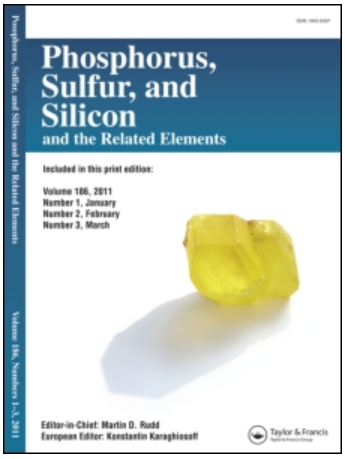

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ENZYME ENGINEERING TOWARDS NOVEL OP-HYDROLASES BASED ON THE HUMAN ACETYLCHOLINESTERASE TEMPLATE

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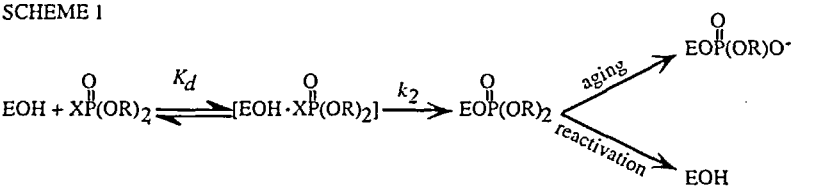
Abstract : Protein engineering technologies were used to generate various derivatives of human acetylcholinesterase (HuAChE). These enzymes were reacted with various organophosphates (OP) and reactivators in order to identify key element in the active center determining the efficiency of phosphylation, reactivation and of the aging process. Based on the results obtained we believe that novel biocatalysts with efficient OP-scavenging and possibly even OP-hydrolase activity may be generated, in the near future.

INTRODUCTION

The catalytic power of AChE and its high reactivity towards OP-inhibitors is believed to be determined by the unique architecture of the AChE active center, consisting of several subsites. Resolution of the 3D structure of *Torpedo* AChE[1], site directed mutagenesis and molecular modeling together with kinetic studies of the AChE muteins with substrates and reversible inhibitors[2-10] are beginning to unveil the functional role of the various active center subsites in the reactivity characteristics of the enzyme: a) the esteratic site containing the active site Ser-203 (human AChE aa numbering system); b) the "anionic subsite"-Trp-86 ; c) the hydrophobic site for the alkoxy leaving group of the substrate includes residues Trp-86, Tyr-337 and Phe-338; and d) the acyl pocket - Phe-295 and Phe-297. Involvement of the same binding elements in AChE reactivity towards organophosphorus inhibitors has been assumed in numerous studies during the last three decades, however a direct experimental evidence for such involvement is still scarce. Due to the high reactivity of AChEs towards OP-inhibitors they have been suggested as exogenous scavengers for sequestration of highly toxic OP-agents[11]. The AChEs react irreversibly and on a molar basis with the OP agents, however, the amounts of AChE required for treatment could be reduced provided that the OP-enzyme conjugates could be efficiently reactivated before the excess OP has reached its physiological target. This goal is difficult to attain especially in cases where the OP-AChE conjugates undergo catalytic

post-inhibitory processes termed aging (see scheme 1). In native AChEs the spontaneous reactivation, through displacement of the phosphyl moiety from the active site, is usually very slow and unable to compete with the aging process, yet efficient enzyme reactivation can be achieved by various oxime nucleophiles[12]. Our goal is therefore to generate enzymes based on the HuAChE template, the OP-adducts of which are more readily reactivated and are resistant to aging, yet still retain their high reactivity towards the OP agents. To meet this challenge, site directed mutagenesis has been utilized to induce small and controlled perturbations of the HuAChE active center functional architecture and their effects on the accommodation of organophosphate inhibitors, reactivators and the aging process were investigated.

SCHEME 1



RESULTS AND DISCUSSION

Contribution of Active Center Structural Determinants to Phosphorylation and Reactivation

Elucidation of the specific HuAChE-inhibitor interactions in the initial complex and during the subsequent nucleophilic process is essential for understanding the reactivity of AChE toward phosphate derivatives. Towards this goal, the apparent bimolecular rate constants for the irreversible inhibition of the HuAChE enzymes were determined under experimental conditions that allow the evaluation of the dissociation constants (K_d) as well as the phosphorylation rate constants k_2 (scheme 1). The organophosphate inhibitors used included: diisopropyl phosphorofluoridate (DFP), diethyl phosphorofluoridate (DEFP) and p-nitrophenyl diethyl phosphate (paraoxon) which allow to test the effects of different leaving groups and alkoxy substituents. The bimolecular rate constant values (ki) for the various enzymes tested extended over 3 orders of magnitude, irrespective of the nature of the inhibitor used. Interestingly, these values could be clustered according to the functional subsites in the HuAChE active center: mutants of the acyl pocket (Phe-295 and Phe-297) exhibiting the highest k_i values of all the enzymes tested; the alkoxy pocket (Trp-86, Tyr-337 and Phe-338) with k_i values close to that of the wild type enzyme; the H-bond network (Glu-202,Glu-450 and Tyr-133) with k_i values consistently lower as compared to the wild type enzyme. The most consistent characteristics of the HuAChE enzymes reactivity pattern toward organophosphates is that structural variations in both the enzyme and the inhibitor affect almost exclusively the stability of the initial

noncovalent complexes. While replacements of selected residues in HuAChE brought about changes in the K_d values of about 4-orders of magnitude, the corresponding phosphorylation rate constants (k_2) remained essentially unchanged. These results imply that the reaction has very stringent steric requirements within the active center and that *noncovalent complexes* which do not meet these requirements are kinetically silent.

The observed similarity of the overall effects of residue replacements at the acyl pocket, on the values of Km for substrates and K_d for phosphates indicate that the acyl pocket serves indeed an analogous purpose in both cases. However, due to the added dimensionality of the phosphates, variations in the K_d values reveal additional effects of the acyl pocket structural modifications. For the substrate, accommodation of the methyl group in this pocket together with the oxyanion hole orient the molecule in plane for the incipient nucleophilic attack. In a similar way, interaction of this subsite with organophosphates helps to orient the molecules for the in-line attack by the catalytic serine. In both cases the respective substituent is projected towards Phe-295 and therefore the corresponding complexes are more sensitive to the volume of residue at this position. Steric interactions with residue at 297 become important for branched alkoxy substituents which present larger volumes to the acyl pocket. Only marginal effects could be observed, upon replacement of the aromatic residues Trp-86, Tyr-337 or Phe-338, on the stability of complexes with DFP and DEFP underscoring the nonspecific nature of the hydrophobic interactions of the alkyl moieties with the enzyme surface. A somewhat more pronounced dependence on the structure of the alkoxy pocket is evident in the case of paraoxon which appears to originate from interactions with the p-nitrophenoxy leaving group. The potential multiplicity of the alkoxy pocket binding elements raises the possibility that both the alkoxy substituent and certain leaving groups are accommodated by this subsite.

Paraoxon inhibited enzymes were also used to gain a better understanding of the reactivation process by monoquaternary and bisquaternary oximes. It appears that the efficiency of the reactivation process depends to a large extent on the integrity of the active center H-bond network (Glu-202 and Glu-450) as well as on the anionic subsite residue Trp-86. A somewhat more surprising result was the observation that amino acids constituting the peripheral anionic site, which is located about 20Å away from the active center, can also affected the efficiency of reactivation by both bisquaternary and monoquatrnary oximes. Although some general patterns of behavior were observed, the extensive kinetic studies have not yet yielded a comprehensive picture regarding the nature of the chemical interactions between the reactivator and the OP-conjugate .

Contribution of Active Center Structural Determinants to Aging

We have recently[6] initiated a study of HuAChE derivative molecules with engineered

resistance to aging (scheme 1). The residues targeted for mutagenesis were in proximity to the catalytic serine and belong the H-bonded network. These preliminary studies demonstrated that indeed the aging process is catalytically assisted through residues in the active center and that in certain mutants of AChE, this process may become so slow (at least 100-fold reduction in first order rate constant compared with wild type) that the OP-conjugates (even for soman conjugates) are fully reactivatable. While this success establishes the feasibility of modulating the aging process, the engineered enzymes were poor scavengers since the same mutations that affected aging had also a major effect on the efficiency of OP-scavenging. To overcome this limitation a more judicious choice of amino acid replacements was made, based on residues remote from the immediate vicinity of the Ser-O-P bond. Mutation of one such residue in the hydrophobic pocket (F338A), generated a biocatalyst which is as competent as the wild type enzyme in scavenging various OP molecules and resistant to aging like the H-bond network mutants.

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