The contribution of the oxanion hole to the functional architecture and to the hydrolytic efficiency of human acetylcholinesterase (HuAChE) was investigated through single replacements of its elements, residues Gly-121, Gly-122 and the adjacent residue Gly-120, by alanine. All three substitutions resulted in about 100-fold decrease of the bimolecular rate constants for hydrolysis of acetylthiocholine; however, whereas replacements of Gly-120 and Gly-121 affected only the turnover number, mutation of residue Gly-122 had an effect also on the Michaelis constant. The differential behavior of the G121A and G122A enzymes was manifested also toward the transition state analog m-(N,N,N-trimethylammonio)trifluoroacetophenone (TMTFA), organophosphorus inhibitors, carbamates, and toward selected noncovalent active center ligands. Reactivity of both mutants toward TMTFA was 2000–11,000-fold lower than that of the wild type HuAChE; however, the G121A enzyme exhibited a rapid inhibition pattern, as opposed to the slow binding kinetics shown by the G122A enzyme. For both phosphates (diethyl phosphorofluoridate, diisopropyl phosphorofluoridate, and paraaxon) and phosphonates (sarin and soman), the decrease in inhibitory activity toward the G121A enzyme was very substantial (2000–7000-fold), irrespective of size of the alkoxy substituents on the phosphorus atom. On the other hand, for the G122A HuAChE the relative decline in reactivity toward phosphonates (500–460-fold) differed from that toward the phosphates (12–95-fold). Although formation of Michaelis complexes with substrates does not seem to involve significant interaction with the oxanion hole, interactions with this motif are a major stabilizing element in accommodation of covalent inhibitors like organophosphates or carbamates. These observations and molecular modeling suggest that replacements of residues Gly-120 or Gly-121 by alanine alter the structure of the oxanion hole motif, abolishing the H-bonding capacity of residue at position 121. These mutations weaken the interaction between HuAChE and the various ligands by 2.7–5.0 kcal/mol. In contrast, variations in reactivity due to replacement of residue Gly-122 seem to result from steric hindrance at the active center acyl pocket.

The catalytic efficiency of acetylcholinesterase (AChE, EC 3.1.1.7) and its high reactivity toward a variety of covalent and noncovalent inhibitors seem to originate from the unique architecture of the active center, currently investigated by x-ray crystallography (1–4) and site-directed mutagenesis (5–10). The x-ray structures of AChE are characterized by a deep and narrow “gorge,” which penetrates halfway into the enzyme and contains the catalytic site at about 4 Å from its base. Several functional subsites in the active center gorge were identified, including the catalytic triad (Ser-203(200), His-447(440), and Glu-334(327)) (1, 5, 11, 12), the acyl pocket (Phe-295(288) and Phe-297(290)) (6, 7, 9), and the “hydrophobic subsite.” The latter accommodates the alcohol portion of the covalent adduct (tetrahedral intermediate) and may include residues Trp-86(84), Tyr-133(130), Tyr-337(320), and Phe-338(331), which operate through nonpolar and/or stacking interactions, depending on the substrate (6, 10, 13). Stabilization of the charged moieties of substrates and other ligands at the active center is mediated by cation-π interactions with the residue at position 86 rather than through true ionic interactions (1, 2, 6, 12, 13). Another important component of the AChE active center functional architecture is an arrangement of hydrogen bond donors that can stabilize the tetrahedral transition enzyme-substrate complex through accommodation of the negatively charged carbonyl oxygen (14). Structural and modeling studies (1, 6, 15) and, in particular, the recent solution of the x-ray structure of the transition state analog TMTFA complexed with TcAChE (3) revealed a three-pronged oxanion hole formed by peptidic NH groups Gly-121(118), Gly-122(119), and Ala-204(201), in contrast to the two-pronged oxanion holes in most of serine and cysteine proteases (16). The contribution of the amide nitrogen of Ala-204, rather than that of the catalytic Ser-203, to the oxanion hole is consistent with the reverse handedness of the catalytic triads in AChE compared with serine proteases (1). Residues Gly-121, Gly-122, and Gly-120 are part of a flexible “glycine loop” which constitutes one of the gorge walls adjacent to the catalytic serine and, due to the narrow dimensions of the gorge bottom, should be in contact with most of the AChE noncovalent ligands (1, 2, 4). The notion that, apart from its role in accommodating the oxanion, this loop is one of the important determinants of the active center geometry was recently supported by the x-ray structure of the huperzine A-TcAChE complex (4). In this structure the confor-
mation of Gly-121 is different from that observed in other TaChE-ligand complexes, demonstrating the flexibility of the loop and the extent of its interaction with the ligand. Such conformational mobility of the glycine loop implies that its function may be modified by single replacements of Gly-120(117), Gly-121(118), and Gly-122(119). It was reported (17, 18) that replacements of some of the analogous glycine residues in butyrylcholinesterase resulted in enzyme (G115(117)/A) exhibiting diminished affinity toward tacrine, but not toward BW284C51, or in enzymes (G117(119)/E and the G117(119)/H) retaining a nearly wild type catalytic activity toward butyrylthiocholine (BTC). In HuAChE, our past attempts to introduce large residues (e.g. histidine, glutamate, and serine) at position 122 yielded no protein, underscoring the different active center void volumes of the two enzymes.

Here we describe the construction of HuAChE enzymes with modified oxyanion hole, through substitution of Gly-121 and Gly-122 by alanine, and examine their reactivity toward substrates and a variety of covalent and noncovalent active center inhibitors. We show that reactivity of the mutants is indeed affected mainly by steric and conformational changes in the glycine loop. Furthermore, the similar changes in catalytic activity due to replacement of the adjacent residue Gly-120, which is not part of the oxyanion hole, may be also attributed to conformational mobility of the glycine loop. These results further define the functional architecture of HuAChE-active center and its role in the enzyme reactivity.

EXPERIMENTAL PROCEDURES

**Mutagenesis of Recombinant HuAChE and Production of Mutants—**
Mutagenesis of AChE was performed by DNA cassette replacement into a HuAChE sequence variant (Ew4) which conserves the wild type (22) coding sequence but carries new unique restriction sites (5). Substitution of residues G120A and G121A and G122A was performed by replacement of an EspI-NarI DNA fragment with synthetic DNA duplexes carrying codon GCC(Ala) at the corresponding mutated positions. All the synthetic DNA oligodeoxynucleotides were prepared using the automatic Applied Biosystems DNA synthesizer. The sequences of all new clones were verified by the dideoxy sequencing method (U. S. Biochemical Corp. Sequenase kit). The recombinant HuAChE mutants were expressed in tripartite vectors which allow expression of the cat reporter gene and the neo selection marker (5, 23). Recombinant HuAChE and its mutants were expressed in HEK 293 cells as described previously (24) using stable recombinant cell clones expressing high levels of each of the mutants (23).

**AChE Ligands—**
Structures of the various AChE ligands are shown in Fig. 1. Acetylcholine iodide (ATC), ethyl(d/-hydroxyphenyl)dime-thylammonium chloride (edrophonium), dip(-allyl-N,N-methylamino-phenyl)pentan-3-one (BW284C51), diisopropyl phosphorofluoridate (DFP), p-nitrophenyl diethylphosphate (paraoxon), physostigmine, and pyridostigmine were purchased from Sigma. S-3,3-Dimethylbutyl thioacetate (TB) was synthesized as described previously (6). Diethyl phosphonofluoridate (DEP) was prepared according to the procedure by Saunders and Stacy (19). Preparation of 2-propyl methylphosphonofluoridate (sarin) and 1,2-dimethylpropyl methylphosphonofluoridate (soman) followed an accepted synthetic procedure using methylphosphonofluoridate (20) and the appropriate alcohol. m-(N,N,N-Trimethylammonio)trifluoroacetophenone (TMTFA) was prepared according to the procedure described by Nair et al. (21).

Kinetic Studies and Analysis of Data—AChE activity was assayed according to E llenman et al. (25) (in the presence of 0.1 mg/ml bovine serum albumin, 0.3 mM 5,5'-dithiobis(2-nitrobenzoic acid), 50 mM sodium phosphate buffer, pH 8.0, and various concentrations of ATC), carried out at 27 °C, and monitored by a Thermomax microplate reader (Molecular Devices).

Values of inhibition constants (Ki) for the noncovalent inhibitors edrophonium, tacrine, and huperzine A were determined from the effects of various concentrations of the inhibitor on Km and Vmax, of the enzyme-catalyzed hydrolysis of ATC. All the HuAChE enzymes examined formed rapid equilibria with the tested inhibitors, allowing for an immediate addition of increasing amounts of enzyme to the ATC/inhibitor mixture (preincubation of the enzymes with huperzine A for 10 min, before addition of the substrate, or simultaneous mixing yielded the same results). The values of Ki were computed from the secondary plots of the values of kcat/Km (determined from slopes of 1/V versus 1/[S]) versus concentrations of the respective inhibitors as described previously (26).

\[
E + CX \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{ECX} \rightarrow E + C \quad \text{(Scheme 1)}
\]

The rate constants of progression of the carbamylation reactions (see Scheme 1) were estimated for at least four different concentrations (and at least 10-fold in ligand concentration, around the estimated value of Ki) of carbamate (CX), by adding substrate at various time intervals, and measuring the enzyme residual activity (E). The apparent bimolecular carbamylation rate constants (k1) at different carbamate concentrations, were computed from the plot of slopes of ln(E) versus time. Only the initial slopes were considered in order to minimize the errors due to reactivation of the carbamylated enzymes. Double-reciprocal plot of kcat versus [CX] were used to compute k1 and Kd from the intercept and from the ratio of the slope and the intercept, respectively, according to the following equations: \(1/k_1 = 1/k_{\text{cat}} + 1/k_{\text{cat}}/[CX] \) (27). Note that when \( k_2 \rightarrow 0 \), k1/Kd approaches the value of dissociation constant for the corresponding Michaelis constant.

Determination of the apparent bimolecular rate constants (k1) for the irreversible inhibition of HuAChE enzymes by organophosphates and organophosphonates as well as estimation of the dissociation constants Ki and the first-order phosphorylation rate constants (k2) for paraoxon and DFP were carried out as described before (10, 13).

The apparent first-order rate constants for the time-dependent inhibition of the wild type and the G122A HuAChE enzymes by TMTFA were determined by periodically measuring the initial rate of substrate hydrolysis of aliquots of the reaction mixture. The inhibitor concentrations used were 7.5–75 mM for the wild type and 5.0–50 μM for the G122A HuAChEs. Following the kinetic treatment of Nair et al. (21) and assuming a two-state inhibition mechanism (Scheme 2), the values of kcat and Kd could be estimated from the linear plots of kcat versus inhibitor concentration according to Equation 1.

\[
k_{\text{cat}} = k_{\text{cat}}^{(\text{TMTFA})} + k_{\text{cat}}^{(\text{pr})} \quad \text{(Eq. 1)}
\]

Since in aqueous solution TMTFA is a mixture of the free ketone (TMTFApr) and the ketone hydrate (TMTFAhydr), corrected values of the association rate constants were obtained from kcat = kcat (1 + [TMTFAhydr]/[TMTFApr]), using the ratio of hydrated and ketone forms of TMTFA (62,500), as determined by 31P NMR (21).

To measure directly the values of kcat, the enzyme was inhibited by excess TMTFA (over 90% inhibition), and the mixture was filtered rapidly through a column (Ultratrace-BioMax30k, Millipore) to remove free inhibitor. Regeneration of enzymatic activity for the wild type and the G122A enzymes followed first-order kinetics, yielding values of the dissociation rate constants (koff). For adduct of the G121A HuAChE, activity was completely restored within processing time required for removal of the free inhibitor. TMTFA behaved toward this enzyme as a rapid reversible inhibitor, and the corresponding inhibition constant (K1) could be determined as described above for noncovalent inhibitors.

**Molecular Modeling—**
Models of the tetrahedral adducts of wild type, G120A, G121A, and G122A HuAChEs with ATC and TMTFA were performed on an Indigo 2 workstation using SYBYL modeling software (Tripos Inc.). Initial models of the substrate adducts were constructed as described before (6), and those of TMTFA were built in analogy to the x-ray structure of the TaChE-TMTFA conjugate (3). The resulting structures were optimized by molecular mechanics using the AMBER and the MAXMIN force fields (with AMBER charge parameters for the enzyme). For most of the starting geometries, for adducts of the G120A and G121A enzymes, a conformational flip of residue at position 121 occurred during the optimization process.

RESULTS

**Modification of HuAChE Hydrolitic Activity—**
Replacement of residue Gly-121 by alanine resulted in an enzyme with a 100-fold lower value of the turnover number (kcat) for both ATC and its noncharged analog TB (Table I; for structures see Fig. 1), as compared with the wild type HuAChE. On the other
hand, this substitution had only a limited effect on the $K_m$ values for both substrates. Past studies with these isosteric substrates demonstrated that in their respective Michaelis complexes with HuAChE the alkoxy substituents are accommodated in a different manner (6). Thus, the lack of significant effect on the values of $K_m$ for either substrate may suggest that during the formation of Michaelis complexes there is no significant stabilization due to interaction of the substrate carbonyl moieties with the oxyanion hole. Replacement of the second oxyanion hole element Gly-122 by alanine also resulted in similar decreases in the values of $k_{cat}$ for ATC and TB (18- and 15-fold, respectively). However, for this enzyme the $K_m$ value for ATC was also affected (6-fold), whereas practically no effect was observed on the corresponding value for TB (Table I). As in the case of the G121A enzyme the nearly equivalent decrease in the turnover numbers for both substrates indicates that the structural modification of the oxyanion hole affects mainly interactions with the substrate acyl moiety. Replacement of Gly-120 by alanine was carried out assuming that although this residue is not a constituent of the oxyanion hole, its substitution may affect the conformation of the glycine loop. Indeed, the effects on the catalytic parameters for ATC are similar to those observed for the G121A enzyme (see Table I), suggesting that the structure of the loop may be similarly affected by the two replacements. However, since the poorly expressed G120A enzyme could be obtained only in extremely low quantities and due to the limited solubility of TB in water, kinetic studies with the noncharged substrate could not be carried out.

### Reactivity toward the Transition State Analog TMTFA—The kinetics of AChE inhibition by TMTFA has been studied extensively since the tetrahedral covalent adduct is believed to mimic the transition state of the acylation process (21, 28, 29). TMTFA was shown to behave as a tight binding time-dependent inhibitor, a behavior characteristic of other trifluoroketone inhibitors of serine proteases (30, 31). In the recently published x-ray structure of TcAChE-TMTFA adduct (3), the bound ligand is thought to provide a constrained analog of ACh tetrahedral intermediate, with the oxyanion projecting toward the NH functions of the oxyanion hole. Therefore, reactivity of TMTFA toward enzymes carrying replacements of the oxyanion hole elements Gly-121 and Gly-122 may provide a sensitive measure of the resulting structural changes in the HuAChE active center.

As for the wild type HuAChE, TMTFA is a time-dependent inhibitor of the G122A enzyme, showing a linear dependence of the pseudo first-order rate constants of inhibition ($k_{obs}$) on inhibitor concentrations (Fig. 2B). The bimolecular rate constants $k_{on}$ and the dissociation rate constants $k_{off}$ were calculated from the relation $k_{obs} = k_{on} [\text{TMTFA}] + k_{off}$ and corrected for hydration of the free ketone (see “Experimental Procedures”). Values of $k_{off}$ for the wild type and the G122A enzymes were also determined directly by monitoring the regeneration of hydrolytic activity from the corresponding adducts and found to be in good agreement with those determined according to Equation 1 (Table II). The value of $k_{on}$ for the G122A enzyme is 300-fold lower than that for the wild type HuAChE (Table II), indicating that formation of the tetrahedral adduct may be hindered by some effect related to the replacement of Gly-122. This is consistent with the observation that the resulting HuA-ChE G122A-TMTFA adduct dissociates at about the same rate as that of the wild type enzyme complex (Table II) and therefore may be stable enough to result in the observed overall kinetic behavior of time-dependent inhibition. Since with regard to oxyanion accommodation formation of the tetrahedral adduct is thought to provide a constrained analog of ACh tetra-

![Chemical formulas of AChE substrates and inhibitors used in this study. A, substrates and transition state analog; B, carbamates; C, phosphates; D, phosphonates; E, noncovalent inhibitors](Image 311x250 to 551x729)

**FIG. 1.** Chemical formulas of AChE substrates and inhibitors. **A**, substrates and transition state analog; **B**, carbamates; **C**, phosphates; **D**, phosphonates; **E**, noncovalent inhibitors.

### Table I

**Kinetic constants for ATC and TB hydrolysis by HuAChE and its derivatives**

| AChE type | $K_m$ \(\times 10^{-5} \text{M}^{-1}\) | $k_{cat}$ \(\times 10^{-9} \text{M}^{-1} \text{cat}^{-1} \text{min}^{-1}\) | $k_{cat}/K_m$ | $\Delta G^\ddagger$ |
|-----------|-----------------------------------|---------------------------------|-----------------|------------------|
| ATC       | TB                                | ATC                             | TB                     | ATC              | TB               |
| WT        | 0.1 0.56                           | 4.0 0.04                        | 0.4 0.003          | 2.75 3.4         |
| G120A     | 0.05 0.06                          | 0.6 0.003                       | 0.4 0.003          | 2.75 3.4         |
| G121A     | 0.1 0.9                            | 0.04 0.003                      | 0.4 0.003          | 2.75 3.4         |
| G122A     | 0.57 0.44                          | 0.22 0.02                       | 0.4 0.05           | 2.75 3.4         |

a The apparent bimolecular rate constant ($k_{app}$) was calculated from the ratio $k_{cat}/K_m$.

b $\Delta G^\ddagger$ represents the change in free energy relative to the wild type enzyme required to reach the transition state (ES*), evaluated from the effect of mutation on the value of $k_{app}$.

c Low availability of the G121A enzyme and limitation of TB aqueous solubility precluded examination of this reaction.

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**TABLE II**

**Modification of the Oxyanion Hole in Acetylcholinesterase**

| Oxyanion Hole Modification | $K_m$ (M) | $k_{cat}/K_m$ | $\Delta G^\ddagger$ | $\Delta S^\ddagger$ | $\Delta H^\ddagger$ |
|----------------------------|-----------|---------------|---------------------|----------------------|---------------------|
| Gly-122A                   | 0.05      | 0.06          | 2.09                | -20%                 | -20%                |
| Gly-121A                   | 0.1       | 0.04          | 2.75                | -20%                 | -20%                |
| Gly-120A                   | 0.05      | 0.02          | 2.75                | -20%                 | -20%                |

$\Delta G^\ddagger$ and $\Delta S^\ddagger$ represent the change in free energy and entropy relative to the wild type.

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**FIG. 2.** Chemical formulas of AChE substrates and inhibitors used in this study. **A**, substrates and transition state analog; **B**, carbamates; **C**, phosphates; **D**, phosphonates; **E**, noncovalent inhibitors.

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**TABLE I**

**Kinetic constants for ATC and TB hydrolysis by HuAChE and its derivatives**

Values represent mean of triplicate determinations with standard deviation not exceeding 20%.

| AChE type | $K_m$ \(\times 10^{-5} \text{M}^{-1}\) | $k_{cat}$ \(\times 10^{-9} \text{M}^{-1} \text{cat}^{-1} \text{min}^{-1}\) | $k_{cat}/K_m$ | $\Delta G^\ddagger$ |
|-----------|-----------------------------------|---------------------------------|-----------------|------------------|
| ATC       | TB                                | ATC                             | TB                     | ATC              | TB               |
| WT        | 0.1 0.56                           | 4.0 0.04                        | 0.4 0.003          | 2.75 3.4         |
| G120A     | 0.05 0.06                          | 0.6 0.003                       | 0.4 0.003          | 2.75 3.4         |
| G121A     | 0.1 0.9                            | 0.04 0.003                      | 0.4 0.003          | 2.75 3.4         |
| G122A     | 0.57 0.44                          | 0.22 0.02                       | 0.4 0.05           | 2.75 3.4         |

a The apparent bimolecular rate constant ($k_{app}$) was calculated from the ratio $k_{cat}/K_m$.

b $\Delta G^\ddagger$ represents the change in free energy relative to the wild type enzyme required to reach the transition state (ES*), evaluated from the effect of mutation on the value of $k_{app}$.

c Low availability of the G121A enzyme and limitation of TB aqueous solubility precluded examination of this reaction.
Modification of the Oxyanion Hole in Acetylcholinesterase

Inhibition and rate constants of HuAChE and its derivatives by the transition state analog TMTFA

| AChE type | $k_{on}$ | $k_{off}$ | $k_{on}/k_{off}$ | $K_i$ | $\Delta G^\circ$ |
|-----------|---------|-----------|------------------|-------|----------------|
|           | $\times 10^{-9}$ | $\times 10^{-6}$ | $\mu$ | kcal/mol |
| WT        | 186     | 6.4       | 4.4             | 2.4$^a$ |                |
| G121A     | $\pm 1500$ | 11,300$^c$ | 11.6            | 4.0   |
| G122A     | 0.6     | 20        | 11.6            | 1,930$^d$ | 4.0 |

$^a$ Values of $k_{on}$ and $k_{off}$ were determined from slope and intercept, respectively, of the plots of $k_{on}$ versus $k_{off}$ (see Table I).

$^b$ Values of $k_{on}$ were determined from the rates of regeneration of hydrolytic activity following removal of free inhibitor.

$^c$ Values of $\Delta G$ were evaluated from the relative values of $K_i$ relative to that of the wild type enzyme. The values represent the relative destabilization of the tetrahedral enzyme-TMTFA adduct due to mutagenesis.

$^d$ Inhibition constants were calculated from the ratio of $k_{on}/k_{off}$.

Inhibition constant was computed from secondary plots of the values $K_i/V_{max}$ (as described under “Experimental Procedures”).

The fast formation of steady state for the HuAChE G121A-TMTFA adduct, which is characteristic to HuAChE complexes with noncovalent inhibitors, suggested that the corresponding dissociation is much faster and that TMTFA no longer behaves as a tight binding inhibitor (32). Indeed, regeneration of the G121A HuAChE hydrolytic activity, following removal of excess inhibitor, was too fast to be determined under conditions of the present study, and therefore only a lower limit of this rate constant could be estimated ($k_{off} \approx 0.15$ min$^{-1}$ as compared with $1.2 \times 10^{-4}$ min$^{-1}$ in the G122A enzyme; see Table II). This finding suggests that the interactions contributing to the stabilization of TMTFA in the covalent adduct are stronger in the cases of the wild type and G122A HuAChE than in case of the G121A enzyme. Moreover, although the relative destabilization of the tetrahedral TMTFA adducts with the G122A and G121A enzymes is similar (4.0 and 5.0 kcal/mol, respectively, see Table II), the differential effects of the two mutations on the values of the rate constants and in particular on those of $k_{off}$ suggest that the destabilizing effects in each case are due to different phenomena.

Reactivity toward Carbamates and Organophosphorous Inhibitors—Carbamate and organophosphate AChE inhibitors react with the enzyme yielding either slowly decomposing or practically stable covalent adducts, and therefore their overall inhibitory potencies are most conveniently measured by the bimolecular rate constants $K_i$ (see Scheme I). Although carbamylation and phosphorylation of the catalytic Ser-203 are quite different reactions, polarization of the respective C=O and the P=O bonds through interactions with the oxyanion hole may be essential in facilitating the nucleophilic addition. On the other hand, the anticholinesterase potencies of carbamates and organophosphates were shown in the past to depend mainly upon their complementarity with the active center. Numerous studies of structure-activity relationships for the various inhibitors (33–37), as well as for AChE enzymes modified by mutagenesis (10), suggested that structural variability affects the affinity toward the enzyme ($K_i$), rather than the rate of the covalent adduct formation ($k_{off}$). In this context it was quite interesting to examine the effects of structural modifications in the oxyanion hole on the inhibition kinetics of HuAChE enzymes by carbamate and organophosphate inhibitors.

Kinetic studies were carried under conditions that allowed group by the anionic subsite Trp-86.

Unlike the cases of the wild type and G122A HuAChE, TMTFA was a rapid reversible inhibitor of the G121A enzyme (see Fig. 2). Inhibition time course of wild type HuAChE (and) by 7.5 nm TMTFA and of G121A HuAChE (○) and G122A HuAChE (●) in the presence of 5.0 μM TMTFA. The fast formation of steady state for the G121A enzyme indicates that steady state is formed rapidly (few minutes). B, dependence of observed first-order inhibition rate constants ($k_{obs}$) on TMTFA concentration. The span of inhibitor concentrations for the wild type HuAChE (left panel) was 5.0–50 nm and for the G122A enzyme (right panel) 0.5–5.0 μM. The rate constants $k_{on}$ and $k_{off}$ were calculated from the slope and intercept, respectively, of these linear fits (see Table II). C, Lineweaver-Burk plots for the G121A enzyme in the absence (△) and in the presence of TMTFA $6 \times 10^{-8}$ (●); $3 \times 10^{-7}$ (●); $4 \times 10^{-7}$ (□); $5 \times 10^{-7}$ (▲); and $7.5 \times 10^{-7}$ (○). D, the value of TMTFA inhibition constant $K_i$ for the G121A enzyme was obtained from secondary plot of the values of $K_i/V_{max}$ versus the corresponding concentrations of the inhibitor (see “Experimental Procedures”).

Adduct with TMTFA should resemble the formation of analogous adduct with substrate like ATC or TB, and the effect of Gly-122 substitution on the value of $k_{obs}$ should be comparable to the corresponding values of $k_{on}$, as shown in Table I and II. The data in Tables I and II show that this is indeed the case for ATC which like TMTFA requires accommodation of the cationic trimethylammonium

![Figure 2](image-url)

**Fig. 2.** Inhibition of the wild type, G121A, and G122A HuAChEs by the transition state analog TMTFA. A, example of inhibition time course of wild type HuAChE (△) by 7.5 nm TMTFA and of G121A HuAChE (○) and G122A HuAChE (●) in the presence of 5.0 μM TMTFA. Note that the inhibition progression curve for the G121A enzyme indicates that steady state is formed rapidly (few minutes). B, dependence of observed first-order inhibition rate constants ($k_{obs}$) on TMTFA concentration. The span of inhibitor concentrations for the wild type HuAChE (left panel) was 5.0–50 nm and for the G122A enzyme (right panel) 0.5–5.0 μM. The rate constants $k_{on}$ and $k_{off}$ were calculated from the slope and intercept, respectively, of these linear fits (see Table II). C, Lineweaver-Burk plots for the G121A enzyme in the absence (△) and in the presence of TMTFA $6 \times 10^{-8}$ (●); $3 \times 10^{-7}$ (●); $4 \times 10^{-7}$ (□); $5 \times 10^{-7}$ (▲); and $7.5 \times 10^{-7}$ (○). D, the value of TMTFA inhibition constant $K_i$ for the G121A enzyme was obtained from secondary plot of the values of $K_i/V_{max}$ versus the corresponding concentrations of the inhibitor (see “Experimental Procedures”).
for derivation of values for $K_d$ and $k_2$ (see “Experimental Procedures” and Scheme 1) for the prototypical AChE carbamate inhibitors physostigmine and pyridostigmine. For the wild type HuAChE, the values of $K_d$ and $k_2$ (Table III) obtained for physostigmine are similar to those measured recently in a comprehensive kinetic study of its reaction with electric eel AChE (37). The bimolecular carbamylation rate constants of the G122A enzyme by physostigmine and pyridostigmine decreased to about the same extent relative to the wild type HuAChE (85- and 56-fold, respectively). For both inhibitors, these changes in the values of $k_2$ were caused by comparable changes in the respective values of $K_d$ and $k_2$ (Table III). A somewhat larger decrease in the bimolecular carbamylation rate constant by physostigmine was observed for the G121A HuAChE (290-fold relative to the wild type enzyme). Unlike the case of the G122A enzyme, this diminished value of $k_2$ is mainly due to loss of affinity toward the inhibitor (the corresponding value of $K_d$ was 65-fold higher than that for the wild type HuAChE). The effect of residue replacement at position 121 on the affinity toward carbamates may be even more dramatically demonstrated for pyridostigmine, since in this case kinetic analysis indicated a rapid reversible inhibition (see Fig. 3A). According to the kinetic model of carbamylation (see Scheme 1), this observation may suggest that the G121A-pyridostigmine Michaelis complex is destabilized to the point where the ratio $k_{-1}/k_2$ precludes observation of the covalent adduct, within the time frame of the kinetic experiment.

Inhibition of the oxyanion hole mutants G122A and G121A by organophosphorous inhibitors was investigated using the phosphates DFP, DEFP, and paraoxon as well as the phosphonates soman and sarin. Comparison of the inhibition rate constants ($k_i$) for the two mutant enzymes clearly demonstrates the different effects of the two replacements on enzyme reactivity toward these inhibitors. Upon replacement of Gly-121 by alanine the reactivity toward all the inhibitors decreased by more than 3 orders of magnitude (see Table IV). On the other hand, for the G122A HuAChE the inhibition rate constants of phosphates decrease 10–100-fold, relative to the wild type enzyme, whereas those of phosphonates are more affected (400–500). Determination of the values of $K_d$ and $k_2$ for paraoxon and DEFP inhibition of the G122A and the G121A HuAChEs has indicated that for the latter enzyme affinity toward the inhibitors rather than the actual rate of the phosphorylation step ($k_i$) is mainly affected (Table V).

Reactivity toward Noncovalent Active Center Inhibitors—As already mentioned, the glycine loop is also a structural element of the active center and therefore may be part of the binding environment for ligands which do not contain the oxyanion moiety. Moreover, its capacity to participate in stabilization of noncovalent complexes is limited due to the absence of side chains. To examine the effect of modified loop structure on stabilization of noncovalent complexes, the inhibitory activities of two active center ligands edrophonium and huperzine A as well as that of the bisquaternary ligand BW284C51, toward the G121A and G122A HuAChEs, have been evaluated.

Replacing either Gly-121 or Gly-122 by alanine had only a minor effect on the inhibitory activity of the bisquaternary ligand BW284C51, showing that the structure of the active center gorge is essentially unchanged (Table VI). For inhibition of the G122A enzyme by edrophonium and more so for inhibition of both mutant enzymes by huperzine A, pronounced effects were observed relative to the wild type HuAChE (Table VI). Since these ligands do not contain groups capable of interacting with the oxyanion hole, the reason for the diminished inhibitory activity is probably steric obstruction due to the modified structure of the glycine loop. For huperzine A such a conclusion is consistent with the recently published x-ray structure of the TcAChE-huperzine A complex where the ligand is tightly fitted against the glycine loop, altering its conformation relative to structures of other noncovalent TcAChE complexes (4).

**DISCUSSION**

The contribution of oxyanion hole to AChE catalytic efficiency was postulated on the basis of analogy to other serine hydrolases and on theoretical studies (38–40). For these hydrolases, and in particular for serine proteases, such structural element has been identified in x-ray structures of covalent adducts that mimic the corresponding tetrahedral intermediates in reactions with substrates (14, 16). In proteases of the chymotrypsin family, the oxyanion hole consists of the backbone NH groups of Gly-193 and Ser-195, whereas in subtilisins one of the H bond donors is the side chain amine group of Asn-155. The three-pronged oxyanion hole identified in the x-ray structure of TcAChE (1, 3) is similar to the corresponding functional motifs observed in the structures of *Candida rugosa* and *Rhizomucor miehei* lipases (41). These lipases share with

**TABLE III**

| Rate and dissociation constants according to Scheme 1 (see “Experimental Procedures”). |
| --- |
| **AChE type** | $k_1$ | $k_2$ | $K_d$ |
| Pyridostigmine | $10^{-4} \times 10^{-4} \times 10^{-4}$ | $10^{-3} \times 10^{-3}$ | 250 |
| G121A | 0.32 | 1.2 | 0.77 |
| G122A | 1.2 | 1.2 | 0.34 |
| Physostigmine | $300^{a}$ | 0.12 | 3.4 |
| G121A | 1.2 | 0.26 | 22 |
| G122A | 1.2 | 1.2 | 0.34 |

* The corresponding values reported for mouse AChE were $k_1 = 49 \times 10^4 M^{-1} min^{-1}$, $k_2 = 4.6 min^{-1}$, $K_d = 9.5 \times 10^{-6} M$ (7). The corresponding values reported from direct stop flow measurements for electric eel AChE were $k_2 = 2.5 min^{-1}$, $K_d = 0.43 \times 10^{-6} M$ (37).
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TABLE IV
Bimolecular rate constants for phosphorylation reactions of HuAChE and its oxyanion hole mutants

| Phosphates | DEFP | DFP |
|------------|------|-----|
| WT         | 97   | 95  | 14 |
| G121A      | 0.03 (2323) | 0.04 (2375) | 0.007 (2000) |
| G122A      | 4.0 (24) | 1.0 (95) | 1.2 (12) |
| kₙ (×10⁻⁴ M⁻¹ × min⁻¹) | 10000 | 2300 |

TABLE V
Kinetic constants for phosphorylation reactions of HuAChE and its derivatives

| Rate and dissociation constant were determined as described before (10). |
|----------------------|------|-----|
| AChE type | kᵢ | hₑ | Kₛ |
| Paraoxon | ×10⁻⁴ × s⁻¹ × min⁻¹ | min⁻¹ | ×10⁶ × s⁻¹ |
| WT | 1.0 | 1.0 |
| G121A | 0.085 | 0.16 | 188 |
| G122A | 8 | 0.2 | 2.6 |
| DFP | 16.6 | 1.6 | 9.8 |
| WT | 0.007 | 0.14 | 2100 |
| G121A | 3.7 | 0.3 | 7.6 |

TABLE VI
Competitive inhibition constants of HuAChE and its derivatives by active center ligands

| AChE type | Edrophonium | BW284C51 | Huperzine A⁺ |
|-----------|-------------|-----------|-------------|
|          | μM | μM | μM |
| WT | 0.8 | 11 | 1.3 |
| G121A | 7.8 | 1.5 | 1300 |
| G122A | 105 | 5 | 1100 |

* Racemic mixture.

AChE the α/β-hydrolase fold (42); however, in the cases of AChE and CRL the oxyanion hole is preformed and does not change significantly upon inhibitor binding (43), whereas that of RML is formed only upon enzyme-ligand complex formation (44).

Despite the importance of these studies in providing structural evidence for the existence of an oxyanion hole, it still remains unclear how interactions of this motif with substrate contributes to catalytic efficiency (16). Henderson (45) suggested a specific binding site for the oxyanion that lowers the free energy of the tetrahedral intermediate favoring its formation. It was argued that stabilization of the oxyanion by pre-aligned dipoles of the oxyanion hole is more effective than in water, as solvent reorganization is avoided (46). More recently, the NH groups of the oxyanion hole were proposed to participate in a “concerted general acid-general base-catalyzed formation of a tetrahedral intermediate” (47). The partial transfer of protons to the forming oxyanion results in short, strong hydrogen bonds which may account for much of the enzymatic rate acceleration.

A direct and quantitative evaluation of the oxyanion hole role in catalysis was carried out for enzymes, such as subtilisin or papain, where one of the hydrogen bonding groups was on a side chain and could be removed through residue replacement (16). For subtilisin, several replacements of residue Asn-155 resulted in significant decreases of the kₙ/Kₛ values (48–50), corresponding to a change in transition state stabilization in the range of 3–5 kcal/mol. Most of the effect could be attributed to a decrease in values of kₙ, suggesting that interactions with the oxyanion hole do not participate in stabilization of the Michaelis complex (16). For subtilisin and for serine proteases in general, such an outcome may not be surprising since the complexes are thought to involve numerous polar and hydrophobic interactions between the enzymes and their bulky peptides (14, 51). However, comparison of these enzymes with AChE raises the question whether the initial accommodation of small substrates and covalent inhibitors by AChE follows the same pattern with regard to oxyanion hole participation.

Functional Role of Residue Gly-121 in Oxyanion Hole—The G121A HuAChE enzyme exhibited impaired catalytic activity toward ATC and TB mainly due to decrease in the respective values of kₙ. Thus, introduction of small side chain onto the HuAChE oxyanion hole residue does not appear to interfere in the step of Michaelis complex formation. Since only limited atomic motion is thought to take place during the transition from planar to tetrahedral substrate geometries (46), this side chain probably does not interfere with formation of the transition state. Therefore, the lower values of the turnover number for the G121A enzyme could be a consequence of relative destabilization of the tetrahedral intermediates due to impaired binding capacity of the oxyanion hole. Moreover, the extent of this destabilization (2.7–3.4 kcal/mol; monitored by the rate constant of the acyl-enzyme formation – kₙ) is in good agreement with that mentioned above for subtilisins mutated at position 155 (49), implying that part of the H-bonding capability of the oxyanion hole in the HuAChE mutant enzyme is lost. The notion that replacement of Gly-121 by alanine changes the structure of the oxyanion hole in HuAChE and consequently affects its H-bonding with the tetrahedral transition state is consistent with the effect of substituting the adjacent residue Gly-120. Although residue Gly-120 is not part of the oxyanion hole, its replacement by alanine had nearly equivalent effect to that of replacing Gly-121, on the kinetic profile of ATC hydrolysis. Since residues Gly-120 and Gly-121 are part of a conformationally mobile glycine loop, the most straightforward rationalization of this similarity is that replacement of either Gly-120 or Gly-121 may bring about the same structural change of the oxyanion hole. The recently reported replacements of residue Gly-115 in butyrylcholinesterase (equivalent to Gly-120 in HuAChE) by alanine and serine also affected mainly the respective values of kₙ (18).

The effect of mutation at position 121 on reactivity toward the transition state analog TMTFA is also consistent with the idea of a partial loss of the oxyanion H-bonding capacity. The inhibition characteristics of the ligand was changed from a slow inhibitor of the wild type HuAChE, into a rapidly equilibrating one for the G121A enzyme (Table II). The main observable difference in kinetic behavior, relative to the adduct of the wild type enzyme, was a significant increase in the dissociation rate (kₛ), indicating a lower stability of the G121A-TMTFA covalent conjugate. Since the tripartite stabilization due to the oxyanion hole (Gly-121, Gly-122, and Ala-204) is a major component of the TMTFA tetrahedral adduct accommodation (3), it
is reasonable to assume that such destabilization may result from partial destruction of the oxyanion hole motif.

The possible functional impairment of the oxyanion hole, in the G120A and G121A enzymes, was also assessed by molecular modeling of the corresponding tetrahedral intermediates with ATC. It appears that introduction of methyl groups results in both cases in a conformational flip of residue at position 121, removing the H-bond donating NH moiety from the vicinity of the oxyanion (see Fig. 4, A and C). In the resulting models, the H-bond interactions with residues Gly-122 and Ala-204, as well as the substrate orientation relative to the active center binding environment, resemble closely those observed in the corresponding model of the wild type enzyme (Fig. 4A). The models suggest also that substitution at positions 120 and 121 by alanine does not result in steric interference in the HuAChE-ATC tetrahedral species (Fig. 4). In addition, the proposed orientation of the Ala-121 methyl group is also consistent with the inhibitory activities of the noncovalent active center ligands, edrophonium and BW284C51 toward the G121A enzyme. The minor differences of $K_i$ values for edrophonium and BW284C51 as compared with those for the wild type HuAChE seem to be a result of residue Ala-121 interaction with the tetramethylammonium moiety of edrophonium and with the aryl ring of BW284C51 (models not shown).

The oxyanion hole is thought to be one of the elements of the AChE functional architecture, participating in formation of Michaelis complexes with organophosphorous inhibitors (10, 15, 52). Indeed, the dissociation constants ($K_i$) of the G121A enzyme complexes with paraoxon and DFP, respectively, were 188- and 214-fold higher than those for the wild type HuAChE (see Table IV). The finding that the dissociation constants for the two phosphates, differing in the bulk of their alkoxy substituents (see Fig. 1), show large but similar variations indicates that the relative destabilization of the corresponding G121A-Michaelis complexes is not due to steric effects but rather due to impaired accommodation of the phosphoryl oxygen. Furthermore, the bimolecular rate constants for inhibition of the G121A enzyme, by all the phosphates or phosphonates examined ($k_i$, see Table IV) show similar decreases relative to the wild type HuAChE. These findings further support the assumption that the only significant functional change in the G121A enzyme is the diminishing of H-bonding capacity of the oxyanion hole and its capability to accommodate the phosphoryl moiety. The overall loss in interaction energy between the G121A enzyme and the organophosphorous inhibitors (4.5–5.2 kcal/mol; calculated from values of the ratio $k_i$mutant/$k_i$wild type shown in Table IV), relative to the wild type HuAChE, is much larger than the corresponding decrease for substrates, suggesting a somewhat different role of the oxyanion hole motif in HuAChE phosphorylation and in acylation by substrates. The reason for such a considerable effect on the enzyme reactivity toward organophosphorous agents may be that polarization of the P=O bond during complex formation is necessary for activating the inhibitor for nucleophilic attack and is facilitated by its tetrahedral geometry.

In view of the proposed difference in the initial accommodation of tetrahedral phosphates and planar substrates by the oxyanion hole, it was interesting to examine the reactivity of the G121A enzyme toward carbamates. For these inhibitors, the planar geometry characteristic to substrates is combined with much lower reactivity of the carbamyl moiety, and thus, activation may be required to assist a nucleophilic addition. Therefore, as in the case of phosphates, activation may be achieved by juxtaposing the carbamyl oxygen with the oxyanion hole so that the C=O bond is strongly polarized already in the Michaelis complex. Indeed, from the values of kinetic constants ($K_i$, $k_i$) for inhibition of the G121A enzyme by physostigmine, relative to the wild type HuAChE (see Table III), it is evident that the mutation affects predominantly the stability of the Michaelis complex. In case of inhibition of the G121A enzyme by pyridostigmine, the dissociation rate of the destabilized Michaelis complex may become much higher than the rate of acylation leading to the observed reversible association with this carbamate.

Functional Role of Residue Gly-122 in Oxyanion Hole—Residue Gly-122 is another element of the HuAChE oxyanion hole located on the flexible glycine loop in the active center. Indication that glycine at position 122 may not be a prerequisite for a fully functional oxyanion hole can be inferred from the sequence of certain cholesterol esterases and lipases, which also belong to the α/β-hydrolase fold (54), and where the motif Gly-120–Gly-121–Gly-122 is replaced by Gly-Gly-Ala. Furthermore, in butyrylcholinesterase the somewhat wider base of the active center gorge (53) allows for substitutions of the analo-
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stERIC interferEnce can be expected also for the tetrahedral intermediates of substrates.

In accordance with the notion that replacement of residue Gly-122 by alanine introduces steric interference in the acyl pocket of HuAChE, we find that this substitution affects differently the rate constants of phosphorylation by phosphates compared with phosphonates (Table III). For the phosphonates, soman and sarin, the decrease in values of $k_\text{cat}$ relative to the wild type enzyme (500- and 460-fold, respectively), was about an order of magnitude larger than for the phosphates. This reactivity pattern appears to be consistent with the proposed restriction of space available in the acyl pocket, since the methyl group introduced to this subsite by phosphonates is more bulky than the alkoxy oxygen presented by the phosphates. The localized nature of this steric perturbation is indicated also by the fact that it similarly affects the values of $k_\text{cat}$ for phosphonates despite the different sizes of their alkoxy substituents. For the phosphates DFP and paraxon most of the effect was observed on the values of $k_\text{cat}$ (about 5-fold for both phosphates), demonstrating that stabilization of the corresponding Michaelis complexes was relatively unaffected.

The diminished reactivity of the G122A enzyme toward physostigmine and pyridostigmine can be also accounted for in terms of a steric constraint in the acyl pocket. Although the size of the molecular moiety introduced to this site by pyridostigmine (dimethylamino) is considerably larger than that of methyl group, its trigonal geometry allows for orientations in which steric conflict with the side chain of Ala-122 is mostly avoided (model not shown).

Conclusions—Kinetic study of catalysis and inhibition of the G120A, G121A, and G122A HuAChE enzymes with variety of covalent and noncovalent inhibitors suggest that site-directed mutagenesis allows us to manipulate specifically the structure and interaction characteristics of an oxyanion hole even though it consists only of main chain NH groups. Furthermore, from the changes in reactivity of the mutant enzymes toward the various ligands and, in particular, toward the transition state analog TMTFA, the extent of structural perturbation introduced by these mutations could be assessed. One of the surprising observations is that formation of HuAChE-substrate Michaelis complexes do not seem to involve substantial interactions with the oxyanion hole. On the other hand, the stability of analogous complexes with covalent inhibitors like organophosphates and carbamates appears to require accommodation by oxyanion hole, probably to activate the respective phosphoryl or carbamyl groups for nucleophilic addition of the catalytic Ser-203.

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FIG. 5. Stereo view of models of TMTFA tetrahedral conjugates with the wild type and G122A HuAChE enzymes. H-bond interaction distances between the oxyanion and components of the oxyanion hole are marked by dashed lines. A, wild type HuAChE-TMTFA conjugate, constructed according to the x-ray structure of the corresponding adduct of TcAChE (3), where the oxyanion is within 3.0, 2.9, and 2.7 Å from the amide NH moieties of Gly-121, Gly-122, and Ala-204, respectively. B, G122A-TMTFA conjugate. Note that accommodation of the trifluoromethyl group of the ligand is hindered by interaction with the methyl group of Ala-122 as demonstrated by partial overlap of their respective volumes (shown as dotted area). The oxyanion hole is within 2.7 Å from the amide NH moieties of Gly-121 and Ala-122 (dashed lines), and within 3.2 Å from the amide nitrogen of Ala-204 (residue not shown).
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