Solutions for the Analytical Cume on Announced Topic:

“Flow Field Penetration in Thin Nanoporous Polymer Films under Laminar Flow by Förster Resonance Energy Transfer Coupled with Total Internal Reflectance Fluorescence Microscopy”,

1. (10) Describe Förster resonance energy transfer. Over what approximate length scales does it report distances and why?

Förster resonance energy transfer describes the direct intermolecular transfer of energy from a higher energy excited state to one lower in energy. The process arises from direct dipole-dipole coupling between the two chromophores, as opposed to emission and subsequent absorption. Because dipole-dipole coupling drops off with the sixth power of the intermolecular spacing, the two molecules must be within a few nm of each other. The efficiency of FRET can be used as an “ruler” to determine intermolecular distances over a few nm.

2. (10) Use Snell’s Law below to describe the conditions necessary for total internal reflection.

\[ n_1 \sin \theta_1 = n_2 \sin \theta_2 \]

\[ \sin \theta_c = \frac{n_2}{n_1} \]  For light entering an interface from medium 1, total internal reflection arises when the angle of incidence \( \theta_1 \) exceeds the critical angle defined by the preceding equation. The equation was generated by setting the angle of refraction \( \theta_2 \) equal to 90° in Snell’s Law. Only incident angles lower than the critical angle produce real-valued refraction angles, with higher incident angles resulting in total internal reflection.

3. (5) Explain the primary advantage of using total internal reflection excitation of the fluorescence.

By using total internal reflection, excitation is limited to molecules within the evanescent field. In total internal reflection, the incident field decays exponentially into the refractive medium, such that excitation arises only within the region close to the interface. The primary advantage in the present study is the suppression of fluorescence background from the bulk solution. Weak absorption and fluorescence by the acceptor can easily overwhelm the surface response when integrating over a large bulk volume.

4. (15) In the work, the authors placed the FRET acceptor in the mobile phase and covalently attached the FRET donor to the substrate. In this configuration, the data had to be corrected for photobleaching.

-Explain why photobleaching would not be as problematic if the acceptor was surface immobilized instead.

-Why, then, did the authors choose to immobilize the donor instead?
If the acceptor were surface immobilized, it would only be weakly excited directly by the incident light, with reduced photobleaching. Furthermore, the mobile phase replenishes the fluorophore, such that photobleaching would likely not be a problem for it either. However, the authors likely chose to live with photobleaching in order to provide background suppression. By immobilizing the donor, the large majority of the signal arises from the relatively few molecules at the interface. It is easier to see a small change on a small background than it is to see a small change on a big background.

5. (10) The authors assume that the cross-sectional flow profile across their flow cell is parabolic in shape. What physical interactions are responsible for curvature in the flow profile? In the absence of slip, the molecules covalently attached to the wall are in intimate contact with those from the solvent immediately adjacent to the walls. Therefore, the flow velocity approaches zero at the walls and changes smoothly to reach a maximum in the center.

6. (15) The effective longitudinal diffusion coefficient $D^*$ is given in Eq. 2. What is the principal difference between $D$ and $D^*$? Draw diagrams that explain the process through which a parabolic flow profile coupled with conventional (Fickian) diffusion along $Z$ results in an increase in the value of $D^*$ relative to $D$. Explain qualitatively why increasing $U$ and $H$ both result in increases in effective longitudinal diffusion, while increasing $D$ results in its decrease? $D$ is the underlying 3D diffusion constant. $D^*$ is the effective diffusion constant along the direction of flow, arising from the combination of underlying diffusion and the additional spreading arising from the vertical diffusion through a parabolic flow profile. Increasing the velocity of flow increases the steepness of the parabola and magnifies the spreading.

7. (15) Explain in detail and in your own words how the results shown in Figure 4 relate to those shown in Figure 5. The shoulder in the fluorescence emission signal in Figure 4 corresponds to acceptor emission in FRET. The data in Figure 5 correspond to the loss in surface-bound donor fluorescence in the blue region of the fluorescence in Figure 4 due to quenching from FRET.
8. (15) Explain, in your own words, what the finite difference method is and how it was used to solve for the effective thickness of the polymer layer unaffected by flow, shown in Figure 7b. What is the significance of Figure 7b?

The finite difference methods is a numerical approach for solving differential equations by starting with initial conditions and propagating the solution in small, finite steps. Because the modified diffusion equation coupling the Taylor-Aris model with Fickian diffusion within the interfacial layer had no closed-form analytical solution, the authors resorted to numerical methods.

Figure 7b is arguably the key figure in the paper. It demonstrates that a correction is needed to account for flow arising within the polymeric film adhered to the interface, with the correction increasing with increasing flow velocity. Under high flow conditions, nearly half the thickness of the brush layer exhibits internal flow, while previous models generally do not consider the possibility of flow within the interfacial regions.

9. (5) Describe in your own words the potential impact of this work on separation science. Difficult to say the long-term impact of fundamental studies, but the findings may lead to better modeling of transport at the polymer/mobile phase interface. The modeling in turn may lead to better understanding and prediction of band broadening in liquid chromatography. If one can accurately model something, one can also accurately optimize.
Biochemistry Cumulative Exam
27 August 2016

Enzymes: Function and Mechanism
12 Questions, 100 Points Total

General instructions: Part I is based on general knowledge. Part II is based on Gersch et al. (2012) J. Biol. Chem. 287:9484, which was supplied to you before this exam and is attached to this booklet. Explanations should typically take only one paragraph of 3-6 sentences or less. Equations and diagrams are encouraged. Your answer must be logical and self-consistent to garner points. Word vomit is not encouraged. Write all answers in the blue exam book.

Part I. General Enzymology (20 points)

1. An enzyme elicits a rate enhancement by lowering the activation energy of the forward reaction.
   1A. How does the enzyme affect the reverse reaction rate? Accelerate
   1B. What happens to Gibbs free energy \( \Delta G \) for the reaction? No Change
   1C. What happens to the equilibrium constant \( K \) for the reaction? No Change

2. Give the name and chemical function of each of the following coenzymes:

   2A.
   ![2A](image)
   A. CoASH – Activation of acyl groups for nucleophilic attack; activation of acyl group \( \alpha \)-carbon for proton abstraction

   2B.
   ![2B](image)
   B. ATP – provide driving force for coupled reactions; activate leaving groups; regulatory posttranslational modifications, etc.

   2C.
   ![2C](image)
   C. NADH – Generally involved in catabolism in the oxidized NAD+ form as an acceptor of hydride equivalents, two-electron transfer

   2D.
   ![2D](image)
   D. SAM – methylation

3. The N-end rule relates the cellular half-life of a protein to its N-terminal amino acid. In mammals, N-end rule pathways are part of the ubiquitin-proteasome system. In bacteria, AAA+ proteases are involved. For example, ClpS is an adaptor protein for recognition and delivery of substrates to the ATP-dependent AAA+ protease ClpAP, which itself consists of the ClpA chaperone (same family as ClpX and ClpC) and the ClpP protease. N-end rule pathways are critical for regulated proteolysis involved in normal physiology as well as control of virulence in S. aureus, as discussed in Gersch et al.

3A) In the context given above and given what you have learned about SaClpP, explain whether or not the link between oligomerization and activity studied by Gersh et al. is physiologically important.

Yes, it is important. Note the preface of the question that "N-end rule pathways are critical for regulated proteolysis." ClpP is a non-specific protease. Without regulation it would non-discriminantly proteolyze substrates, especially as monomers containing an easily accessible active site. Oligomerization ensures that active sites are competent for chemistry only when the ClpP tetradecamer is properly formed, creating in catalytic chamber with regulated access that prevents wanton proteolysis.

3B) Explain the physiological importance of ClpS and ClpA.

Again, this is in the context of regulated proteolysis. If ClpS is an adaptor, it implies that ClpS contributes to regulation by determining substrate specificity and access to the ClpP proteolytic chamber. As mentioned in the introduction of Gersch et al., ClpA is an unfoldase that assists in unfolding and delivering protein substrates to ClpP. This is particularly important since through the paper we learn that some ClpP's like SaClpP are not efficient at degradation of proteins much larger than peptide fragments.

4. Gersch et al. observed that in the compact SaClpP structure the handle domain exhibited high crystallographic B-factors. They interpreted this as a conformation with high structural flexibility. In terms of atomic position, explain what is a crystallographic B-factor and why it could indicate structural flexibility.

A crystallographic B-factor is related to the mean square displacement of an atom in a protein crystal. Therefore, if the B-factor is large, then for individual proteins within the crystal population, the atom of interest samples a large range of positions. This large range of positions could indicate either static or dynamic disorder in the proteins within the crystal, suggesting flexibility. In contrast, low disorder and a rigid conformation would lead to only small displacements from the population mean for the atom interest across individual proteins.

5. Gersch et al. observed that sulfate increases the activity of SaClpP. Suppose that the presence of the sulfate anion only increases the catalytic turnover efficiency. Explain how $K_M$ and $k_{cat}$ parameters would differ in KCl versus Na$_2$SO$_4$. 
Efficiency = $k_{\text{cat}} / K_M$. In the presence of Na$_2$SO$_4$, $k_{\text{cat}} / K_M$ is larger relative to efficiency in the presence of KCl. There are therefore three possibilities: (1) $k_{\text{cat}}$ increases, (2) $K_M$ decreases, or (3) $k_{\text{cat}}$ increases and $K_M$ decreases. If you think about this as the opposite of non-competitive inhibition by KCl, then you expect that primarily $k_{\text{cat}}$ increases. Formally, if you are dealing with simple Michaelis-Menten kinetics, then we know $k_{\text{cat}} = k_2$ and $K_M = (k_1 + k_2)/k_1$. If the chemistry step is rate limiting and $k_2 << k_1$, then $k_{\text{cat}} = k_2$ will increase while $K_M$ approximately stays the same. However, if $k_2 = k_{\text{cat}}$ increases enough that the chemistry step is no longer rate limiting, than both $k_{\text{cat}}$ and $K_M$ will increase.

6. Gersch et al. used size-exclusion chromatography (SEC) to determine the molecular mass of SaClpP oligomers. Below is a hypothetical calibration curve for the protein molecular weight standards they used.

6A) On a single SEC plot, draw the chromatographic traces for wildtype (WT) SaClpP in the presence of KCl (solid line) versus Na$_2$SO$_4$ (dotted line). Assuming you collect peak fractions, explain which peaks contain active or inactive enzyme.
6B) On a single SEC plot, draw the chromatographic traces for the D170A SaClpP mutant in the presence of KCl (solid line) versus Na₂SO₄ (dotted line).

7. Gersch et al. used thermal shift assays to compare relative protein stabilities. Thermal shift assays were performed using SYPRO Orange.

7A) Explain how SYPRO Orange functions in this assay.

SYPRO Orange is a fluorescent dye that reports protein unfolding. As temperature increases, proteins unfold exposing hydrophobic patches that bind SYPRO Orange. When SYPRO Orange binds, its fluorescence increases, indicating unfolding/denaturation.

7B) On a single plot, draw protein thermal melt curves for WT (solid line), R171A (dotted line), and E137A (dashed) SaClpP. Draw a legend for the traces for clarity.

7C) Suppose you have a high-affinity, non-covalent inhibitor. On the same plot above, draw a thermal melt curve for the WT SaClpP-inhibitor complex and explain your choice.

A high-affinity inhibitor will form a thermodynamically favored complex, contributing to stabilization in part from binding energy and, thus, melting temperature increases.
8. Gersch et al. used the PDBePIA server to computationally estimate the free energies of dissociation for the SaClpP oligomers. Explain why $\Delta G_{\text{diss}}$ is non-negative.

Just like in Question 7c above: Binding involves stabilization of a complex in part from binding energy. For a protein complex like SaClpP, if a stable complex forms, it implies that it is thermodynamically favorable (at least locally). Therefore, the equilibrium constant $K_{\text{assoc}} = [\text{complex}] / [\text{monomer}] > 1$ and $\Delta G_{\text{assoc}} < 0$. Thus, conversely, $K_{\text{dissoc}} < 1$ and $\Delta G_{\text{dissoc}} \geq 0$, that is, dissociation requires energy input.

9. Gersch et al. observed that the pH-rate profile of SaClpP has a peak at pH 7.0 with an apparent pK$_a$ of 6.5 on the rising (left) phase of the peak profile. Suppose this pK$_a$ is associated with residue S98. How does the pK$_a$ of the nucleophile here in the protein context compare to the expected pK$_a$ of the nucleophile free in solution? If they differ, explain why.

The pK$_a$ of the hydroxyl of the serine side chain would be about $\sim 13$ free in solution. In classic serine proteases like ClpP, the pK$_a$ of the serine nucleophile is lowered through the hydrogen bonding network of the catalytic triad. Specifically, in SaClpP the pK$_a$ of the nucleophilic S98 hydroxyl is perturbed through H-bonding with the imidazole of the general base H123.

10. The D3 alkyne-containing β-lactone is an example of activity-based protein profiling (ABPP) reagent. Draw an electron/arrow pushing mechanism that explains how the D3 lactone inhibitor works on SaClpP.
11. Draw a general 1-step reaction for the copper-mediated click reaction - no arrow pushing, just structures. Write the fluorophore name used for detection in lieu of its chemical structure.

The copper-mediated click reaction is an azide-alkyne cycloaddition that results in the 1,4-isomer.

12. Gersch et al. observed that D170A and R171A SaClpP mutants showed no reactivity to the D3 β-lactone reagent. They interpreted this as “a malfunction of the active site which impairs the nucleophilic attack.” Essentially, in the context of their structural information they suggest the D170A and R171A mutations affect the chemistry step in the proteolytic process. However, if this observation were made in the absence of any structural data, at least one other major interpretation could be made. What reasonable alternative interpretation is there? Explain how would you test it.

Many answers. For example, an alternative interpretation is that the D170A and R171A mutations interfere with substrate binding. Loss of binding could be due to local perturbation of the SaClpP binding site or a global change or loss of tertiary/quaternary structure, for example - if the active site catalytic triad were intact but a conformational change occluded substrate access. If substrate cannot bind, product cannot be formed. To assay specifically for loss of binding, we could use a labeled substrate peptide. For example a radioisotope tagged peptide. You could also label a substrate with a fluorophore for a fluorescence polarization assay.
Inorganic I

A.

calc: S = 0 and all B-B bonds equidistant
D$_{3h}$ symmetry

B. $6(\sigma) + 2(\text{lone pair}) + 2(\pi) = 10\ e^-$

$X_3$ neutral: $9\ e^- \Rightarrow 3\ \text{for } X = B$

C. $2\ \pi + 1 \text{ lower energy } \sigma$ bonding orbital

$\sigma$ bonding orbital

version
D. N-Heterocyclic Carbene

Correct name: 1,3-bis(2,6-diisopropylphen-1-yl)imidazole-2-ylidene

E. $sp^3$ hybrids $5s + 5p_x \alpha = x, y, z$

\[ 107^\circ \]

\[ \Theta \]

4 pairs $\equiv T_d$

F. 6 e- pairs @ S

$sp^3$ pyramidal

axial or equatorial L

most likely equatorial