TD 8: EF_Tu-dependent binding of amino acylated tRNA to ribosomes
Techniques: Fluorescence assays and pre-steady state kinetics
References: Rodnina et.al., EMBO J, 1998, 17, 7490

I. Background:

Previous work (pre-1998) established the basic model, shown in **Figure 1** of Rodnina et.al., EMBO J, 1998, 17, 7490. However, measurements of individual steps were done in different assay buffers (varied [Mg2+]) and at different temperatures. To compare the steps to each other (which are fast, slow rate-determining?), need to do all measurements under the same conditions.

Here: 5mM MgCl, 20degC, and 10mM MgCl, 20degC

II. Overall approach:

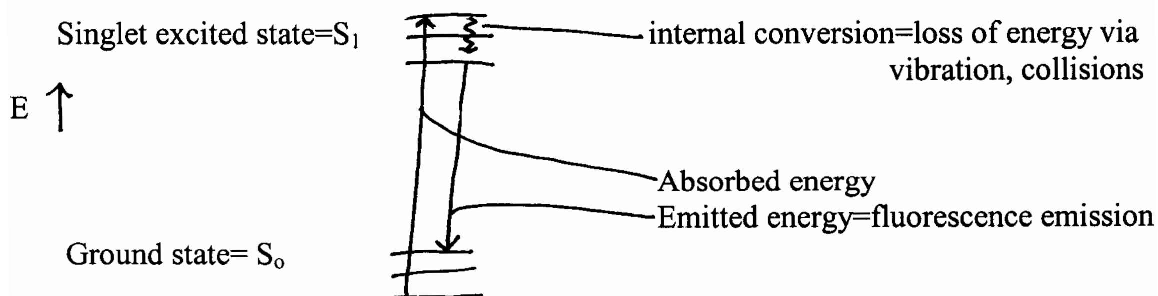
Use/develop clever assays that allow us to measure only 1 or 2 of these steps at a time, and use pre-steady state analysis techniques to detect transient intermediates.

III. Caution:

It is a big assumption that the model above is correct and is the only pathway used by the ribosome. In reality, probably multiple pathways and much more complex.

IV. Technique I: Fluorescence assays

Often used in biochemistry due to high sensitivity (easy to detect only a few photons) and specificity (can give us info about specific conformation changes and biochemical events)
Definition: Fluorescence = light emitted by a molecule that has been excited by light
Fluorophore = molecule that exhibits fluorescence
Luminescence= emission of light from electronically excited state



Types of luminescence

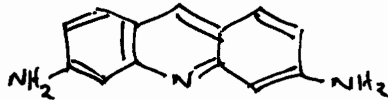
Fluorescence – excitation from light, emission from S_1, fast ~10^8 s^{-1} (example- highlighter)
Phosphorescence- excitation from light, emission from T1, slow (example- glow in dark toy)
Chemiluminescence- excitation from chemical energy (example- glowstick)
Bioluminescence- form of Chemiluminescence in organisms, all known examples from luciferase oxidation of luciferins (example – firefly)

For fluorescence, the energy of emmited light is always less than energy of absorbed light
$E_{em} < E_{abs/ex}$

$\lambda_{em} > \lambda_{ex}$

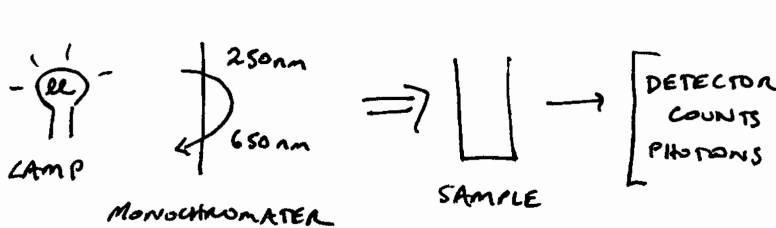
because $E=hv=hc/\lambda$

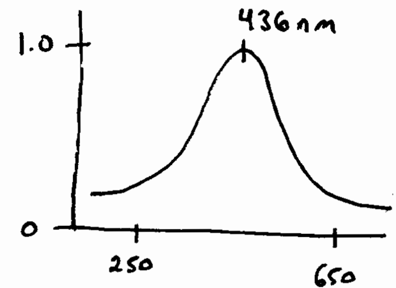
Example- **proflavine** (Pf) excitation (ex) λ = 436 nm, emission (em) λ = 510 nm



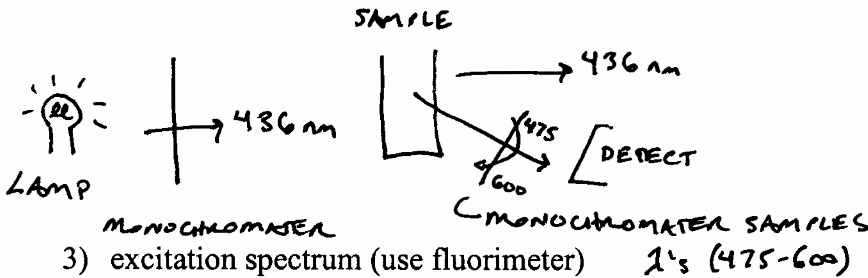
3 most common measurements made on fluorophores

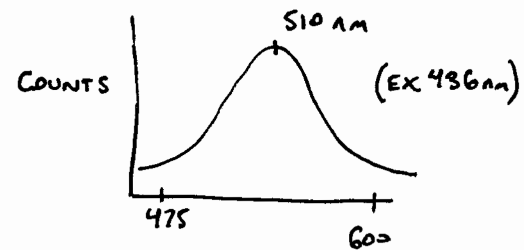
1) Absorbance spectrum (use UV-vis spectrophotometer)



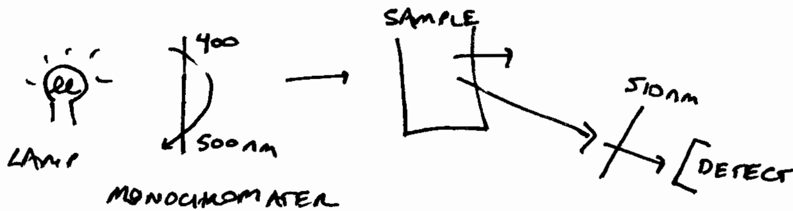


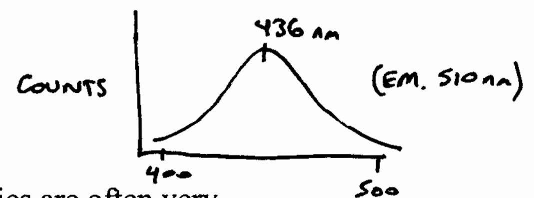
2) emission spectrum (use fluorimeter)



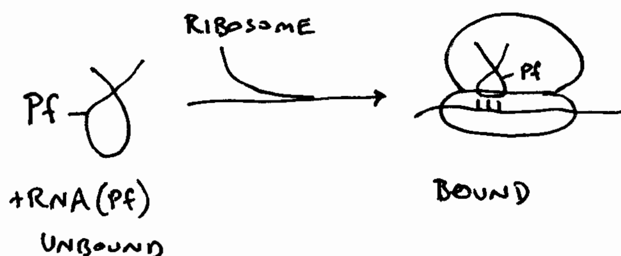


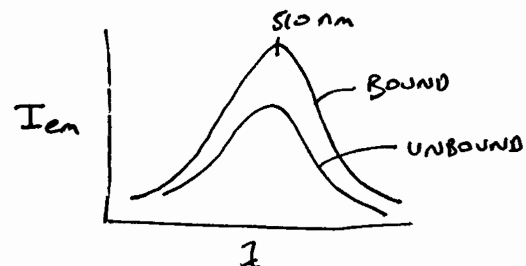
3) excitation spectrum (use fluorimeter)





Fluorophores are useful biochemical probes because their properties are often very sensitive to environment (hydrophobicity, pH, aromatic stacking, redox potential, distance to other fluorophores). λ^{max}em, λ^{max} ex, I_{em} (intensity) can all change as a function of environment
Example- proflavine attached to tRNA, I_{em} changes when tRNA binds the ribosome





V. Technique 2: Pre-steady state kinetics

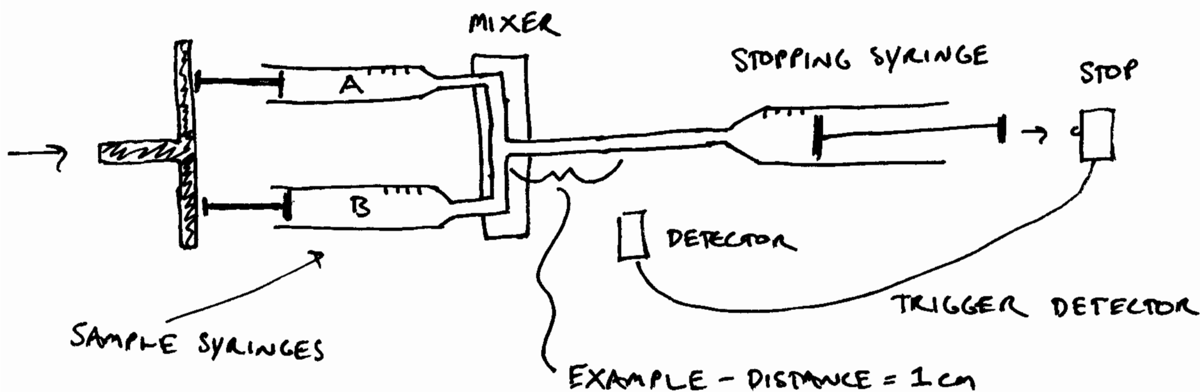
Steady state kinetic measurements give K_m and k_{cat} for overall reaction, usually a combination of several steps. Need pre-steady state analysis to determine individual rate constants and to detect transient intermediates

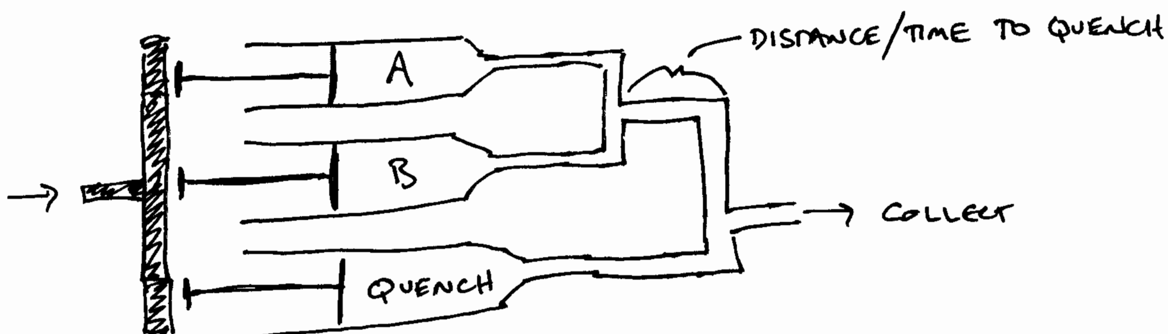
The key: initiate reaction quickly and make measurement quickly
K_{cat} is usually 1-> 10^7 s^{-1}, must make measurements in time range of 1-10^{-7} sec

Three methods:

1. rapid mixing and sampling (used by Rodnina et al in our example)
2. flash photolysis (uncage a reaction with light)
3. measure rate of relaxation to equilibrium

"Stopped flow" device to rapidly mix 2 samples and then detect reaction product ~1msec later (10^{-3} sec)
See a picture on p. 282 of Voet& Voet Biochemistry, John Wiley & sons, Inc, 2004 Volume 1. Or see my drawings below.
If the distance from the mixing point to the detector is 1cm, and the flow rate is 10m/s, then the sample is only 1 msec old when the detector first sees it.
A "quenched flow" device allows you to rapidly mix 2 samples and ten quench the reaction flow a few msec later-> this is useful if you need to analyze the reaction products in some way other than fluorescence (for example- TLC or HPLC separation needed)





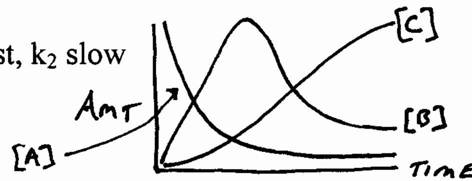
Analysis of pre-steady state kinetics
-gets very complicated for anything but the simplest reactions- usually done w/ software programs (e.g. MicroMath, TableCurve)

For a simple irreversible reaction:
A -> B with rate constant = k
$d[B]/dt=k[A]$ and $d[A]/dt=-k[A]$
solving by integrating $[A]_t=[A]_o e^{-kt}$ where $[A]_o$ = the initial concentration of A
and $[B]_t=[A]_o(1-e^{-kt})$

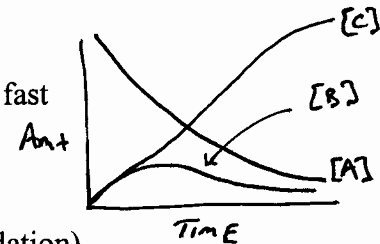
For a reversible reaction A-> B with rate constant k_1, B-> A with rate constant k_{-1}
$[A]_t= ([A]_o/(k_1+k_{-1}))(k_{-1}e^{(-(k_1+k_{-1})t)} + k_1)$
$[B]_t= ([B]_o/(k_1+k_{-1}))(k_{-1}e^{(-(k_1+k_{-1})t)} + k_1)$

For two consecutive reactions A->B with rate constant k_1, and B->C with rate constant k_2
$[A]_t=[A]_o e^{-k_1 t}$
$[B]_t= ([A]_o k_1/(k_2*-k_1))(e^{-k_1 t}- e^{-k_2 t})$
$[C]_t=[A]_o(1+(1/k_1-k_2)(k_2 e^{-k_1 t}-k_1 e^{-k_2 t}))$

Graphs for k_1 fast, k_2 slow

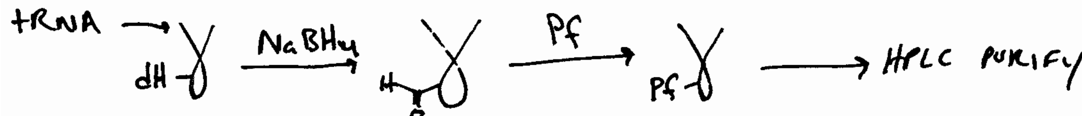


for k_1 slow, k_2 fast

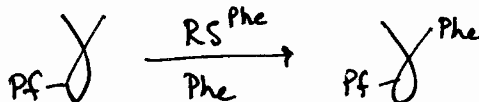


VI. <u>Steps 1,2 and 6 (initial binding, codon recognition, and accommodation)</u>
No assay for step 1 alone or step 2 alone, so must be analyzed together, and separate kinetic constants teased out

Assay:
1) prepare proflavine (Pf)-labeled tRNA
Label at dihydrouridine(dH) using $NaBH_4$, purify with HPLC
*controls show that Pf label doesn't mess up tRNA structure/function



2) charge with Phe using tRNA synthetase (RS), get "Phe-$tRNA^{Phe}$(Pf16/17)"



3) form ternary complex with EF-Tu·GTP
use size exclusion to purify "EF-Tu·GTP· Phe-$tRNA^{Phe}$(Pf16/17)"

4)prepare poly-U loaded ribosome

5)mix ternary complex (0.1microM) with poly-U-ribosome(0-3microM) in stopped flow device

6) record I_{em} of Pf over time

Get Graph of I_{em} over time for 10 and 5mM Mg^{2+}
See Rodnina et.al., EMBO J, 1998, 17, 7490 **Figure 2A**

Previous experiments have shown
-initial binding causes I_{em} of Pf to increase
-codon recognition causes further increase of I_{em}
-No change in I_{em} for GTP activation, hydrolysis, or Ef-Tu conformational change
-accomodation step gives I_{em} decrease
-peptide bond formation=no I_{em} change
Thus: Iem increase represents steps 1 and 2 (initial binding and codon recognition) Iem decrease represents accommodation (step 6)
Note: would not be able to see initial peak under steady state conditions

Fit data to equation with 2 exponentials (for rise and fall), get out 2 "apparent rate constants"
K_{app1}= steps 1 and 2 ($60s^{-1}$ @10mM Mg^{2+}, $40s^{-1}$ @5mM Mg^{2+})
K_{app2}= step 6 ($8s^{-1}$ @10mM Mg^{2+}, $10s^{-1}$ @5mM Mg^{2+})

VII. Steps 3 and 8: Ef-Tu activation and dissociation from ribosome

Assay: use **mant-dGTP** (ex. 362nm, em. 448nm)- behaves biochemically like GTP

1. prepare Ef-Tu·mant-dGTP· Phe-$tRNA^{Phe}$ ternary complex
2. add to poly-U loaded ribosome in stopped flow device
3. record I_{em} of mant Fluorophore over time

Get **Figure 3A** from Rodnina et.al., EMBO J, 1998, 17, 7490
Showing I_{em} of mant over time for 5 and 10 mM Mg^{2+}

Previous studies have shown
-no change in Iem during initial binding and codon recognition (steps 1&2)
-Iem increases during GTPase activation (step 3)
-no change when GTP is hydrolyzed (step 4)
-Iem decreases when Ef-Tu·GDP dissociates from ribosome (step 8)

Thus: 2 exponential fitting gives 2 k_{app}'s
$k_{app}1$ (fluorescence increase) assigned to steps 1-3, ending in GTPase activation
$k_{app}2$ (fluorescence decrease) assigned to step 8, Ef-Tu dissociation
$k_{app}1 = 55s^{-1}$ @ 10 mM Mg^{2+}, $25s^{-1}$ @ 5 mM Mg^{2+}
$k_{app}2 = 3\text{-}5s^{-1}$ independent of [Mg^{2+}]

VIII. Step 4- GTP hydrolysis

Assay: γ-^{32}P-GTP

1. create ternary complex Ef-Tu· γ-^{32}P-GTP · Phe-tRNA^{Phe}
2. add to poly-U ribosomes in quenched flow apparatus
3. quench reaction after 5msec-1sec
4. run reaction out on TLC plate to quantify how much GTP has been hydrolyzed to GDP + Pi (image ^{32}P with phosphorimaging)

Get: **Figure 4** from Rodnina et.al., EMBO J, 1998, 17, 7490, showing amount of GTP hydrolyzed over time

Fit to single exponential, get
k_{app} = 55s^{-1} @ 10 mM Mg^{2+}, 23s^{-1} @ 5 mM Mg^{2+}
represents rate for steps 1-4

IX. Step 7: peptidyl transfer

Assay: use ^{3}H-Phe-tRNA^{Phe}

1. prepare radiolabeled tRNA
tRNA^{Phe} + [^{3}H]-Phe + RS^{Phe} -> product-> purify
2. use this to make ternary complex Ef-Tu· GTP · ^{3}H-Phe-tRNA^{Phe}
2. combine with poly-U loaded ribosomes in quenched flow device
3. quench reaction after 5msec-1sec w/ 0.6M KOH (hydrolyzes amino acylated tRNAs)
4. inject onto HPLC to separate ^{3}H-Phe from ^{3}H-Phe- ^{3}H-Phe dipeptide
5. count fractions with scintillation counter

Get: **Figure 5** from Rodnina et.al., EMBO J, 1998, 17, 7490, showing dipeptide synthesized over time

Fit to single exponential, get
k_{app} = 7-8s^{-1} independent of [Mg^{2+}] for steps 1-7

Note: rate ~same as for accommodation step, suggesting accommodation is rate-;imiting and after accommodation, peptide formation occurs instantaneously

X. Dissociation constants for steps 1 and 2

Assay for k-2 measurment:

1. prepare Ef-Tu· GDPNP · Phe-tRNA^{Phe} ternary complex
GDPNP= non-hydrolyzable analog of GTP (NH prevents hydrolysis)
2. Also prepare labeled version with Pf-tRNA
Ef-Tu· GDPNP · Phe-tRNA^{Phe}(Pf16/17)
3. prepare ribosome – polyU complex w/ Ef-Tu· GDPNP · Phe-tRNA^{Phe}(Pf 16/17) remains stuck after codon recognition

4. In a stopped flow instrument combine: Ribosome·polyU·Ef-Tu· GDPNP · Phe-tRNA^{Phe}(Pf 16/17)
   + 10 fold excess of unlabeld ternary complex
5. monitor Pf Iem over time

Get: **Figure 6** from Rodnina et.al., EMBO J, 1998, 17, 7490, showing Iem of Pf decreasing over time

Fit to single exponential, get
$k_{-2} = 0.2s^{-1}$ @ 10 mM Mg^{2+}, $2s^{-1}$ @ 5 mM Mg^{2+}

previous studies showed $k_{-1} = 25s^{-1}$ @ 10 mM Mg^{2+}, $30s^{-1}$ @ 5 mM Mg^{2+}

XI. Global fitting to determine individual rate constants

-use software

See Table I from Rodnina et.al., EMBO J, 1998, 17, 7490 for a summary of individual rate constants

Conclusions:
1. only k_3 and k_{-2} are strongly Mg^{2+} dependent
k_3 may be because Mg^{2+} is required for GTp hydrolysis
k_{-2} may be because without Mg^{2+} Ef-Tu conformation causes weaker binding of tRNA to mRNA
2. Initial binding is faster than what one would predict by random collision: is there some pre-organization? Something guiding the ternary complex into the ribosome?
3. GTP hydrolysis very fast
4. Accommodation step is slow, peptide bond formation instantaneous
5. slowest step overall is EF-Tu dissociation