

Problem 1:

	Fetal form	Adult form
V_{\max}	$8.6 \times 10^{-7} \text{ M s}^{-1}$	$5.6 \times 10^{-7} \text{ M s}^{-1}$
K_m	0.31 mM	0.16 mM
k_{cat}	$5.1 \times 10^2 \text{ s}^{-1}$	$4.1 \times 10^2 \text{ s}^{-1}$
Specificity constant	$1.7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$	$2.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$

You need to assume that all of the enzyme is active, since k_{cat} reflects the number of substrate molecules processed *per active site* if [S] was infinite (i.e. saturating). Inactive enzyme is just dead weight and would throw off the calculation of $[E]_{\text{total}}$, and therefore the calculation of k_{cat} .

The main difference is that the fetal form has a slightly higher k_{cat} , meaning it can catalyze more reactions when fully saturated (which never happens). (Note that while k_{cat} is an inherent property of the enzyme, V_{\max} is **not**; any discussions of comparing V_{\max} 's are sheer nonsense.) The adult form has a lower K_m , meaning it can has somewhat more "affinity" for the substrate. This gives the adult form a slightly higher specificity constant. Perhaps the substrates are at lower concentration in adults than in the fetus. However, these differences are pretty small, and some might say they were trivial and uninteresting. Sometimes differential expression of different isozymes under different circumstances makes sense and can be rationalized. Sometimes not.

EDTA chelates metals, and phosphohexose isomerase requires Mg^{2+} for activity (see page 533 of your book). You need to stop the reaction while its still in the initial phase. It is important to be sure that the forward reaction you are measuring is always favorable during the time course. If product accumulates too much, you are no longer in the initial phase, and Michaelis-Menten kinetic analysis cannot be used. It should be obvious that the reactions having the lowest substrate concentrations will have the highest *extent* of conversion (not the highest *rate*). If you calculate it, you will find that the fetal and adult forms have converted 7.3% and 7.9% of the G-6-P, respectively, to F-6-P after 30 seconds. From this, you can calculate the free energy change right at the point of termination. When the reaction starts, $\Delta G'$ of the reaction will be negative and **very large** (because there is no product yet), and will increase continuously as F-6-P accumulates and G-6-P drops until it rises to 0 at equilibrium. Of course, you have to stop the reaction *long before* it gets to that point. To calculate $\Delta G'$ at the point of termination, use the familiar formula:

$$\Delta G' = \Delta G^{\circ'} + RT \ln([F-6-P]/[G-6-P])$$

$$\Delta G^{\circ'} = +1.7 \text{ kJ mol}^{-1} \text{ (see page 533)}$$

$$R = 8.315 \text{ J K}^{-1} \text{ mol}^{-1}$$

$T = 298 \text{ K}$ (this was not specified, but it will not make much difference what you pick, as long as it is somewhere in the physiological range of 280 – 310 K)

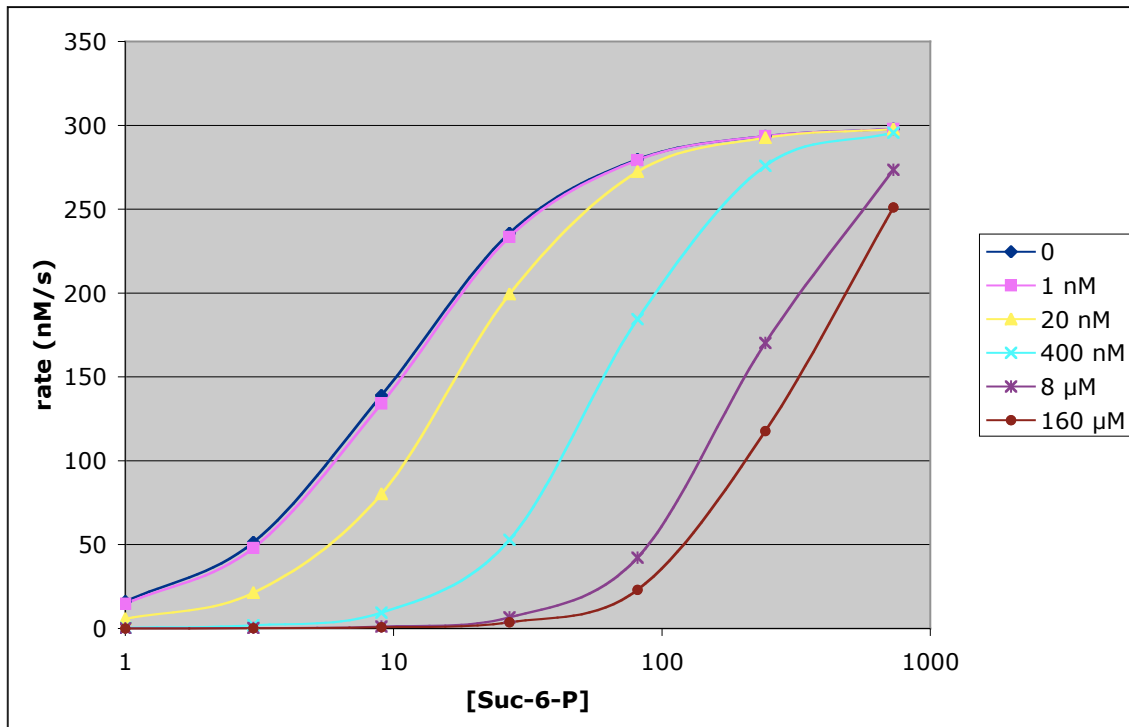
Thus, the $\Delta G'$ s will be -4.6 and -4.4 kJ mol^{-1} at the point of termination.

Problem 2:

If you plot $1/V$ vs. $1/[S]$, you will get lines converging on the same y-intercept. Thus, TR-54 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. If you re-calculate all of the intercepts without the $1 \mu\text{M}$ Suc-6-P points, then they are almost identical. 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 doesn't, just bounces around, and the highest $[I]$ gave the lowest intercept, proving that it cannot be mixed. Because of this, if you try to calculate a K_i' , you get a *negative* number, which should clue you in – this has no physical meaning. In the real universe, biochemical constants like K_d , K_m , K_i *must* be positive – how can you have a *negative concentration* of ligand, substrate, or inhibitor?? (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.)

$$\begin{aligned}V_{\max} &= 0.4 \mu\text{M/s} \\K_m &= 22 - 23 \mu\text{M} \\K_i &= 94 \text{ nM}\end{aligned}$$

In the second part, the retainin data should be plotted simply: V vs. $[S]$. If you go straight to the double reciprocal plot, you will miss something – the curves are *sigmoidal* rather than hyperbolic. Obviously, Michaelis-Menten kinetics do not apply here, and you cannot use a Lineweaver-Burke plot.



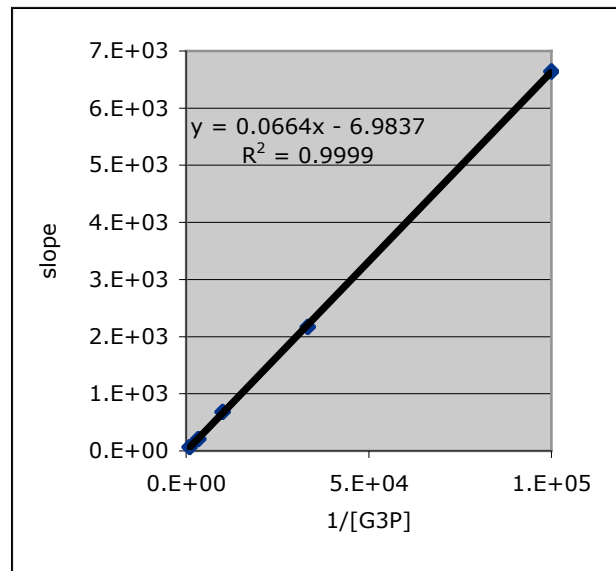
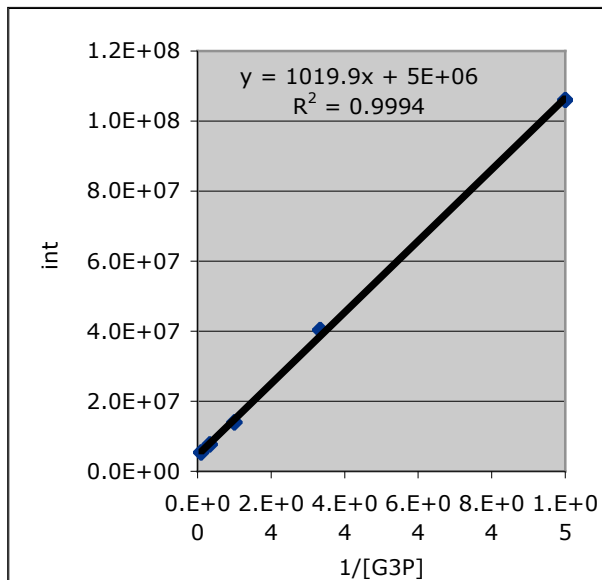
Note how higher concentrations of retainin shift the curve to the right, so that more and more substrate is required to get the half-maximal rate. You've seen this behavior before – an allosteric inhibitor such as BPG binding to hemoglobin shifts its O₂-binding curve in a similar way. But you need a multimeric enzyme for this type of behavior. The simplest explanation is that there are 2 possible conformations of the dimer, T and R, the low and high affinity forms, and it is an “all or nothing” change. Normally the equilibrium strongly favors the R (high affinity) form, so that you might even miss it in the absence of the effector. Retainin should bind to the T form, stabilizing it, and shifting the equilibrium. This has the effect of lowering the apparent affinity of the enzyme for substrate, as you observe in the curves.

Problem 3:

First of all, if you consider the forward reaction (FBP → G3P + DHAP), it should be pretty obvious that aldolase *cannot* work by a Ping-Pong mechanism. Thus, the reverse reaction (G3P + DHAP → FBP) has to be via a ternary complex. This is quickly confirmed once you do a double-reciprocal plot – the lines are obviously not parallel. The only question is whether the ternary complex is formed by an ordered or random pathway. And that will only be apparent after double-reciprocal analysis. In fact, the simplest way to proceed is to assume that it is random and then see what the numbers are. Then you have the dilemma of deciding which is S1 and which is S2. If it is random, it will not make any difference; but if it is ordered, it will.

Situation 1: You pick DHAP as S1 and G3P as S2.

Get the slopes and intercepts of 1/V vs. 1/[DHAP], and then plot the slopes and intercepts vs. 1/[G3P]. You will find that the intercept of the intercepts gives you 1/V_{max}, from which you can get k_{cat} (220 s⁻¹). The slope of the intercepts vs. 1/[G3P] gives you Km₂/V_{max}, and you can calculate that the Km for G3P = 220 μM.

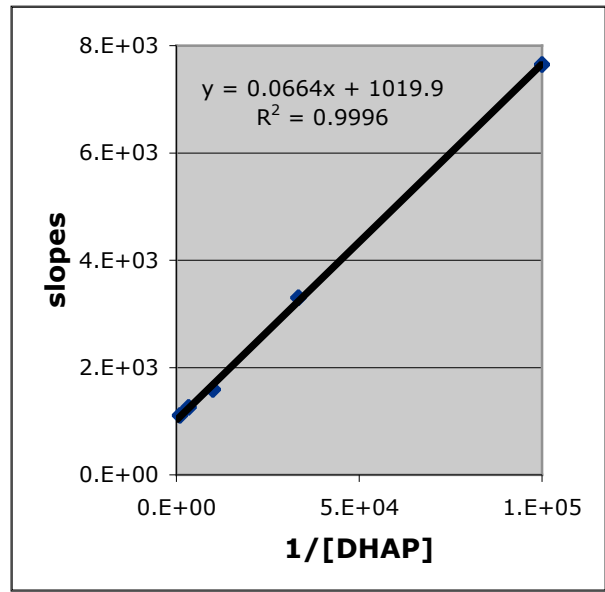
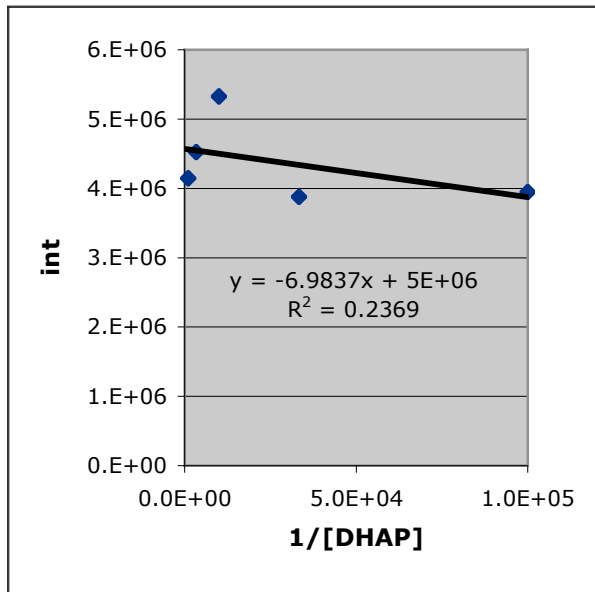


The key is the intercept of the slopes vs. 1/[G3P]. This is very close to zero; in fact the calculation gives -7 s, which would give a Km₁ of -1.5 μM/s. Obviously a negative number makes no sense, and therefore Km₁ is meaningless. (Some people thought it had

to be *exactly zero* to be considered ordered. When working with real data, the chance of getting exactly zero is infinitesimally small – you should expect to get a number very close to zero, either positive or negative. Considering that this line had y-values from 60 to 6000, getting an intercept of -7 isn't bad!) The slope of the slopes gives you K_s1K_m2/V_{max} , from which you can get the K_d for DHAP = $65 \mu M$.

Situation 2: You pick G3P as S1 and DHAP as S2.

Get the slopes and intercepts of $1/V$ vs. $1/[G3P]$, and then plot the slopes and intercepts vs. $1/[DHAP]$:



It should be obvious that the plot of intercepts is *not* a linear relationship – you even get a negative slope, which would give rise to a negative K_m2 (for DHAP), again demonstrating that DHAP cannot bind second. (Interestingly, the intercept gives you a V_{max} , which is about the same as before. This makes some sense if you think about it, since those were calculated using $1/[G3P]$.) The slopes plot will actually give you correct values for K_m1 (G3P) and K_s2 (DHAP), which also makes sense. It is K_m2 and K_s1 that have no meaning.

So, DHAP must bind first and G3P must bind second in the reverse reaction. That means that G3P must be released first and DHAP second in the forward reaction catalyzed by aldolase:
 $FBP \rightarrow G3P + DHAP$

Why would this be so? If you look at the structure, it becomes somewhat obvious. As you know, FBP has to bind in the open chain form. Many of the residues of the enzyme have to interact with the “upper part” of FBP (i.e. corresponding to DHAP). The B subunit of aldolase has DHAP practically buried in the enzyme, bound at the end of a short channel in the protein, with the phosphate moiety at the bottom. This likely explains why DHAP must exit last.

Problem 4:

This problem was graded individually, since everyone had a unique protein. Six points just for following directions and filling out the preliminary information.

Problem 5:

- a) Enolase is a homodimer. Each subunit has two domains. The N-terminal domain starts with a 3-strand anti-parallel β -sheet followed by 3 β -helices. Because of their distinctness, this could accurately be called an “ $\beta+\beta$ ” class domain. The fourth β -helix serves as a transition to the C-terminal domain. This is an α/β barrel (“ β/β ” class domain) with a parallel β -sheet in the center containing 8 strands. [Note that there is no β -helix between the first and second β -strands, just a loop, so the second β -strand is the only one that is not parallel with the rest, but it is followed by 2 β -helices (running in opposite directions) so that the next strand is anti-parallel to it (i.e. same as the other 6). This also gives the barrel equal numbers of β -strands and β -helices (8). If you had not looked carefully, you would think it was a normal α/β barrel made up completely of $\beta\alpha\beta\alpha$ motifs.]
- b) The majority of the dimerization interface is made up of the N-terminal 3-strand β -sheet (first ~37 residues) and the first and last β -helices of the α/β -barrel (residues ~178-202 and ~402-402). There is also some contribution from a short helix and loop right after the first one (up to ~ residue 213) and a loop preceding the penultimate helix of barrel (residues ~374-381). Much of interaction is between the N-terminal sheet of one subunit with the helices of the other subunit, although there is some interaction between the loops after the first barrel helix.
- c) Here are the residues I identified as being most important:

Residue (amino acid & number)	In secondary structure element? ¹	Likely role	Distance to nearest atom of metal or substrate ²
Lys345	No (loop)	Abstracts H ⁺ from C-2	3.5 Å from C-2 to N _β
Glu211	turn	Donates H ⁺ to C3-OH of 2-PGA Abstracts H ⁺ from water near PEP	2.4 Å from C3-OH to O _β 2.7 Å from H ₂ O(745), which is 3.1 Å from C-3
Glu168	β -sheet	Near C3-OH (might serve same role as Glu211?)	2.3 Å from O ₃ to O _β
Glu295	β -sheet	Binds Mg ²⁺	1.9 Å from Mg ²⁺ to O _β
Asp320	β -helix/turn	Binds Mg ²⁺	2.3 Å from Mg ²⁺ to O _β
Asp246	β -sheet	Binds Mg ²⁺	2.2 Å from Mg ²⁺ to O _β
Lys396	β -sheet	ionic interaction with carboxylate	2.9 Å from O ₂ to N _β

Asp321	turn	Interacts with water molecules complexed with Li ⁺ (represents 2 nd Mg ²⁺ ?)	2.5, 3 Å from carboxylate O's to the 2 H ₂ O's
The following are less obvious			
His373	No (loop)	Near C3-OH	3.1 Å from O ₃ to C _β
Arg374	No (loop)	Ionic interaction with phosphate	3.6 Å from P to N _{H2}
Ser375	turn	Near phosphate (2 potential H-bonds)	Sidechain -OH is 2.6 Å from P _i O Amide N is 2.9 Å from P _i O
Ala38	No (loop)	H-bond/dipolar interaction with phosphate	Amide N is 2.3 Å from P _i O
Ser39	No (loop)	H-bond/dipolar interaction with phosphate	Amide N is 2.8 Å from P _i O
His159	□-helix	Near phosphate	3.2 Å from O ₂ to N _β
Gln167	No (loop)	Near carboxylate (but cannot H-bond)	2.7 Å from O ₁ to N _β
Arg14	turn	Ionic interaction with phosphate	5.6 Å from Pi

¹If yes, mention what kind of secondary structure (□ helix, □ sheet, or turn).

²Indicate which atoms you used to measure the distance (of the amino acid and the substrate or metal).

Problem 6:

There are several clues that this enzyme must use a Ping-Pong mechanism, primarily the fact that there is only 1 CoA-binding site and only room for 1 acetyl-CoA to react at a time and the fact that an acetylated Cys (C89) is an integral part of the reaction cycle. Thus, you could divide the reaction into 2 stages:

- 1) $E + \text{CoA-S-acetyl} \rightarrow E\text{-C89-S-acetyl} + \text{CoA-SH}$ (acetylation reaction)
- 2) $E\text{-C89-S-acetyl} + \text{CoA-S-acetyl} \rightarrow E + \text{acetoacetyl-CoA}$

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

- 1) In order to make the Cys89 thiol a better nucleophile, some base will abstract its proton, allowing it to attack the carbonyl carbon the acetyl-CoA. There will be a transient tetrahedral oxyanionic state before the C-S bond (to CoA) is broken; this would likely be favored by proton donation to the S of CoA. Then CoA-SH will dissociate.

- 2) A new acetyl-CoA binds. It will have to bind somewhat differently than the first, due to the acetyl group. The second reaction is also different from the first, because now a base must abstract a proton from C-2 of the acetyl moiety (which would be more difficult than abstraction from a thiol). This is made possible by the thioester with CoA, which promotes enolization, and stabilizes the carbanion through resonance with the enolate form. Nucleophilic attack of the C-2 carbanion on the carbonyl carbon of the acetyl group attached to Cys will give another tetrahedral oxyanionic intermediate. This is resolved by breakage of the C-S bond (to Cys89), giving the product acetoacetyl-CoA. The breakage would be stimulated by proton donation to the S of Cys89.

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

- 1) Cys387 is likely the proton abstractor in the acetylation reaction. It is placed appropriately, and mutation to Ala lowers overall activity and acetylation activity by similar amounts. This argues that its primary role is in acetylation, although it may play a minor role in the second stage (perhaps the proton donor back to Cys89?)
- 2) His272 probably abstracts a proton from Cys387, allowing it to act as the base to take the proton from Cys89. Using the same argument as above, it is primarily involved in the acetylation reaction. Thus, Cys387 and His272 act as a “proton relay”. The distance may be long enough to allow His272 to serve as the proton donor to the S of CoA, allowing it to leave as CoA-SH. This group is probably the one with the lower pKa (~6), which inactivates acetylation when protonated.
- 3) The amide Ns of Gly90 and Gly92 probably serve as part of an “oxyanion hole”, in analogy with the serine proteases, to allow the nucleophilic attack on the carbonyl carbon of the acetyl group. This oxyanion hole would be used in each stage. Thus, one would expect to see that its disruption would affect both stages, but the overall reaction (in which it is used twice) would be much more strongly affected than acetylation (in which it is used only once). However, how do you mutate a backbone nitrogen? The only way to get affect it is to mutate to Pro – this removes the proton from the nitrogen (it is now attached to the side chain instead), this removing the ability to serve as a H-bond donor. However, such a change may not be tolerated by the protein; it may not fold properly. Evidently, this is what happened upon mutation of Gly90 to Pro. Fortunately, the Gly92 to Pro mutation allows you to analyze the result.
- 4) Lys113 is only involved in the second stage; it has no role in acetylation. It is obviously doing more than just providing a positive charge, since conversion to Arg destroys activity. However, conversion to His allows some activity. This is consistent with the idea that it may be acting as a base. Thus, it is likely that the role of Lys113 is to abstract the proton from C-2 of the second acetyl-CoA, like Lys345 does in enolase. This would be the group with a pKa near ~8. Obviously the environment of the Lys has lowered its pKa significantly.

- 5) The role of the Zn^{2+} is most likely as an electrophile to stabilize the enolate formed after abstraction of the proton from C-2. (The Cl in the inhibitor takes the place of the oxygen.)