Reversibly Bound and Covalently Attached Ligands Induce Conformational Changes in the Omega Loop, Cys\textsuperscript{69}–Cys\textsuperscript{96}, of Mouse Acetylcholinesterase*

We have used a combination of cysteine substitution mutagenesis and site-specific labeling to characterize the structural dynamics of mouse acetylcholinesterase (mAChE). Six cysteine-substituted sites of mAChE (Leu\textsuperscript{76}, Glu\textsuperscript{81}, Glu\textsuperscript{84}, Tyr\textsuperscript{124}, Ala\textsuperscript{262}, and His\textsuperscript{287}) were labeled with the environmentally sensitive fluorophore, acrylodan, and the kinetics of substrate hydrolysis and inhibitor association were examined along with spectroscopic characteristics of the acrylodan-conjugated, cysteine-substituted enzymes. Residue 282, being well removed from the active center, appears unaffected by inhibitor binding. Following the binding of ligand, hypsochromic shifts in emission of acrylodan at residues 124 and 287, located near the perimeter of the gorge, reflect the exclusion of solvent and a hydrophobic environment created by the associated ligand. By contrast, the bathochromic shifts upon inhibitor binding seen for acrylodan conjugated to three omega loop (Ω loop) residues 76, 81, and 84 reveal that the acrylodan side chains at these positions are displaced from a hydrophobic environment and become exposed to solvent. The magnitude of fluorescence emission shift is largest at position 84 and smallest at position 76, indicating that a concerted movement of residues on the Ω loop accompanies gorge closure upon ligand binding. Acrylodan modification of substituted cysteine at position 84 reduces ligand binding and steady-state kinetic parameters between 1 and 2 orders of magnitude, but a similar substitution at position 81 only minimally alters the kinetics. Thus, combined kinetic and spectroscopic analyses provide strong evidence that conformational changes of the Ω loop accompany ligand binding.

Jianxin Shi, Aileen E. Boyd‡, Zoran Radic, and Palmer Taylor§

From the Department of Pharmacology, University of California, San Diego, La Jolla, California 92093

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‡ Present address: Dept. of Oral and Maxillofacial Surgery, University of California, San Francisco, CA 94153-0440.

§ To whom correspondence should be addressed: Dept. of Pharmacology, University of California, San Diego, San Diego, La Jolla, CA 92093. Tel.: 858-534-1366; Fax: 858-534-8248; E-mail: pwtaylor@ucsd.edu.

\textsuperscript{1} The abbreviations used are: AChE, acetylcholinesterase; mAChE, mouse acetylcholinesterase; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); MEPQ, 7-[(methylthioxoyphosphinyl)-oxy]-1-methylquinolinium iodide; TFK\textsuperscript{1}, m-N,N,N-trimethylammonio trifluoromethyl acetoephone; TFK\textsuperscript{2}, m-tert-butyl trifluoromethylacetophenone; acrylodan, 6-acryloyl-2-dimethylaminonaphthalene.

Acetylcholinesterase (AChE),\textsuperscript{1} a serine hydrolase in the α/β-fold hydrolase protein superfamily (1), terminates nerve sig-
We examined the kinetics of substrate catalysis and inhibitor association with the modified enzymes, and we correlate these kinetic parameters with the spectroscopic changes in the conjugated acrylodan upon ligand association. Fluorescence measurements reveal changes in conformation reflected in the substituted side chains well removed from the active center gorge. The results suggest that ligand binding at the catalytic site allosterically alters the conformation of a specific segment of the $\Omega$ loop whereby gorge closure occurs and residue side chain positions distal to the binding site are affected.

**EXPERIMENTAL PROCEDURES**

**Inhibitors and Substrates**—Acetylthiocholine iodide, 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), dithiothreitol, tacrine (9-amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate), BW286c51, decamethonium, and edrophonium were purchased from Sigma. $m$-(N,N,N-trimethylammonio)trifluoromethylacetophenone (TFK$^-$) was synthesized as described (21) and kindly provided by Dr. Daniel Quinn, University of Iowa, Iowa City, IA. 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, San Diego, CA) containing an encoding cDNA where the AChE sequence was terminated at position 548. The plasmid was transfected into HEK293 cells. Cells were selected with G418 to obtain stable producing cell lines, and AChE was expressed as a secreted soluble enzyme in serum-free media (20). Mutant enzymes were generated by standard mutagenesis procedures, and cassettes containing the mutation were subcloned into pCDNA 3 (20). Nucleotide sequences of the cassettes were confirmed by double-stranded sequencing to ensure that spurious mutations were not introduced into the coding sequence. Affinity chromatography using $m$-(aminophenyl)trimethylammonium linked through a long chain to Sepharose CL-4B resin (Sigma) permitted one-step purification of AChE. From 4 to 6 liters of media, mutant and wild type enzymes were purified in quantities ranging between 5 and 25 mg, as described previously (22–24). Purity was ascertained by SDS-PAGE and by measurements of specific activity.

**FIG. 1.** Locations of introduced cysteines for fluorophore modification. Residues 76, 81, and 84 are at the tip (76) and outer portion (81, 84) of the $\Omega$ loop. Residues 124 and 287 are on an opposing face of the gorge and make up part of the peripheral anionic site. Residue 262 is on a peripheral disulfide loop and in the crystal has a large thermal factor. A–D, Connolly surface representations of structure. A, unliganded AChE (6); B, TFK$^-$ conjugated with AChE; note partial exposure of the white molecule, TFK$^-$, at the base of the gorge (33); C, fasciculin 2 bound AChE at the mouth of the gorge (5); D, fasciculin 2 complex with AChE, rotated 90°. Acrylodan conjugated to E84C is shown in yellow; and acrylodan conjugated to E81C is shown in green. (Note in D the proximity between arginine 11 on Fas 2 and the acrylodan side chain at position 84).
TABLE I

| Enzyme  | $K_m$  | $K_{SS}$ | $b$  | $k_{cat}/min$ | $k_{cat}/K_m$ |
|---------|--------|----------|------|---------------|--------------|
| WT      | 54 ± 16| 14 ± 5   | 0.2 ± 0.07 | 1.6 ± 0.4 | 3.0 |
| Y124C   | 65 ± 17| 20 ± 14  | 0.2 ± 0.09 | 1.4 ± 0.3 | 2.2 |
| H286C   | 58 ± 7 | 12 ± 6   | 0.2 ± 0.06 | 1.8 ± 0.2 | 3.1 |
| A262C   | 59 ± 4 | 11 ± 1   | 0.2 ± 0.04 | 1.6 ± 0.1 | 2.7 |
| L76C    | 97 ± 19| 17 ± 1   | 0.2 ± 0.03 | 1.8 ± 0.1 | 1.9 |
| E81C    | 57 ± 6 | 11 ± 1   | 0.2 ± 0.03 | 1.6 ± 0.1 | 2.9 |
| E84C    | 190 ± 9| 26 ± 2   | 0.2 ± 0.05 | 1.9 ± 0.4 | 1.0 |

* Data are from Ref. 20.

__Assay of Catalytic Activity__—The spectrophotometric method of Ellman was used (25), and kinetic constants for acetylcholinesterase hydrolysis were determined by fitting the observed rates as described (26). Titration of active sites with known concentrations of the irreversible phosphorylating agent, MFEOQ, was accomplished by the method of Levy and Ashani (27).

__Acrylodan Labeling__—Mutant enzymes were pretreated with 0.25 mM dithiothreitol for 30 min at room temperature to ensure reduction of the introduced cysteine. Excess dithiothreitol was removed by use of a G-50 spin column (Pharmacia Fine Chemicals) equilibrated in 10 mM Tris, 100 mM NaCl, 40 mM MgCl$_2$, pH 8.0. A volume of 1 ml of acrylodan at 100 times the enzyme concentration was slowly mixed with the enzyme to achieve an ~5-fold molar excess of acrylodan to mutant enzyme. Labeling was allowed to proceed for at least 12 h at 4 °C, and unreacted acrylodan was removed by size exclusion chromatography using Sephadex G-25 (Amersham Pharmacia Biotech) in 0.1 M sodium phosphate buffer, pH 7. Concentrations of acrylodan-labeled enzyme were determined from the maximal acrylodan absorbance found between 360 and 380 nm (ε = 18,400 M$^{-1}$ cm$^{-1}$). Stoichiometry of labeling of the various preparations, estimated from a comparison of enzyme concentration by protein (280 nm) to acrylodan (360–380 nm) absorbance, ranged as follows: L76C, 0.7–0.8; E81C, 0.79–1.0; E84C, 0.77–1.0; Y124C, 0.79–1.0; A262C, 0.69–0.85; and H286C, 0.82–0.88. Specificity of labeling was assessed by comparison of areas under the fluorescence emission curves for acrylodan-treated mutant and wild type enzymes. Specific labeling for each mutant was as follows: L76C, 97–95%; E84C, 93–95%; Y124C, 83–90%; A262C, 80–90%; H286C, 70–76%.

__Tritfluoroacetophenone Inhibition__—Picomolar amounts of enzyme in 0.01% bovine serum albumin in 0.1 M sodium phosphate buffer, pH 7.0, were reacted with TFK$^+$ in the absence of substrate. Inhibition was monitored by measuring residual enzyme activity by removal of aliquots during the course of the reaction. Bimolecular rate constants of inhibition were determined by nonlinear fits of the data (28).

__Spectrofluorometric Assays__—Steady-state emission spectra were measured at room temperature using a Jobin Yvon/Spex FluoroMax II spectrofluorometer (Instrument S.A., Inc., Edison, NJ) with the excitation and emission bandwidths set at 5 nm. The excitation wavelength for acetylcholinesterase was set at 359 nm, and emission was monitored between 420 and 600 nm. Equilibrium dissociation constants, $K_d$, for BW286c51 and edrophonium with the acrylodan-labeled enzyme were obtained by titration of a fixed quantity of labeled enzyme (54–120 nM) with various concentrations of indicated inhibitors. $K_d$ values were obtained by monitoring the fractional decrease in the total area under the fluorescence emission curve from 420 to 600 nm for the acrylodan-labeled E84C or a limited segment of the emission between 450 and 485 nm (ε = 18,400 M$^{-1}$ cm$^{-1}$). Stoichiometry of labeling of the various preparations, estimated from a comparison of enzyme concentration by protein (280 nm) to acrylodan (360–380 nm) absorbance, ranged as follows: L76C, 0.7–0.8; E81C, 0.79–1.0; E84C, 0.77–1.0; Y124C, 0.79–1.0; A262C, 0.69–0.85; and H286C, 0.82–0.88. Specificity of labeling was assessed by comparison of areas under the fluorescence emission curves for acrylodan-treated mutant and wild type enzymes. Specific labeling for each mutant was as follows: L76C, 97–95%; E84C, 93–95%; Y124C, 83–90%; A262C, 80–90%; H286C, 70–76%.

__Schematic 1__—In this scheme substrate can combine at two discrete sites to form two binary complexes, ES and SE (where S is substrate; E is enzyme; and P is product). Only ES results in substrate hydrolysis. For simplicity, S is assumed to combine equally well with E and ES. The efficiency of substrate hydrolysis of the ternary complex SES, as compared with ES, is reflected in the value of the parameter, b, the relative catalytic turnover of the ternary complex.

__RESULTS__

__Characterization of Substrate Hydrolysis and Fasciculin 2 Inhibition__—The cysteine-substituted enzymes show kinetics of acetylcholinesterase hydrolysis similar to wild type enzyme (Table I and Scheme 1) suggesting that all mutant enzymes fold correctly despite the presence of the newly introduced cysteine. The $K_m$ value of E84C shows slightly less than a 4-fold increase, whereas the change in turnover rate, $k_{cat}$, is minimal. Similar changes in kinetic constants were observed previously for E84Q mAChE (28). Since $K_m$, in diffusion limited catalysis, depicts the initial encounter between substrate and enzyme, an increase in $K_m$ likely arises from the reduction of negative charge that electrostatically steers the cationic substrate into the active center gorge. Interestingly, a similar E81C mutation has little or no effect on substrate hydrolysis. Not all negatively charged residues around the active center appear to be involved equivalently in electrostatic steering.

Association and dissociation rates of fasciculin with A262C, H286C, and Y124C mutant enzymes were also found to be close to the rates with wild type enzyme (20). Fasciculin, at low concentrations, is also capable of associating with the mutant enzymes after acrylodan conjugation (Fig. 2). In addition, enzyme activity measurements of fasciculin-bound acrylodan conjugates show greater than 99% inhibition (data not shown).

__Influence of Residue Modification on Inhibition by m-Tri- methylammoniotrifluoroacetophenone__—TFK$^+$ binding to cysteine-substituted enzymes, both free and modified with acrylodan, was also examined (Table II). For E81C and E84C, the association rate constants ($k_{on}$) for TFK$^+$ were obtained from measurements of enzyme activity. Although positions 81 and 84 are both spatially removed from TFK$^+$-binding site, $k_{on}$
for E84C is slightly slower than that for wild type enzyme. By
contrast, E81C shows no difference in the kinetic constants.
Conjugation of acrylodan, a neutral naphthalene derivative,
with E84C reduces \( k_{\text{on}} \) of TFK \(^*\) 7-fold compared with unconju-
gated E84C, whereas conjugation of E81C with acrylodan only
reduces \( k_{\text{on}} \) of TFK \(^*\) slightly. For acrylodan-labeled mutants,
\( k_{\text{on}} \) was measured from the time-dependent decrease of fluo-
rescence signal (Fig. 3).

Influence of Residue Modification on Inhibition by Nonco-
valent Active Site Inhibitors—A similar trend in inhibition kinetics was seen with noncovalent active site inhibitors such as
edrophonium and BW286c51 (Table II). An increase over wild
type \( K_d \) of 2-fold occurs for edrophonium binding to E84C, and
an 18-fold increase in \( K_d \) is observed for BW286c51 binding.
Similar increases in \( K_d \) of edrophonium and BW286c51 were
seen for E84Q human AChE (18). By comparison, E81C showed
no alterations in ligand binding constants. For acrylodan-la-
beled mutants, \( K_d \) was measured from the fluorescence signals of
an equilibrium titration (Fig. 4). Acrylodan-labeled E84C shows
\( K_d \) increases of 10-fold for edrophonium and 3-fold for
BW286c51 compared with unreacted E84C. For acrylodan-la-
beled E81C, only a slight increase in \( K_d \) is seen for both ligands.
The high concentration of acrylodan-labeled E81C required for
equilibrium titrations precludes an accurate estimate of \( K_d \)
for high affinity ligands such as BW286c51.

Effect of Fasciculin on Acrylodan Fluorescence Emission—
The peptide toxin, fasciculin, inhibits AChE by tightly coupling the
mouth of active center gorge (Fig. 1) (11, 30–32). Table III shows
changes in emission maxima of acrylodan-labeled AChE mutants in the presence of fasciculin. There is no discernible
change in fluorescence emission of acrylodan-conjugated
A262C (20), consistent with the position 262 being distal to the
fasciculin-binding site. The large hypsochromic shifts seen at
both the 124 and 287 positions reflect solvent exclusion and an
increase in hydrophobicity experienced by the fluorophores in
the gorge upon fasciculin binding (20). For the \( \Omega \) loop mutant,
L76C, fasciculin binding produces a 40% increase in quantum
yield but no change in emission maximum. Bathochromic shifts
are found at both the 81 and 84 positions, with position 84
producing a shift of larger magnitude (Fig. 2 and Table III).

Effect of Covalently Conjugated Active Site Inhibitors on Ac-
rylodan Fluorescence Emission—Changes in emission maxima of
acrylodan-labeled AChE mutants in the presence of conjugat-
ing trifluorocacetophenones are shown in Table IV. The tri-
fluorocacetophenones inhibit the enzyme by conjugating to form
a hemiketal at active site serine without dissociation of leaving group (33). Both the isoester neutral and cationic trifluoro-
ketones (TFK\(^0\) and TFK\(^+\)) produced no discernible changes in
emission spectra of acrylodan conjugated at H287C and A262C,
consistent with a fluorophore position distant from gorge base
and hence not in direct contact with ligand. Remarkably, both
TFK\(^0\) and TFK\(^+\) produce a substantial bathochromic shift (at
least 30 nm) with acrylodan-E84C. The trifluoroketones also
produce spectral shift of intermediate value (20 nm) for E81C
and a much smaller change (4–6 nm) for L76C. Interestingly,
neutral TFK\(^0\) produces a large 22 nm of hypsochromic shift
with the \( \Omega \)124C acrylodan conjugate.

\( O,O\)-Dimethyl-O-(2,2-dichlorovinyl)phosphate, a small achiral
organophosphonate, phosphorylates the active site serine of
mAChE, with subsequent departure of the dichlorovinyloxy
fluoroacetophenones inhibit the enzyme by conjugating to form
a hemiketal at active site serine without dissociation of leaving group (33). Both the isoester neutral and cationic trifluoro-
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and a much smaller change (4–6 nm) for L76C. Interestingly,
neutral TFK\(^0\) produces a large 22 nm of hypsochromic shift
with the \( \Omega \)124C acrylodan conjugate.

**Table II**

| Enzyme | \( k_{\text{on}} \) \( \times 10^9 \) \( \text{m}^{-1} \text{min}^{-1} \) | \( k_{\text{on}} \) WT | \( k_{\text{on}} \) mutant | \( K_d \) | \( K_d \) mutant | \( K_d \) WT | \( K_d \) WT |
|--------|------------------|---------------|----------------|------|---------------|------|------|
| Wild type | 150 | 1 | 150 | 250\(^a\) | 2.0\(^a\) | 150 | 250\(^a\) |
| E81C | 150 | 1 | 150 | 640 | 6.9 | 150 | 640 |
| E81C-acrylodan | 94 | 1.6 | 94 | 650 | 33\(^a\) | 94 | 650 |
| E84C | 93 | 1.6 | 93 | 6300 | 18 | 93 | 6300 |
| E84C-acrylodan | 13 | 11 | 13 | 130 | 65 | 13 | 130 |

\(^a\) Data are from Ref. 29.

\(^b\) Equilibrium dissociation constants are derived from the ratio of \( k_{\text{off}}/k_{\text{on}} \) using stopped-flow measurement of tryptophan fluorescence quenching.
spectra. Indeed, acrylodan conjugated at positions 124, 262, and 287 showed very little or no change in spectrum. However, bathochromic shifts at positions 81 and 84 were observed, although of smaller magnitude for E84C when compared with other ligands (Table IV).

Effect of Noncovalent Active Site Inhibitors on Acrylodan Fluorescence Emission—Noncovalent active site inhibitors, such as edrophonium, tacrine, and huperzine, associate primarily with the choline subsite at the base of active site gorge. Crystal structures of inhibitors bound to Torpedo californica AChE revealed that these ligands should have no direct contact with the conjugated fluorophore at all six cysteine-substituted sites (36, 37). Upon edrophonium, tacrine, or huperzine association, alteration of acrylodan emission maxima is undetectable for positions 124, 287, and 262 (Table V). However, as seen for other ligands, acrylodan conjugated at E84C surprisingly shows a bathochromic shift of 33 nm (from 477 to 510 nm) upon inhibitor binding. A change of smaller magnitude is seen in the case of acrylodan-L76C (from 505 to 509 nm) and acrylodan-E81C (from 480 to 510 nm) with noncovalent active site inhibitors. Ligand binding results in a common emission maximum ($\lambda_{\text{max}}$) for acrylodan at the three loop positions.

Effect of Bisquaternary Inhibitors on Acrylodan Emission Spectrum—Extended bisquaternary inhibitors, such as BW286c51 and decamethonium, belong to a class of inhibitors...
that interact with two binding sites of AChE simultaneously (32, 38–39). The quaternary ammonium moiety on one end of the molecule associates with the Trp 86 residue that characterized the choline-binding site, whereas the other end resides near Trp286 at the active site gorge rim. Table VI shows changes in emission maxima of acrylodan-labeled AChE mutants in the presence of bisquaternary inhibitors. No changes are observed at position 262. By contrast, both decamethonium and BW284c51 caused a pronounced hypsochromic shift and increase in quantum yields with acrylodan conjugated at Y124C and H287C. Addition of decamethonium produced a hypsochromic shift of 35 nm at position 124, and a modest 7 nm shift at position 287. BW284c51 has a similar effect; for the /H9024 loop mutants, L76C, E81C, and E84C, bathochromic shifts of similar magnitude to the monoquaternary ligands were observed (Tables V and VI).

**DISCUSSION**

**Characteristics of Fluorescence from Acrylodan-conjugated Cysteine Residues**—Fluorescence emission of acrylodan is exquisitely sensitive to the dielectric constant of the solvent. In general, the fluorescence emission spectrum of acrylodan shifts toward the red (bathochromic) shift, and the quantum yield decreases as the polarity of solvent increases (20, 40–42). This sensitivity to solvent polarity arises from the interaction of the excited state of acrylodan with its surrounding solvent. The excited state is more polar than the ground state and, as such, will interact with a polar solvent so as to align solvent dipoles. This alignment lowers the energy of the excited state and causes the red shift of the emission spectrum. Hence, an acrylodan-labeled enzyme with an emission maximum of 510–525 nm likely reflects exposure of the side chain to solvent (20, 42). On the other hand, acrylodan emission maxima in the range of 475–500 nm likely reflect solvent exclusion and a more hydrophobic environment surrounding the fluorophore. The time course of TFK/H11001 reaction with acrylodan-E84C (Fig. 3) reveals a large spectral shift from 477 to 512 nm, indicating acrylodan conjugated at this position has moved to a more hydrophilic environment with TFK/H11001 bound. The large spectral shift yields a clear isoemissive point, which arises when only two distinct emitting species are present, in this case the free enzyme and the TFK/H11001 conjugate.

**Influence of Residue Modification on Ligand Binding**—The changes in emission spectra of acrylodan-labeled Ω loop residues 81 and 84 have been exploited to monitor ligand binding
TABLE VI
Fluorescence emission parameters of acrylodan-labeled mouse AChE mutants in the presence of bisquaternary ligands

Data are shown as mean values of at least three determinations. Relative quantum yields were determined by comparison of areas of the fluorescence emission curves. Data for the unliganded enzymes are found in Table III.

| Enzyme  | Saturating Decamethonium | Chromic Shift (nm) | Relative Quantum Yield |
|---------|--------------------------|-------------------|------------------------|
| L76C    | 508                      | 3                 | 1.13                   |
| E81C    | 510                      | 21                | 0.98                   |
| E84C    | 512                      | 35                | 0.47                   |
| Y124C   | 487                      | -13               | 1.05                   |
| A262C   | 517                      | 0                 | 0.97                   |
| H287C   | 510                      | -14               | 2.13                   |

(126) We observe that cysteine substitution and acrylodan conjugation at position 84 affect ligand binding kinetics but not at position 81. Cysteine substitution at position 84 has little influence on catalytic parameters derived from steady-state catalysis (Table I). The $K_m$ of E84C increases less than 4-fold compared with the wild type enzyme. By contrast, a similar substitution at position 81 has no effect on $K_m$ steady-state kinetics. Faerman (32, 33) observed that cysteine substitution and acrylodan conjugation at position 84 affect ligand binding kinetics but not at position 81. Although a cysteine substitution at position 81 has no effect on $K_m$, influence on catalytic parameters derived from steady-state kinetic parameters with structural perturbations. Our site-directed fluorophore labeling provides a physical assessment of the localized conformational change in the $\Omega$ loop. In cases where the fluorophore makes direct contact with the ligand, as for acrylodan-labeled Y124C and H287C with fasciculin, the energetic perturbations from substitution are larger, since complementarity of the binding site may be altered through the insertion of acrylodan side chain at the interface between the ligand and its binding site (20).

Acrylodan Modification at a Site Distal to the Active Center Core—We chose the A262C modification as a positional reference for a site distal to the active center. This residue is also located at the tip of a disulfide loop but is located −30 Å away from the rim of the active center gorge. Crystallographic studies show this region to have a high temperature coefficient (B factor), indicative of substantial molecular motion of this surface residue. In fact, the position of this residue and its immediate neighbors is only secured in crystal forms where proximity of the symmetry-related AChE molecule limits its movement in the crystal structure (6).

Acrylodan substitutions at this position show a low wavelength emission ($\lambda_{\text{max}} = 517$ nm) indicative of exposure to a hydrophilic environment (Table III). Moreover, none of the ligands studied, whether they are covalently attached to the active center (TFK or alkylphosphates), reversibly bound to the active center (edrophonium), span between the active center and peripheral site (decamethonium and BW286c51), or bind only to peripheral site (fasciculin), affect the spectroscopic properties of acrylodan conjugated at site 262 (Tables III–VI). This pattern indicates a lack of global conformational change affecting residue environments in a disulfide loops well removed from the active center (Fig. 1).

Residues Residing on the Active Center Gorge in Apposition with the $\Omega$ Loop—Residues 124 and 287 lie in close proximity to the $\Omega$ loop with H287C at the rim of the gorge and Y124C, residing just below the rim in the gorge interior (Fig. 1). The crystal structure of the complex shows fasciculin to “cap” these residues, and our previous studies show hypsochromic shifts of acrylodan upon fasciculin binding (20). None of the reversibly bound active center ligands (edrophonium, huperzine, and tacrine) induce a spectral shift at position 124 or 287. However, modest quenching is observed at position 124 upon binding of these active center ligands. The bisquaternary ligands, which should approach or come in close apposition with these residues, cause significant hypsochromic shifts. The large shift for decamethonium at position 124 may reflect the ability of the cluster of aromatic residues to collapse around the methylene chain of decamethonium enclosed within the active center gorge. Crystalllographic studies show one quaternary ammone of decamethonium to be consistently positioned in the vicinity of Trp$^8_{124}$; however, both the flexible side chain and the outermost quaternary group are found to associate multiple positions in the decamethonium-AChE complexes studied to date (6, 29).

The distinct spectra observed for the two isosteric trifluoroacetophenone conjugates is surprising (Table IV). Covalent inhibition of cationic trifluoroacetophenone (TFK$^+$) produces very little spectral shift of acrylodan at either position 124 or 287. This is consistent with the crystal structures where the trimethyl ammonio moiety of TFK$^+$ forms a cation−π interaction with Trp$^8_{124}$, and the trifluoroacetophenone moiety forms a hemiketal bond with the active center serine 203 (33). Howev,er, the isosteric $t$-butyl congener (TFK$^t$) shifts the envirom-

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ment of residue 124 to that resembling a hydrophobic state. This difference suggests that the orientation of this hemiketal conjugate differs where the t-butyl group extends toward the gorge exit. TFK\(^{c}\) inhibits the wild type enzyme 70-fold slower than TFK\(^{a}\), presumably due to lack of cation-π interaction and slightly different ligand orientation (21). Alkyl phosphorylation with small alkyl groups also has little influence on the environment at position 124 (Table IV).

Ω Loop Substitutions—Our greatest surprise emerged from studies on the outer portion of the Ω loop, defined by residues between Cys\(^{69}\) and Cys\(^{96}\), where we have examined three positions extending from the near tip of the loop (Leu\(^{76}\)) at the gorge rim descending toward the active center (Glu\(^{81}\) and Glu\(^{84}\)). The residues modified are all on the outer surface and do not form the inner gorge wall. Since residues 81 and 84 carry acidic side chains, they might be expected to show solvent exposure in the native enzyme and not be involved in the internal stabilization of the loop, as is evident in the crystal structure of the mouse enzyme (5, 6). In the absence of ligand, the spectra of the conjugated acrylodan moiety reveal different degrees of solvent exposure with the acrylodan at position 84 being the most protected in an hydrophobic environment, acrylodan at 81 being intermediate, and acrylodan at 76 being most exposed. Examination of crystal structures of mouse enzyme revealed a surface cavity near the side chain of the 84 site (5, 6). The observed \(\Delta\lambda_{\text{max}}\) likely reflects acrylodan buried in this surface cavity when conjugated to the 84 site (Fig. 1).

The presence of fasciculin causes a large bathochromic shift of acrylodan fluorescence at both the 81 and 84 positions, as well as increase in quantum yield of acrylodan at 76. The lack of a shift in emission seen for acrylodan at the 76 position may simply reflect a balance between a small environmental change at 76 upon ligand binding in general and partial solvent occlusion at this position by fasciculin. In the case of Glu\(^{84}\), the bathochromic shift likely reflects Arg\(^{11}\) of fasciculin loop I coming in van der Waals contact with the 84 side chain and displacing acrylodan into a more polar environment. However, an explanation of the bathochromic shift at position 81 requires a more involved analysis. Although 81 is removed from the fasciculin-binding site, fasciculin has a sufficient molecular dimension to restrict the Ω loop so that the entire loop freezes or closes upon fasciculin binding. Thus, fasciculin binding may confer strain on the α-carbon backbone structure of the Ω loop such that the acrylodan side chain at positions 81 and 84 becomes exposed to the hydrophilic environment. The fact that substitutions at both positions yielded acrylodan spectra with equivalent emission maxima after ligand binding suggests a conformational involvement of the entire loop.

Similar to fasciculin, small ligands that bind to the active center produce a similar strain. All of the small ligands, whether reversibly bound or covalently attached, elicit marked changes in acrylodan emission with the largest spectral shift seen for ES4C, an intermediate value seen for ES1C, and only small change observed for L76C. In each case the conformational change induced by the ligand causes the acrylodan to move into a region of higher dielectric constant, presumably being more solvent-exposed. The pattern is remarkably consistent among the ligands, and only the small organophosphates when conjugated induces a shift of smaller magnitude. A likely explanation for the observed conformational changes is that ligand binding to the active center induces gorge closure, which is mediated throughout the Ω loop. The strain placed on the α-carbon backbone upon gorge closure causes the side chains to shift positions and become exposed to hydrophilic environment.

DeFarri et al. (43) have noted that the peripheral site inhibitor, thioflavin T, when bound to AChE, shows a large enhance-
15. Schrag, J. D., and Cygler, M. (1993) J. Mol. Biol. 230, 575–591
16. Grochulski, P., Li, Y., Schrag, J. D., Beuthiller, F., Smith, P., Harrison, P., Ruhin, B., and Cygler, M. (1993) J. Mol. Biol. 230, 72843–72847
17. Grochulski, P., Li, Y., Schrag, J. D., and Cygler, M. (1993) Protein Sci. 3, 82–91
18. Velan, B., Barak, D., Ariel, N., Leitner, M., Bino, T., Ordentlich, A., and Sussman, J. (1996) FEBS Lett. 395, 22–28
19. Faerman, C., Ripoll, D., Ben, S., Lefèvre, Y., Morel, N., Massoulie, J., Sussman, J., and Silman, I., (1996) FEBS Lett. 386, 65–71
20. Boyd, J. D., and Young, M. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 395–398
21. Nair, H. K., Seravalli, J., Arbuckle, T., and Quinn, D. M. (1994) Biochemistry 33, 8566–8576
22. Marchot, P., Ravelli, R. B., Raves, M. L., Bourne, Y., Vellom, D. C., Kanter, J., Camp, S., Sussman, J. L., and Taylor, P. (1996) Protein Sci. 5, 672–679
23. Berman, J. D., and Young, M. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 395–398
24. De la Hoz, D., Doctor, B. P., Ralston, J. S., Rush, R. S., and Wolfe, A. D. (1986) Life Sci. 39, 195–199
25. Ellman, G. L., Courtney, K. D., Andres, V. J., and Featherstone, R. M. (1961) Biochem. Pharmacol. 7, 88–95
26. Radic, Z., Pickering, N. A., Vellom, D. C., Camp, S., and Taylor, P. (1993) Biochem. Pharmacol. 42, 12074–12084
27. Levy, D., and Ashani, Y. (1986) Biochem. Pharmacol. 35, 1079–1085
28. Radic, Z., Kirchhoff, P. D., Quinn, D. M., McCammon, J. A., and Taylor, P. (1997) J. Biol. Chem. 272, 23265–23277
29. Taylor, P., and Radic, Z. (2001) J. Biol. Chem. 276, 4622–4633
30. Radic, Z., Duran, R., Vellom, D. C., Li, Y., Cervenansky, C., and Taylor, P. (1994) J. Biol. Chem. 269, 11233–11239
31. Taylor, P., and Radic, Z. (1994) Annu. Rev. Pharmacol. Toxicol. 34, 281–320
32. Marchot, P., Khelif, A., Ji, Y. H., Mansuelle, P., and Bougis, P. E. (1993) J. Biol. Chem. 268, 12458–12467
33. Harel, M., Quinn, D. M., Nair, H. K., Silman, I., and Sussman, J. L. (1996) J. Am. Chem. Soc. 118, 23440–23446
34. Wilson, I. B. (1960) in The Enzymes (Boyer, P. D., Lardy, H., and Myrback, K., eds) Vol. 4, 2nd Ed., pp. 501–520, Academic Press, New York
35. Wong, L., Radic, Z., Bruggemann, R. J., Hosea, N., Berman, H. A., and Taylor, P. (2000) Biochemistry 39, 5750–5757
36. Harel, M., Schalk, I., Ehreetsabatier, L., Bouet, F., Goldner, M., Hirth, C., Axelsen, P. H., Silman, I., and Sussman, J. L. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9031–9035
37. Raves, M. L., Harel, M., Pang, Y. P., Silman, I., Kozikowski, A. P., and Sussman, J. L. (1997) Nat. Struct. Biol. 4, 57–63
38. Taylor, P., and Lappi, S. (1997) Biochemistry 14, 1989–1997
39. Taylor, P., and Jacobs, N. M. (1974) Mol. Pharmacol. 10, 93–107
40. Lakowicz, J. R. (1999) Principles of Fluorescence Spectroscopy, 2nd Ed., pp. 185–210, Kluwer Academic Publishers and Plenum Publishing Corp., New York
41. Lew, J., Coruh, N., Tsigelny, I., Garrod, S., and Taylor, S. S. (1997) J. Biol. Chem. 272, 1507–1513
42. Prendergast, F. G., Meyer, M., Carlson, G. L., Iida, S., and Potter, J. D. (1983) J. Biol. Chem. 258, 7541–7544
43. De Ferrari, G. V., Mallender, W. D., Inestrosa, N. C., and Rosenberry, T. L. (2001) J. Biol. Chem. 276, 23262–23267
44. Millard, C. B., Kryger, G., Ordentlich, A., Greenblatt, H. M., Harel, M., Raves, M. L., Segall, Y., Barak, D., Shafferman, A., Silman, I., and Sussman, J. L. (1999) Biochemistry 38, 7032–7039
45. Tara, S., Elcock, A. H., Kirchhoff, P. D., Briggs, J. M., Radic, Z., Taylor, P., and McCammon, J. A. (1998) Biopolymers 46, 465–474
46. Tai, K., Shen, T., Bjerjesson, U., Philippopoulos, M., and McCammon, J. A. (2001) Biophys. J. 81, 715–724
