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7.344 Directed Evolution: Engineering Biocatalysts □ □
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Session 13 Lecture Notes

1. PTE – we have seen this enzyme before – is phosphotriesterase. Its capabilities to hydrolyze organophosphates like sarin, VX and soman make it of particular interest. The enzyme uses a binuclear zinc metal center to activate a water molecule. This water molecule acts as a nucleophile toward the substrate and cleaves the e- group leaving a phosphate (Not harmful) behind. Why are these organophosphate materials not harmful to PTE? It is water that does the chemistry, not an enzyme side chain so the substrates do not act as suicide inhibitors. The zinc residues are held in place by two histidines each and two aspartates that bridge the zinc ions. See Figure 1 panel A.
2. The authors use structure-guided cassette mutagenesis to mutate the residues above the binding pocket (H254, H257) as they are known to contribute to substrate selectivity. They get a library of 400 variants and screen 1200 colonies using HTS for desired catalytic activity. The authors choose this method because much is already known about the enzyme mechanism. They then use their best guys and make a new site-saturation library at another position selected using structural data – iterative cassette mutagenesis.
3. Active mutants are screened for the ability to hydrolyze a substrate analog containing a chromophore. Is this a good measure of WT activity? Depends on where that bulky group lies...Should re-screen mutants with desired substrate to follow up. They don't actually do this, so maybe none of their designed mutants would actually work – big downer for this paper!
4. The results are that they identify a series of single double and triple mutants that have much better than wt activity. (see Figure 3) The triple mutant has activity that is 3 orders of magnitude better than wt. Also, they have made a protein that has a completely different active site – this is amazing! It is now a three metal center making the bound water molecule even more nucleophilic. Look at crystal structure.
5. Do we agree with the statement, “There is clearly a significant amount of synergism between the three sites that would have been missed had the mutagenesis proceeded sequentially,”?
6. NAL is a useful enzyme for synthesizing variants of sialic acid from an aldehyde and pyruvic acid. These compounds are potentially inhibitors of NA – show mechanism and why this is significant. It is really hard to make SA, so this offers an easier way.

7. The authors test activity using a coupled assay for product cleavage. They assay for the production of pyruvic acid by way of oxidation using NADH using lactate dehydrogenase. (Standard assay) Is this meaningful to assay for cleavage instead of product formation? They say yes based on the principle of microscopic reversibility. Let's look at this... Enzyme might not have the same affinity for the product as for the starting material, even in the reaction is reversible. This would slow the reaction rate, right? It would be really nice to see that they check up on their mutants with an assay for product formation, especially since they use a coupled assay, not SM detection for their initial screen. Since they are using a screen they are limited in their library size. At least
8. Library is generated by making site-saturation libraries of three different sites known by crystal structure analysis to be part of the second shell of residues. Residues involved in glycerol binding are known from previous structures. They do not, however, make any combinatorial libraries – this is too bad since they have a very nice screen and it would only be 8000 total variants for a combined library – or they could do it in parallel and then see what they get. They are missing mutations with synergistic effects.
9. The authors show that only one of their libraries has any mutants of significant activity. Take a look at figure 3. More importantly, they find a mutant E192N that has 50 fold-greater activity toward their substrate and increased specificity as compared to SA. Interestingly, the mutant prefers big, bulk groups over the smaller ones – this could be a problem in the forward reaction – there is a lot of energy release making the two smaller substrates.
10. Big problem, no assay making the desired substrate.
11. CASTing relies on the idea that synergistic mutations between two relevant sites in an enzyme are more beneficial than single site mutations – do the authors prove this with their own results? We'll see... They use a crude model to determine which residues nearby should be mutagenized and then design cassettes that contain all possible mutants organized into separate libraries for each pair. They do have a later paper that does shuffling between libraries. The advantage is that if you have an enzyme with no good selection, but you can run a screen and you have lots of structural data, this is easy and fast and potentially gives you more bang for the buck, but the same amount of work as SSM.
12. They want to mutagenize a lipase to accept bulky side chains (we have seen this before, back in YSD). They use the crystal structure of the active site

with PNP-methyldecanoate docked to choose synergistic residues. Libraries are screened using PNP substrates.

13. Results: Mutants had very diverse activities toward the range of substrates. 3 cases of double mutants with better activity and 5 cases of single mutants with better activity – we don't really get a good comparison of all of this because the mutant's specificity is so different, but they say that the double mutants are better. Key point: "the simple expectation that..." CASTing can be used to improve enantioselectivity as well. (The authors seem surprised with the, but this isn't surprising.)
14. Marginal benefit over traditional SSM methods.