Photoreversible Inhibition of Cholinesterases: Catalytic Serine-Labeled Caged Butyrylcholinesterase

Sandra Loudwig,[a] Yvain Nicolet,[b] Patrick Masson,[c] Juan C. Fontecilla-Camps,[b] Suzanne Bon,[d] Florian Nachon,[c] and Maurice Goeldner*[a]

The photoregulation of the catalytic activity of butyrylcholinesterase (BChE) was investigated by treating the enzyme with a newly developed carbamylating reagent, N-*methyl*-N-*(2-nitrophenyl)carbamoyl chloride, which has proved to be an efficient photoremovable alcohol-protecting group. BChE was efficiently inhibited in a time- and concentration-dependent manner, and the enzyme could be protected against inhibition by active-site-specific ligands (that is, tacrine). The inactivation kinetics showed a biphasic character, which can be analyzed as the result of the existence of two conformational states in solution. Pseudo-irreversible inactivation of BChE, which results from catalytic serine carbamylation, was suggested by recovery of the enzyme activity after dilution and was demonstrated by X-ray crystallography. Remarkably, the 3D structure of the carbamylated BChE conjugate showed a non-ambiguous carbamylation of the catalytic serine residue as the only chemical modification on the protein. The photoreversibility of the enzyme inactivation was analyzed by irradiating the inactivated enzyme at 365 nm and was shown to reach completion in some conditions. The efficient and specific "caging" of BChE, together with the availability of carbamylated BChE crystals, will offer a unique possibility to study the catalytic properties of this enzyme by kinetic crystallography after cryophotolytic uncaging of the enzyme conjugate crystals.*

KEYWORDS:

enzyme inactivation · carbamates · inhibitors · kinetics · photolysis

Introduction

Acetylcholinesterase (EC.3.1.1.7; AChE) is a serine hydrolase which terminates cholinergic transmission by efficient hydrolysis of the neurotransmitter acetylcholine.[1] The related butyrylcholinesterase (EC.3.1.1.8; BChE), despite having no identified endogenous substrate, plays a key role in detoxification, by degrading esters like succinylcholine[2] and cocaine.[3] The recently resolved X-ray crystal structure of BChE[4] allowed a direct comparison with AChE;[5] this revealed quasi similar 3D structures, in agreement with the high amino acid sequence identity (54%). Both enzymes display a buried catalytic triad at the bottom of a deep and narrow gorge. The main differences reside in the absence of several aromatic residues at the peripheral site, at the gorge entrance, and in the "acyl pocket" of BChE compared to those of AChE; thus, BChE has a larger active-site cavity.[6] Despite numerous studies into their catalytic mechanism, the great efficiency of cholinesterases (ChEs), especially in the way the hydrolysis products exit from the enzyme, remains unexplained in view of their 3D structures. Time-resolved macromolecular crystallography[7] offers a new opportunity to study a catalytic mechanism at the atomic level. In the case of the ChEs it might be useful for the depiction of a rapid change in enzyme conformation; such a change is proposed in a hypothesis that has been forwarded to explain these exceptional hydrolytic properties (that is, the "back door" hypothesis which accounts for the exit of hydrolysis products).[8] For time-resolved studies it is necessary to trigger the enzymatic activity with precise time control, which can be achieved by a photochemical release of caged substrates or enzymes.[9] Besides other technical difficulties inherent to Laue crystallography, this method requires an efficient and synchronous photofragmentation reaction throughout a protein crystal. To overcome these technical difficulties we developed an alternative cryophotolytic

[a] *M. Goeldner, S. Loudwig*
Laboratoire de Chimie Bioorganique, UMR 7514 CNRS
Faculté de Pharmacie
Université Louis Pasteur Strasbourg
B.P. 24, 67401 Illkirch Cedex (France)
Fax: (+33) 390-244-306
E-mail: goeldner@bioorga.u-strasbg.fr

[b] *Y. Nicolet, J. C. Fontecilla-Camps*
Laboratoire de Cristallographie et de Cristallogénèse des Protéines
Institut de Biologie Structurale Jean-Pierre Ebel, CEA/CNRS
41, rue Jules Horowitz, 38027 Grenoble Cedex 1 (France)

[c] *P. Masson, F. Nachon*
Unité d'Enzymologie
Centre de Recherches du Service de Santé des Armées
B.P. 87, 38702 La Tronche Cedex (France)

[d] *S. Bon*
Laboratoire de Neurobiologie Cellulaire et Moléculaire, UMR 8544 CNRS
Ecole Normale Supérieure
46 rue d'Ulm, 75230 Paris Cedex 05 (France)

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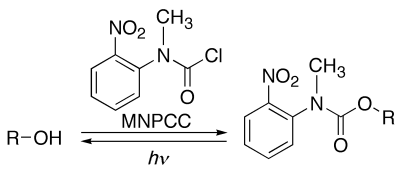
method.[10] By operating at cryogenic temperatures (100 – 150 K) to ensure complete inertness of the enzyme, the photolytic reaction can be pushed to completion. Subsequently, by application of a controlled temperature profile, some catalytic intermediate states could be trapped and characterized with classical monochromatic crystallography.

For the purpose of these studies, photolabile precursors of the ChE enzymatic product and substrate analogues have been synthesized and tested as reversible and site-specific inhibitors, to allow fast and efficient photochemical triggering of ChE activity in solution.[11] Clearly, it is more advantageous to cage the enzyme itself because a stoichiometric irreversible modification of the protein leads to a stable inactivated enzyme that requires a minimum amount of photons for its reactivation. Only a few examples of protein caging are found in the literature, due to the difficulty in targeting a suitable caging group to the desired site of the protein. This has been successfully achieved by selective modification of strategic cysteine residues, either natural[12, 13] or incorporated by site-directed mutagenesis.[14] Alternatively, unnatural amino acid mutagenesis enabled direct incorporation of photolytic precursors of various amino acids at a precise position in the sequence.[15] The extension of protein caging to residues other than cysteines is more difficult and requires a site-directed targeted modification. The first example of caged catalytic serine enzymes was described by the group of Porter, who used cinnamate esters or phenylbenzoylbenzoates to acylate the catalytic serine residue of serine proteases.[16]

The present article describes the inactivation of BChE, as well as its photolytic reactivation, by using *N*-methyl-*N*-(2-nitrophenyl)carbamoyl chloride (MNPCC), a new carbamylating reagent that has been described as a photoremovable alcohol-protecting group[17] (Scheme 1). Selective pseudo-irreversible carbamylation of the catalytic serine residue of BChE, as characterized by X-ray crystallography, together with a complete photolytic recovery of the activity of MNPCC-inactivated BChE, exemplifies the efficient caging of this enzyme. This light-activated protein therefore opens new possibilities for mechanistic studies on ChEs.



Scheme 1. N-*Methyl*-N-*(2-nitrophenyl)carbamoyl chloride (MNPCC) as a photolabile alcohol-protecting group.*

Results

Analysis of the biphasic nature of the MNPCC-induced BChE inactivation

Treatment of the natural human plasma BChE tetrameric form (Figure 1 a) or recombinant BChE (results not shown) with MNPCC led to time- and concentration-dependent inhibitions. Recombinant human BChE, engineered for crystallization purposes, is monomeric and displayed kinetic characteristics similar to natural BChE.[18] Several mechanisms can account for nonlinear inactivation of enzymes in solution.[19] Human BChE has been found to be composed of two enzyme forms, E and E′, in slow equilibrium[20] and there was no evidence for modification of several residues in the crystal, so the observed biphasic inhibition kinetics can be described by a sum of two first-order processes for pseudo-irreversible modification of a single residue in E and E′ (Scheme 2; further details are given in the Supporting Information). Inactivation was performed at pH values of 7.4 and 6.5 and the kinetic parameters are listed in Table 1.

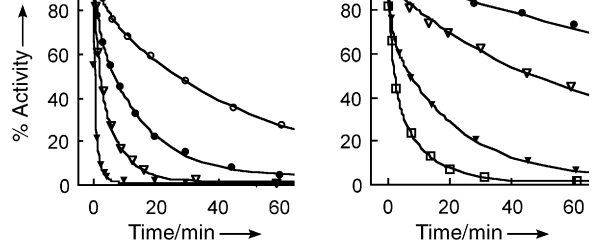
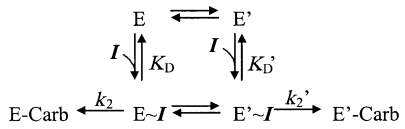


Figure 1. *a) Inhibition of natural human BChE by MNPCC at concentrations of ○ 5 μM, ● 10 μM, ▽ 20 μM, and ▼ 50 μM. b) Protection provided by tacrine at concentrations of □ 0 M, ▼ 0.05 μM, ▽ 0.1 μM, ● 0.2 μM, and ○ 0.5 μM against the inhibition of human BChE by 20 μM MNPCC.*



Scheme 2. *Kinetic scheme for the inactivation of BChE by MNPCC. The enzyme displays two conformational enzymatic forms (E and E′) in equilibrium in solution. Further details are given in the text.* K_D *= the dissociation constant.*

Table 1. *Biphasic inhibition constants of human BChE by MNPCC at pH 7.4 and 6.5.*

	pH 7.4	pH 6.5
k_2 [min^{-1}]	0.4	0.4
k'_2 [min^{-1}]	4	5
K_D [M]	6×10^{-5}	2×10^{-4}
K'_D [M]	5×10^{-5}	3×10^{-4}

Analysis of the selectivity of labeling

MNPCC-induced inactivation of BChEs could be efficiently prevented by tetrahydroaminoacridine hydrochloride (tacrine), a reversible active-site-specific inhibitor for BChEs. Figure 1 b shows the results of inhibition of human BChE in the presence of various concentrations of tacrine. Inhibition constants (K_i) for tacrine were determined from Lineweaver – Burk plots as previously described.[21] The following values were obtained: $K_i = 9 \pm 2$ nM for natural human BChE; $K_i = 13.6 \pm 0.9$ nM for

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recombinant human BChE. Inactivations were of mixed type: $\alpha K_i = 16.2 \pm 2$ nM for natural human BChE and 17.7 ± 1 nM for recombinant BChE, respectively. Complete protection against inactivation by tacrine was observed at a concentration of about 500 nM for both BChEs.

Pseudo-irreversible character of BChE inactivation by MNPCC

Dilution by 500-fold (see the Materials and Methods section) led to a slow (300 h) but complete reactivation of the MNPCC-inactivated BChE. Treatment by different reactivators, including pralidoxime chloride (1 mM), did not increase the reactivation rate of BChE.

X-ray crystal structure of the carbamylated enzyme conjugate

Recombinant BChE crystals were grown as previously described.[18] Crystals of carbamoylated BChE were obtained by soaking crystals of native BChE in the mother-liquor solution (0.1 M 4-morpholinoethanesulfonic acid (MES) buffer (pH 6.5) containing 2.3 M ammonium sulphate) containing 1 mM MNPCC for 90 min. The crystals were subsequently soaked in a solution containing 20% glycerol as a cryoprotector prior to being flash-cooled in a nitrogen gas stream. Data were collected at a resolution of 2.0 Å at the ID14eh1 beamline (ESRF-Grenoble, France) and processed with the programs MOSFLM, SCALA, and TRUNCATE from the CCP4 suite.[22] Data collection statistics are presented in Table 2. The unit cell parameters are isomorphous with the native ones previously determined. The X-ray crystal structure was solved by using the native BChE model,[4] in which the S198-bound butyrate group, water, and glycerol molecules were removed. The fit was then improved by using the rigid-body refinement procedure from CNS.[23] Subsequent refinement and manual rebuilding cycles with CNS and TURBO[24] led to a model with a working *R* factor and a free *R* factor of 0.2016 and 0.2319, respectively (Figure 2). Refinement statistics are presented in Table 3.

Table 2. Data collection statistics for the carbamylated human BChE crystal.[a]

space group	I422
unit cell:	
$a = b$, c [Å]	154.257, 128.087
$\alpha = \beta = \gamma$ [°]	90
resolution range [Å]	41.5 – 2.0
R_{sym}	0.068 (0.349)
I/σ	7.7 (2.2)
completeness [%]	99.4 (98.4)
multiplicity	9.1 (7.3)

[a] Values in brackets correspond to the higher resolution shell.

Photochemical reactivation analyses

The photoreversibility of the inhibitions was tested by irradiating the inactivated (≥90%) enzyme samples at 365 nm and measuring the enzyme activities as a function of time, by using the Ellman test[25] (see the Materials and Methods section). The reactivation reactions were tested either directly or after addition of 0.1 M hydroxylamine to eliminate excess reagent and the byproduct of photolysis. Alternatively, the photochemical reactivation was tested after treatment of the inactivated enzyme with charcoal to remove excess reagent from the medium.

The photochemical regenerations were very high both for natural and recombinant BChEs in all conditions tested (Figure 3). Without any preliminary treatment, the recovery reached 80%; this could be increased to 90% recovery after charcoal treatment, and a complete recovery was obtained in the presence of hydroxylamine.

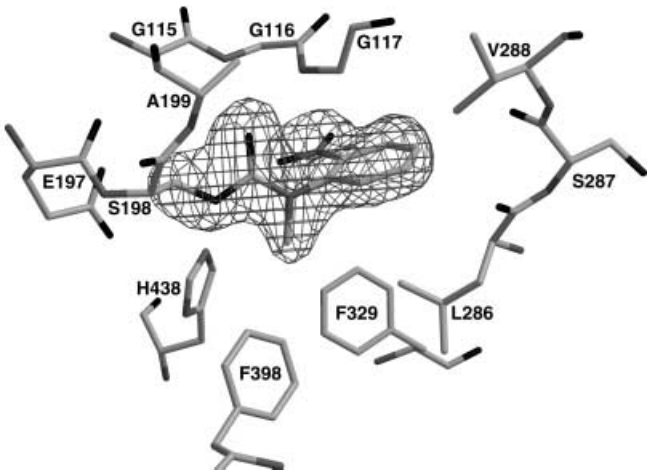

Figure 2. X-ray crystal structure (2.1 Å resolution) of the active site of recombinant human BChE carbamylated by MNPCC. Further details are given in the text and the Supporting Information.

Table 3. Refinement statistics for the carbamylated human BuChE crystal.

R factor	0.2016
free *R* factor	0.2319
number of atoms:	
protein	4228
solvent	554
other	176
root-mean-square deviation:	
bonds [Å]	0.009
angles [°]	1.55
number of reflections used	48 124

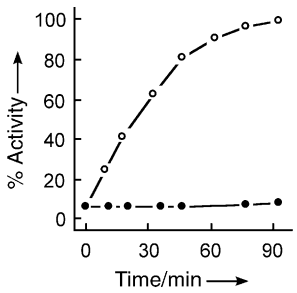

Figure 3. ○: Photoregeneration ($\lambda = 365$ nm) of natural human BChE activity after inhibition by 20 μM MNPCC (20 min) and subsequent treatment with 0.1 M NH_2OH. ●: Control experiment without light.

Discussion

The photoregulation of protein activity (protein caging) finds applications in cellular or structural biology, owing to the possibility of triggering the activity in a time- and space-controlled

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manner. Enzyme caging can be achieved by modifying, with a photolabile group, an amino acid residue essential for activity. The photochemical masking of a catalytic serine residue in an enzyme has been described with site-directed irreversible inhibitors.[16] The recent description of *N*-methyl-*N*-(2-nitrophenyl)carbamates as photoremovable alcohol-protecting groups[17] prompted us to test the possibility of modifying the catalytic serine residue of ChEs by *trans*-carbamylation reactions. Unfortunately none of the synthesized carbamylating reagents, including carbamates containing quaternary ammonium groups, followed the desired *trans*-carbamylation reaction; instead they generated reversible inhibitors (results not shown). Accordingly, we tested the corresponding photoactive carbamoyl chloride derivative MNPCC. A very efficient BChE inactivation reaction occurred in a time- and concentration-dependent manner (Figure 1 a). Surprisingly, the enzyme-inactivation kinetics showed a clear biphasic character, which could not be explained by simple interpretations. We conducted several control experiments under the incubation conditions. First, the reagent was stable ($t_{1/2} > 68$ h), so that its concentration did not change during the inactivation kinetics. Second, the enzyme(s) is (are) stable in the absence of reagent. Finally, the resulting carbamylated residues of the enzymes are not hydrolyzed to the free serine residues during the time course of the experiment, so the formation of a steady-state equilibrium is avoided. Several explanations could account for the observed biphasic inactivation kinetics.[19] Multistep enzyme inactivation processes can be either series (for example, consecutive steps with partially active intermediates) or parallel (inactivation of distinct isozymes). The mechanistic model, that is, the number of intermediate states or molecular species, can be difficult to validate from multiexponential analysis of inactivation kinetics. However, inactivation of BChE by MNPCC can be clearly described by a biexponential model. Biphasic inactivation kinetics of BChE may result from the occurrence of an equilibrium between two interconverting conformational states of the enzyme (E and E′). Such a model has been recently proposed for this enzyme.[20] Thus, if we assume that rapid and reversible formation of complexes $E \sim \boldsymbol{I}$ and $E' \sim \boldsymbol{I}$ is followed by carbamylation (E-Carb) and (E′-Carb), the equation shown in Scheme 2 can result in a biphasic inactivation process for BChE. The calculated values ($K_D = 60$ μM, $K'_D = 50$ μM, $k_2 = 0.4$ min^{-1}, $k'_2 = 4$ min^{-1}) clearly show similar affinity constants and two distinct inactivation rate constants. This indicates that the two enzymatic forms have the same affinity for the inhibitor, but differ in the catalytic efficiency of their carbamylation. The kinetic data obtained at pH 6.5 (Table 1) led to similar conclusions.

The specificity of the inactivation reactions was analyzed by using site-specific ligands as protectors. Tacrine, a high-affinity active-site-specific inhibitor,[21] was selected as the protector. In the presence of increasing concentrations of tacrine, the inactivation kinetics were progressively slowed down; this led eventually to full protection against inactivation (Figure 1 b). The inactivation processes are thus predominantly directed to the enzyme's active site. To further assess the occurrence, or otherwise, of an active-site-specific carbamylation induced by MNPCC, we tested the possibility of reversing the inactivations by strongly diluting the inhibited enzymes. Even though the hydrolytic reactivation of carbamylated BChE is known to be slow and often only partial, we were able to recover, very slowly (300 h), the full enzyme activity of natural BChE after a 500-fold dilution from an initial concentration of 20 μM MNPCC reagent (results not shown). Thus, the inactivation of BChE appears to be clearly related to carbamylation of the catalytic serine residue.

We intended to characterize the carbamylation of BChE more clearly. In the absence of a radiolabeled probe it is difficult to assess the stoichiometry of enzyme modification, while the high degree of glycosylation (nine carbohydrate chains per monomer for the natural tetrameric protein) hinders accurate electrospray MS analyses. Therefore, X-ray crystallography was used to define precisely the alkylation site(s) on BChE. The crystallographic determination of the 3D structure of BChE was achieved on a monomeric recombinant enzyme lacking four glycosylation sites.[18] The soaking of BChE crystals in a buffered solution containing the MNPCC reagent led to enzyme modification, which was analyzed by X-ray diffraction. Chemical modification occurred specifically on a single residue, the catalytic serine (S198), as illustrated in Figure 2; the electron density observed was in agreement with a covalent bond between the serine oxygen atom and the carbonyl group of the MNPCC reagent.

Knowing that the carbamylation of the active serine residue was responsible for enzyme inhibition, we tested the possibility of a photochemical regeneration of the BChE activity. We recently showed that photolysis of *N*-methyl-*N*-(2-nitropenyl)-carbamates[17] generated the corresponding free alcohol together with *N*-methyl-2-nitrosoaniline. Although the mechanism of the photochemical fragmentation remains to be elucidated, we tested the photochemical reaction on the inactivated BChEs. Irradiation at 365 nm, with a Xe – Hg light source, showed very high recovery of the activity for the BChEs, from 80 – 100 % under various reaction conditions (Figure 3). The photorecovery of enzymatic activity after inhibition with 20 μM MNPCC and without removal of excess reagent reached 80 % (results not shown). Alternatively, the photochemical reactivation was tested after treatment of the inactivated enzyme with charcoal, to remove excess reagent from the medium and to enable a more efficient photolytic reaction. In this case, the activity of the BChEs could be regenerated up to 90 % (results not shown). Finally, the use of 0.1 M NH_2OH quenched the excess reagent, and under these conditions we observed a more rapid onset of the reactivation of BChE, which allowed the reactivation to reach completion (Figure 3).

The high recoveries of enzyme activity indicate that the formed photolytic nitroso byproduct[17] does not interfere with the enzyme during this photolytic reaction. This result is in agreement with the low affinity of this chemical for the ChEs (IC_{50} value of about 0.3 mM; results not shown), so that it should readily depart from the active site after photolysis.

Within the time course of this study we also analyzed the effect of MNPCC on AChE for a possible photochemical regulation of its activity (results not shown). Although highly efficient time- and concentration-dependent inactivation kinetics could be observed, also displaying a biphasic character, the analyses of the kinetics could not demonstrate the specific

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involvement of the catalytic serine residue in the inactivation processes. While a possible contribution by peripheral residues has been proposed from studies with mutant enzymes in particular, the photolytic reactions with the inactivated AChE were particularly disappointing and displayed only very partial enzyme activity recoveries.

The observed inactivation/photoreactivation processes for BChE fit nicely with a specific carbamylation reaction of the catalytic serine residue and subsequent efficient photolytic decarbamylation. All the presented experiments, time- and concentration-dependent inhibitions, efficient protection by tacrine, and reversibility of the inhibition by dilution, are fully consistent with a carbamylation of the catalytic serine. A two-state enzyme model was proposed to explain the biphasic inactivation kinetics. Finally, the specific carbamylation reaction was demonstrated on the recombinant enzyme by X-ray crystallography of the carbamylated enzyme conjugate. The photochemical reversibility of the enzyme activity is remarkable in its extent, since a full recovery of the activity was possible by irradiating the inactivated enzyme in the presence of NH_2OH. This efficient photoregulation of BChE will be tested on crystals of the carbamylated BChE conjugate in cryophotolytic conditions[10] to further explore the mechanistic properties of BChE.

Materials and Methods

Chemicals and materials: *N*-Methyl-*N*-(2-nitrophenyl)carbamoyl chloride (MNPCC), [*N*-methyl-*N*-(2-nitrophenyl)],[2-(*N*,*N*,*N*-trimethylammonium)ethyl] carbamate, and [*N*-methyl-*N*-(2-nitrophenyl)], (benzyl) carbamate were synthesized as previously described.[17] Butyrylthiocholine iodide, acetylthiocholine iodide, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), tetrahydroaminoacridine hydrochloride (tacrine), and bovine serum albumin (BSA) were purchased from Sigma/Aldrich. Sodium dihydrogen phosphate monohydrate was from Merck; sodium hydroxide and dioxane (purex grade) were from Carlo Erba. Water was purified through a Millipore column system.

Enzymes sources: Natural human butyrylcholinesterase (BChE) was purified from plasma. Recombinant monomeric BChE, lacking four out of nine *N*-glycosylation sites and the C-terminal oligomerization domain (WHAT), was expressed in stably transfected CHO cells, as previously described.[18] Natural and recombinant BChEs were purified by affinity chromatography on procainamide-Sepharose gel and by ion-exchange chromatography.[18] Both natural and recombinant BChEs showed similar catalytic properties. Acetylcholinesterase (AChE) was obtained from electric organs of *Torpedo marmorata* (Station de Biologie Marine, Arcachon, France), solubilized by a mild proteolytic procedure, and purified by affinity chromatography.

Enzymatic activity measurement: Enzymatic activities were assessed by the method of Ellman,[25] at 25 °C and in 50 mM phosphate buffer (pH 7.4) containing 2.5 mM DTNB (1 mg mL^{-1}), with 1 mM butyrylthiocholine for BChE. Aliquots of BChE media were added to a final assay volume of 1 mL, and the increase in absorbance (ΔA min^{-1}), corresponding to thiolate formation ($\varepsilon_{(412\,nm,\,pH\,7.4)} = 13\,600$ M^{-1} cm^{-1}), was monitored at 412 nm for 30 s, by using a UVIKON XL UV spectrophotometer from Bio-Tek, equipped with a kinetic program. The enzyme aliquots were chosen so that the measured activity was 0.7 ΔA min^{-1}, which corresponds to 0.05 U. One unit (U) corresponds to the amount of enzyme that hydrolyses 1 μmole of substrate per minute. Under those conditions, 1 ΔA min^{-1} corresponds to 0.073 U.

Inhibition of cholinesterases by MNPCC: Inhibitions were performed at 4 °C. BChE was diluted to 5 U in 50 mM phosphate buffer (pH 7.4) containing BSA (1 mg mL^{-1}). Aliquots of 200 μL were inhibited with 5, 10, 20, or 50 μM MNPCC added at $t = 0$. Aliquots of 10 μL were taken at different times for activity measurements. $[act]_0$, the measured increase in absorbance (ΔA min^{-1}) of an aliquot without inhibitor, was taken as 100% activity. The control enzymes were stable during the inhibition experiments.

Protection against inhibition by tacrine: Protection reactions were performed at 4 °C. BChE was diluted to 5 U in 50 mM phosphate buffer (pH 7.4) containing BSA (1 mg mL^{-1}). Aliquots were mixed with 0, 0.05, 0.1, 0.2, or 0.5 μM of tacrine. Three aliquots of 10 μL were taken for each concentration to determine $[act]_0$, which was taken as 100% activity. 20 μM MNPCC was added at $t = 0$.

Photoreactivation of carbamylated BChE: Aliquots (500 μL) of BChE (5 U) in 50 mM phosphate buffer (pH 7.4) containing BSA (1 mg mL^{-1}) were inhibited for 20 min with 20 μM MNPCC, up to complete loss of activity. Excess MNPCC was then quenched with 0.1 M hydroxylamine and 300 μL of this solution was irradiated at 365 nm in a quartz cell at 10 °C, with magnetic stirring. The light source was a Hg – Xe 1000 W lamp, fitted with a monochromator. The remaining solution was kept as a control, to check that no hydroxylamine-mediated reactivation of the enzyme was observed in the absence of irradiation. Aliquots were taken at different times to monitor the progressive increase of activity. Another aliquot (300 μL) of noninhibited BChE was irradiated for the same time, to show that no damage was caused to the enzyme by irradiation.

Reactivation of carbamylated BChE by dilution: BuChE (50 μL; 1250 U) was inhibited by 20 μM MNPCC for 20 minutes. After inactivation, aliquots were diluted 500-fold in 50 mM phosphate buffer (pH 7.4) containing BSA (1 mg mL^{-1}). Aliquots (20 μL) of this solution were taken to monitor the recovery of activity, which was complete within 300 h. A control experiment on an aliquot (50 μL) of the same 1250-U enzyme solution was carried out; the noninbibited enzyme samples were kept for 20 min at 4 °C and then diluted 500 times: no loss of activity occurred during the 20 min experiment.

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[1] J. Massoulié, L. Pezzementi, S. Bon, E. Krejci, F. M. Vallette, *Prog. Neurobiol.* **1993**, *41*, 31 – 91.
[2] O. Lockridge, *Pharmacol. Ther.* **1990**, *47*, 35 – 60.
[3] G. N. Carmona, R. A. Jufer, S. R. Goldberg, D. A. Gorelick, N. H. Greig, Q.-S. Yu, E. J. Cone and C. W. Schindler, *Drug Metab. Dispos.* **2000**, *28*, 367 – 371.
[4] Y. Nicolet, O. Lockridge, P. Masson, J. C. Fontecilla-Camps, F. Nachon, unpublished results.
[5] J. L. Sussman, M. Harel, F. Frolow, C. Oefner, A. Goldman, L. Toker, I. Silman, *Science* **1991**, *253*, 872 – 879.
[6] M. Harel, J. L. Sussman, E. Krejci, S. Bon, P. Chanal, J. Massoulié, I. Silman, *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 10 827 – 10 831.
[7] K. Moffat, *Chem. Rev.* **2001**, *101*, 1569 – 1581.
[8] M. K. Gilson, T. P. Straatsma, J. A. McCammon, D. R. Ripoll, C. H. Faerman, P. H. Axelsen, I. Silman, J. L. Sussman, *Science* **1994**, *263*, 1276 – 1278.
[9] K. Curley, D. S. Lawrence, *Curr. Opin. Chem. Biol.* **1999**, *3*, 84 – 88.
[10] A. Specht, T. Ursby, M. Weik, L. Peng, D. Bourgeois, M. Goeldner, *ChemBioChem* **2001**, *2*, 845 – 848.
[11] L. Peng, I. Silman, J. L. Sussman, M. Goeldner, *Biochemistry* **1996**, *35*, 10 854 – 10 861.

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[12] G. Arabaci, X. C. Guo, K. D. Beebe, K. M. Coggeshall, D. Pei, *J. Am. Chem. Soc.* **1999**, *121*, 5085 – 5086.
[13] G. Marriott, M. Heidecker, *Biochemistry* **1996**, *35*, 3170 – 3174.
[14] H. Bayley, C. Y. Chang, W. T. Miller, B. Niblack, P. Pan, *Methods Enzymol.* **1998**, *291*, 117 – 135.
[15] a) V. W. Cornish, D. E. Mendel, P. G. Schultz, *Angew. Chem.* **1995**, *107*, 677 – 690; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 621 – 633; b) M. W. Nowak, J. P. Gallivan, S. K. Silverman, C. G. Labarka, D. A. Dougherty, H. A. Lester, *Methods Enzymol*. **1998**, *293*, 504 – 529.
[16] A. D. Turner, S. V. Pizzo, G. Rozakis, N. A. Porter, *J. Am. Chem. Soc.* **1988**, *110*, 244 – 250.
[17] S. Loudwig, M. Goeldner, *Tetrahedron Lett.* **2001**, *42*, 7957 – 7959.
[18] F. Nachon, Y. Nicolet, N. Viguie, P. Masson, J.-C. Fontecilla-Camps, O. Lockridge, *Eur. J. Biochem.* **2002**, *269*, 630 – 637.
[19] J. P. Henley, A. Sadana, *Biotechnol. Bioengn.* **1986**, *28*, 1277 – 1285.
[20] P. Masson, M.-T. Froment, S. Fort, F. Ribes, N. Bec, C. Balny, L. M. Schopfer, *Biochim. Biophys. Acta* **2002**, *1597*, 229 – 243.
[21] H. A. Berman, K. Leonard, *Mol. Pharmacol.* **1991**, *41*, 412 – 418.
[22] Collaborative Computational Project, Number 4, *Acta Crystallogr. D* **1994**, *50*, 760 – 763.
[23] A. T. Brunger, P. D. Adams, G. M. Clore, W. L. DeLano, P. Gros, R. W. Grosse-Kunstleve, J. S. Jiang, J. Kuszewski, M. Nilges, N. S. Pannu, R. J. Read, L. M. Simonson, T. Warren, *Acta Crystallogr. D: Biol. Crystallogr.* **1998**, *54*, 905 – 921.
[24] A. Roussel, C. Cambilleau, C. Turbo-Frodo, in *Silicon Graphics Geometry Partners*, Discovery Silicon Graphics, Mountain View, CA, **1989**, 77 – 79.
[25] G. L. Ellman, K. D. Courtney, V. Andres, M. R. Featherstone, *Biochem. Pharmacol.* **1961**, *7*, 88 – 95.

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