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A step toward the reactivation of aged cholinesterases – Crystal structure of ligands binding to aged human butyrylcholinesterase

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ABSTRACT

Organophosphorus nerve agents irreversibly inhibit cholinesterases. Phosphylation of the catalytic serine can be reversed by the mean of powerful nucleophiles like oximes. But the phosphyl adduct can undergo a rapid spontaneous reaction leading to an aged enzyme, i.e., a conjugated enzyme that is no longer reactivable by oximes. One strategy to regain reactivability is to alkylate the phosphylic adduct. Specific alkylating molecules were synthesized and the crystal structures of the complexes they form with soman-aged human butyrylcholinesterase were solved. Although the compounds bind in the active site gorge of the aged enzyme, the orientation of the alkylating function appears to be unsuitable for efficient alkylation of the phosphylic adduct. However, these crystal structures provide key information to design efficient alkylators of aged-butyrylcholinesterase and specific reactivators of butyrylcholinesterase.

1. Introduction

Cholinesterases (ChEs) are enzymes that regulate neurotransmission in cholinergic synapses and neuromuscular junctions. They are the targets of a broad range of toxicants notably organophosphorus nerve agents (OPs). OPs exert their acute toxicity by phosphylating the catalytic serine of ChEs. Hydrolysis of the phosphyl adduct is extremely slow but inhibited ChEs conjugates can be readily reactivated by oximes [1] so that they form the basis of the treatment of OP intoxications [2]. The oximate form of reactivators makes a nucleophilic attack on the phosphorus atom of ChE–OP conjugate leading to the formation of a pentacoordinated transition state. After cleavage of the serine phosphorus bond, the phosphyloxime dissociates from the reactivated enzyme. But, for most OP, there is a spontaneous and time-dependent reaction occurring after the initial adduct formation, called "aging". Aging generally corresponds to a dealkylation of the phosphylated conjugate [3]. Aging rate can be very fast depending on the nature of the OP. For example, soman adducts have a half-life time of a few minutes [4]. No oxime is capable of reactivating the aged forms of OP-inhibited AChE due to the poor reactivity of the phosphonic acid adduct. We hypothesize that dealkylation could be reversed using an electrophile to obtain a realkylated phoshonylconjugate prone to reactivation by oximes. Since pyridiniumaldoxime 2-PAM is shown to bind in the active site of aged acetylcholinesterase [5], we chose the pyridinium scaffold to design specific alkylating agents: A pyridinium moiety substituted by an alkylating function. We tested two potential alkylating compounds (Fig. 1). Aged human ChEs were incubated with saturating concentrations of **1** and **2** (up to 2 mM), and eventually adding 0.5 mM HI-6 at different time points over many hours in the attempt to reactivate the potentially realkylated enzyme. We observe no evidence of reactivation.

In the present study, our initial goal was to collect structural data from complexes formed by soman aged-human AChE or BChE with **1** and **2**. We only succeeded in obtaining complexes with aged human BChE, no density corresponding to the ligands was present in the structures of aged human AChE. The alkylators tested bind near the phosphonylserine and are stabilized by aromatic stacking and cation-π interactions with aromatic residues of the active site (Tyr^{332} and Trp^{82}). The distance between the electrophilic carbon of the alkylator and targeted oxyanion of the phosphonyl serine remains too large to allow the alkylation reaction of aged BChE. However, these structural data are useful to further develop innovative

Abbreviations: ChE, cholinesterase; AChE, acetylcholinesterase; BChE, butyrylcholineserase; OP, organophosphate; CHO, Chinese Hamster Ovary; DMSO, dimethyl sulfoxide; 2-PAM, pralidoxime; ATC, acetylthiocholine iodide; BTC, butyrylthiocholine iodide; PDB, Protein Data Bank; TLS, translation libration screw motion.

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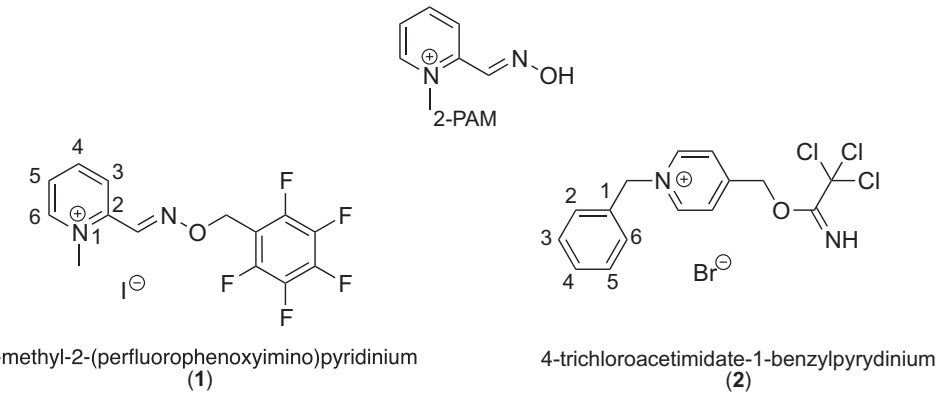

Fig. 1. Chemical structures of three ligands forming complexes with soman-aged human butyrylcholinesterase: 2PAM, methyl 2-(pentafluorobenzyloxyimino)pyridinium iodide and benzyl pyridinium-4-methyltrichloroacetimidate bromide.

specific alkykating molecules aimed at improving the reactivability of aged BChE, and also provide structural clues to design specific BChE reactivators.

2. Material and methods

Caution: Soman is highly toxic and is classified as a schedule one chemical as defined in the Chemical Weapons Convention. The handling of soman is dangerous and requires suitable personal protection, training, and facilities.

2.1. Chemicals

Soman, pinacolyl methylphosphonofluoridate, was obtained from the DGA Maîtrise NRBC (Vert-le-Petit, France). Pyridine-2-carboxaldoxime methiodide (2-PAM) was obtained from Lancaster Synthesis (Alfa Aesar®). The synthesis of methyl 2-(pentafluorobenzyloxyimino)pyridinium iodide (1), benzyl pyridinium-4-methyltrichloroacetimidate bromide (2), and intermediate compounds is described in detail in the supporting information. All other chemicals were from Sigma (Saint Quentin Fallavier, France).

2.2. Recombinant human acetylcholinesterase and butyrylcholinesterase

Recombinant BChE was truncated monomer containing residues 1–529 whose tetramerization domain and 4 *N*-glycosylation sites were deleted [2]. Recombinant AChE was also a truncated monomer containing residues 1–543 [6]. Both recombinant enzymes were expressed in Chinese hamster ovary (CHO) cells and secreted into serum-free culture medium, purified by affinity and exclusion chromatography as previously described [7,8].

2.3. Determination of inhibition constant (Ki) of ligands for non-inhibited human cholinesterases

Inhibition constants (Ki) were determined by incubating about 1 nkat of recombinant human AChE or BChE at 25 °C with various concentrations of ligands 1 or 2 in 100 mM sodium phosphate buffer, pH 7.4, containing 1 mg/mL BSA. The AChE activity was measured on aliquots using ATC ranging from 20 to 750 μM. The BChE activity was measured on aliquots using BTC ranging from 5 to 240 μM. Measurements were performed at least in duplicates. Ki was determined by fitting the data using equation modeling either a non-competitive inhibition (1) or a competitive inhibition (2) and Pro Fit (Quantum Soft):

$$V_i = \frac{V_{max} \times [S]}{K_S + [S]} \times \frac{K_i}{K_i + [I]} \quad (1)$$

$$V_i = \frac{V_{max} \times [S]}{[S] + K_S \times \left(1 + \frac{[I]}{K_i}\right)} \quad (2)$$

where V_i is the initial rate; V_{max}, the maximal rate; [S] the concentration of substrate (BTC); [*I*], the concentration of inhibitor; Ks the association constant of substrate and Ki, the inhibition constant.

2.4. Crystals of soman-aged BChE conjugates in complex with ligands

The soman stock solution was at 10 mM in 2-propanol. The ligands solutions were stocked at 10 mM in DMSO and 2-PAM solution at 100 mM in distilled water. Crystals of soman-aged BChE were obtained from a solution of BChE first inhibited by 0.4 mM of soman in 5 mM MES buffer, pH 6.5. BChE crystallized at a concentration of 7 mg/ml from 0.1 M MES buffer, pH 6.5, supplemented with 2.1 M ammonium sulfate, using the hanging drop system [9]. Then, the crystals of soman-aged BChE were soaked into a solution containing 1 mM of 2-PAM or 0.5 mM of 1 or 2 and washed with a cryoprotectant solution (0.1 M MES buffer pH 6.5, with 2.3 M ammonium sulfate, containing 20% glycerol) before flash-cooling in liquid nitrogen.

2.5. X-ray data collection and structure of BChE conjugates

Diffraction data were collected at the European Synchrotron Radiation Facility (ESRF, Grenoble, France), at the ID23-1 beam line (λ = 1.004 Å). All data sets were processed with XDS [10]. The structures were solved by use of the CCP4 suite [11]. An initial solution model was determined by molecular replacement, starting from the recombinant BChE structure (PDB entry 1P0I). For all diffraction data sets, the model was refined with REFMAC5 [12], then Phenix software suite [13]. An initial rigid body refinement was followed by iterative cycles of model building with Coot [14,15], and then restrained & TLS refinement was carried out. The ligands and their descriptions were built using the Dundee PRODRG 2.5 server including energy minimization using GROMOS 96.1 force field. TLS group were defined with the help of the TLS Motion Determination server (http:// skuld.bmsc.washington.edu/~tlsmd/index.html) [16]. Refined TLS parameters are included in the deposited PDB file for each entry. Protein structures were illustrated using the program PyMOL (Schrödinger).