminoacyl-tRNA into the ribosomal A site and peptide bond formation, however, a direct interaction between the closed L1 stalk and the newly deacylated tRNA residing in the hybrid P/E tRNA binding configuration is established spontaneously; this event involves coupled movements of the L1 stalk and the ribosome-bound tRNAs, as well as ratcheting of the ribosome. Our data reveal that the entire pre-translocation complex fluctuates between two global conformations: global state 1 (GS1) characterized by an open L1 stalk, classically-bound tRNAs and a non-ratcheted ribosome and global state 2 (GS2), characterized by a closed L1 stalk, hybrid-bound tRNAs, and a ratcheted ribosome. Binding of the ribosomal translocase, elongation factor G (EF-G), shifts the GS1/GS2 equilibrium towards GS2, promoting the intermolecular L1 stalk-tRNA interaction. Pre-steady state smFRET experiments reveal that the L1 stalk-tRNA interaction persists throughout the translocation reaction, suggesting that the L1 stalk allosterically collaborates with EF-G in order to direct tRNA movements during translocation.

2985-Pos Board B32

Dynamic Mode Switching and Loosely Coupled Conformational Events Observed in Single Ribosomes

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During protein synthesis transfer RNA (tRNA) and messenger RNA (mRNA) translocate through the ribosome in a rapid and stepwise fashion, a process that is catalyzed by the GTPase elongation factor G (EF-G). Single-molecule fluorescence resonance energy transfer (smFRET) measurements show that the ribosome complex can spontaneously adopt a key translocation intermediate in the absence of nucleotide hydrolysis. By obtaining smFRET data from multiple structural perspectives, we observe that tRNA and EF-G restructure the ribosome energy landscape, increasing the rate at which this intermediate is achieved. In particular, EF-G binding induces a fast dynamic mode of the L1 stalk, which facilitates rapid and reversible formation of the translocation intermediate. Bulk measurements indicate that the extent of conformational coupling is important in determining the rate of translocation. When the extent of coupling is increased, a faster rate of translocation is achieved. The rate of translocation is decreased when the conformational changes are further decoupled, such as by the addition of the antibiotic viomycin. Thus, the extent of coupling of conformational processes on the ribosome may present a new mechanism for the regulation of gene expression.

2986-Pos Board B33

An Allosteric Pathway Revealed in the Ribosome Binding Stress Factor BipA

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BipA is a highly conserved prokaryotic GTPase that functions as a master regulator of stress and virulence processes in bacteria. It is a member of the translational factor family of GTPases along with EF-G, IF-2 and LepA. Structural and biochemical data suggest that ribosome binding specificity for each member of this family lies in an effector domain. As with other bacterial GTPases, the ribosome binding and GTPase activities of this protein are tightly coupled. However, the mechanism by which this occurs is still unknown. A series of experiments have been designed to probe structural features of the protein to see if we can pinpoint specific areas of BipA, perhaps even individual residues, which are important to its association with the ribosome. Included in the list are the C-terminal effector domain of the protein, which is distinct to the BipA family of proteins, and amino acid residues in the switch I and II regions of the G domain. Using sucrose density gradients, we have shown that the C-terminal domain is required in order for BipA to bind to the ribosome. Moreover, deletion of this domain increases the GTP hydrolysis rates of the protein, likely through relief of inhibitory contacts. Additional evidence has revealed an allosteric connection between the conformationally flexible switch II region and the C-terminal domain of BipA. Site directed mutagenesis, sucrose gradients and malachite green assays are being used to elucidate the details of this coupling.

2987-Pos Board B34

Rare Codon Clustering: Implications for Protein Biogenesis

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Most amino acids are encoded by more than one codon. These synonymous codons are not used with equal frequency: in every organism, some codons are used more commonly, while others are more rare. Though the encoded protein sequence is identical, selective pressures favor more common codons for enhanced translation speed and fidelity. However, rare codons persist, presumably

due to neutral drift. Here, we determine whether other, unknown factors, beyond neutral drift, affect the selection and/or distribution of rare codons. Such selective pressures could be used to control, for example, the rate of appearance of the nascent polypeptide, influencing co-translational folding pathways. We have developed a novel algorithm that evaluates the relative rareness of a nucleotide sequence used to produce a given protein sequence. We show that rare codons, rather than being randomly scattered across genes, often occur in large clusters. These clusters occur in numerous eukaryotic and prokaryotic genomes, and are not confined to unusual or rarely expressed genes: many highly expressed genes, including genes for ribosomal proteins, contain rare codon clusters. We show experimentally that such a rare codon cluster can impede ribosome translation of the rare codon sequence. These results indicate additional selective pressures govern the use of synonymous codons, and specifically that local pauses in translation can be beneficial for protein biogenesis.

2988-Pos Board B35

Analysis Of Ribosomal Dynamics As Revealed By Cryo-EM And Flexible Fitting

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Ribosomes are the molecular machines that translate the genetic message into nascent peptides, through a complex dynamics interplay with mRNAs, tRNAs, and various protein factors. A prominent example for ribosomal dynamics is the rotation of the small ribosomal subunit with respect to the large subunit, characterized as the ''ratchet motion'', which is triggered by the binding of several translation factors. Based on density maps of ribosomal complexes obtained by cryo-EM, we analyzed two kinds of ribosomal ratchet motions, induced by the binding of EF-G and RF3, respectively. By using the flexible fitting technique (real-space refinement) (1) and an RNA secondary structure display tool (coloRNA) (2), quasi-atomic models of the ribosome were obtained in these ratchet-motion-related functional states. The observed differences in rRNA were further mapped onto the highly conserved RNA secondary structure diagram. Comparisons between the two sets of ratchet motions revealed that, while the overall patterns of the RNA displacement are very similar, several local regions stand out in their differential behavior, including the highly conserved GAC (GTPase-associated-center) region. We postulate that these regions are important in modulating the general ratchet motion and bestowing it with the dynamic characteristics required for the specific function.

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2989-Pos Board B36

Cryo-em Study Of Trna Hybrid States Stabilized By Viomycin

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Translocation is the step in translation where the peptidyl A-site tRNA on the ribosome moves to the P site and the deacylated P-site tRNA moves to the E site. Recently, several single-molecule FRET studies and cryo-EM studies have confirmed the existence of the tRNA hybrid states (A/P and P/E) and the spontaneous ratchet motion of the ribosome from Macrostate I to Macrostate II prior to translocation (Agirrezabala et al., 2008; Ermolenko et al., 2007a,b; J. Fu, J.B. Munro, S. Blanchard, J. Frank, unpublished). In one of the studies, antibiotic viomycin, which is known to block translation, was shown to promote MS II and the hybrid states the tRNA (Ermolenko et al., 2007B). To determine the mechanism by which viomycin blocks translation, and to further understand the intermediate states during translocation, we studied a pre-translocational complex prepared with viomycin. Single-particle reconstruction was used to determine the structure of the complex. Subsequent classification resulted in two distinct ribosome complexes in MS I and II. We believe that MS II represents the state in which translation is stalled by viomycin. Subsequent analysis revealed that the A-site tRNA is in the A/P hybrid state and the P site tRNA in a novel position in which it makes extensive contacts with the L1 stalk. The results confirmed the observation, by the single molecule FRET study, that viomycin locks the ribosome in the hybrid state. We are setting out for a more detailed analysis to understand the molecular details of the viomycin-induced hybrid states.

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2990-Pos Board B37

Polyelectrolyte Behavior And Kinetics Of The Aminoacyl-trna On The Ribosome

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A coarse-grained model is utilized to examine the changes in flexibility of aminoacyl-transfer RNA when it binds to elongation factor-Tu and guanosine-5′-triphosphate GTP. We predict that under appropriate conditions mode-coupling speeds-up the barrier-crossing rate for cognate (three base pairs that are matched) relative to near-cognate (one base pair mismatch) ternary complexes. We estimate the torque acting on the cognate ternary complex due to induced wrapping of the 30S subunit around the decoding site after correct codon-anticodon recognition. We predict by all atom grand Monte Carlo simulations the magnesium binding sites in tRNA-EF-Tu complex at low magnesium concentration. The prediction is in agreement with binding sites observed in x-ray structure (grown under high salt concentrations). We have used high level ab initio calculations to unravel the nature of interaction energy of magnesium with site-specific tRNAPhe bases. We find noticeable non-electrostatic contributions to the total interaction energy of the magnesium-base complex in gas phase and in polar solvent. Finally, we have developed stochastic techniques to elucidate fundamentally important rare events involving large thermal fluctuations along reaction pathways. These techniques will allow us to investigate the probability of forming contacts to stabilize GTPase activated state that involve configurational searches in the tail end of probability distribution.

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2991-Pos Board B38

Four Amino Acids, Two Kinetic Steps, No Synthetase: The Original Genetic Code?

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Considering a theoretical genetic system with only four codon-anticodon pairs and four amino acids randomly assigned to the tRNAs, we show that an elementary form of translation allows the system to display coding rules for particular values of kinetic constants and reactants concentrations. We show that these values compare well with experimental data. The analysis suggests that only two types of amino acids could be efficiently differentiated at that level. While adding the contribution of a plausible form of tRNA aminoacylation inferred from studies on ribozymes, we show that the combination of both steps would allow this polymerization process to differentiate the four amino acids without aminoacyl-tRNA synthetase. Features of the genetic code support our analysis.

Protein Conformation

2992-Pos Board B39

Surface Modification Affects the Heme Planarity and Accessibility in Horseradish Peroxidase

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Some noncovalently linked hemes like those in the peroxidases have highly conserved characteristic distortions in the porphyrin plane. Conservation occurs even for some proteins with a large natural variation in the amino acid sequence. Thus it is reasonable to anticipate that nonplanar porphyrins and protein-induced changes in the planarity may provide a mechanism for protein modulation of biological properties.

We previously reported that covalent modification of three accessible charged lysines (Lys-174, Lys-232, Lys-241) to the hydrophobic anthraquinolysine residues successfully improves electron transfer properties, catalytic efficiency, and stability of HRP.