Photoregulation of cholinesterase activity

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Abstract: The study of the functioning of cholinesterases and in particular the identification of the clearance of enzymatic products from the active site, is undertaken by two different photoregulatory methodologies. A mechanism-based photo-inactivation of the cholinesterases is proposed to map this route of clearance. The use of radiolabeled probes will permit to identify the modified amino acids on the 3-D structure of the enzyme. Secondly, a time-resolved crystallographic study could fully describe the conformational changes occurring during enzyme catalysis. This work requires the use of adapted photochemical triggering of a dynamic reaction within the enzyme crystal. The synthesis, the photochemical and the biochemical evaluations of photolabile cholinergic probes are described as potential triggers of the release of cholinergic effectors from the enzyme active site.

Hydrolysis of the neurotransmitter acetylcholine (Ach), by the cholinesterases (ChEs), acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) is a fast enzymatic process (Fig.1) i.e. AChE operates with a turnover number approaching 20,000s-1(ref. 1).

\[
\begin{align*}
\text{Acetylcholine} & \quad \rightarrow \quad \text{Enz-OH (ChE)} \\
\text{Tetrahedral Intermediate} & \quad \rightarrow \quad \text{Choline} \\
\text{Enz-OH} & \quad \rightarrow \quad \text{H}_2\text{O}
\end{align*}
\]

Fig. 1: Acetylcholine hydrolysis by cholinesterases

The description of the 3-D structure of AChE (ref.2) as well as several AChE-inhibitor complexes (refs.3-6) has permitted a better understanding of structure-function relationships for these enzymes. It has however raised new questions concerning the traffic of substrate and products to and from the active site. Thus, the description of a deep and narrow gorge, with the catalytic triad located near its bottom, appears to be inconsistent with the high turnover rate of the ChEs. In addition, the description of unusual electrostatic characteristics of AChE i.e. the existence of a large dipole which may serve to attract the cationic substrate towards the bottom of this gorge (ref.7), raised also the possibility that alternative routes to the active site might exist through the walls of the enzyme and may be involved in the clearance of products (ref.8).

Site-directed mutagenesis was used to challenge the existence of alternative routes for the traffic of the substrate and the products, to and from the active site. In particular the search of mutations which would seal the so called "back-door" failed up to now (refs.9,10) and the topic has aroused considerable controversy (refs.11,12). To address this question, we propose two independent approaches based on the photoregulation of the ChE activity.

The first approach uses a mechanism-based photo-inactivation of ChEs which should irreversibly label these enzymes after the catalytic reaction has occurred. The identification of the photo-cross-linked amino acid residues might inform us on the existence and the localization of this alternative route for the clearance of enzymatic products.
The second approach is based on a potential time-resolved crystallographic study of ChEs which requires suitable triggering mechanism being monitored, to ensure efficient and synchronized initiation of a dynamic process within the enzyme crystals. Photochemical triggering methods are best adapted for investigating such fast enzymatic reactions, provided that photochemical release of the effector (enzymatic product or substrate) is fast and efficient.

1. Mechanism-based photo-inactivation of ChEs

The suicide or kcat inhibitors (ref.13) are enzyme substrates that are converted through the catalytic reaction to reactive species which inactivate the enzyme by an alkylation reaction occurring at the enzyme active site, provided that nucleophilic amino acid residues are present at this site, to quench the formed species. Similarly, a mechanism-based photo-inactivation of an enzyme requires the generation of a photosensitive species within the enzyme active site after the catalytic step. This can be achieved either by transforming a non-photosensitive substrate to a photosensitive enzymatic product or as proposed here, by generating, from a photosensitive substrate, a photosensitive enzymatic product which can be activated at a specific wavelength. Such type of photo-inactivation reaction belongs to the photosuicide inhibitory processes (ref.14).

1.1 The search for suitable chromophores

The hydrolysis of p-aryldiazonium esters generates the corresponding 4-diazocyclohexa-2,5-dienones which causes a substantial chromophore shift of their \( \lambda_{\text{max}} \) when compared to their parent diazonium salts. These diazocyclohexadienone molecules represent photosensitive probes (ref 15) which generate highly reactive carbene species able to label efficiently amino acid residues (ref 16). Figure 2 illustrates the chromophore shift on the hydrolytic reaction of p-butyroxybenzene diazonium salt (1).

This diazonium salt (1) interacts differently with the ChEs, while being a substrate for BuChE, it has been shown to be a very efficient affinity label for AChE (ref.17). As expected, such aryldiazonium salts are chemically reactive, as illustrated by the AChE affinity labeling reaction, and require for their potential use as ChEs mechanism-based photochemical inactivators, increased chemical stability towards spontaneous hydrolysis. TABLE 1 summarises the chemical modifications introduced for this purpose, leading to the description of the p-anisoate ester (2) which showed an important stabilisation in addition to favourable enzymatic kinetic parameters for BuChE. This molecule was considered further for a photochemical inactivation of BuChE.

1.1.1 Photochemical inactivation of BuChE using ester (3)

Irradiation around 360nm of an incubation mixture of BuChE, purified from human plasma, with ester (3) shows a time-dependent enzyme inactivation. The observed pseudo-first order inactivation kinetics at
photoregulation of cholinesterase activity

**Table 1**: Chemical stability (half-lives in min) and enzymatic parameters of various synthesized \(p\)-phenyldiazonium esters.

<table>
<thead>
<tr>
<th>(t_{\text{1/2}}) min.</th>
<th>25</th>
<th>50</th>
<th>60</th>
<th>100</th>
<th>220</th>
<th>160</th>
<th>1400</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_m) ((\mu)M)</td>
<td>100</td>
<td>50</td>
<td>40</td>
<td>30</td>
<td>110</td>
<td>50</td>
<td>80</td>
</tr>
<tr>
<td>(V_m) ((\mu)mol/min/mg)</td>
<td>25</td>
<td>25</td>
<td>170</td>
<td>45</td>
<td>15</td>
<td>10</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Different inhibitor concentrations in addition to a wavelength-dependence of the rates of inactivation, corresponding to the action spectrum of the enzymatic reaction product (4) (dimethyl-3,5-diazo-4-cyclohexa-2,5-diene) argue in favour of a mechanism-based photo-inactivation process. This inactivation is protectable by cholinergic inhibitors such as tacrine or decamethonium.

The 2.6 position of the aromatic ring of BuChE, after photolabeling at 360nm in the presence of 

**2. Photo-regulation of cholinesterase activity for time-resolved crystallographic studies**

The study of fast conformational changes in proteins requires the use of appropriate time-resolved experimental approaches including time-resolved macromolecular crystallography (refs 18,19). This methodology requires a fast and efficient triggering of a dynamic process within the enzyme crystal to be synchronized with a synchrotron diffraction light source. The development of photolabile triggers is a prerequisite for such studies and we conceived different photolabile probes, all containing \(o\)-nitrobenzyl moieties to release photochemically cholinergic effectors within the enzyme active site.

2.a Photolabile precursors of ChEs effector

Figure 3 illustrates several photochemical precursors of respectively choline (ref. 20) (5), carbamylcholine (ref 21) (6) and Nor-acyetylcholine (7) which have been synthesized and evaluated for their biochemical and photochemical properties.

Fig. 3 Photolabile cholinergic effectors

2.b Photochemical cleavage reactions

The mechanism of the photochemical fragmentation reaction of \(o\)-nitrobenzyl derivatives has been initially established by Trentham and co-workers (ref 22) on the photochemical release of ATP from the \(o\)-nitrobenzyl \(p^2\)-ester of ATP demonstrating that the decay of an "aci nitro" intermediate could be correlated to the formation of ATP. The kinetics of this photolytic reaction was analyzed by monitoring the formation (\(\lambda_{\text{max}}\) around 400nm) and the decay of this intermediate around 400nm. Figure 4 illustrates such photochemical fragmentation reaction on a cholinergic probe.
Fig. 4 Photochemical fragmentation reaction of o-nitrobenzyl ether of choline

2.3 Spectral properties, photochemical reaction data and biochemical data

TABLE 2 summarizes the spectral properties, the quantum yields, the half-time of photo-fragmentation and the reversible inhibition constants for AChE and BuChE of selected probes (5), (6) and (7). The quantum yields were determined by comparison with the photolysis of the carbamylcholine derivative as reference compounds (ref 23) and the half-times of photofragmentation were determined by analyzing the decays of the corresponding aci-nitro intermediates at 405nm after laser flash photolysis.

<table>
<thead>
<tr>
<th></th>
<th>(5) (ref. 20)</th>
<th>(6) (ref. 23)</th>
<th>(7)</th>
</tr>
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<tbody>
<tr>
<td>( \lambda_{max} ) (nm)</td>
<td>261</td>
<td>262</td>
<td>254</td>
</tr>
<tr>
<td>( \varepsilon_{max} ) (M(^{-1}).cm(^{-1}))</td>
<td>5300</td>
<td>5200</td>
<td>3900</td>
</tr>
<tr>
<td>( t_{1/2} ) (μsec)</td>
<td>10</td>
<td>24</td>
<td>25</td>
</tr>
<tr>
<td>( \Phi )</td>
<td>0.27</td>
<td>0.25</td>
<td>Not Determined</td>
</tr>
<tr>
<td>( K_r, \text{AChE (μM)} )</td>
<td>13.0 ± 0.3</td>
<td>44.0 ± 0.9</td>
<td>Not Determined</td>
</tr>
<tr>
<td>( K_r, \text{BuChE (μM)} )</td>
<td>11.1 ± 0.1</td>
<td>78.4 ± 1.5</td>
<td>Not Determined</td>
</tr>
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</table>

Conclusion

The photosuicide inhibition of ChEs using radiolabeled probe (2) might give us new structural information on the clearance of the corresponding diazocyclohexadienonyl species (4) from the enzyme active site. In particular, the identification of amino acid residues involved in such conformational movement, will have to be confirmed by site-directed mutagenesis experiments. Among the different cholinergic o-nitrobenzyl derivatives which have been synthesized and analyzed, probes (5) and (6) have been demonstrated (ref 24) to be of potential use for time-resolved crystallographic studies on ChEs. Their photochemical properties, in particular, their fast kinetics of photo-fragmentation are suitable for the study of the fast enzymatic reactions involving ChEs. They generate choline, within the active site of the enzymes, in two complementary ways: the first by direct photo-fragmentation and the second by enzymatic hydrolysis of a substrate generated by photo-cleavage. The biochemical properties of probe (7) are presently investigated to complete this panel of photolabile cholinergic probes, before analyzing the properties of all these probes when soaked into crystals of AChE and performing the time-resolved crystallographic studies.
Acknowledgments

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References