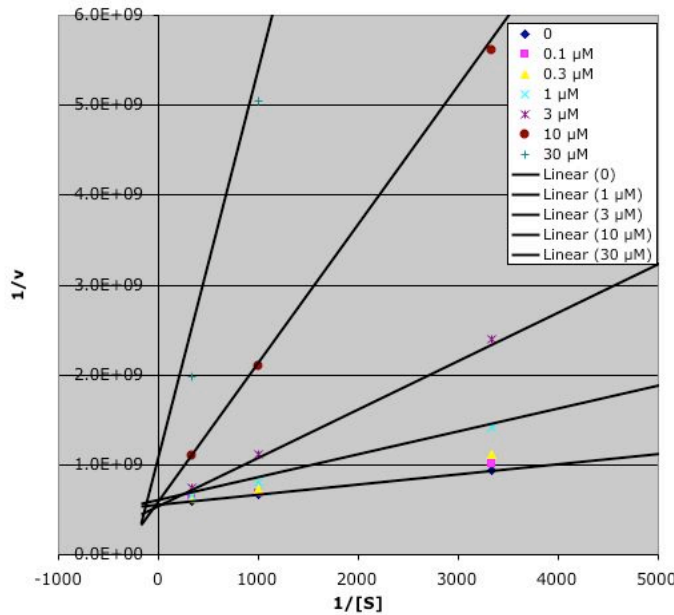


Problem 1a:

V_{\max}	$1.8 \times 10^{-9} \text{ M s}^{-1}$
K_m	0.2 mM
$[E]_{\text{total}}$	2 nM
k_{cat}	0.9 s^{-1}
Specificity constant	$4.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$

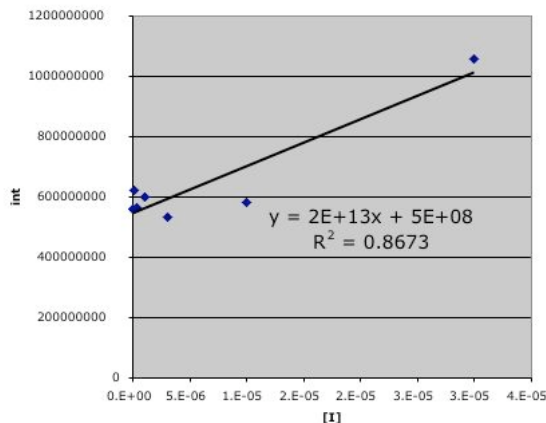
Obviously the k_{cat}/K_m indicates that this lysozyme is far from catalytic perfection (at least for *this* substrate), in which case it would be close to $10^8 \text{ M}^{-1} \text{ s}^{-1}$.

1b: If you plot $1/V$ vs. $1/[S]$ at each inhibitor concentration, you will get lines converging on the same y-intercept:



Thus, LSCI is a **competitive** inhibitor. Note that the highest inhibitor concentration gives a slightly different intercept, but this is mainly due to the point from the lowest substrate concentration – the lowest rate by far, where small errors will become significant. This illustrates a key point in double reciprocal plots: the outermost points can really skew the line, but since these came from the lowest substrate concentrations and rates, they have the largest errors (as a %), so be wary of it. Some people thought that, since the intercepts were somewhat different, it had to be a mixed inhibitor, but further analysis should prove this wrong – there is *no trend* like you'd expect from a mixed inhibitor. The intercept should continuously increase as $[I]$ increases, but it does not:

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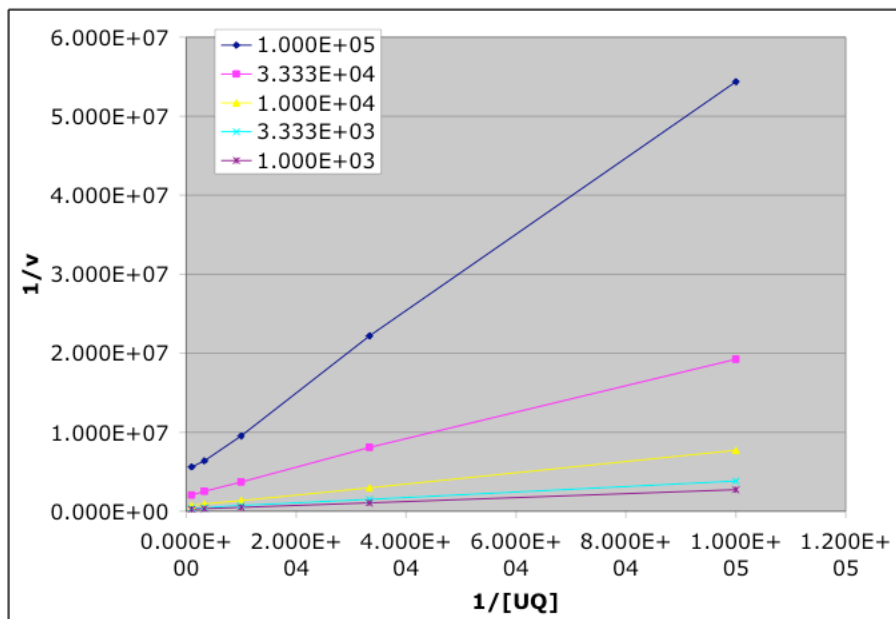
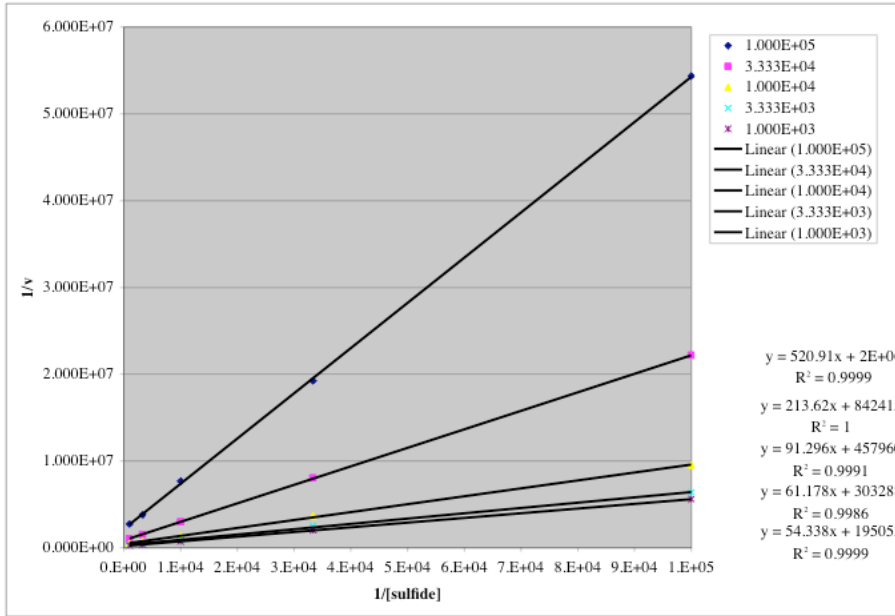


Because of this, if you try to calculate a K_I' , you get a value of about $35 \mu\text{M}$, which is out of the limits you tested. You should always worry when the numbers you pull out of an analysis are beyond the range of concentrations you tested, (i.e. Just plugging data into formulae and cranking out numbers is not enough – you have to *think* about the results and if they make sense.)

$K_I = 0.8 \mu\text{M}$

Problem 2:

You can pick one of the substrates as S1 and the other as S2. Keeping S2 constant, you plot $1/V$ vs. $1/[S1]$. Here I have done this for both sulfide and UQ:



You see very quickly that the lines are not parallel, ruling out a Ping-Pong mechanism. The only question is whether the ternary complex is formed by an ordered or random pathway. If its random, then it does not matter which one is S1 or S2; it is ordered, then it will make a big

difference. As you can tell above, it does not, but I will go ahead and analyze both situations in detail.

Situation 1: You pick sulfide as S1 and UQ as S2.

Get the slopes and intercepts of $1/V$ vs. $1/[S^2]$, and then plot the slopes and intercepts vs. $1/[UQ]$. You will find that the intercept of the intercepts gives you $1/V_{\max}$ ($4.6 \mu\text{M s}^{-1}$), from which you can get k_{cat} ($\sim 4600 \text{ s}^{-1}$). The slope of the intercepts vs. $1/[UQ]$ gives you K_{m2}/V_{\max} , and you can calculate that the K_m for $UQ = 90 \mu\text{M}$.

The key is the intercept of the slopes vs. $1/[UQ]$, which should give K_{m1}/V_{\max} . This is definitely non-zero, so that means that K_{m1} is also non-zero, which means that it is a random pathway. The K_m for $S^2 = 220 \mu\text{M}$. You also get the K_d for $S^2 = 245 \mu\text{M}$ from the slope of the slopes ($=K_{S1}K_{m2}/v_{\max}$). Note that, since $K_{S1}K_{m2} = K_{S2}K_{m1}$, you can also calculate that the K_d for UQ is $99 \mu\text{M}$.

Situation 2: You pick UQ as S1 and S^2 as S2.

Get the slopes and intercepts of $1/V$ vs. $1/[UQ]$, and then plot the slopes and intercepts vs. $1/[S^2]$. Obvious, you get a normal linear relationship in this case also. Using similar analysis as the above, you first get the $V_{\max} = 4.6 \mu\text{M/s}$ (i.e. same as before), and that the K_m for sulfide = $221 \mu\text{M}$ from the plot of the intercepts vs. $1/[S^2]$. Then, using the plot of slopes vs. $1/[S^2]$, you get the K_m for $UQ = 90 \mu\text{M}$. You also get the K_d for $UQ = 99 \mu\text{M}$, and from that you get K_d for $S^2 = 243 \mu\text{M}$.

As you can see, the answers are remarkably similar no matter which way you start.

Problem 3:

This problem was graded individually, since everyone had a unique protein. Two points each for enzyme name, EC #, and reaction (written out fully), and filling out PDB codes/species names. I took off 4 points if the species were not different and 8 points for choosing an enzyme we covered in class. (In other words, follow directions!). I gave 5 points for full and accurate descriptions of each protein, and 6 points for the comparison. In each case, I looked at your proteins and analyzed them myself. I figure that if I can find out more in 5-10 minutes than you could, you probably could have done better...

Problem 4:

- a) This should have been fairly obvious, but some people never seemed to get it. Here is the equivalence of the polypeptide chains to subunits:
- $A = \alpha 1$
 - $B = \beta 1$
 - $C = \alpha 2$
 - $D = \beta 1$

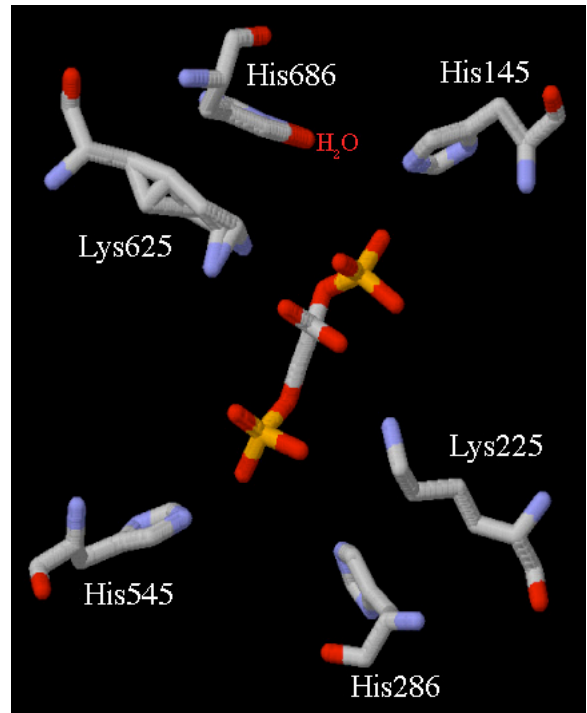
The point was to look for interactions *between* subunits (not within a subunit) that exist in the T state, but not the R state. As it turns out, if you had just tracked down the 3 ionic interactions mentioned in the book, you would have gotten most of the points. I also found a fourth one. I awarded 2.5 points for each ionic interaction (4 of them) and 1 point for each potential H-bond (which are weaker). Note that, because of symmetry, each interaction occurs twice; I awarded the points only once for each symmetry-related pair. I also took off 1 point for each bogus interaction: interactions that are not changed upon transitions from T to R state or interactions that did not really exist in the T state (examples: H-bonds where the distance was $> 4\text{\AA}$ or where the geometry would not allow such an interaction, or ionic interactions with *aliphatic* sidechains, etc. – hey, it took me *time* to track those down!)

There were almost no changes within the α_1/β_1 (α_2/β_2) heterodimers – the structures of each heterodimer were practically superimposable between R and T states. This is expected, since the change is mainly a movement of the 2 heterodimers in relation to each other. (However, one eagle-eyed student did spot a potential H-bond breakage between α -His103 and β -Asn108, which is not listed below.) Strangely, there did not seem to be any interactions between the β subunits affected by the change.

Residue (subunit, amino acid & number)	Residue (subunit, amino acid & number)	Type of interaction (<i>e.g.</i> H- bond, ion pair, <i>etc.</i>)	Distance between them (in terms of the most-important atoms ¹)
Interactions between α_1 and α_2 :			
α -Arg141	α -Asp126	Ionic (between sidechains)	$\sim 2.7\text{\AA}$
α -Arg141 (C-terminus)	α -Lys127	Ionic (between Lys sidechain & carboxy-terminus)	$\sim 2.7\text{\AA}$ (*Not in book)
α -Arg141 (C-terminus)	α -Val1 (N-terminus)	Ionic (between amino-terminus & carboxy-terminus)	$\sim 4.4\text{\AA}$
Interactions between $\alpha_1 - \beta_2$ (and $\alpha_2 - \beta_1$):			
α -Lys40	β -His146 (C-terminus)	Ionic (between Lys sidechain & carboxy-terminus)	$\sim 2.3\text{\AA}$
α -Tyr142	β -Asp99	Potential H-bond	2.5\AA
α -Asp94	β -Trp37	Potential H-bond	2.8\AA
α -Arg141	β -Val34	Potential H-bond (to carbonyl O of Val)	2.9\AA
α -Arg92	β -Arg40	Potential H-bond (to carbonyl O of Arg92)	$\sim 3.3\text{\AA}$ (<i>a bit long...</i>)

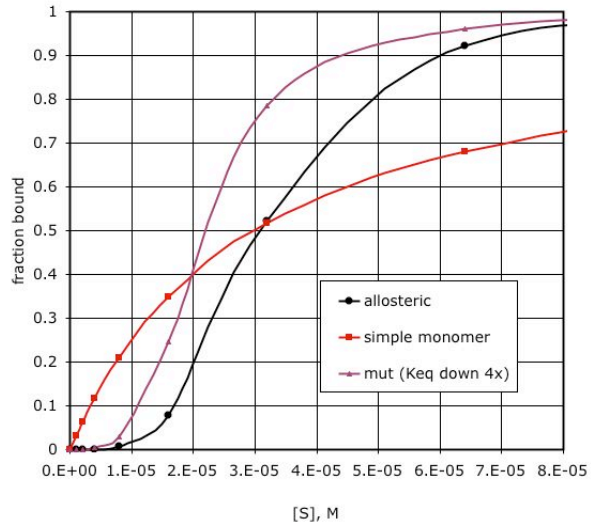
- b) Using RasMol, the closest residues to BPG are shown below. The complication is that the people who did this structure decided to change the numbering system (for unfathomable reasons – perhaps they wanted each residue to be unique?). If you examine the sequences (“show sequence”), you can see the new numbering system.

If you look at those in the R-state structure, it is obvious that they cannot accommodate BPG. (It is *possible*, however, that the His143s *might* still be able to bind the BPG.)



Subunit (α , β , etc)	Residue (amino acid type and residue number)	Type of interaction (charge-charge, H-bond, etc)
β_1	His145 (= His2)	Ionic &/or H-bond
β_1	Lys225 (= Lys82)	Ionic (most likely); might be H-bond
β_1	His286 (= His143)	Ionic (cannot be H-bond – too far and it is H-bonded to a water)
β_2	His545 (= His2)	Ionic &/or H-bond
β_2	Lys625 (= Lys82)	Ionic (most likely); might be H-bond
β_2	His686 (= His143)	Ionic (cannot be H-bond – too far and it is H-bonded to a water)

c) Note that Asn can also H-bond (the O can accept 2 H-bonds, and the N can donate two), so mutation of Asp to Asn is not necessarily going to break a H-bond. It will, however, destroy the ionic interaction between Asp126 of one α subunit and Arg141 of the other α subunit. This will have the effect of destabilizing the T state. This is *not* the same as destabilizing the R state, but it will shift the equilibrium between T and R to favor R more. This will have the effect of shifting the curve to the left – it will bind O_2 better at lower $[O_2]$. Here I have simulated the effect of increasing K_{eq}^{TR} 4-fold. Note that breaking this one interaction would *not* be expected to convert hemoglobin to a nonallosteric binding (*i.e.* hyperbolic curve).



d) The equilibrium constant of $T(O_2)_4 \rightarrow R(O_2)_4 = [R(O_2)_4]/[T(O_2)_4]$

What we need to do is relate $[T(O_2)_4]$ to $[T]$ and $[R]$ to $[T(O_2)_4]$ and then use the K_{eq}^{TR} to link them together: $[R(O_2)_4]/[T(O_2)_4] = [T]/[T(O_2)_4] * [R]/[T] * [R(O_2)_4]/[R]$

By definition:

$$K_D^T = [T][O_2]/[T \cdot O_2] = [T \cdot O_2][O_2]/[T \cdot (O_2)_2] = [T \cdot (O_2)_2][O_2]/[T \cdot (O_2)_3] = [T \cdot (O_2)_3][O_2]/[T \cdot (O_2)_4]$$

$$K_D^R = [R][O_2]/[R \cdot O_2] = [R \cdot O_2][O_2]/[R \cdot (O_2)_2] = [R \cdot (O_2)_2][O_2]/[R \cdot (O_2)_3] = [R \cdot (O_2)_3][O_2]/[R \cdot (O_2)_4]$$

Thus,

$$(K_D^T)^4 = [T][O_2]/[T \cdot O_2] * [T \cdot O_2][O_2]/[T \cdot (O_2)_2] * [T \cdot (O_2)_2][O_2]/[T \cdot (O_2)_3] * [T \cdot (O_2)_3][O_2]/[T \cdot (O_2)_4]$$

$$= ([T]/[T(O_2)_4])([O_2]^4)$$

By the same token, $(K_D^R)^4 = ([R]/[R(O_2)_4])([O_2]^4)$ and $(K_D^R)^4 = ([R(O_2)_4]/[R])([O_2]^{-4})$

Thus, we can equate $[T]/[T(O_2)_4] = (K_D^T)^4 [O_2]^{-4}$ and $[R(O_2)_4]/[R] = (K_D^R)^4 [O_2]^4$

So, $[R(O_2)_4]/[T(O_2)_4] = [T]/[T(O_2)_4] * [R]/[T] * [R(O_2)_4]/[R]$

$$= (K_D^T)^4 [O_2]^{-4} * K_{eq}^{TR} * (K_D^R)^4 [O_2]^4$$

$$= K_{eq}^{TR} * (K_D^T/K_D^R)^4$$

$$= (3 \times 10^4)(8 \times 10^{-4} \text{ M} / 2 \times 10^{-6} \text{ M})^4$$

$$= (3 \times 10^4)(400)^4 = 7.7 \times 10^6$$

Thus, as you should expect, ligand binding stabilizes the R form. That is, when hemoglobin is fully loaded with oxygen, the R form is heavily favored. (There is practically no T form.)

Problem 5:

Just based on first principles, you can imagine how the reaction must proceed:

- 1) The Ser –OH makes a nucleophilic attack upon the β -P_i of CDP-DAG. This would be sped up a great deal if a base abstracted the hydroxyl proton (think of the Ser protease mechanism).
- 2) After the attack, there is a transient intermediate with a pentavalent phosphorous. The β -P_i will now have a different geometry – triangular bipyramidal as opposed to tetrahedral. (The enzyme would speed up the overall reaction if it stabilized this intermediate.) Two of the oxygens will be anionic and the other 3 will be bridging to CMP, DAG, and (now) Ser.
- 3) This *ménage à trois* will be resolved by breakage of one of the linkages. CMP would be the best leaving group, in general, as an anionic phosphate moiety is much more stable than an alkoxide. Although not strictly necessary (in the case of phosphate), breakage would be accelerated by proton donation.

Given the clues, we can make some intelligent guesses as to the roles of different groups within the enzyme during the mechanism:

- 1) Asp31 and Lys 44 make up the Ser binding site. Their charged R groups make ionic interactions (and perhaps also H-bonds) with the amino and carboxylate groups of Ser, positioning it appropriately. Mutation results are consistent with their role of binding Ser without a major role in catalysis. His84 also accepts a H-bond from the Ser hydroxyl, which is important for catalysis (see next step).
- 2) His84 has got to be the proton abstractor. It is placed appropriately, and mutation of this residue has a huge effect upon k_{cat} . It also explains the loss of activity as pH drops. The pK_a of activity loss (5.5) is quite close to that of the His imidazole.
- 3) Note that this does not explain the importance of the Ser carboxylate. If it was just allowing it to bind properly, then modification (*e.g.* amidation, esterification) of it should be no different than modification of the amino group (*e.g.* acetylation). However, modification or loss (ethanolamine) of this group has an effect upon catalysis. If you notice, the carboxylate is also close to the imidazole (the other N). Thus, this could form a “catalytic triad” like that in the Ser protease mechanism. The Ser carboxylate accepts a H-bond from the His. It might be a strong H-bond or even abstract the proton. In any case, it will make the His a better base, so that it can abstract the proton from Ser. If you think about it, this is a cute way to make the enzyme very specific for Ser as opposed to ethanolamine, which is just a Ser without the carboxylate. The substrate activates its own deprotonation. (If you wanted to convert it to a PE synthase, you could just add an Asp or Glu in the active site to activate the His.)
- 4) Then the deprotonated Ser attacks the β -P and you get the pentavalent intermediate. This is what the VMI-1 inhibitor is mimicking. Now this is a very important point (which most people missed): much of the mechanism is involved with *stabilizing this intermediate*.

There are a lot of interactions with the nucleotide that you can pick out from the co-crystal structure with VMI-1:

- a. Trp77: π -stacks with the cytosine ring
- b. Ala98: carbonyl O accepts H-bond from $-\text{NH}_2$ of cytosine
- c. Thr99: donates H-bond to N3 of cytosine
- d. Asn107: donates H-bond to carbonyl O of cytosine
- e. Gln134: H-bonds to 2'- & 3'-OHs
- f. Mg^{2+} : stabilizes the extra negative charge and is coordinated by the anionic oxygens of the $\beta\text{-P}_i$.

But this is the important point: these interactions are in the intermediate state. They either don't exist or (more likely) are weaker before that is attained. In other words, they stabilize the intermediate. It appears that CDP-DAG cannot adopt the right geometry to optimize the interaction of the CDP part with the enzyme, probably because DAG is sterically blocked from entering the site. The geometry of the pentavalent $\beta\text{-P}_i$ allows the cytidine nucleoside to enter its binding niche.

- 5) Now the pentavalent intermediate is resolved. There are several ways this could happen, but the important thing is to direct breakage to $\beta\text{-P}_i\text{-}\alpha\text{-P}_i$ linkage and away from the $\beta\text{-P}_i\text{-DAG}$ linkage. It is clear that Glu172 and Arg185 play a key role in directing breakage to the right place, and it is not too difficult to see how they accomplish this. The negative charge on Glu172 would inhibit formation of an alkoxide anion nearby, while the positive charge on Arg185 would promote formation of an anion on the nearby $\alpha\text{-P}_i$.

Of course, if Glu172 were protonated, then it could serve as a proton donor to the phosphoester oxygen linking DAG to CDP. However, since the pK_a of Glu is below that of His, this could not happen in the pH regime under which the enzyme is active.

His84 (which is now protonated) could be a proton donor to the $\alpha\text{-P}_i$ oxygen either during the breakage event or right afterward. In the end, this residue must be protonated before the next cycle, anyway.