Nanosecond Dynamics of the Mouse Acetylcholinesterase Cys\(^{69}\)–Cys\(^{96}\) Omega Loop*

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Jianxin Shi‡§, Kaihsu Tai†¶, J. Andrew McCammon†, Palmer Taylor‡** and David A. Johnson‡‡
From the ‡Department of Pharmacology, University of California, San Diego, La Jolla, California 92093-0636, ¶Howard Hughes Medical Institute and Departments of Pharmacology and of Chemistry and Biochemistry, University of California, San Diego, La Jolla, California 92093-0365, and §§Division of Biomedical Sciences, University of California, Riverside, California 92521-0121

The paradox of high substrate turnover occurring within the confines of a deep, narrow gorge through which acetylcholine must traverse to reach the catalytic site of acetylcholinesterase has suggested the existence of transient gorge enlargements that would enhance substrate accessibility. To establish a foundation for the experimental study of transient fluctuations in structure, site-directed labeling in conjunction with time-resolved fluorescence anisotropy was utilized to assess the possible involvement of the omega loop (Ω loop), a segment that forms the outer wall of the gorge. Specifically, the flexibility of three residues (L76C, E81C, and E84C) in the Cys\(^{69}\)–Cys\(^{96}\) Ω loop and one residue (Y124C) across the gorge from the Ω loop were studied in the absence and presence of two inhibitors of different size, fasciculin and huperzine. Additionally, to validate the approach molecular dynamics was employed to simulate anisotropy decay of the side chains. The results show that the Ω loop residues are significantly more mobile than the non-loop residue facing the interior of the gorge. Moreover, fasciculin, which binds at the mouth of the gorge, well removed from the active site, decreases the mobility of 5-(((2-acetyl)amino)ethyl)aminonaphthalene-1-sulfonic acid reporter groups attached to L76C and Y124C but increases the mobility of the reporter groups attached to E81C and E84C. Huperzine, which binds at the base of active-site gorge, has no effect on the mobility of reporter groups attached to L76C and Y124C but increases the mobility of the reporter groups attached to E81C and E84C. Besides showing that fluctuations of the Ω loop residues are not tightly coupled, the results indicate that residues in the Ω loop exhibit distinctive conformational fluctuations and therefore are likely to contribute to transient gorge enlargements in the non-ligated enzyme.

Acetylcholinesterase (AChE),\(^1\) ranking among the most catalytically efficient enzymes known, catalyzes the hydrolysis of the neurotransmitter acetylcholine with a turnover number of 10\(^4\) s\(^{-1}\) (1, 2). Curiously, catalysis shows high efficiency despite the cross-sectional dimension of acetylcholine is nearly equal to the width of the narrowest portion of the 20-Å-long and tortuous gorge leading to the catalytic center (Fig. 1A) (3–7). This paradox suggests the existence of an enlarged solution conformational state(s) of the active-center gorge. Molecular dynamics simulations support the existence of breathing or gating motions that could enhance substrate accessibility to the active site (8–10).

One segment of the gorge that may play a major role in gorge enlargement is the large Ω loop (defined by the Cys\(^{69}\)–Cys\(^{96}\) disulfide bond) that corresponds to the activation loop (Cys\(^{60}\)–Cys\(^{77}\)) of Candida rugosa lipase, a related carboxyl esterase with an α,β-hydrolase fold (11, 12). X-ray crystallographic analysis of the apo form of this lipase shows the activation loop occluding the active site in the absence of substrate and folding backward allowing substrate access when lipid is bound. We previously demonstrated by examining the steady-state emission from selective acrylodan-labeled side chains that AChE-inhibitor binding induces distinctive conformational changes in certain regions of the mouse AChE Cys\(^{69}\)–Cys\(^{96}\) Ω loop (13).

To determine whether the Cys\(^{69}\)–Cys\(^{96}\) Ω loop could contribute to gorge enlargement, we used site-directed labeling with IAEDANS at various positions in conjunction with time-resolved fluorescence anisotropy to compare the backbone flexibility of three residues in the Ω loop (L76C, E81C, and E84C) with the flexibility of a residue in the active-site gorge, but not part of the Ω loop (Y124C). We also examined the influence of two inhibitors on conformational flexibility, one of which interacts directly with the Ω loop at the gorge entry (fasciculin), whereas the other (huperzine) associates with side chains of the loop near the gorge base. IAEDANS replaced acrylodan in this study, because it is conjugated more readily to active-site gorge residues than thioreactive fluorescein derivatives, and because its relatively long emission lifetime allows better resolution of whole body from local depolarization processes. Additionally, molecular dynamics simulations of side chain motions of the four individual residues where cysteine was substituted showed agreement with anisotropy decay parameters of the wild type (wt) enzyme. We found that the conjugated Ω loop residues were significantly more flexible than the non-
loop residue. Also, the effects of the inhibitors were consistent with the flexibility of the Ω loop contributing to transient gorge enlargement that could enhance substrate accessibility and product egress.

EXPERIMENTAL PROCEDURES

Materials—Acetylthiocholine iodide, 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman’s reagent), and diithiothreitol were purchased from Sigma. H-1-Huperzine A was purchased from Calbiochem. 5-((2-bis(acetylamino)ethylamino)phthalene-1-sulfonic acid (IAEDANS) was obtained from Molecular Probes (Eugene OR). Fasculin 2 (purified from the venom of Dendroaspis angusticeps) was a gift of Dr. Pascal Marchot (University of Marseille, France). Drs. Yacov Ashani and Bhupendra P. Doctor (Walter Reed Army Research Center, Washington, D.C.) kindly provided procainamide-linked Sepharose CL-4B resin. All other chemicals were of the highest grade commercially available.

Expression, Mutagenesis, and Purification of mAChe—Mouse AChE was produced by transfection of expression plasmid (pcDNA3, Invitrogen) containing an encoding cDNA where the AChE sequence was terminated at position 548. The plasmid was transfected into human embryonic kidney (HEK293) cells. Cells were cotransfected with G418 to obtain stable producing cell lines, and AChE was expressed as a secreted soluble enzyme in serum-free media. Mutant enzymes were detected while the samples were excited with orthogonally polarized light and the mission monitored with a polarizer oriented in the vertical and orthogonal direction (G = 0.9936).

Emission anisotropy, r(t), is given by the expression shown in Equation 1:

\[ r(t) = \frac{I(t) - G^2 I_0(t)}{I(t) + 2G^2 I_0(t)} \]  
(Eq. 1)

From this and the expression for the total emission, S(t), for a macroscopically isotropic sample,

\[ S(t) = I(t) + 2G^2 I_0(t) \]  
(Eq. 2)

was deconvolved simultaneously from the individual polarized emission components expressed as shown in Equations 3 and 4,

\[ I(t) = \frac{S(t)}{3} (1 + 2rt(t)) \]  
(Eq. 3)

and

\[ I_0(t) = \frac{S(t)}{3} (1 - r(t)) \]  
(Eq. 4)

Thus, both I(t) and I_0(t) were determined by the same fitting functions, S(t) and r(t), and fitting parameters.

Fluorescence lifetimes were determined by initially generating a total emission profile from I(t) and I_0(t) with Equation 2, and then fitting this decay profile to a biexponential decay expression with the Globals UnlimitedTM (Laboratory for Fluorescence Dynamics, Urbana, IL) software package. The resulting lifetimes were entered and fixed in the second step of the analysis process where I(t) and I_0(t) were simultaneously analyzed for the parameters of S(t) and r(t) with the Globals UnlimitedTM program. Here r(t) is a nonassociative anisotropy decay function as shown in Equation 5,

\[ r(t) = r_{f} e^{-\tau (t)} (1 - f_{d}) e^{-\mu (t)} \]  
(Eq. 5)

where \( r_{f} \) is the amplitude of the anisotropy at time 0, \( f_{d} \) is the fraction of the anisotropy decay associated with the fast decay processes, and \( \phi \) is rotational correlation time of the anisotropy decay. Rotational correlation times are measured for the two (fast and slow) processes. This nonassociative model assumes that the rotational correlation times are common to each of the emission relaxation times. Goodness of fit was evaluated from the value of \( \chi^{2} \) and visual inspection of the difference between the experimental data and the empirical anisotropy decay.

| Mutant | Control | Huperzine | Fasculin | Relative quantum yieldb |
|--------|---------|-----------|----------|-------------------------|
| L76C   | 485     | 485       | 478      | 1.0                     | 1.11                        |
| E81C   | 489     | 494       | 492      | 0.82                    | 0.90                        |
| E84C   | 484     | 491       | 486      | 0.77                    | 0.97                        |
| Y124C  | 482     | 482       | 469      | 1.0                     | 1.55                        |

a The excitation wavelength was 340 nm.

b Relative quantum yields were determined by comparison of areas under the emission spectra of each labeled mutant in the absence of ligand with its spectra in the presence of the indicated ligand. Concentrations of fasciculin and huperzine were 1.3 and 4 μM, respectively.

**Table I**

Effect of huperzine and fasciculin on steady-state emission parameters of IAEDANS-labeled mouse AChE

Data are shown as mean values of at least three determinations.
model. (It is worth noting that the observed $r_0$ values typically are less than the fundamental anisotropy of the reporter group (0.33). This most probably reflects the fact that the very fast depolarizing motions produced by tether arm movements are not resolvable by commercial instruments.)

Molecular Dynamics Simulation of Anisotropy Decay—The decay of anisotropy because of side chain and segmental motions was simulated utilizing the approach developed by Ichiye and Karplus (15). Specifically, the anisotropy decay of a fluorophore conjugated to a protein side chain was represented as the decay in the time correlation function shown in Equation 6,

$$\rho(t) = \langle P_2(\hat{\mu}(t) \cdot \hat{\mu}(t - \tau)) \rangle, \quad (Eq. 6)$$

where $\hat{\mu}$ is a normalized vector properly chosen to represent the local motion being reported by the fluorophore. In our calculations, $\hat{\mu}$ was chosen to be a normalized vector representing the direction that goes from the $\alpha$-carbon to an atom near the tip of the wild type residue. To wit, for the glutamate residue, we used the average of the two normalized $C_\alpha-O$ vectors; for leucine, the normalized $C_\alpha-H$ vector; and for tyrosine, the normalized $C_\alpha-O_H$ vector.

For this study, the previously reported 10-ns molecular dynamics simulation of the unliganded wt mAChE (16) was extended to afford a 15-ns trajectory. Frames (snapshots of the trajectory containing the coordinates of all the atoms) were calculated at 1-ps time intervals, and the rotational degrees of freedom were removed by superimposing all frames into a reference frame, minimizing the root mean squared deviations. With these trajectories on a nanosecond time scale, it was possible to simulate anisotropy decay due to just the combination of side chain and segmental motions; deconstruction of the vectors to resolve the side chain and the segmental motions was not attempted.

RESULTS AND DISCUSSION

Characterization of Labeled Mutants—Acetylcholine hydrolysis kinetics of the cysteine-substituted mutants were shown previously to be similar to the wt enzyme suggesting that all mutant enzymes fold correctly despite the presence of the substituted cysteines (13). Moreover, the specific labeling for each mutant was estimated to be 71–80%, 89–93%, 79–80%, and 74–84% for L76C, E81C, E84C, and Y124C, respectively. Substrate and inhibitor recognition by the IAEDANS-labeled mutants is evident from both fasciculin (1.3 $\mu$M) and huperzine (4 $\mu$M) producing greater than 95% inhibition of the rate of acetylcholine hydrolysis (data not shown). This is not surprising, because previous kinetic studies showed that $k_{on}$ and $k_{off}$ of fasciculin were unaltered in the cysteine mutants (17).

In the absence of ligand, the emission maxima of the four IAEDANS-labeled mutants were similar ranging between 482 and 489 nm (Table I). The active-site ligand, (−)-huperzine A, a herbal alkaloid used in Chinese traditional medicine, binds at the base of active-site gorge and should have no direct interac-

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**FIG. 1.** Locations of substituted cysteines for fluorophore conjugation. A, cutaway view of mAChE crystallographic structure showing an active-site gorge 20 Å in depth (PDB code 1MAA). The view is taken from the side with catalytic triad (Ser203, Glu334, and His447) on the bottom left. B, Connolly surface presentation of mAChE looking into the gorge entry with catalytic serine highlighted in red at its base. Residues 76, 81, and 84 displayed in blue are at the tip (76) and outer portion (81, 84) of the Cys69–Cys96 loop. Residue 124 displayed in green is on an opposing face of the gorge and makes up part of the peripheral anionic site. C, model of mAChE in complex with huperzine based on crystal structure of huperzine: *Torpedo californica* AChE (PDB code 1VOT). Huperzine binds at the bottom of active-site gorge and makes surprisingly few specific contacts in the active-site gorge. D, fasciculin 2 complexed to mAChE adapted from PDB code 1KU6. Note: hydrogen bonding between the guanidine nitrogen moieties of Arg$^{37}$ and Arg$^{39}$ in fasciculin and carbonyl oxygen moieties of Glu$^{44}$ (3.3 Å) and Leu$^{76}$ (3.24 Å) on mAChE, respectively.
tion with all cysteine-conjugated sites based on the x-ray crystal structure of the huperzine-AChE complex (Fig. 1C) (18). Huperzine only affected the steady-state emission properties of the E81C- and E84C-labeled mutants, red shifting the emission maxima 5 and 7 nm and decreasing the relative quantum yields by 18 and 23% respectively (Table I). This pattern suggests that the huperzine increases the polarity of the microenvironment around the Glu81 and Glu84 but not around the Leu76 and Tyr124 side chains.

Fasciculin, a peptic peripheral site inhibitor that caps the mouth of the active-site gorge, influences the microenvironment of the substituted positions in a complex manner. The crystal structure of fasciculin-mAChE complex shows hydrogen bonding between the guanidino moieties of Arg11 and Arg37 in fasciculin and the carbonyl oxygens of Glu84 and Leu76 on the AChE, respectively (Fig. 1D) (4). Met13 of fasciculin also is in close proximity but not “in van der Waals contact” with Tyr124 in the AChE. The largest spectral changes were observed with the labeled Y124C mutant where fasciculin blue-shifted the emission maxima 13 nm and increased the relative quantum yield by 55% (Table I). A similar pattern was seen with the L76C conjugate, but the blue shift was 7 nm, and the relative quantum yield enhancement was 11%. The emission properties of E81C- and E84C-labeled mutants were much less affected by fasciculin binding; the emission maxima were red-shifted 3 and 2 nm, and the relative intensities were decreased by 10 and 3%, respectively (Table I). These results indicate that fasciculin dramatically decreased the polarity around the reporter groups attached to Y124C and to a lesser extent around L76C. Also, fasciculin slightly increased the polarity around the reporter groups attached to E81C and E84C, which is qualitatively the result found using acrylodan instead of IAEDANS (13).

Emission and Anisotropy Decay—For brevity, just the parallel and perpendicular emission decay profiles of one labeled mutant (E84C) along with the corresponding anisotropy decay profile are illustrated in Fig. 2. The total emission decay profiles (S(t), Equation 2) of all the mutants were best fit to a three-exponential decay function whose geometric averaged lifetimes are summarized in Table II and ranged from 10.0 to 14.6 ns. The ranges of values of the short, intermediate, and long lifetimes of the various conjugates studied were 1.1–3.4, 9.3–16.0, and 18.2–27.9 ns, respectively (data not shown).

Anisotropy decay profiles for the IAEDANS conjugates, with the exception of the labeled Y124C mutant complexed to fasciculin, were well fit by a nonassociative biexponential model (Equation 5). These profiles are illustrated in Fig. 3, and the best fit parameters are summarized in Table II. Except for the huperzine-bound E84C mutant, the processes associated with the “slow” rotational correlation times accounted for 60–80% of the total resolvable anisotropy decay, and the values of these rotational correlation times ranged between 69 and 132 ns (Table II). This range of values is greater than what might be predicted from a protein with 547 amino acids and three N-linked oligosaccharides of average mass determined by matrix-assisted laser desorption ionization-mass spectrometry. However, sedimentation equilibrium measurements for this protein correlate best with a molecular mass of 122,000 Da, suggesting that at higher concentrations the molecule may form a reversibly associating dimer.2 The longest emission lifetimes (18.2–27.9 ns) are long enough to allow estimation of a global rotational correlation time. The crystal structure shows mAChE to have limited dimensional asymmetry so only a single rotational correlation would be resolvable. Because faster emission decay step(s) are also present, deconvolution of components of the intermediate and slow phase is subject to uncertainty. Consequently, slow internal fluctuations contributing to the decay rate cannot be ruled out. For the case of huperzine-bound E84C mutant, the Φslow values ranged between 80 and 260 ns. This broader range of values probably reflects a greater uncertainty in Φslow measurements when the slow decay processes represent a small fraction of the total resolvable anisotropy decay, as was the case for the huperzine-bound E46C mutant (31%; Table II).

Comparison of the fast anisotropy decay components of the apo form of the labeled mutants show two features. First, reporter groups attached to substituted cysteines whose native side chains in the crystal structure project into the active-site gorge (L76C and Y124C) are less mobile than reporter groups attached to Ω loop-substituted cysteines located on the outer surface of the enzyme (E81C and E84C). This conclusion is seen in the time 0 anisotropy and fslow/Φfast values (Table II). The time 0 anisotropy values (r0), which likely reflect very fast and unresolvable tether-arm motions (19–21), are higher for the L76C (0.27) and Y124C (0.28) than the E81C (0.25) and E84C (0.25) labeled mutants (Table II). This would suggest, not surprisingly, greater hindrance to the tether-arm movements of the gorge-residing residues. The fslow/Φfast values of reporter groups attached to the residues that project into the gorge are smaller (L76C, 0.07 and Y124C, 0.05) than the fslow/Φfast values of reporter groups that are on the outer surface of the enzyme (E81C, 0.13 and E84C, 0.15; Table II). Because the fslow/Φfast ratio is on the time scale of backbone motions and can be analyzed in terms of the diffusion rate in a “diffusion-in-a-cone model” (22), these results indicate that the ranking of segmental mobility of the residues examined is as follows: E84C > E81C ≫ L76C > Y124C. The second feature that emerges from a comparison of the fast anisotropy decay parameters, r0 and fslow/Φfast, is that the reporter groups attached to the substituted cysteines in the Cys69-Cys96 Ω loop (L76C, E81C, and E84C)
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Table II

Effect of huperzine and fasciculin on the anisotropy decay parameters of mouse AChE-labeled with IAEDANS

| Mutant   | Inhibitor | \( r_0^{a} \) | \( \phi_{fast}^{c} \) | \( \phi_{slow}^{d} \) | \( \phi_{fast}^{c} \) | \( \phi_{slow}^{d} \) |
|----------|-----------|----------------|---------------------|---------------------|---------------------|---------------------|
| L76C     | None      | 0.27 ± 0.01    | 3.5 ± 0.3           | 0.07 ± 0.01         | 84–111              | 1.2                 |
| Huperzine| 0.28 ± 0.02| 0.26 ± 0.01    | 3.6 ± 0.6           | 0.07 ± 0.01         | 84–119              | 1.3                 |
| E81C     | None      | 0.25 ± 0.01    | 3.6 ± 0.3           | 0.05 ± 0.01         | 92–118              | 1.2                 |
| Fasciculin| 0.29 ± 0.01| 0.21 ± 0.01    | 3.2 ± 0.2           | 0.13 ± 0.01         | 76–105              | 1.7                 |
| Huperzine| 0.26 ± 0.01| 0.40 ± 0.01    | 2.3 ± 0.1           | 0.18 ± 0.01         | 84–118              | 1.3                 |
| E84C     | None      | 0.25 ± 0.01    | 4.2 ± 0.2           | 2.9 ± 0.1           | 0.15 ± 0.01         | 90–132              | 1.3                 |
| Fasciculin| 0.22 ± 0.02| 0.68 ± 0.02    | 2.8 ± 0.2           | 0.25 ± 0.03         | 80–260              | 1.2                 |
| Huperzine| 0.26 ± 0.01| 0.24 ± 0.02    | 4.6 ± 0.8           | 0.05 ± 0.01         | 69–87               | 1.1                 |
| Y124C    | None      | 0.28 ± 0.01    | 6.6 ± 0.6           | 0.05 ± 0.01         | 75–89               | 1.2                 |
| Huperzine| 0.29 ± 0.02| 0.18 ± 0.01    | 4.0 ± 0.7           | 0.04 ± 0.01         | 74–91               | 1.4                 |

\[ a] The time 0 anisotropy.

\[ b] The fraction of the observed anisotropy decay associated with the “fast” depolarization processes.

\[ c] Fast rotational correlation time.

\[ d] The range of the “slow” rotational correlation times that yield \( \chi^2 \) values 5% above the minimum.

\[ e] The reduced \( \chi^2 \).

\[ f] Geometric averaged lifetimes (\( \Sigma \phi_r \), where \( \Sigma \phi_r = 1 \)).

\[ g] ND, not determined.

are more mobile than the reporter group attached to the non-Ω loop residue (Y124C) (Table II).

Ligand Binding and Anisotropy Decay—The influence of huperzine and fasciculin on the anisotropy decay parameters of the labeled mutants studied is complex and ligand-dependent. Huperzine, which should not interact with the modified Ω loop residues (Fig. 1C), selectively increased the mobility of just the labeled E81C and E84C residues without affecting the mobility of the labeled L76C or Y124C residues. This is evidenced in the \( f_{fast}/\phi_{fast} \) values of the labeled-E81C and E84C mutants that increased upon huperzine binding from 0.13 to 0.18 and from 0.15 to 0.25, respectively (Table II: Fig. 3). The fact that the fluctuations of just the E81C and E84C were perturbed, and not L76C in the Ω loop, is indicative of a lack of conformational coupling between Leu76 and Glu84/Glu84 positions in the Ω loop. Hence, the Ω loop does not behave as a rigid flap.

Consistent with the crystal structure showing fasciculin hydrogen bonding (from the guanidino moiety of Arg77) to the carbonyl oxygen of Leu76 (2.83 Å; Fig. 1), the \( f_{fast}/\phi_{fast} \) value of the labeled L76C mutant decreases upon fasciculin binding from 0.07 to 0.05 (Table II) suggesting decreased segmental mobility around L76C. For the E81C mutant, where the wild type crystal structure does not predict any direct interaction with fasciculin, the \( f_{fast}/\phi_{fast} \) value increased from 0.13 to 0.16 (Table II), indicating a modest increase in segmental mobility of the E81C residue, a behavior similar to that seen for huperzine. Combining hydrophobic and electrostatic interactions, the fasciculin-mAChE complex encompasses an interface of 1100 Å. It is surprising that, despite strong suppression of mobility throughout, we observed enhanced dynamic motion around E81C.

For the E84C mutant, whose side chain may come in closer contact with fasciculin than E81C, fasciculin binding may introduce multiple factors that control decay of anisotropy. Fasciculin slowed the fast anisotropy decay processes suggesting a reduced segmental mobility (\( f_{fast}/\phi_{fast} \) decreases from 0.15 to 0.05) and accelerated the slow anisotropy decay processes (the range of \( \phi_{slow} \) values decreased from 90–132 to 69–87 ns; Table II). These seemingly complex decay profile emerges without direct occlusion between the fluorophore and toxin as evidenced by the fact that fasciculin binding produced no significant change in \( r_0 \) and only a minimal change in the steady-state and emission lifetime parameters (Tables I and II). However, the fasciculin molecule should stabilize the Ω loop near the rim of the gorge, and Arg77 on fasciculin may electrostatically interact with the sulfonic acid moiety of the conjugated AEDANS. A possible explanation for the complex decay profile at Glu84 is that fasciculin induced large amplitude, slow backbone fluctuations around E84C that both slowed the segmental fluctuation rate and increased its amplitude. Such internal fluctuations might occur if the C-terminal half of the Ω loop starting at about Glu84 became less tethered to the core of the molecule and underwent larger angular excursions on a time scale between 10 and 50 ns. Such conformational fluctuations would accelerate the observed slow rotational correlation time. From this perspective, fasciculin has a disordering effect at both E81C and E84C. Clearly, additional studies are required to establish the mechanistic basis for these increased fluctuations.

For the labeled Y124C mutant, the anisotropy decay could not be reasonably fit to a simple decay model. Instead of continuously decreasing from time 0, the anisotropy increased very slightly for a few nanoseconds and then decreased. Moreover, the total depolarization process was much slower than any other decay profiles examined (Fig. 3). This pattern of decay probably results from a complex association of emission lifetimes with specific rotary diffusional processes (23). Combined with the very substantial (55%) increase in the total emission described above and the close proximity of Tyr124 to fasciculin in the crystal structure of mAChE (PDB code 1KU6), which would presumably be closer in the 5(((acetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (AEDANS)-labeled mutant, the complex anisotropy decay of the fasciculin-bound and labeled Y124C mutant strongly suggests a direct reporter group-fasciculin interaction and subsequent reduction in the mobility of Y124C. This direct interaction makes problematic an assessment of the effect of fasciculin on the segmental motion around the Tyr124 residue in the wild type enzyme.
Comparison of Experimental and Simulated Anisotropy Decay—To validate further fluorescence anisotropy decay to measure segmental mobility, the anisotropy parameters were compared with the calculated decay in the time correlation function $\langle \rho \rangle$ (where $\rho$ is time correlation function of a vector representing the direction that goes from the $\alpha$-carbon to an atom near the tip of the wt side chain) for each mutated site. The results of this comparison for the apoenzyme are illustrated in Fig. 4. Because $\rho$ has the characteristics of a time correlation function, it becomes less trustworthy as $\tau$ increases and the number of samples diminishes. Therefore, only the first one-third of the 15-ns $\rho(\tau)$ profile is shown (Fig. 4B). For the unliganded mAChE, the simulated mobility ranking for the four sites of interest was E81C > E84C > L76C > Y124C. This is similar, but not identical, to the experimental anisotropy measures of segmental mobility, $f_{\text{fast}}/f_{\text{slow}}$, whose mobility ranking was E84C > E81C > L76C > Y124C.

Why the theoretical calculations show the motions of residue
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81 randomizing more rapidly than residue 84, whereas the experimental data show the opposite, is unclear. However, there are several explanations to be considered. First, experimental anisotropy decay is dependent upon the orientation of the emission transition dipole relative to the major directions of the movement of the reporter group, and the correlation function is not. Second, cysteine substitutions and fluorophore conjugation could have perturbed the local environment differentially affecting the mobilities of the substituted side chains. Finally, the experimental $f_0/\Delta f_{0\infty}$ ratio is not measuring the same motions as $\rho(\tau)$. $\rho(\tau)$ reflects both side chain and segmental motions of the studied residue, whereas $f_0/\Delta f_{0\infty}$ is more reflective of ensemble averaged segmental motions of the fluorophore conjugated and adjacent amino acid residues (20).

**General Conclusions—**As discussed above, several lines of evidence point toward the existence of transient gorge enlargements that would facilitate substrate binding and product egress. These include molecular dynamics simulations that predict breathing or gating movements (8–10) and the para- doxically high substrate turnover number in relation to the small diameter of the active-site gorge when compared with the cross-sectional size of acetylcholine. This report adds further credence to the existence of transient gorge enlargements by experimentally demonstrating that the outer wall of the active-site gorge, the Cys$^{69}$–Cys$^{96}$ Ω loop, is conformationally flexible and capable of undergoing residue-specific and ligand-dependent changes in backbone motions. The fact that all published AChE crystal structures depict only a narrow active-site gorge does not provide conclusive evidence for a static, narrow active-site gorge in solution (5–7). A growing body of evidence suggests that the conditions of AChE crystallization simply favor a narrow gorge conformational state (13).

Although we only have a fragmentary experimental picture of the conformational dynamics of the mAChE, this report and an ongoing study using site-directed labeling with fluorescein maleimide of three additional sites near the gorge shows only a partial degree of coupling of the fluctuations between residues. If this reflects a general feature of AChE conformational dynamics, then it would suggest that the transient gorge enlargements result from random or near random (non-concerted) fluctuations that periodically result in gorge enlargement. These near random fluctuations leading to periodic gorge enlargements would presumably occur in a time frame of nanoseconds or subnanoseconds (24). Despite the high catalytic efficiency of AChE with a turnover number of 10$^4$ s$^{-1}$, this time interval for gorge fluctuations is short in relation to the time required for individual catalytic steps and appreciable diffusion of substrate.

In summary, we examined the possibility that the Cys$^{69}$–Cys$^{96}$ Ω loop, which forms the outer wall of the active-site gorge of mAChE, plays a role in transient gorge enlargement by using site-directed labeling in conjunction with time-resolved fluorescence anisotropy. A molecular dynamics simulation of the side chain and segmental motions of the mutated residues was also performed to further validate this approach. The results indi-

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