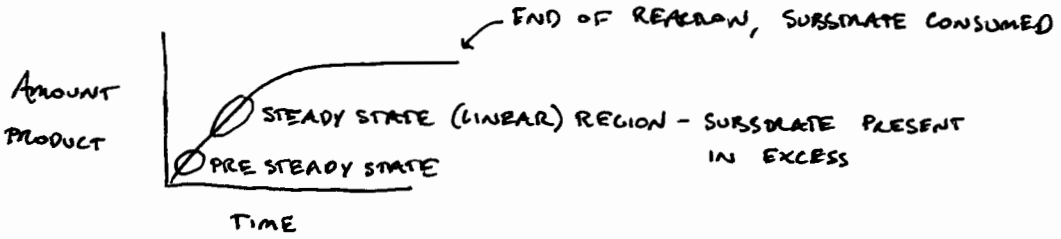
TD 9
Techniques: Review of Steady State kinetics, FRET
References: Nature (2001) 411, 1065 (FRET)
Annu Rev Biochem (1998) 67, 509 (GFP)

I. Review of Steady State Kinetics

If any of this is at all unfamiliar, review a basic biochemistry text like Voet&Voet

E+S E•S E + P

E= enzyme, S= substrate, P = product
Graph of product produced over time



Steady State assumption: [E•S] is constant, d[E•S] /dt = 0

Steady state rate equation: $v = k_{cat}([E]_o[S]/(K_m + [S]))$
$K_m=(k_{-1} + k_{cat})/ k_1$ (definition)

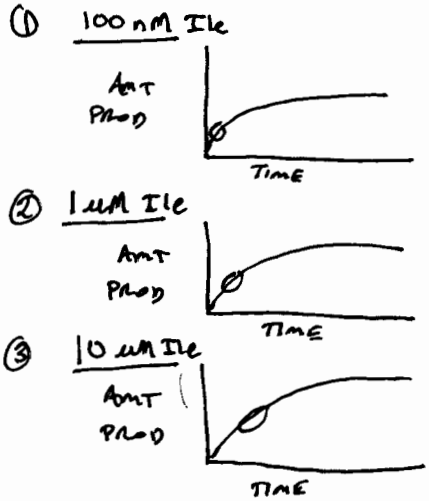
Note that as [S] -> infinity, $v_{max} = k_{cat}[E]_o$

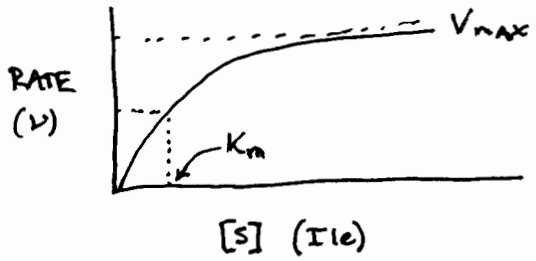
Also, when $K_m = [S]$, $v = \frac{1}{2} v$ max

Example: how to measure K_m & k_{cat} for RS^{Ile} :

Ile + ATP + RS^{Ile} RS^{Ile}•Ile-AMP RS^{Ile} + tRNA-Ile + AMP

There are 3 substrates (Ile, ATP, and tRNA), therefore, there are 3 K_m's. To measure K_m for Ile, provide excess of tRNA and ATP (saturate) and titrate in various amounts of Ile. Plot the amount of product produced over time, and calculate the reaction velocity from the linear region (slope = reaction velocity). Then plot reaction rate against substrate concentration- K_m & k_{cat} can be easily taken from this graph





II. FRET (fluorescence resonance energy transfer)

A way to estimate distances between two fluorophores

-done when emission λ (em) of 1 fluorophore (donor) overlaps with excitation λ (ex) of another (acceptor)

-NOT a reabsorption (no intermediate photon) – dyes are coupled by a dipole-dipole interaction- the excited states of 2 fluorophores

NOT radiative

Example: Fluorescein and Rhodamine

Fluorescein ex= 494nm, em = 514nm (donor)

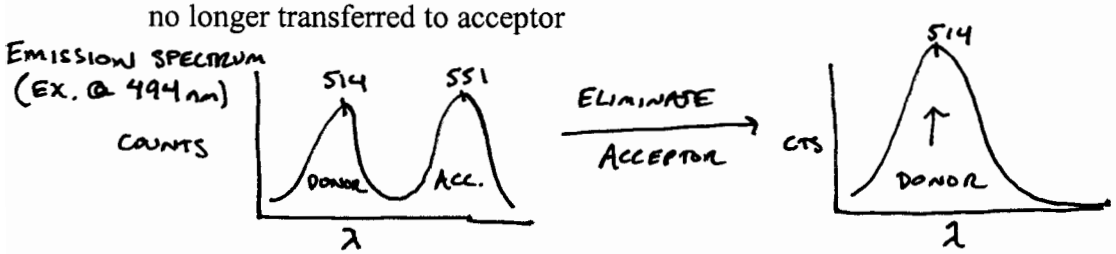
Rhodamine ex= 528nm, em = 551nm (acceptor)

Attach both fluorophores to a protein

Emission spectrum when the two fluorophores are excited at 494nm shows two peaks.

Separate the fluorophores by digesting the protein (trypsin), or eliminate the acceptor by photobleaching

Now see only one peak, corresponding to the donor, intensity (I) increased since energy no longer transferred to acceptor



FRET efficiency = E= 1- (I_{donor}- before bleach/ I_{donor} –after bleach)

High FRET= big increase after bleach= small fraction= E near 1.0

Low FRET= small increase after bleach = big fraction = E near 0

Can also get E from lifetime measurements

E= 1- (τ_{donor}- before bleach/ τ_{donor} –after bleach)

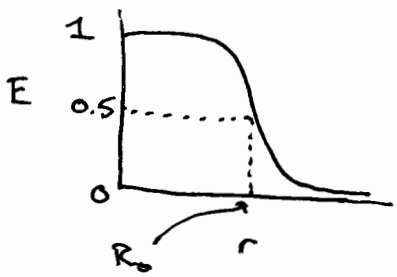
To get distances, use FRET theory $E= R_o^6/ (R_o^6 + r^6)$

where r= distance between donor and acceptor

R_o = "Forster radius"- distance at which E=0.5

A plot of E vs r shows that you can get the most info about distance at around R_o (30-60 angstroms) for most dyes- conveniently about the diameter of an average protein

R_o is specific to the fluorophores used



III. GFP- green fluorescent protein

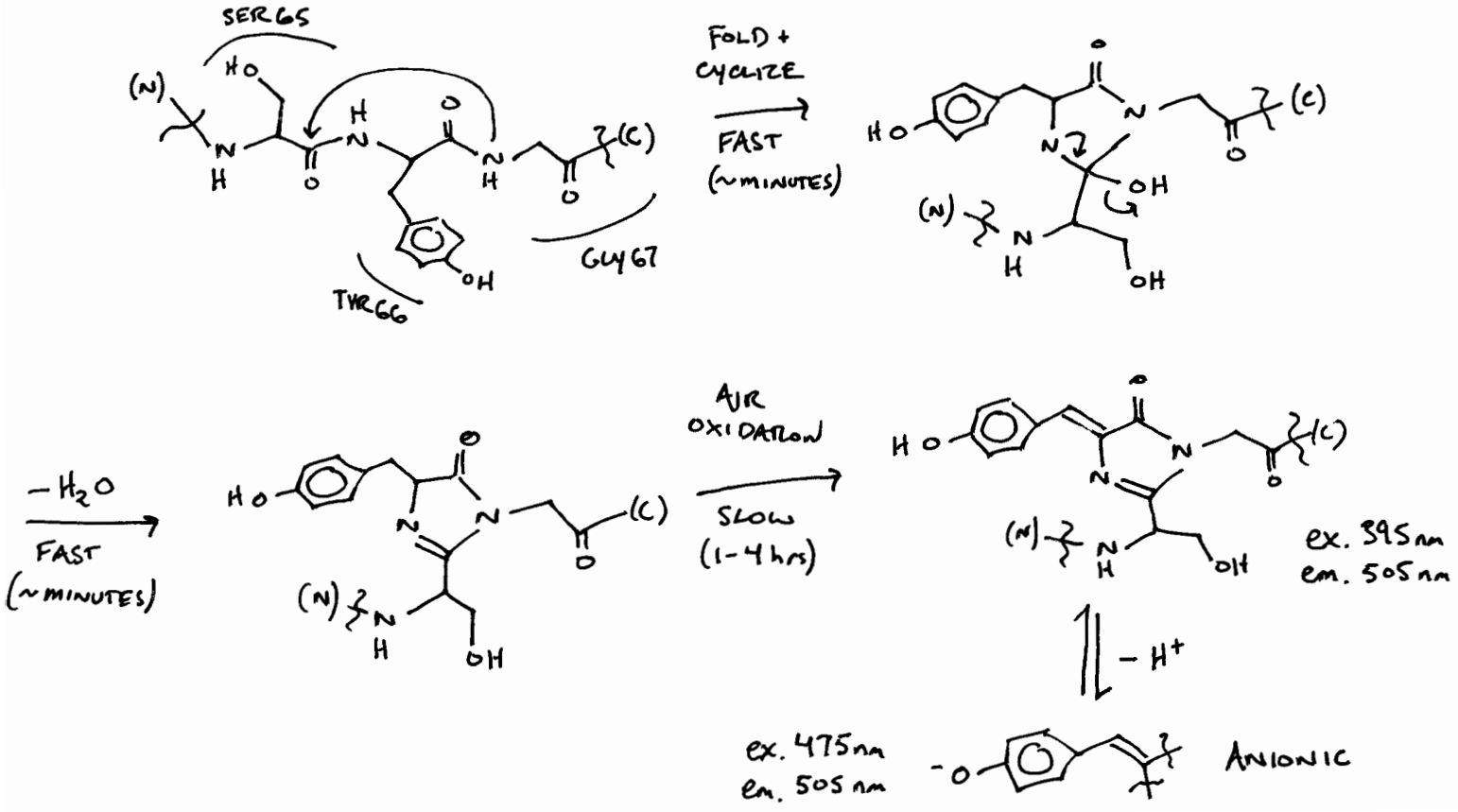
Why? – to label proteins for optical imaging with perfect specificity
features: from jellyfish (GFP= FRET acceptor for auquorin->convets blue light to green)
238 amino acids
11 strand beta-barrel (anti-parallel)
Ser65-Tyr66-Gly67 form chromophore
(ex=395nm, em=505nm; anionic form ex=475nm, em=505nm)
maturation time after expression (1-4 hrs)
slow step: oxidation
imidazolinones spontaneously auto-oxidize in air
No fluorophore develops in anaerobic conditions

RFP= red fluorescent protein
features-from coral
1st becomes green, then red ~12 hrs later
mass spec shows red protein 2 mass units smaller than green intermediate
obligate tetramer

other rationally designed colors: yellow(YFP), blue(BFP), cyan(CFP)
EGFP=enhanced GFP, has only one ex peak, @ 475nm (S65T mutant) -> destroys H bond with Glu222, forcing it to be protonated-> stabilizes anion form of Tyr 66

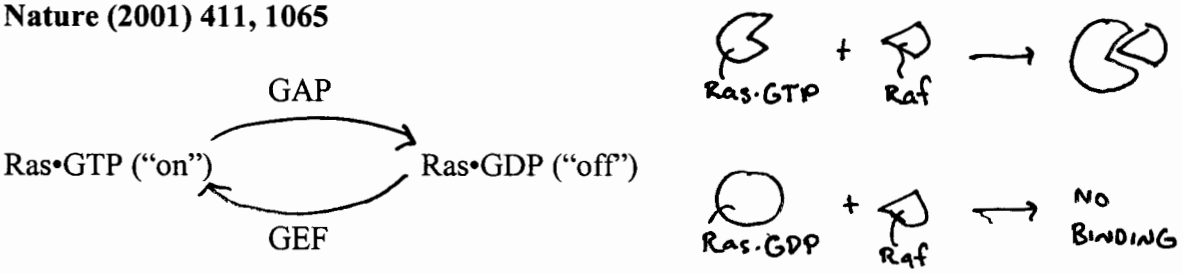
advantage- GFPs are genetically encoded (can attach to desired protein with perfect specificity)
disadvantage- REALLY big

GFP chromophore- Annu Rev Biochem (1998) 67, 509



IV. Example of use of FRET- Ras activation

Nature (2001) 411, 1065



Ras ia a GTPase that acts as a molecular switch in many signaling cascades
When "on," Ras•GTP interacts with many downstream effectors, including Raf
Raf binds only to "on" Ras

Approach:
make DNA for intramolecular complex of CFP(cyan)-Raf-linker-Ras-YFP(yellow)
introduce into cells
express protein
image
See **figure 1a** in Nature (2001) 411, 1065 for a cartoon image of the protein construct in the GTP and GDP bound state

To image: excite CFP, collect YFP emission
excite CFP, collect CFP em
divide YFPem/CFPem -> ratio image

In the figures, red=high YFP/CFP ratio-> high FRET
blue = low YFP/CFP ratio-> low FRET

Compare 2 reporters: Ras activation reporter "Raichu-Ras"
Rap1 reporter "Raichu-Rap"
Rap1 is another GTPase
Goal- use FRET to compare *in vivo* spatial and temporal activation of these GTPases
Experiment : add EGF (epidermal growth factor)-> Ras or Rap1construct-> cell growth
observations: Ras activated @ plasma membrane 1st
Rap1 activated @ perinuclear membrane 1st
Ras activated @ free edges of cell
Conclusions: Ras and Rap1 act by different mechanisms
small subpopulation of each are activated by EGF, most stays initially inactive
again, in Nature (2001) 411, 1065
Figure 1b shows emission spectra of Ras and Rap1, red line= GTP bound (high FRET), blue= GDP bound (low FRET), green = protein digest control
Figure 1a compares cells expressing Ras and Rap1- notice that Ras is first activated at free edges of cell (as indicated by red high FRET), and Rap1 is activated at perinuclear membrane.
Figure 2c- compares location of red high FRET areas (from Ras) to border of cell, notice activation at free cell edge