** This examination is open book, but is to be worked on **independently**. You may not discuss or otherwise communicate **any** aspect of the exam with **anyone** other than C. Martin. This includes any discussions with anyone after you are done with the exam, but before the exam’s due date and time. This is **very important**.

Due in class, 9:30am, Tuesday, February 25

Show your work for full credit. Be concise, but complete. 
Avoid long rambling answers which indicate that you don’t really understand the question.

1. a) (5 points) Explain the following statement, recently found in an abstract of a manuscript on fluorescence of retinal:

   “The radiative $S_1$ state lifetimes are found to be in the 100-200 ns range, indicating that the $S_1$ to $S_0$ transition is strongly forbidden”

   Typical fluorescence lifetimes are in the 1-10 nsec range. For a lifetime this long, all the rates of the pathways out of the excited state must be very slow. That is, $k_f$ must be very small. “Strongly forbidden” is another way of saying that the transition is not very probable, that it’s rate is very small.

b) (5 points) Speaking very generally, explain what might be happening in the following:

   “With decreasing polarity the rate of the $S_1$ state decay decreases”

   Solvent polarity can induce red or blue shifts, but this is **completely separate** from solvent effects on excited state lifetimes. Do not confuse the effects. This was a common mistake on the exam, which makes me sad 😞: I tried very hard to emphasize this distinction.

   This was taken directly from a paper. “Decreasing polarity” usually refers to a study in which the polarity of the solvent/medium is varied, as we discussed in class. This statement suggests that nonradiative pathways out of the excited state ($k_{ic}$) must involve interactions with polar solvent molecules. Decreasing the polarity, decreases these interactions and therefore slows the rate of decay.

2. As you saw in the practice material, the chromophore involved in both bacteriorhodopsin and in the visual systems of higher organisms is retinal. Consider the lower molecule at right, “all trans retinal, not quite.”

   a) (25 points) Treating this as a particle in a box, calculate the size of the box, given that the lowest energy electronic transition is about 400 nm.

   Many of you tried to make this much harder than it was. It was supposed to be one of the easier questions on the exam. Treat it as a simple particle in a box problem. Done. You all know how to do this one! Remember 12 electrons!

   The six double bonds contribute a total of 12 electrons to the $\pi$ system. Filling them into the energy levels, yields a HOMO of n=6 and a LUMO of n=7. So the lowest energy transition will be from n=6 to n=7. This allows us to calculate d:
\[ E = \frac{hc}{8m} \left( \frac{n_x^2}{a^2} + \frac{n_y^2}{b^2} \right) \]

\[ d = 12.5 \text{ Å} \]

b) (15 points) Your colleague criticizes your answer (after the deadline for the exam, of course, since she shouldn’t be talking with you about the exam until after the exams are handed in next Tuesday). She notes that this should more correctly be viewed as a two dimensional particle in the box, with a second dimension of 1-2 Å. Explain (with arguments based on relevant equations) why this would not change your answer. The energy of the levels for an arbitrary two dimensional “box” are given by:

\[ E = \frac{hc}{8m} \left( \frac{n_x^2}{a^2} + \frac{n_y^2}{b^2} \right) \]

where \( n_x = 1, 2, 3, \ldots \) and \( n_y = 1, 2, 3, \ldots \)

Very many of you tried to do exactly what was done in class. This part of the problem was meant to get you to extend what you’ve learned. The diagram presented in class does not work here because \( a \neq b \). So you need to use the equation to figure out the energy levels for this question. It’s very straightforward if you simply use the equation.

This gets trickier than in the practice exam, in that \( a \neq b \). So let’s assume that the other dimension (b) is 2.0 Å (you could have used 1.0 Å, it would work even better).

Note that

\[ E = \frac{hc}{8m} \left( \frac{n_x^2}{a^2} + \frac{n_y^2}{2.0} \right) \]

where \( n_x = 1, 2, 3, \ldots \) and \( n_y = 1, 2, 3, \ldots \)

So increasing \( n_y \) increases the energy level much less than does increasing \( n_x \). This is illustrated at right. So, in fact, the lowest energy transition is \((n_x=6, n_y=1)\) to \((n_x=7, n_y=1)\), similar to the one dimensional case.

\[ E = \frac{hc}{8m} \left( \frac{n_x^2}{a^2} + \frac{n_y^2}{b^2} \right) \]

This is exactly what we saw for the one dimensional case. The (long) size of the box will be the same.
3. The trp repressor exists as a dimer of identical monomers, and each monomer contains two Trp (W) residues. The following describes the environment around each Trp, as determined by x-ray crystallography:

- **W99**
  - Less buried
  - Nearby charged residues
  - Indole ring sandwiched between Glu & Gln
  - Near Gln 32 from opposite subunit

- **W19**
  - More buried
  - Nearby nonpolar residues
  - Indole ring sandwiched between Phe & His

Additionally, W99 is on helix F, connected to the D and E helices, which form the helix-turn-helix DNA binding motif of this protein. In the absence of DNA (as in these experiments), the D and E helices are only partially ordered.

The following two mutants of this protein have been prepared (W19F refers to a WÆF mutation at position 19, think carefully about what that means for the Trp fluorescence you will observe in that mutant). The properties of these mutants are compared below:

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$\ell$ (ns)</th>
<th>$k_{\text{radiative}}$ (ns$^{-1}$)</th>
<th>$k_{\text{nonradiative}}$ (ns$^{-1}$)</th>
<th>Obsrv</th>
</tr>
</thead>
<tbody>
<tr>
<td>W19F</td>
<td>333</td>
<td>0.069</td>
<td>1.1</td>
<td>0.063</td>
<td>0.85</td>
</tr>
<tr>
<td>W99F</td>
<td>319</td>
<td>0.168</td>
<td>4.1</td>
<td>0.041</td>
<td>0.20</td>
</tr>
</tbody>
</table>

a) (10 points) Discuss which features agree with the general trends we covered in class and which do not. What are the values of $k_{\text{nonradiative}}$ for the two mutants? Another bit of general information is that more electronegative groups are often better excited state quenchers than less electronegative groups.

Here I just wanted you to think about environment and the various processes.

Environment can effect both the energies of the orbitals (and so the wavelength) and the lifetimes of the excited state. But some of you thought that changing the energies of energy levels would necessarily alter excited state lifetimes. The two concepts are completely distinct – this is important!

Taken from *Biophys. J.* 63, 741-750 (1992). This is the Trp repressor.

Mutant W19F contains fluorescence solely from Trp99 and W99F contains only that from Trp19. This simple concept is essential to your answer.

W99 is less buried and in a more polar environment and so “should” be more red shifted than the buried, nonpolar surrounded W19. This is seen. The fluorescence quantum yield from W99 (W19F) is lower than that from W19 (W99F), and the nonradiative decay rate is much higher for W99. One might have thought that the electronegative His near W19 would have quenched better than the groups near W99.

b) (5 points) The fluorescence lifetime from mutant W19F shows a distinct biphasic decay.

Propose an explanation. How might you test your proposal?

Proposed expt’s should test your hypothesis for why, not simply verify that it's biphasic.

Helix F is connected to helices E and D, which are partially ordered. The folding or unfolding of these helices could create two subpopulations of molecules, with different subtle environments around W99. It might only involve the rotation of an efficient quencher such as Gln 32.

To test, one might look at the fluorescence lifetime in the presence of the ligand Trp (or possibly the target DNA sequence). These interactions could tighten up the E and D helices, removing the disordered subpopulation.
c) (10 points) At low concentrations, the fluorescence quantum yield from mutant W19F increases. Explain why this might be so. Propose a spectroscopic measurement to test your hypothesis. Propose a new protein mutation for another test. Explain.

This result is made up - it’s not in the paper. However, it is proposed that Gln 32 is involved in the quenching of W99. At low concentrations of protein, a significant population of monomers would form, such that Gln 32 would no longer be nearby to provide quenching of the excited state. This would increase the quantum yield from W99 at low protein concentrations.

To test this proposal, we might introduce a mutation of Gln to Ala or to Glu. Remove the electronegative functionality on Gln. This should result in an increase in the quantum yield from W99. Alternatively, we could simply look at the time course of excited state decay as a function of protein concentration. As monomers form, we will see a sub population with a longer excited state lifetime - in other words, we will see biphasic decay kinetics.

4. Human fibrinogen is a very large and unusually elongated molecule that forms long fibers. In a study of fibrinogen, the linear dichroism of a triplet (excited) state was used to measure the rotational correlation time of the protein. In this experiment, a short (15 ns) flash (frequency $u_1$) of linearly polarized light is used to generate an excited triplet state. The resulting linear dichroism was then measured with a second (probe) beam (frequency $u_2$).

This problem is derived from Biochemistry 31, 7580-6 (1992).

a) (10 points) There is a perfectly good fluorescence signal associated with the $S_0 \rightarrow S_1$ transition, why didn't the authors use the fluorescence anisotropy of this signal?

Note that the protein forms long fibers. This means that at least in one direction, the rotational correlation time will be very long (slow rotation). If the fluorescence lifetime is short (typically nsec to µsec range), then the singlet state will have decayed long before any appreciable rotation has occurred. The molecule appears “static” to a fluorescence measurement.

b) (5 points) Can you guess why they might not have used phosphorescence?

In other words, triplet state lifetimes are typically much longer than singlet lifetimes. So we may be able to measure the slow rotation of the fibers if we can measure something from the triplet state. Phosphorescence would be one approach, but the authors didn't use this - perhaps the phosphorescence quantum yield is too small (remember that the phosphorescence transition is “forbidden”). Also, the phosphorescence life time might be too long (slow rotation could appear "fast" relative to a very long phosphorescence life time). If there is triplet state at an appropriate energy distance above the $T_0$ state, then we could measure an absorption transition (in other words, linear dichroism).

c) (10 points) Why excite with a brief, polarized laser flash?

The key here: Polarized excitation yields "selective excitation" which provides the orientation required for linear dichroism.

Remember that we have a randomly oriented solution, but to do linear dichroism we need some way of orienting or preferentially exciting a subset of the chromophores. Although the protein fibers in solution are randomly oriented, the initial laser flash excites a subset of the fibers which are all aligned with their symmetry axis in one direction (in an analogous way to 3-dimensional rotation for standard fluorescence anisotropy). We have created an
ordered system in one dimension, which will of course, then show a linear dichroism as usual. As the molecules rotate, this linear dichroism will decay, with a lifetime reflecting the rotational rate along the axis of symmetry. Decay of the linear dichroism measures this effect.

This example combines the concepts of linear dichroism (an absorbive process which requires only partial order) with preferential excitation to achieve that order. Many of the principles we have learned are at work here.