Progress Towards BChE Tethered With a Reactivating Ligand: a Pseudo-Catalytic Nerve Agent Bioscavenger

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Introduction and hypothesis

Human butyrylcholinesterase (hBChE) constitutes a promising alternative to presently available protection against organophosphate (OP) poisoning. This biological scavenger avoids the reaction of the OP with human acetylcholinesterase (hAChE) by covalent sequestration. One major drawback of the stoichiometric nature of action is the high dose and associated costs that are needed in order to provide adequate protection. Co-administration of the currently approved oximes, such as HI-6 and pralidoxime, to reactivate the OP-inhibited BChE and to establish a pseudo-catalytic function failed as it was demonstrated that the reactivation of OP-inhibited BChE by these oximes is too slow. We are currently exploring an alternative approach that comprises the chemical engineering of hBChE with its own reactivating chemical functionality that will effectively hydrolyze the chemical bond between the active site serine and the organophosphate moiety by a proximity-driven reaction (Figure 1).

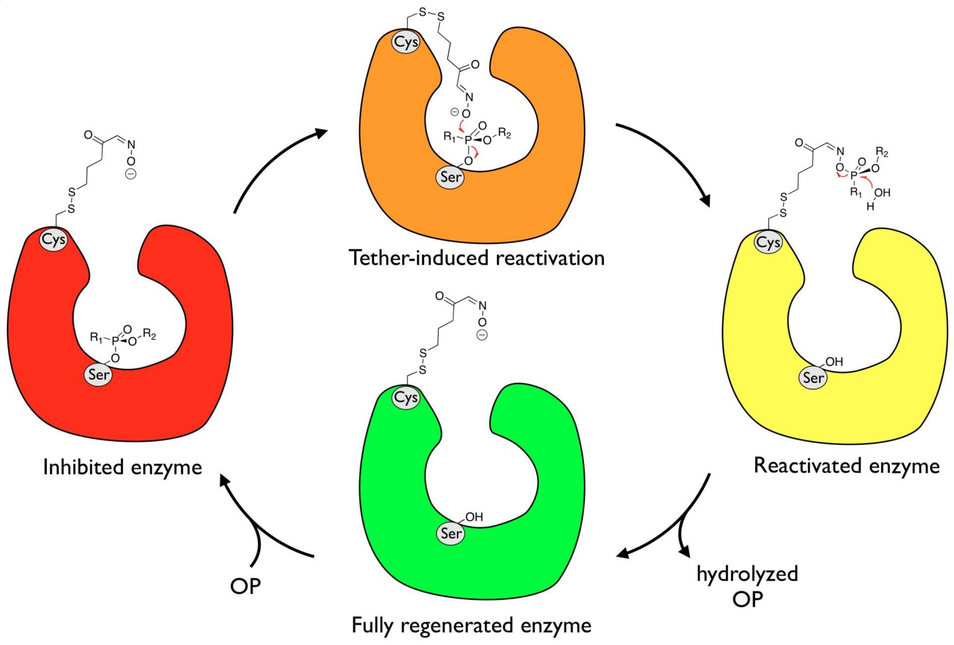
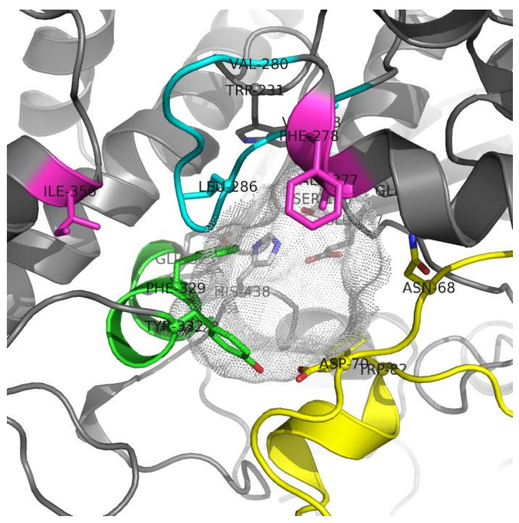


Figure 1. Schematic representation of the idea. A reactivating tether is engineered close to the active site of BChE (green). After OP sequestration (red) the tether will reactivate the inhibited enzyme (orange). The resulting reactivator-OP intermediate (yellow) should spontaneously hydrolyze to sustain catalytic activity.

Enzyme design, production and catalytic activity

A cysteine-mutation in hBChE is required because cysteine-thiols can be selectively functionalized with a chemical reaction in the presence of other amino acid residues. The position of this mutant cysteine residue must be carefully chosen to fulfill specific requirements:

- *It must be located at the protein surface to remain accessible for chemical functionalization.*
- *It must be located close to the gorge entrance, but sufficiently far from the active site to avoid permanent crowding of the site by the tethered reactivator.*
- *The mutation should not be located in a region of the enzyme known to be critical to the catalytic activity of the enzyme.*



Three mutant sites were chosen (Figure 2) and these enzymes were generated by site directed mutagenesis and expressed in Chinese hamster ovary cells. The enzymatic constants of the mutant enzymes (Table 1), determined by using the model described by Radic, were very close to those of the WT-hBChE, showing that the mutations did not affect the enzymatic properties of the protein.

Table 1. Enzymatic parameters of WT and mutant hBChE

	Km (µM)	Kss (mM)	b factor
hBChE-WT	10.2 (+/- 5.3)	0.82 (+/- 0.23)	2.99 (+/- 0.45)
hBChE-A277C	22.0 (+/- 3.8)	0.9 (+/- 0.1)	2.30 (+/- 0.16)
hBChE-I356C	11.0 (+/- 2.0)	0.5 (+/- 0.1)	2.78 (+/- 0.21)
hBChE-F278C	12.6 (+/- 5.0)	1.11 (+/- 0.29)	2.92 (+/- 0.33)

Figure 2. Looking into the gorge of hBChE (top view). The gorge surface is represented as white dots. The yellow, green and blue loops cannot be altered without affecting enzyme activity. The mutant positions of interest to anchor the tethers are shown in magenta.

Molecular dynamics based design and synthesis of tethers

The tethers were designed (Figure 3) to comprise a sulfhydryl-reactive group, required to link the tether to the enzyme, a tunable amide-based spacer to bridge the distance between the cysteine mutant site and the active site and different types of nucleophiles as the reactivating moiety.

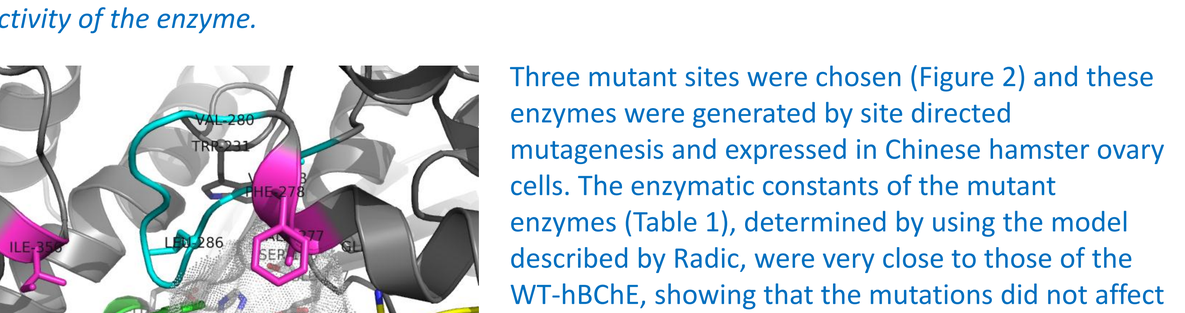


Figure 3. Left: Example of a tether as used in molecular dynamics simulations. Right: schematic representation of the molecules to be synthesized

The design was aided by molecular dynamics simulations. To this end, the cysteine mutations were *in silico* introduced into the X-ray structure of hBChE inhibited by VX (pdb 2xqk). The tethers were introduced *in silico* to the mutant sites having their nucleophilic part docked into the active site as the starting position. Molecular dynamics simulations were carried out and the distance over time between the oxygen atom of the nucleophile and the phosphorus atom of the phosphonyl moiety was monitored (Figure 4) providing an image of the evolution of this distance in time for each combination of mutant, spacer (n=2,3,4) and nucleophile.

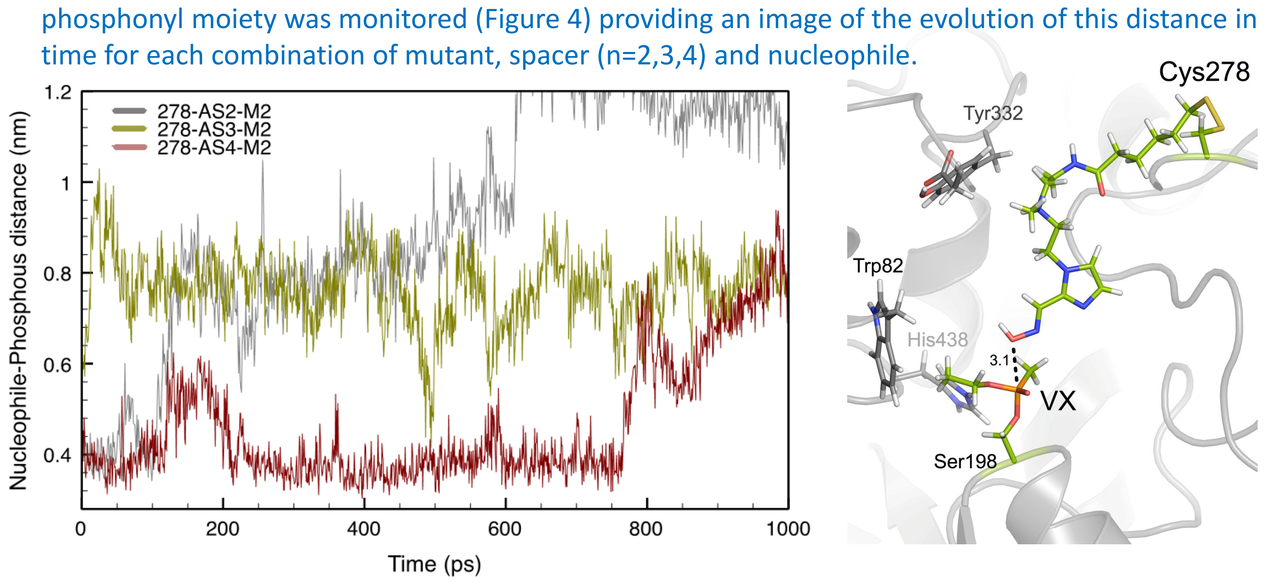


Figure 4. Example of the results of a molecular dynamics simulation, using Asn-M2 tethers of different lengths connected to the hBChE-F278C mutant. The graph (left) shows the evolution over time (total time = 1ns) of the distance between the oxime oxygen and the phosphonyl posphorus atoms. The frame that showed the shortest distance during this time is depicted on the right, showing the orientation and position of the nucleophile w.r.t. the phosphonyl moiety.

Synthesis procedures were developed to prepare these tethers. The structures of tethers synthesized so far are shown in Figure 5.

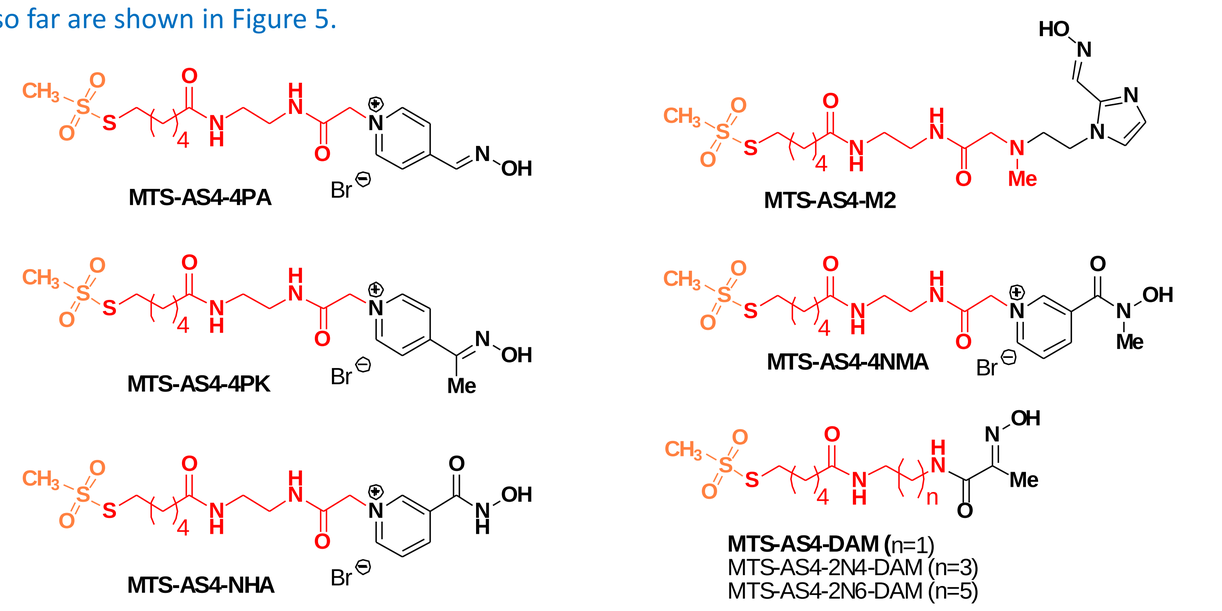


Figure 5. Tethers synthesized so far. For the synthesis of ASn tethers (n=2,3) the same synthetic procedures can be used.

Enzyme tethering and analysis

Tethering experiments have been carried out using the hBChE-I356C mutant and MTS-AS4-PA and MTS-AS4-M2 (Figure 6). The enzyme was subjected to mild DTT treatment to reduce any oxidized sulfhydryl groups, prior to the tethering reaction. Next, the enzyme was treated with iodoacetic acid to carboxymethylate any unreacted sulfhydryl groups. Finally, the enzyme was digested using trypsine and the digest was analyzed by LC-MS. In both cases, the modified T31 peptide fragment was found containing the Cys-356 connected to the tether. The intensity of the corresponding iodocarboxymethylated fragment was less than 5% of the total ion count, suggesting excellent conversion of available sulfhydryl groups.

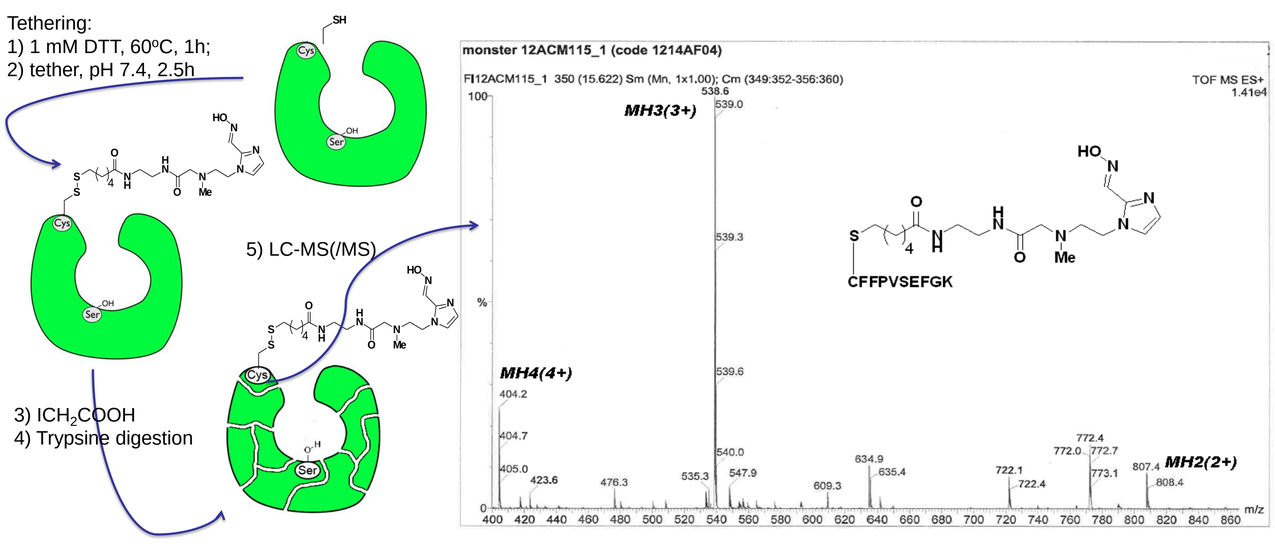


Figure 6. Enzyme tethering and subsequent trypsine digestion for analyis. This example shows the MS spectrum of the decamer peptide fragment carrying the AS4-M2 tether. At this stage, 2 tethers have been succesfully connected to the hBChE-I356C mutant.

Conclusions

Three cysteine-mutants of hBChE were prepared that showed comparable enzymatic activity as the wild type hBChE. We have designed and synthesized several tethers containing various nucleophiles and a MTS-group for covalent linkage to these mutant enzymes. Succesful tethering was demonstrated for the I356C mutant and two tethers.

Acknowledgements

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