Allosteric Modulation of Acetylcholinesterase Activity by Peripheral Ligands Involves a Conformational Transition of the Anionic Subsite†

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ABSTRACT: Replacement of residues Asp74, Trp286, and Tyr72, which are constituents of the peripheral anionic site (PAS) of human acetylcholinesterase (HuAChE), affected similarly both the binding and the inhibition constants of the PAS-specific ligand propidium, demonstrating that changes in the inhibitory activity are a direct consequence of altered binding to the PAS. In contrast, the active center HuAChE mutants W86A and Y133A show respective 350- and 25-fold increased resistance to inhibition by propidium but no change in binding affinities, demonstrating that the allosteric mechanism of PAS-mediated inhibition involves a conformational change of these Trp86 and Tyr133 residues rather than physical obstruction of substrate access by the inhibitor itself. These findings support the recent proposal that the allosteric mechanism operates via transition between active and nonactive conformations of the anionic subsite Trp86 and that replacement of Tyr133 by alanine may stabilize a nonactive Trp86 conformation that occludes the active center [Ordentlich et al. (1995) *J. Biol. Chem. 270*, 2082]. In further support of this mechanism and the role of Tyr133, we find that (a) the dissociation constants (K_d) for the noncovalent complexes of the irreversible inhibitors diisopropyl phosphorofluoridate or paraoxon with Y133A HuAChE are increased 20−500-fold, relative to either wild-type enzyme or its Y133F or W86A mutants; and (b) access of substrates such as 3,3-dimethylbutyl thioacetate is restored by removal of Trp86 from the Y133A enzyme (i.e., the W86A/Y133A mutant). We suggest that the conformational transition of Trp86 is coupled to the motions of the cysteine loop (Cys69−Cys96) of HuAChE and is inherent to the dynamics of the native enzyme.

One of the more intriguing features of acetylcholinesterase (AChE)[1] reactivity is the modulation of its catalytic activity following ligand binding to a peripheral site (PAS) at the enzyme surface (Changeux, 1966; Hucho et al., 1991). The significance of such modulation for the enzymatic function is probably in adjusting the catalytic activity to the rapid fluctuations in the concentration of neurotransmitter and other charged species, like the cations Ca^{2+}, Mg^{2+}, and Na^{+}, in the synaptic cleft (Berman & Nowak, 1992). While evidence exists that peripheral site ligands affect the conformation of the active center (Berman et al., 1981; Taylor & Radic, 1994), implying an allosteric effect, direct evidence is still lacking, and the actual mechanism of this modulation is yet unknown. The resolution of the enzyme structure by X-ray crystallography (Sussman et al., 1991) did not yield any clues as to this mechanism but rather rendered it more enigmatic by placing the active site within a narrow "gorge" about 20 Å away from the periphery.

Site-directed labeling (Weise et al., 1990), mutagenesis studies, and molecular modeling (Shafferman et al., 1992a,b; Ordentlich et al., 1993; Radic et al., 1993; Barak et al., 1994) allow for the identification and localization of the PAS residues at the protein surface and near the rim of the active center gorge. These include aromatic residues Tyr72, Tyr124, Trp286, and Tyr341 that together with the acidic residue Asp74 constitute an array of partially overlapping but distinct binding sites (Barak et al., 1994) capable of binding structurally diverse ligands like propidium, gallamine, or fasciculin (Rosenberry, 1975; Quinn, 1987; Radic et al., 1994). The relative locations of the active center and the PAS, in the molecular model of HuAChE (Barak et al., 1992; based on the X-ray structure of *Torpedo californica* acetylcholinesterase, TcAChE), are consistent with the suggestion that bisquaternary ligands, typified by decamethonium, bind to the enzyme by bridging the two sites (Harel et al., 1993; Barak et al., 1994). The location of PAS, relative to the gorge entrance, implies also that association of AChE with the prototypical PAS ligand propidium may affect the access of substrates to the active center by either physical obstruction or charge repulsion imparted by the cationic ligand (Taylor & Radic, 1994). Thus, the way by which PAS-specific ligands like propidium exert their inhibitory effects is not yet clear.

The nearly equal affinities of propidium toward free and methanesulfonylated TcAChE (Taylor & Lappi, 1975)

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[1] Abbreviations: AChE, acetylcholinesterase; HuAChE, human acetylcholinesterase; TcAChE, *Torpedo californica* acetylcholinesterase; ACh, acetylcholine; ATC, acetylthiocholine; TB, 3,3-dimethylbutyl thioacetate; DFP, diisopropyl phosphorofluoridate.

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indicate that modifications at the active site do not affect the binding properties of PAS. In addition, the failure of reversible active center inhibitors like edrophonium or *N*-methylacridinium to displace propidium has been regarded as evidence for formation of ternary complexes (Taylor & Radic, 1994). On the other hand, HuAChE mutant enzymes, in which the anionic subsite at the active center was altered by replacement of either residue Trp86 or the adjacent residue Tyr133 (both of which are about 15 Å away from the PAS) by alanine, are practically refractory to inhibition by propidium (Ordentlich et al., 1993, 1995). These findings imply either that the binding affinity of propidium to PAS has been indirectly affected by these replacements or that the allosterically induced conformational changes in the active center that abolish catalytic activity are no longer possible. The first alternative is compatible with the notion that physical blocking of the active site gorge, by the PAS ligand, contributes to the inhibitory activity of propidium. Such a conclusion is also consistent with the molecular model of the HuAChE−propidium complex (Barak et al., 1994) in which the ligand obstructs the access to the active site. On the other hand, inhibition through conformational flexibility of the side chain of residue Trp86 is consistent with the differential catalytic activities of the W86A and Y133A enzymes toward the charged substrate ATC and its noncharged isostere TB, relative to the wild-type HuAChE (Ordentlich et al., 1993, 1995).

In this study, we further investigate the role of the active center residues Trp86 and Tyr133 in the inhibition of HuAChE catalytic activity, through ligand association with the PAS. We provide evidence that the affinity of W86A enzyme for propidium is close to that of the wild-type HuAChE, suggesting that the inhibitory effect of propidium is not a direct consequence of its association with the PAS. We demonstrate that the reactivity properties of the double mutant W86A/Y133A of HuAChE are consistent with the notion that the conformational transition of residue Trp86 constitutes the structural response to the occupation of the PAS. Finally, we propose that the conformational state of Trp86 is governed by the dynamic behavior of the cysteine loop (Cys69−Cys96) on the protein surface and that this mobility plays an important role in the mechanism of the allosteric effect in AChE.

EXPERIMENTAL PROCEDURES

Mutagenesis of Recombinant HuAChE and Construction of Expression Vectors. Mutagenesis of AChE was performed by DNA cassette replacement into a series of HuAChE sequence variants which conserve the wild-type (Soreq et al., 1990) coding specificity, but carry new unique restriction sites (Shafferman et al., 1992a). Generation of mutants W86A(W*84*),[2] D74N(*72*), Y133A(*130*), Y133F(*130*), Y341A-(*334*), and W286A(*279*) was described previously (Shafferman et al., 1992b; Ordentlich et al., 1995). The double mutant W86A/Y133A HuAChE was formed by substituting the *Eco*RV−*Esp*I fragment and the *Esp*I−*Bst*EII fragment of the human *ache* DNA with the respective fragments of the W86A and Y133A mutant DNAs. All the synthetic DNA oligodeoxynucleotides were prepared using an Applied Biosystems DNA synthesizer. The sequences of all new clones were verified by the dideoxy sequencing method (USB sequenase kit). The rHuAChE cDNA mutants were expressed in tripartite vectors which allow expression of the *cat* reporter gene and the *neo* selection marker (Kronman et al., 1992; Shafferman et al., 1992a).

Establishment of Stably Expressing Cell Pools and High-Expressor Cell Clones. Human embryonal kidney 293 cells were transfected by the calcium phosphate method as described previously (Kronman et al., 1992). Following overnight incubation in the transfection mix, cells were refed with DMEM containing 10% ChE-depleted FCS (PAT) after which the cells were incubated for an additional 48 h at 37 °C in the presence of 5% CO_2. Transient expression levels of the various rHuAChE mutants were determined by AChE activity measurement complemented by specific enzyme-linked immunoadsorbent assay (ELISA; Shafferman et al., 1992a). The latter allows assessment of AChE production by mutants in which enzymatic activity is impaired under normal conditions.

Cells in which stable integration of the plasmid has occurred were selected by exposure to G418 for a period of 3−4 weeks. In a typical experiment, 100−200 stable G418-resistant cell colonies were formed. To assist confluent pool formation, cell colonies were trypsinized and allowed to resettle in the cell culture dish. Stably transfected cell pools were assayed for enzyme activity and protein mass (ELISA). Individual clones of the rHuAChE mutants Y72A, D74N W86A, Y133A, W286A, Y341A, and Y72A/W286A were isolated by limiting dilution of the stable pool cells followed by seeding onto 96-well microtiter plates. The arising cell clones (usually between 200 and 500 per AChE type) were subjected to screening procedures. In accordance with the data gathered at the transient expression phase and from the stable cell pools, the method of screening for high-producer clones was adopted. In all cases, the selected high-producer clones displayed an enzyme secretion level higher than 1 μg per 10^6 cells during a 24 h period. In the cases of W286A and Y72A/W286A, especially high levels of secreted enzymes were observed [10−25 μg (10^6 cells) (24 h)$^{-1}$].

Purification of HuAChE Enzymes. The crude HuAChE enzymes were purified by affinity chromatography on monoclonal antibody AE-1 (Fambrough et al., 1982) coupled to Sepharose. Activation of Sepharose CL-4B was carried out according to Wilchek and Miron (1982). Purified AE-1 monoclonal antibodies were coupled to the activated Sepharose at a ratio of 15 mg of protein/g wet resin. The enzyme binding capacity of the column was 0.5−1.5 mg of enzyme/mL of gel. Following elution by 4 M $MgCl_2$ in 1 M glycine buffer, pH 6.0, the purified enzyme was dialyzed, concentrated by 70% ammonium sulfate precipitation, resuspended in 10 mM phosphate buffer, pH 8.0, and dialyzed against either 10 mM phosphate buffer, pH 8.0, or 1 mM Tris-HCl buffer, pH 8.0.

Substrates and Inhibitors. Acetylthiocholine iodide (ATC), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 3,8-diamino-5,3′-(trimethylammonium)propyl-6-phenylphenanthridinium iodide (propidium), 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284C51), *p*-nitrophenyl diethyl phosphate (paraoxon), and diisopropyl phosphorofluoridate (DFP) were all purchased from Sigma. 3,3-Dimethylbutyl thioacetate (TB) was synthesized and purified as described previously (Ordentlich et al., 1993).

[2] Amino acids and numbers refer to HuAChE; the numbers in parentheses refer to the position of analogous residues in TcAChE, according to the recommended nomenclature (Massoulie et al., 1992).

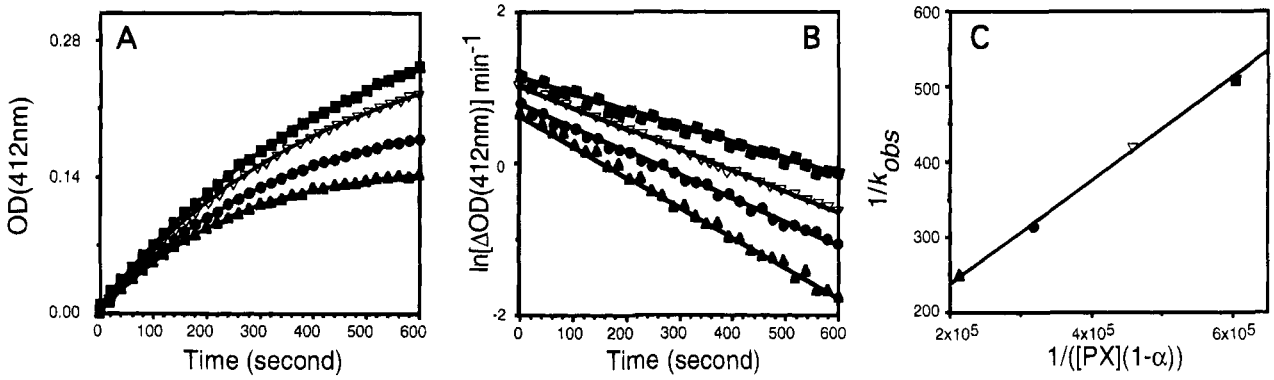


FIGURE 1: Progressive inhibition curves and derivation of the kinetic parameters for phosphorylation of HuAChE by paraoxon in presence of substrate (method B; see Experimental Procedures). Concentrations of inhibitor shown are 7×10^{-6} M (■), 9×10^{-6} M (▽), 1.25×10^{-5} M (●), and 1.75×10^{-5} M (▲). (A) Time course of hydrolysis of ATC in the presence of paraoxon. (B) Linear time dependence of the slopes (ln values) of the progressive inhibition curves shown in panel A. The slopes (Δ ln $v/\Delta t$) of these plots yield the values of k_{obs} for the corresponding concentrations of inhibitor. (C) Double-reciprocal plot of k_{obs} vs inhibitor concentration corrected for the presence of substrate ($1 - \alpha$; where $\alpha = [S]/K_m + [S]$). The plot allows us to derive the ratios K_d/k_2 and $1/k_2$ (see Scheme 1) from the slope and the intercept, respectively.

Determination of HuAChE Activity and Analysis of Kinetic Data. Catalytic activities of the recombinant HuAChE and its mutant derivatives collected from transiently or stably transfected cells were assayed according to Ellman et al. (1961). Assays were performed in the presence of 0.1 mg/mL BSA, 0.3 mM DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] in 5 or 50 mM sodium phosphate buffer, pH 8.0, and varying ATC (0.01−0.6 mM) concentrations. The assays were carried out at 27 °C and monitored by a Thermomax microplate reader (Molecular Devices). Michaelis−Menten constants (K_m) and the apparent first-order rate constants (k_{cat}) were determined as described before (Shafferman et al., 1992a,b). The apparent bimolecular rate constants (k_{app}) were calculated from the ratio k_{cat}/K_m.

Kinetic data for inhibition by propidium and determination of the competitive inhibition constants (K_i) were derived as described previously (Ordentlich et al., 1993, 1995) according to the kinetic treatment developed by Barnet and Rosenberry (1977) and Berman and Leonard (1990).

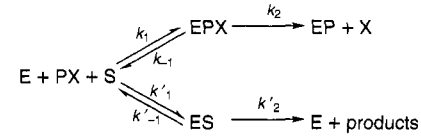
The apparent bimolecular rate constants for the irreversible inhibition of HuAChE enzymes by organophosphates DFP and paraoxon (k_i) were determined by two methods:

Method A. Phosphorylation under pseudo-first-order conditions, with respect to the inhibitor concentrations, was carried out, and the residual enzymatic activity was monitored at various times. The bimolecular rate constants k_i^A were computed from slopes of ln *E versus* time at different inhibitor concentrations (*E* is the concentration of free enzyme).

Method B. Determination of the bimolecular rate constants for phosphorylation (k_i^B) was carried out following the double-reciprocal method of Hart and O'Brien (1973). Apart from bimolecular rate constants, this method allows evaluation of the dissociation constant for EPX, the noncovalent enzyme−inhibitor complex ($K_d = k_{-1}/k_1$), and the phosphorylation rate constant of the reaction (k_2, see Scheme 1). The enzyme is reacted simultaneously with an excess of inhibitor and substrate that ensures pseudo-first-order conditions with respect to both reactions. The kinetic data were analyzed according to the reaction depicted in Scheme 1.

A typical progressive inhibition curve is shown in Figure 1A. Slopes of the tangents at each 20 s were obtained as part of fitting a cubic spline curve through the experimental

Scheme 1



points (Rogers & Adams, 1990). Semilogarithmic plots of these slopes against time resulted in linear correlations for all the concentrations of the inhibitors used. The slopes (Δ ln $v/\Delta t$) were determined by linear regression (Figure 1B). These values are related to the kinetic parameters of the inhibition process according to the expression:

$$\frac{1}{k_{obs}} = \frac{K_d}{k_2}\frac{1}{[PX](1-\alpha)} + \frac{1}{k_2} \qquad \alpha = \frac{[S]}{K_m + [S]}$$

where [PX] is the inhibitor concentration. Plotting $\Delta t/\Delta$ ln v against $1/[PX](1 - \alpha)$ yields the ratios K_d/k_2 and $1/k_2$ as the slope and the ordinate intercept (Figure 1C), respectively.

Equilibrium Fluorescence Titrations. Equilibrium titrations of purified W86A, D74N, Y133A, Y341A, and Y72A/W286A enzymes with propidium were carried out essentially as described before (Taylor et al., 1974; Taylor & Lappi, 1975). A SLM 500 spectrofluorometer was used to monitor ligand fluorescence at 625 nm, upon excitation at 535 nm. All fluorescence titrations were performed at 1 mM sodium phosphate buffer, pH 8.0, at 25 ± 0.2 °C. Dissociation constants (K_D) were obtained from Scatchard plots. The concentrations of free (P_f) and bound (P_b) propidium are obtained from the relations (Mooser et al., 1972):

$$[P_f] = (I_o - \gamma'[P_o])/(\gamma - \gamma') \qquad [P_b] = [P_o] - [P_f]$$

where I_o is the observed fluorescence intensity, $[P_o]$ is the total concentration of propidium, and γ and γ' are fluorescence proportionality coefficients of free and bound propidium, respectively (Taylor et al., 1974). The coefficient γ' is evaluated from the initial limiting slope of the fluorescence titration curve where the concentration of the enzyme is much higher than that of propidium. In all cases, except except for one of the mutants, a saturable profile of the fluorescence titration curves was obtained. The contribu-

tion of nonspecific binding was estimated from fluorescence titration of the wild-type enzyme in the presence of 10-fold molar excess of the nonfluorescent PAS ligand BW284C51.

Generation of Alternative Conformations of HuAChE by Molecular Simulations. All simulations were performed on a SUN-Sparc 10 workstation using the AMBER 4.01 suite of programs with the all-atom parameter set (Pearlman et al., 1991). Characterization and visual examination of the molecular structures were done using the molecular modeling package SYBYL 6.03 (Tripos, 1994) running on an IRIS 4D/70GT workstation. The starting conformation of the enzyme was the HuAChE model (Barak et al., 1992). The cysteine loop (Cys69−Cys96) as well as the active center gorge was solvated by adding a spherical cap of water (using the SOL option in AMBER, a total of 619 molecules were added). The cap waters were restrained by soft half-harmonic potential to avoid evaporation without affecting the protein movement. The HuAChE residues included in the simulation (using the belly option in AMBER) were comprised of the cysteine loop (Cys69−Cys96) as well as residues in or around the active site gorge (a total of 96 residues). The simulation protocol consisted of the following: (a) initial solvent relaxation followed by minimization of the entire structure (Laughton, 1994); (b) gradual heating to 1000 K (at the rate of 50 K/3 ps) and a production run of molecular dynamics carried out for 60 ps, with weak coupling to a temperature bath (Björkstén et al., 1994); (c) from different starting points along this trajectory, the system was cooled down to 300 K (at a rate of 50 K/3 ps; Brünger & Krukowski, 1990); (d) the structural stability of the resulting structures was assessed via a molecular dynamics run at 300 K for 50 ps, followed by averaging of the structures (using the MDANAL module of AMBER) and minimization.

RESULTS

Effects of Peripheral and Active Center Anionic Site Mutations on Inhibition and Binding by Propidium. Recently several residues, at the rim of the AChE active site gorge, were identified as constituents of the PAS array, participating in interactions with a variety of PAS ligands (Shafferman et al., 1992a,b; Radic et al., 1993; Barak et al., 1994). The molecular model of the HuAChE−propidium complex, constructed according to these studies, indicates that the inhibitor does not interact with the active center residues Trp86 and Tyr133 (the tetraalkylammonium group of the ligand is 9 Å away from Trp86; Barak et al., 1994). However, replacement of these residues by aliphatic amino acids resulted in enzymes almost refractory to inhibition by propidium. It was therefore suggested that the aromatic residue at position 86 is involved in the inhibitory process through allosteric interactions with the periphery (Barak et al., 1994; Ordentlich et al., 1995). In order to gain a better understanding of the way propidium exerts its allosteric inhibitory activity, we have studied the binding profiles of propidium to selected HuAChE enzyme derivatives. The experimental method utilizes the spectroscopic characteristics of propidium which upon complexation with AChE shows a significant red shift in its absorption spectrum concomitant with fluorescent enhancement (Taylor et al., 1974). These properties were used in the past (Taylor & Lappi, 1975; Berman et al., 1981; Radic et al., 1991) to demonstrate a correspondence between the kinetic value of the inhibition

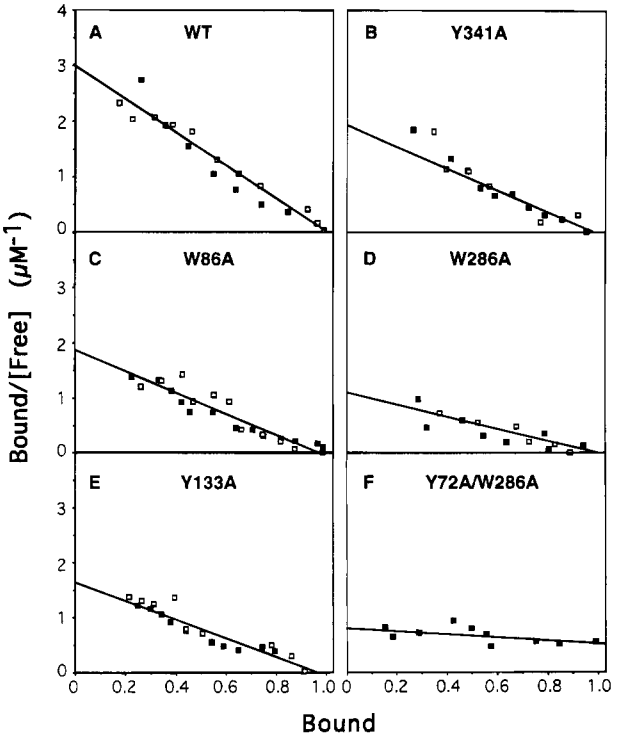


FIGURE 2: Scatchard plots of propidium association with wild-type HuAChE and some of its derivatives. The enzyme identity and the concentrations of enzyme used in the titrations are shown in the corresponding panels. For the double mutant Y72A/W286A (panel F), titration at the lower enzyme concentration did not exhibit measurable binding and was identical to that measured for the wild-type enzyme in presence of excess BW284C51. Enzyme concentration range: empty squares (0.6−0.8 μM); full squares (1.6−2.0 μM).

constant (K_i) and the value of the dissociation constant (K_D) for interaction of propidium with TcAChE.

Titrations were performed on HuAChE enzymes carrying replacement of residues at either the active center or the PAS and compared to the wild-type enzyme. Since such titrations require purified enzymes in relatively large quantities, we generated stable 293 clone cell lines expressing constitutively high levels of the various HuAChE mutants. The screening for high-producer cell clones of W286A, D74N, Y341A, and Y72A/W286A enzymes was possible through quantitative measurements of the catalytic activity, while for those expressing the relatively unreactive W86A and Y133A enzymes through quantitative immunological assays (ELISA). Since most of the mutants were not bound to the procainamide affinity columns, which are usually employed for rHuAChE purification (Kronman et al., 1992), an alternative affinity column was developed, based on monoclonal antibodies (AE-1) as described under Experimental Procedures.

The concentrations of purified enzymes used in the fluorescence titrations with propidium were in the range of $(0.6-2.0) \times 10^{-6}$ mol equiv of HuAChE monomeric subunit. With the exception of the double mutant Y72A/W286A, the concentrations of the enzyme used corresponded within ±5% to the concentrations derived from the inflection points in the fluorescence titration curves (Taylor et al., 1974). This stoichiometric ratio is clearly demonstrated in the Scatchard plots (Figure 2), again with the exception of the double mutant (Figure 2F). We note that in the case of Y72A/W286A enzyme saturation of binding was not observed in the fluorescence titration curve, even at high propidium

Table 1: Dissociation Constants and Competitive Inhibition Constants of Propidium for HuAChE and Its Derivatives

HuAChEs	K_D (μM)[a]	K_i (μM)[b]
wild type	0.26 ± 0.07[c]	0.20 ± 0.02
W86A	0.48 ± 0.20	70 ± 35
Y133A	0.47 ± 0.17	5.0 ± 2.5
D74N	0.42 ± 0.24	0.83 ± 0.07
Y341A	0.30 ± 0.14	0.60 ± 0.10
W286A	1.00 ± 0.16[d]	2.15 ± 0.15
Y72A/W286A	≥3.1[e]	35 ± 5.0

[a] Values calculated from Scatchard plots of six independent measurements at different concentrations of catalytic subunits (0.6−2.0 μM); ± refers to standard deviations (Figure 2). [b] Values represent mean of triplicate determinations. [c] Value in agreement with that reported by Barak et al. (1994). [d] Reported by Barak et al. (1994). [e] This lower limit value corresponds closely to that obtained for the wild-type HuAChE in the presence of a large excess of the PAS ligand BW284C51 (see text).

concentrations (up to 8 μM), and from the corresponding Scatchard plot, it appears that the value of K_D is at least 10-fold higher than that of the wild-type enzyme. Results similar to those for the double mutant were obtained in control experiments where fluorescence titrations of the wild-type enzyme were carried out in the presence of saturating concentrations of the PAS bisquaternary ligand BW284C51, which should effectively compete out the specific binding of propidium to the PAS. The propidium fluorescence enhancement, under such conditions, was therefore regarded as nonspecific binding, and it determined the upper limit of the method for evaluation of the dissociation constants.

To evaluate the correlation between propidium inhibitory activity and its affinity toward the various HuAChE enzymes, inhibition experiments were carried out at an ionic strength similar to that used for the binding studies. Resulting values of the inhibition constants, K_i (Table 1), are an order of magnitude lower than those evaluated previously at higher buffer concentration (Barak et al., 1994). The average K_D value for the wild-type HuAChE (0.26 μM) is similar to the values reported for TcAChE (0.1−0.37 μM) under similar ionic strength conditions (1 mM Tris buffer, pH 8; Taylor et al., 1974; Taylor & Lappi, 1975; Radic et al., 1991), and is in excellent agreement with the corresponding value of K_i (Table 1). It may be noted that the values of the dissociation constants K_D, determined for TcAChE at high ionic strength (3.4−4.7 μM; Berman et al., 1987; Berman & Leonard, 1992), are about an order of magnitude higher. This behavior is consistent with the effect of ionic strength on the values of inhibition constants K_i and indicates the electrostatic nature of the interaction of the positively charged ligand with the negative electrostatic field of the enzyme surface.

Examination of the corresponding values of K_D and K_i for the HuAChE enzymes reveals a clear distinction between the behavior of enzymes carrying replacements at the PAS and those enzymes mutated at the active center (positions 86 and 133). For the D74N and Y341A mutant HuAChEs, the minor effects on inhibition constants K_i, relative to the wild-type HuAChE, are reflected by comparable changes in the values of K_D. In the case of W286A, the 10-fold increase in K_i corresponds to the 4-fold increase in the value of K_D. The 170-fold increase in the value of K_i, for the double mutant Y72A/W286A, is again in complete accordance with our inability to measure high K_D values by the fluorescence titration method. Although suggested many times in the past, the identity of the locus interacting with propidium, at the AChE surface, was never before established by a direct measurement. The variation of the K_D values, for HuAChE enzymes carrying replacements at the periphery, demonstrates that residues Trp286, Tyr72, and Asp74 indeed constitute the binding site of propidium. Furthermore, the correspondence of the respective K_D and K_i values, for these HuAChE enzymes, demonstrates that the changes in the inhibitory activity of propidium are a direct outcome of an altered binding to the PAS. The aromatic residues, at positions 72, 124, and 286, were also identified recently as constituents of the binding site for fasciculin (Radic et al., 1994), a PAS-specific ligand competitive with propidium.

In contrast with the good correspondence between K_D and K_i values observed for the HuAChE PAS mutants, for the HuAChE enzymes carrying replacements at the active center, the values of K_D are similar to that of the wild-type HuAChE (less than 2-fold increase). Yet K_i values are 350-fold (for W86A) and 25-fold (for Y133A) higher than that for the wild-type enzyme. Consequently, these results demonstrate that the resistance of the active center mutants W86A and Y133A to inhibition by propidium *is not a result of reduced binding but rather of an impaired inhibition mechanism.*

Effects of Replacement of Trp86 and Tyr133 on Reactivity toward Organophosphates. Replacement of Tyr133 by alanine, but not by phenylalanine, was shown to compromise the reactivity of the corresponding HuAChE toward both charged and noncharged substrates (Ordentlich et al., 1995). We have recently suggested that such reduced reactivity is due to a conformational transition of residue Trp86 in the active center of the Y133A HuAChE mutant enzyme, resulting in destabilization of the initial noncovalent complexes with substrates (Ordentlich et al., 1995). A similar transition was invoked with respect to the mechanism of inhibition induced by binding of propidium (Ordentlich et al., 1995). To further investigate this putative mechanism, we studied in detail the reactions of organophosphate covalent inhibitors with HuAChE enzymes carrying mutations at positions 86 and 133. The organophosphate inhibitors DFP and paraoxon associate with AChE through formation of transient noncovalent complexes, followed by covalent attachment to the catalytic serine resulting in stable tetrahedral adducts. These reactions are analogous to the acylation step in AChE reaction with substrates and therefore should be similarly affected by structural changes in the active center. Treatment of the irreversible inhibition kinetic data, according to the method by Hart and O'Brien (1973; method B, see Experimental Procedures), allows evaluation of the dissociation constants (K_d) of the initial noncovalent complexes EPX as well as the phosphorylation rate constants k_2 (see Scheme 1). Since in this method the K_d values are calculated from the apparent bimolecular rate constant of inhibition ($k_i^B = k_2/K_d$), we confirmed these values by an alternative method (method A; see Experimental Procedures) where the bimolecular rates were determined under pseudo-first-order conditions with respect to the inhibitor concentrations. Kinetics of phosphorylation reactions were studied for the wild-type, Y133A, Y133F, and W86A HuAChE enzymes. According to our model, the affinity of the Y133A enzyme toward the organophosphate derivatives should be much lower (higher values of K_d) compared either to that of

Table 2: Kinetic Constants for Phosphorylation of HuAChE and Its Derivative by DFP[a]

HuAChEs	k_i^A [b] ($\times 10^{-4}$ M^{-1} min^{-1})	k_i^B ($\times 10^{-4}$ M^{-1} min^{-1})	K_d (μM)	k_2 (min^{-1})
wild type	14.0 ± 1.0	8.4 ± 0.3	11.9 ± 1.4	1.0 ± 0.1
W86A	2.6 ± 0.1	6.5 ± 0.13	11.3 ± 0.46	0.73 ± 0.02
Y133A	0.03 ± 0.01	0.05 ± 0.004	275 ± 45	0.15 ± 0.02
Y133F	1.4 ± 0.07	4.6 ± 0.1	9.0 ± 0.4	0.41 ± 0.01

[a] k_i^A and k_i^B are the bimolecular rate constants for phosphorylation determined by methods A and B, respectively (see Experimental Procedures); K_d and k_2 can be derived only by method B. [b] Values are in agreement with those reported by Ordentlich et al. (1995).

Table 3: Kinetic Constants for Phosphorylation of HuAChE and Its Derivative by Paraoxon[a]

HuAChEs	k_i^A ($\times 10^{-4}$ M^{-1} min^{-1})	k_i^B ($\times 10^{-4}$ M^{-1} min^{-1})	K_d (μM)	k_2 (min^{-1})
wild type	96.6 ± 1.6	96.7 ± 2.2	0.75 ± 0.1	0.72 ± 0.1
W86A	5.0 ± 0.7	7.5 ± 0.3	11.7 ± 1.8	0.88 ± 0.16
Y133A	0.16 ± 0.01	0.12 ± 0.01	388 ± 42	0.48 ± 0.04
Y133F	12.5 ± 1.0	20.5 ± 0.74	4.3 ± 0.5	0.87 ± 0.16

[a] k_i^A and k_i^B are the bimolecular rate constants for phosphorylation determined by methods A and B, respectively (see Experimental Procedures); K_d and k_2 can be derived only by method B.

the wild-type HuAChE or to those of Y133F and W86A enzymes.

The values of bimolecular inhibition rate constants k_i^A and k_i^B for either DFP (Table 2) or paraoxon (Table 3), as determined by the two independent methods, are very similar. This similarity corroborates the reliability of the values for K_d and k_2 derived from k_i^B. Examination of the K_d values for the HuAChE enzymes shows, as predicted, that while W86A and Y133F exhibit nearly wild-type-like affinity toward DFP, that of Y133A is 25-fold lower. An even more dramatic increase of the K_d value for Y133A HuAChE is observed in the case of paraoxon (517-fold). However, in this case, the respective K_d values are also somewhat higher for both W86A and Y133F (15-fold and 6-fold, respectively), indicating that for paraoxon the steric requirements during complex formation with the enzyme are more stringent. The values of phosphorylation rate constants k_2 show little variation irrespective of the inhibitor or the HuAChE enzyme tested. The most notable deviation from the wild-type HuAChE value is the 7-fold decrease of k_2 for the phosphorylation of Y133A enzyme by DFP. This finding suggests equivalent orientation of the inhibitor, relative to the nucleophilic serine, in the noncovalent complexes with the HuAChE enzymes.

The reactivity patterns for Y133A and Y133F enzymes toward organophosphates confirm our predictions and are consistent with the molecular models of Y133A and Y133F mutants (Ordentlich et al., 1995). According to these models, the only major difference, distinguishing the Y133A mutant from Y133F or the wild-type enzymes, is the altered conformation of residue Trp86 in the active center.

Reactivity of the HuAChE Double Mutant W86A/Y133A toward Charged and Noncharged Substrates. If as we propose the main effect of replacing Tyr133 by alanine is steric occlusion of the active center through conformational transition of Trp86, it should be prevented by removal of the bulky indole side chain from position 86 in the Y133A mutant. In addition, the resulting double mutant HuAChE would lack the anionic subsite in the active center.

Table 4: Kinetic Constants for ATC and TB Hydrolysis by W86A/Y133A HuAChE Compared to Enzymes Substituted at Positions 86 and 133[a]

HuAChEs	K_m (mM)		k_{cat} ($\times 10^{-5}$ min^{-1})	
	ATC	TB	ATC	TB
wild type[b]	0.14	0.30	4.0	0.55
Y133A[b]	26.0	[c]	0.4	[c]
W86A[b]	93.0	0.55	0.8	0.18
W86A/Y133A	[c]	0.07	[c]	0.005

[a] Values represent the mean of triplicate determinations with the standard deviation not exceeding 20%. [b] Values correspond closely to those reported by Ordentlich et al. (1993) and Ordentlich et al. (1995). [c] Activity could not be detected.

Indeed, generation of the W86A/Y133A enzyme and evaluation of its hydrolytic activity demonstrate that, like the W86A enzyme, the double mutant is not active toward the charged substrate ATC (Table 4). Furthermore, the W86A/Y133A enzyme exhibits hydrolytic activity toward the noncharged substrate TB with a K_m value similar to that of the wild-type enzyme (Table 4). Thus, the reactivity pattern of the double mutant W86A/Y133A is clearly distinct from that of the Y133A enzyme for which no reactivity toward TB could be observed. It is interesting to note that the double replacement, at positions 86 and 133, has a substantial effect on the catalytic machinery as manifested by the 100-fold decrease of the k_{cat} value, relative to the wild-type HuAChE (Table 4). In part, this may be related to the removal of the H-bond acceptor moiety from position 133 since the corresponding k_{cat} for the Y133F enzyme is 10-fold lower (Ordentlich et al., 1995). Thus, the W86A/Y133A enzyme appears to exhibit binding characteristics that discriminate between charged and noncharged ligands, resembling that of the W86A HuAChE. The fact that substitution of Trp86 by alanine within the Y133A enzyme *restores* the catalytic activity toward TB and in particular that the value of K_m, of the W86A/Y133A mutant, is similar to that of the wild-type enzyme is consistent with the notion that Trp86 is indeed involved in restricting the access to the active center of the Y133A HuAChE. This is further demonstrated by the increase of K_d values for the organophosphate inhibitors, in the case of the Y133A enzyme.

Alternative Conformations of the Cysteine Loop (Cys69–Cys96) in HuAChE. Residue Trp86, and Asp74 which is an important constituent of the PAS (Barak et al., 1994), is part of the sequence comprising the cysteine loop (Cys69–Cys96). The notion that the conformational mobility of residue Trp86 may be coupled to the occupation of the PAS, through rearrangement of this loop (Ordentlich et al., 1995) prompted us to examine its conformational mobility. The conformational properties of the cysteine loop (Cys69–Cys96) in HuAChE were assessed by simulated annealing. Analysis of the several structures, resulting from the simulation protocol (see Experimental Procedures), shows that the loop can assume a range of similar conformations that are different from that of the initial HuAChE model. One of these conformations is shown in Figure 3. The results indicate that (a) the cysteine loop (Cys69–Cys96) is the most mobile part of the structure, (b) the observed conformational changes affect the positioning of Trp86, abolishing its function as the "anionic" locus in the active center, and most significantly (c) the enhanced mobility is always confined to the same loop segment, although the simulated confor-

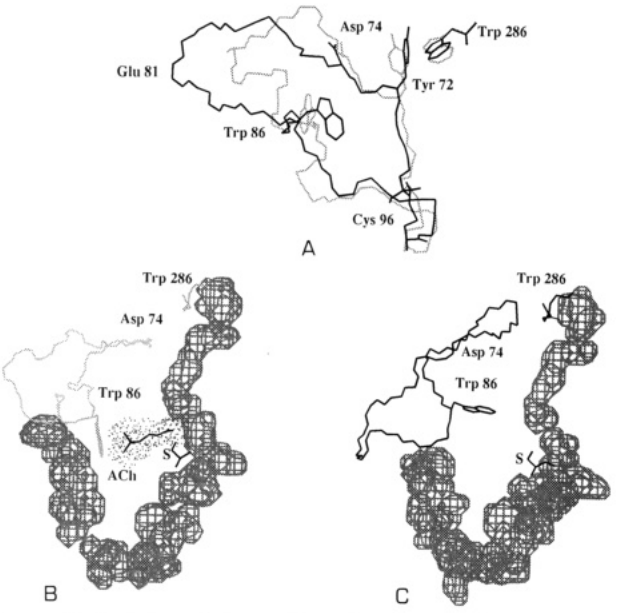


FIGURE 3: Relation of the conformational state of residue Trp86 to the overall conformation of the cysteine loop (Cys69−Cys96) and its effect on accessibility of the HuAChE active center. (A) Superposition of the conformation derived from the X-ray structure of AChE (light line) and a representative computed alternative conformation (heavy line) of the loop obtained from simulated annealing of HuAChE (Ariel et al., in preparation). Note that the major movement occurs in the stretch spanning 16 amino acids from Asp74 to Arg90. The position of the indole moiety of Trp86 relative to the active site gorge is provided in panels B and C, where a side view cross-section of the gorge is represented by volume contours along with the respective conformation of the loop. (B) The functional conformation of Trp86, allowing the substrate ACh (shown with its van der Waals volume) to access the active center and interact favorably with the indole moiety (anionic site). S denotes the location of the active site Ser203. (C) Nonfunctional conformation of Trp86, blocking access of substrates and other ligands (e.g., DFP, paraoxon, etc.) to the active center.

mational changes cannot be classified as a rigid-body motion. A detailed analysis of the simulated structures of HuAChE is to be presented elsewhere (Ariel et al., in preparation).

DISCUSSION

How Does Propidium Inhibit HuAChE? The molecular mechanism of allosteric modulation of AChE activity, following ligand binding to peripheral sites, has been a subject of considerable interest ever since it was proposed almost 30 years ago (Changeux, 1966). Early studies established that structurally diverse ligands like propidium, tubocurarine, and gallamine can occupy binding sites which are distinct from the active site, all eliciting similar but not identical changes in the active center (Berman et al., 1981). These changes were associated with the inhibitory effects on AChE catalytic activity and were analyzed in terms of two-state models whereby the ligands alter the distribution between reactive and unreactive conformations of the enzyme (Pattison & Bernhard, 1978; Tomlinson et al., 1980). Recently, the involvement of specific residues in the binding site array, referred collectively as PAS, has been suggested by site-directed mutagenesis and kinetic studies of inhibition by PAS-specific ligands as well as by X-ray crystallography (Shafferman et al., 1992a; Radic et al., 1993; Harel et al., 1993; Barak et al., 1994). Elements of this array are located at or near the rim of the active site gorge and include Trp286 and a cluster of vicinal residues (Tyr72, Tyr124, and Glu285) as well as residues Asp74 and Tyr341 located on the other side of the gorge entrance. A common feature of these sites is a core comprised of residues Trp286 and Asp74 (Shafferman et al., 1992a; Barak et al., 1994). The propidium fluorescence titration experiments of D74N, W286A, Y341A, and Y72A/W286A HuAChE enzymes reported here (Figure 2) demonstrate the correlation between affinities toward the modified PAS sites and the inhibitory activity (Table 1). This correlation provides direct evidence that inhibition by propidium results from specific binding to a single PAS site and is consistent with the proposed molecular model of the HuAChE−propidium complex (Barak et al., 1994). On the other hand, neither the value of K_D, from fluorescence titration experiments, nor the molecular model is consistent with the previously observed resistance of W86A HuAChE toward inhibition by propidium (Ordentlich et al., 1993; Table 1). To account for this discrepancy, the inhibition characteristics of W86A HuAChE, together with the conformational flexibility of the Trp86 side chain, were interpreted as an indication of a functional "cross-talk" between the periphery and the active center whereby the conformational state of Trp86 depends on the interaction of ligands with the remote PAS (Ordentlich et al., 1995). An alternative explanation can be offered, in which propidium interacts directly with Trp86. This is inconsistent with the relative affinities of propidium and of its decamethylene homolog decidium toward the AChE−pyrenebutyl methylphosphonate adduct (Berman et al., 1987). The affinity of decidium, which interacts with both the active center and the PAS, toward the free AChE is 100-fold higher than toward the adduct. Yet propidium was shown, in that study, to bind with equivalent affinity to both the adduct and the free AChE. This comparison demonstrates that the affinity toward decidium, but not toward propidium, is affected by steric occlusion of the active center by the bulky phosphonate moiety, indicating that propidium indeed does not interact with the active center residue Trp86. This conclusion is further supported by the failure of reversible active center ligands like edrophonium and *N*-methylacridinium to displace propidium (Taylor & Radic, 1994).

Due to the location of the PAS, near the rim of the active site gorge, the bound ligand may hinder the access of substrates to the active center by physical obstruction of the gorge entrance and/or by charge repulsion imparted by the association of the cationic ligand. While this suggestion is consistent with the molecular model of the propidium−HuAChE complex in which the gorge entrance is indeed blocked by the ligand (Barak et al., 1994), it is not supported by the available experimental data, pertaining to inhibition of AChE by propidium and other reactivity characteristics of the enzyme. The rate of phosphonylation of AChE, by noncharged methylphosphonates, is only marginally affected by addition of saturating concentrations of propidium (Berman & Leonard, 1990). In addition, AChE complexed with the slowly dissociating PAS ligand fasciculin can still react with the phosphorylating agent DFP (Marchot et al., 1993). Finally, as indicated by the corresponding value of K_D, binding of propidium does not affect significantly the reactivity of W86A enzyme toward charged and noncharged substrates, although the location of the bound ligand relative to the gorge entrance should resemble that of the wild-type HuAChE complex. Thus, it appears that steric obstruction of the inward path for substrates and inhibitors does not

contribute in a major way to the inhibitory effect of propidium. Finally, the lack of effect of propidium on the reactivity of the W86A HuAChE toward ATC indicates also that charge repulsion is of no consequence to the propidium inhibitory mechanism. This conclusion is also supported by the reported reactivities of AChEs, chemically labeled at the PAS by charged reagents (Haas et al., 1992; Duran et al., 1994), toward ATC.

Role of Residue Trp86 in Allosteric Modulation of HuAChE Reactivity. As suggested recently, on the basis of molecular modeling of the wild type, for the W86F and the W86A enzymes, the side chain of Trp86 can occupy two conformational states: one that is functional as the anionic subsite while the other occludes the active center and should reduce catalytic activity (Ordentlich et al., 1995). Induction of conformational change of Trp86 by occupation of the PAS is consistent with our results as well as with the report regarding the spectral changes induced in the pyrenebutyl phosphonate−AChE adduct that arise from occupation of the PAS (Berman et al., 1981). The increase in the polarizability of the pyrenebutyl environment was interpreted as resulting from torsional movements of aromatic side chains. These studies imply not only that the conformational change of Trp86 is coupled to binding of the PAS ligand but also that such change can be induced irrespective of the state of occupation of the active center.

We proposed (Ordentlich et al., 1995) that the involvement of residue Trp86 in the allosteric mechanism of full or partial inhibition of the enzyme is apparently through destabilization of the noncovalent complexes which are formed, in the active center, following the initial enzyme−ligand encounter. The reactivity properties of the Y133A and Y133F enzymes, and in particular those of the double mutant W86A/Y133A HuAChE, appear to support this hypothesis. The affinity of Y133A HuAChE toward ATC is considerably reduced, relative to the wild-type enzyme, as is evident from the 185-fold respective increase in the value of K_m. In addition, this enzyme is refractory to inhibition by the active center ligand edrophonium (Ordentlich et al., 1995). If this decrease in affinity results from steric occlusion of the active center, a similar effect could be expected for the noncharged substrates and inhibitors. Indeed, the bimolecular rate constants of phosphorylation of the Y133A enzyme (but not of the Y133F or W86A enzymes) by two organophosphate derivatives, DFP and paraoxon, are 170- and 805-fold lower, respectively, compared to those of the wild-type enzyme, while corresponding values of the dissociation constants of the noncovalent complexes (K_d) are 23- and 517-fold higher. Thus, the reduced reactivity of Y133A toward noncharged ligands is largely due to the relative destabilization of the initial noncovalent complexes.

Since an altered conformation of the Trp86 side chain is the only major difference between the modeled structures of Y133A and the wild-type HuAChE, it could be expected that other structural elements of the active center should not contribute significantly to such interference. In this case, one could expect that removal of the bulky residue from position 86, in both the Y133A enzyme and the HuAChE−propidium complex, should relieve the steric occlusion at the active center and restore reactivity toward noncharged, but not toward charged, ligands. Such expectation is borne out both by the observed behavior of the W86A−propidium complex toward TB (Ordentlich et al., 1993) and by the reactivity of the W86A and W86A/Y133A enzymes toward this substrate (Table 4, K_m values similar to that of the wild-type enzyme).

Although the conformational flexibility of Trp86 and its effects on the catalytic activity provide a very plausible mechanism for the "cross-talk" between the peripheral sites and the active center, the relay path of the allosteric signal is still unclear. Clues to a possible way for signalling the incidence of binding at the periphery to the active center and for inducing motion of Trp86 can be found in the fact that the central binding element of the peripheral site, Asp74, which was also implicated in the "cross-talk" mechanism (Shafferman et al., 1992b), is part of the sequence comprising the cysteine loop (Cys69−Cys96) (see Figure 3A) which also includes Trp86. It is possible that the alternative conformation of Trp86 is not a result of a local motion of the indole moiety but rather a part of a larger conformational change involving the entire cysteine loop (Cys69−Cys96). This loop, which constitutes the thin portion of the gorge wall in AChE, is a structurally conserved element (loop $L_{b3,2}$; Cygler et al., 1993) in the lipase/esterase family of proteins sharing the α/β-hydrolase fold (Ollis et al., 1992). In lipases from various sources, conformational flexibility of the corresponding loops was implicated in the mechanisms of interfacial activation and substrate accommodation. Moreover, participation of surface loops in conformational changes, upon association with substrates or inhibitors, has been observed in a wide variety of biochemical systems (Kempner 1993; Gerstein et al., 1994). The possible participation of the cysteine loop (Cys69−Cys96) of AChE in some sort of conformational adjustment is suggested both by virtue of the evolutionary constraint on the conservation of this structural motif in the lipase/esterase family (Ollis et al., 1992) and by the X-ray structure of TcAChE which indicates that such adjustment may be essential for the enzymatic activity (Axelsen et al., 1994). The crystallographic dimensions of the active site gorge (Sussman et al., 1991) should preclude the access of ACh or larger ligands, like THA (Harel et al., 1993), to the active center *in contrast* to the experimental evidence. Furthermore, according to the model of the HuAChE−propidium complex (Barak et al., 1994), built on the basis of these X-ray results, occupation of the PAS by either propidium or fasciculin should block the gorge entrance, again in contrast to experimental observations (Radic et al., 1994). Indeed, molecular dynamics simulations of TcAChE (Axelsen et al., 1994) as well as our preliminary results from simulated annealing suggest that the structure of unliganded enzyme differs from that observed in X-ray crystallography (Figure 3). It is possible that the crystallographic structure may represent the ligand-bound enzyme since it remains virtually unchanged in complexes with edrophonium and THA (Harel et al., 1993; Axelsen et al., 1994). In conclusion, it appears that AChE can exist in at least two conformational states differing with respect to the shape and dimensions of the entrance to the active site gorge. Interaction with charged substrates or active center inhibitors will stabilize the catalytically functional state, as reflected in the X-ray structure (Sussman et al., 1991; and Figure 3B), while association with PAS ligands such as propidium may shift the equilibrium in the direction of a nonfunctional state (Figure 3C). Although the extent of structural difference between the functional and the nonfunctional states is not

known, kinetic studies and molecular dynamics simulations (Ariel et al., in preparation) suggest that the main difference, in the architecture of the active center, is in the conformational state of residue Trp86. From structural analogies to other enzymes of the lipase/esterase family and from molecular dynamics simulations, it appears that the surface loop $L_{b3,2}$ is one of the most mobile structural motifs in AChE and that this mobility constitutes part of the transition between the reactive and the nonreactive states. Thus, the relay path of the allosteric signal, in the cross-talk between the periphery and the active center, may be comprised of shifting the *natural* equilibrium between the different conformational states of the cysteine loop (Cys69−Cys96) in AChE.

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