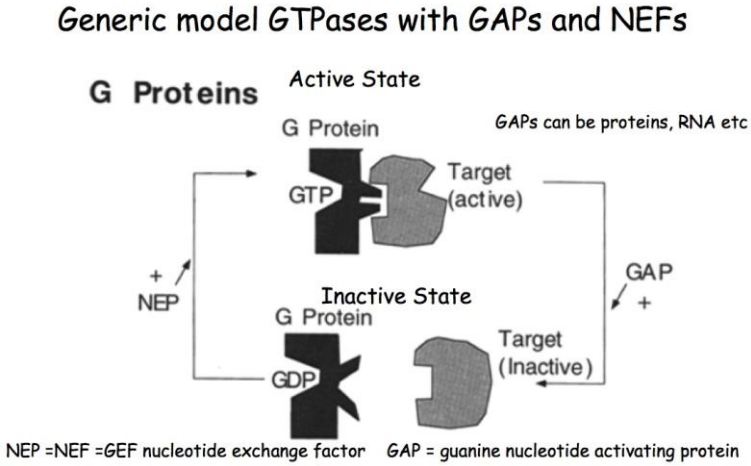
Chem 5.08 Recitation 2 **Week 2 Feb 10-12**

Recitation 2 Presteady state kinetic analysis: Required reading EMBO J (1999) 18, 3800. The model presented also uses data referenced in this paper but published in 1995, 1996 and 1998.

Overview of small G proteins: Small G proteins regulate many different biological processes. EF-Tu and EF-G are both examples of these proteins.



I. Introduction to the methods and experimental design:

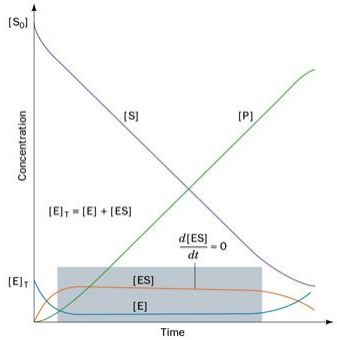
The Michaelis Menten equation (Eq 1) describes most enzymatic reactions and requires the steady state assumption. Review the assumptions made to obtain this equation in your introductory biochemistry course notes.

Eq 1: $v = V_{max}[S]/(K_m + [S])$

k_{cat} and k_{cat}/K_m are the two important kinetic parameters:

k_{cat} the turnover number ($time^{-1}$), tells you how good a catalyst you have.

k_{cat}/K_m is the specificity constant ($M^{-1}s^{-1}$, catalytic efficiency). Both parameters are composed of a number of first order rate constants that depend on the mechanism and in general cannot be evaluated by steady state analysis. To understand a reaction one wants to be able to measure the rate constants describing each step. In an effort to achieve this goal we turn to presteady state kinetics. The basic equations are the same, but the steady state assumption is not made.



presteady state ↑ ↑

Figure 1. The presteady state is the "short time" (usually ms) before the steady state is achieved.

In steady state kinetics, small amounts of enzyme (μM to nM) and large amounts of substrate are usually used. In the presteady state, a large amount of enzyme is used so that you can "monitor" the first turnover of the reaction.

For example recall Beer's law $A = lc\varepsilon$

If the protein is 10^{-8} M and the extinction coefficient (ε) of the substrate or product being monitored is $10^4 M^{-1}cm^{-1}$ and l = 1 cm, then, the total change in A in the first turnover would be 0.0001 units. This small change is challenging to measure. If, however, the enzyme is present at 10^{-4} or 10^{-5} M the absorption change is now 1 or 0.1, and is easy to measure.

If large amounts of enzyme are used, since the rate of your reaction is proportional to the concentration of catalyst, the rate is very fast. If you were pipetting your reagents by hand, the reaction would be over by the time you mixed them and place them in the cuvette and into the spectrophotometer. Thus, an apparatus such as a stopped flow (SF) or a rapid chemical quench (RCQ) instrument is needed that mixes the solutions for you. The limitation of measurable rate constants is set by the efficiency of mixing. In commercial instruments, the mixing dead time is 1 to 3 ms. If the rate constant of interest (k_{obs}) is 500 s^{-1}, then the half life of the reaction ($t_{1/2} = 0.693/k_{obs}$) would be 1.3 ms. Thus the reaction is more than 60% complete in the dead time of the instrument. At 1000 s^{-1}, the half life is 0.7 ms and the reaction would be over before you started to record data. Typically one likes to follow the reaction for 4 or 5 half lives. Recently mixing devices with dead times of 100 microsec have been described, but they are not yet commercially available. One can lower the rate of the reaction by changing the temperature (37 vs 4 °C). However, in biological systems, reactions are often rate-limited by conformational changes, that is, they are physically gated, and thus one would like to study the reaction at physiological temperatures if this is possible.

- Apparatus

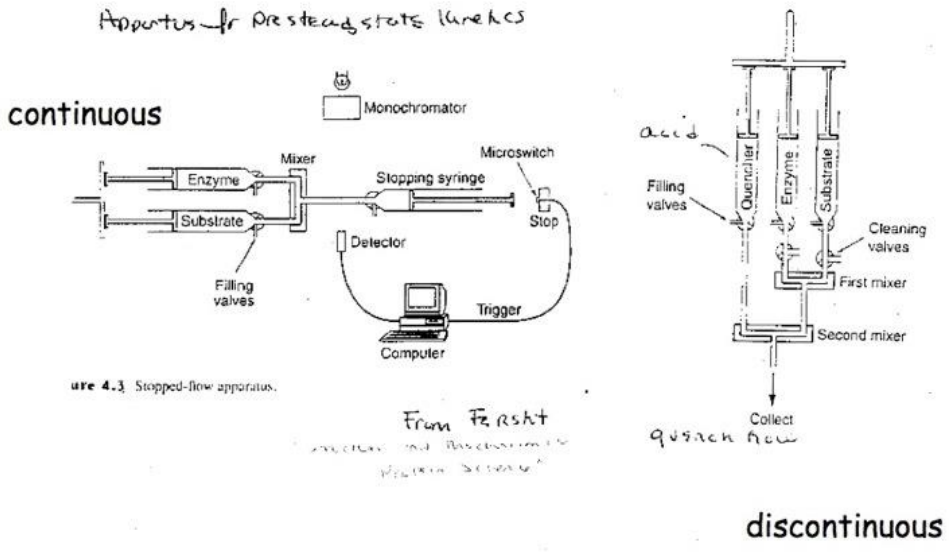


Figure 1. The SF (left) and RCQ-(right) instruments.

The **SF methods** allow **continual** monitoring of the reaction as a function of time, if there is either a **unique change** in the absorption or fluorescence between the starting materials and products. The rapid chemical quench method is **discontinuous**, that is, the reaction must be stopped RAPIDLY (~ms, so that stopping is not rate-limiting) and this is usually done with H^+ or EDTA (ethylene diamine tetraacetic acid) if the enzyme requires Mg^{2+}. Many nucleic acid polymerases require $Mg^{2+}$and thus this is the method of choice. Once your reaction is quenched, you must then develop a method to separate products from starting material at each time point by HPLC or TLC and quantitate the amounts. Typically radioactively labeled starting material is used as it has great sensitivity. The use of radioactivity will be discussed in the next recitation.

II Introduction to the EF-Tu switch and the model derived from many different kinds of experiments.

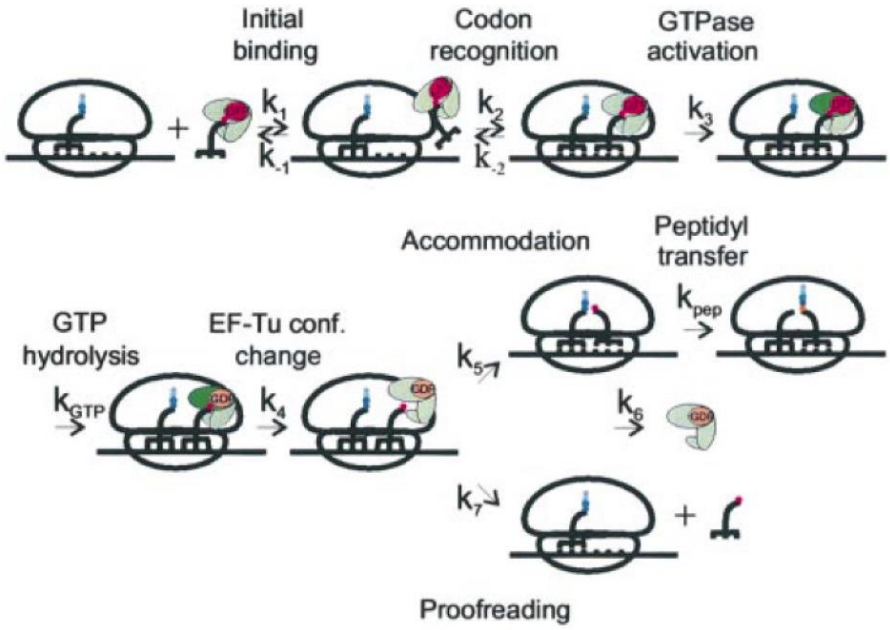


Figure 2. The kinetic model for the role of EF-Tu in elongation from all of the Rodnina papers based on SF and RCQ data with global analysis of data from all sources. The red ball is the GTP bound state of EF-Tu (light green) and the pink ball is the GDP bound state. EF-Tu is light green and the G domain of EF-Tu is dark green. Taken from the required reading.

In this model, initial (weak) binding of the charged tRNA• EF-Tu to the ribosome is given by k_1/k_{-1}. Codon recognition is described by k_2/k_{-2} and GTPase activation is described by k_3, a conformational change followed by very rapid GTP hydrolysis (k_{GTP}). An EF-Tu conformational change then releases itself from the charged tRNA, k_4. There is a switch in

the conformation of EF-Tu when GTP is hydrolyzed to GDP (you will examine this change in conformation in PS 2). In the next step called accommodation (k_5), the charged amino acid must be placed in the appropriate conformation for the final step, peptide bond formation (k_{pep}). Alternatively if the codon/anticodon interaction is NOT cognate, then proofreading (k_7) can occur. Thus the ability to choose between cognate, near cognate, and non-cognate can occur at k_{-2} vs k_3 and k_5 vs k_7.

In this proposed mechanism for EF-Tu, the conformational changes are the key steps. **These conformational changes are coupled to rapid irreversible chemical steps. One cannot measure the rate constants for the chemical steps as the conformational changes limit them.**

III. The model for fidelity looking at cognate and near cognate:
1) EF-Tu•GTP•aa-tRNA^{aa} is a stable ternary complex, (like all GTP binding proteins, it has very low GTPase activity, 0.0005 s^{-1}). The ternary complex is so stable that it can be purified by Sephadex chromatography.

2) initial selection (k_1/k_{-1}, k_2/k_{-2} vs. k_3)
k_{-2} vs. k_3
The protein senses the cognate interaction and if it is correct, a conformational change occurs followed by a rapid irreversible chemical step (GTP conversion to GDP and Pi). (Note if the conformational change is slow and GTP hydrolysis is fast, the rate of GTP hydrolysis is identical to that for the conformational change, that is the chemical step is kinetically masked.)

selection for the correct interaction:

	near-cognate	cognate
k_{-2}	17s^{-1}	0.2 s^{-1}
k_3	50 s^{-1}	500 s^{-1}
difference	2.5 fold	2000 fold

As noted above, in the absence of GAP (GTPase activating protein, which is ribosomal RNA for EF-Tu!), the rate of the GTPase is only 0.0005 s^{-1}. It is accelerated by a factor of 10^6 fold when the "GAP" (the ribosome) is present. In the case of a non-cognate-aa-tRNA^{aa}, dissociation of the EF-Tu•GTP•non-cognate-aa-tRNA^{aa} occurs without ever hydrolyzing GTP. Thus NO energy is wasted.

3. proofreading

EF-Tu•GDP is present and the charged tRNA has been delivered.
k_7 vs k_5 (conformational change, followed by a rapid irreversible chemical step which is peptide bond formation)

	near cognate	cognate
k_5	0.1 s^{-1}	7 s^{-1}
k_7	6 s^{-1}	0.3 s^{-1}

If proofreading occurs in this step, you have sacrificed a GTP
"Book-keeping" of GTP hydrolysis is complicated, because GTP can be consumed in proofreading as well as peptide bond formation. The model system used by Rodnina et al exhibits **chemically and kinetically competent peptide bond formation** of 7 s^{-1} (this number is similar to peptide bond formation *in vivo*, rate ~10 s^{-1}).

For global analysis of all of your kinetic data using the probes described below, the early versions of the KINSIM software is free, while the more advanced versions require a license. KINSIM is a standard program used for integration of all the data from your experiments. http://jplusconsulting.com/products/reactlab-kinsim/ Several new programs are now available http://www.kintek-corp.com/kinetic_explorer/ http://www.kintek-corp.com/pdf/kintek_explorer_instructions_v2.2-indexed.pdf. For references describing these methods that are now widely used see: Methods in Enzymology 467, 601 Chap 23 (2009); Anal Biochem 387 2029 and 30-41 (2009)

IV. Where did this model come from? Many different types of experiments!. Global analysis of all kinetic data (from all the different experiments) with computer software, give kinetic simulations that accommodate available information and are the basis for the current working hypothesis (summarized in Table 1).

Different types of probes:

Rapid Chemical Quench and use of radioisotopes

a. GTP[γ-^{32}P] → ^{32}Pi + GDP; one can measure the rate of ^{32}Pi formation. ^{32}P is a radioisotope and a strong β emitter. A scintillation counter is used.
b. [^{3}H]-aa tRNA$^{[3H]\text{-}aa}$, one quenches the reaction with HO^-. This quench hydrolyses the ester linkage between the aa and the tRNA and releases the amino acid and the dipeptide from the tRNA. One then needs a TLC or HPLC system to separate the radiolabeled aa from the radiolabeled peptide.

Stopped Flow fluorescence:

c. conformational changes (use fluorescent probes)
i. proflavin labeled tRNA^{aa}
ii. EF-Tu labeled with a fluorescent probe using site directed mutagenesis to place a cysteine at a desired position using structural information.
iii. GTP analogs- fluorescently labeled mant-GTP with the fluorescent label attached to the 2'-OH of the nucleotide.

Additional tools:

d. Non-hydrolyzable GTP analogs that prevent GTP hydrolysis and allow monitoring of the first two steps in kinetic scheme (binding and conformational changes) and prevent chemistry.
e. Fluorescently labeled mRNA can also be obtained.

Data and figure legends taken from the required reading:

Fig. 2. Time courses of A-site interactions of Leu ternary complex. (**A**) Conformational changes of aa-tRNA monitored by the fluorescence of Leu-tRNA$_2^{Leu}$(Prf16/17/20). Parameters of two-exponential fits: k_{app1} = 65/s, A_1 = 22%, k_{app2} = 6/s, A_2 = –16%. (**B**) Conformational changes of EF-Tu monitored by mant-dGTP fluorescence. Parameters of two-exponential fits: k_{app1} = 44/s, A_1 = 37%, k_{app2} = 2/s, A_2 = –35%. (**C**) GTP hydrolysis in EF-Tu·[γ-^{32}P]GTP·Leu-tRNA$_2^{Leu}$ (●; k_{app} = 37/s) and EF-Tu·[γ-^{32}P]GTP·Phe-tRNA^{Phe} (○; k_{app} = 55/s). (**D**) Dipeptide formation with [^{3}H]Leu-tRNA$_2^{Leu}$ (●) or [^{3}H]Phe-tRNA^{Phe} (○). Concentrations after mixing were 0.1 μM ternary complex and 2 μM ribosomes. Note the different ordinates for Phe and Leu in (D). Smooth lines show time curves calculated from elemental rate constants (Table I).

Let us look at some of the primary data where the mRNA is polyU and Phe is the cognate amino acid F-tRNA^F and leucine L-tRNA^L is near cognate. Acylated F-tRNA^{acF} is loaded into the P site of the ribosome. The concentration of the ribosomes and the Mg^{2+} are controlled. The rate of release of $^{32}P_i$ for cognate and near cognate are given above and look very similar (C, above). However, the lag phase, prior to release of $^{32}P_i$ is substantially different. This is an example of how it is hard to "intuit" pre-steady state kinetics. The observed rate constant is composed of all of the steps k_1, k_{-1}, k_2, k_{-2} etc and the model needs to account for the lag phase as well as the apparent k_{obs} for Pi release.

In the case of dipeptide formation, the cognate dipeptide (FF) is formed in substantially larger amounts than the near cognate dipeptide (FL) formation (look at the differences in the y axes for each experiment, D above). The cognate dipeptide is produced at 10x higher levels than the near cognate.

In the experiments shown in A and B above, two different fluorescent probes were used: in A a fluorescent Leu-tRNA^{Leu} is used and in B, mant-dGTP is used. As described in detail in the paper, additional information obtained previously by these coworkers played an important role in interpretation of this data. These are just samples of some of the many experiments that these investigators have carried out. All the information in this paper and

prior papers is put together and analyzed to provide the model and rate constants in Table 1 in the paper.

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