Probing the Active Center Gorge of Acetylcholinesterase by Fluorophores Linked to Substituted Cysteines*

Received for publication, January 27, 2000, and in revised form, April 13, 2000
Published, JBC Papers in Press, April 21, 2000, DOI 10.1074/jbc.M000606200

Aileen E. Boyd, Alan B. Marnett‡, Lilly Wong, and Palmer Taylor§

From the Department of Pharmacology (0636), School of Medicine, University of California, San Diego, La Jolla, California 92039

To examine the influence of individual side chains in governing rates of ligand entry into the active center gorge of acetylcholinesterase and to characterize the dynamics and immediate environment of these residues, we have conjugated reactive groups with selected charge and fluorescence characteristics to cysteines substituted by mutagenesis at specific positions on the enzyme. Insertion of side chains larger than in the native tyrosine at position 124 near the constriction point of the active site gorge confers steric hindrance to affect maximum catalytic throughput ($k_{cat}/K_m$) and rates of diffusional entry of trifluoroketones to the active center. Smaller groups appear not to present steric constraints to entry; however, cationic side chains selectively and markedly reduce cation ligand entry through electrostatic repulsion in the gorge. The influence of side chain modification on ligand kinetics has been correlated with spectroscopic characteristics of fluorescent side chains and their capacity to influence the binding of a peptide, fasciculin, which inhibits catalysis peripherally by sealing the mouth of the gorge. Acrylodan conjugated to cysteine was substituted for tyrosine at position 124 within the gorge, for histidine 287 on the surface adjacent to the gorge and for alanine 262 on a mobile loop distal to the gorge. The 124 position reveals the most hydrophobic environment and the largest hypsochromic shift of the emission maximum with fasciculin binding. This finding likely reflects a sandwiching of the acrylodan in the complex with the tip of fasciculin loop II. An intermediate spectral shift is found for the 287 position, consistent with partial occlusion by loops II and III of fasciculin in the complex. Spectroscopic properties of the acrylodan at the 262 position are unaltered by fasciculin addition. Hence, combined spectroscopic and kinetic analyses reveal distinguishing characteristics in various regions of acetylcholinesterase that influence ligand association.

Acetylcholinesterase (AChE), a serine hydrolase in the α/β fold protein superfamily (1), functions at cholinergic synapses to terminate nerve signals by catalyzing ester hydrolysis of the neurotransmitter acetylcholine (2, 3). To enhance synaptic efficiency, AChE has evolved to function rapidly and can catalyze acetylcholine hydrolysis at near diffusion-limited rates (4, 5). Several crystal structures of AChE have been solved, revealing notable features of the tertiary structure (6–8). The active site serine resides at the bottom of a deep and contorted gorge lined primarily with aromatic amino acid side chains. This seemingly less accessible position of the serine raises questions regarding ease of substrate entry and product dissociation, which have been addressed, but incompletely resolved, through molecular dynamics simulations and site-directed mutagenesis studies (9–12). Furthermore, co-crystallization of the tight binding snake toxin fasciculin with mouse and Torpedo californica AChE shows complete occlusion of the active site gorge (7, 13) despite small inhibitors remaining accessible to react with the active site serine of the complex, albeit at reduced rates (14, 15). These residual rates suggest either the presence of alternative entry points for these inhibitors or breathing motions that open a gap between the fasciculin and AChE interfaces.

Fluorescent ligands can be used to probe structural characteristics of proteins in solution. The prototypic AChE peripheral site ligand propidium, for instance, elucidated the nature of a peripheral binding site for inhibitors, remote from the active site serine residue (16). Furthermore, fluorescent phosphonates, which conjugate with the active site serine, were utilized to measure the hydrophobicity of the active site gorge, the contribution of charge to active center accessibility, and the distance between the reactive serine and the peripheral site years before a crystal structure was solved (17, 18). Potentially, site-directed mutagenesis on recombinant DNA-derived AChE should render a broader range of discrete positions available for selective fluorescence labeling on the enzyme surface.

Cysteine substitution mutagenesis, followed by labeling the resulting reactive thiol with methanethiosulfonate (MTS) compounds or fluorescent probes, has been utilized to identify and characterize functionally important residues in several enzyme and receptor systems (19–21). A monomeric form of mouse AChE, in which the C-terminal cysteine is removed, leaving the remaining six cysteines linked through three disulfide bonds in a stable structure (1), presents an ideal candidate for cysteine substitution mutagenesis. In this study, we substitute cysteine into three positions on the enzyme: within the active site gorge (Tyr124), at the gorge rim (His287), and on the enzyme surface.
removed from the gorge entrance (Ala262). The cysteines were then modified with cationic, neutral, and anionic substituents. Substrate and inhibitor binding kinetics were examined to assess the influence of electrostatic and steric parameters on ligand association kinetics. Acrylodan, a fluorescent ligand offering a neutral side chain substitution (22), enabled us to assess the influence of electrostatic and steric parameters on ligand association kinetics. Acrylodan, a fluorescent ligand of AChE complex and relate these physical parameters to the evaluate polarity of the probe environment and solvent accessibility of a neutral side chain substitution (22), enabled us to ligand association kinetics. Acrylodan, a fluorescent ligand of AChE complex and relate these physical parameters to the evaluate polarity of the probe environment and solvent accessibility of a neutral side chain substitution (22), enabled us to ligand association kinetics. Acrylodan, a fluorescent ligand of AChE complex and relate these physical parameters to the evaluate polarity of the probe environment and solvent accessibility of a neutral side chain substitution (22), enabled us to ligand association kinetics. 

**EXPERIMENTAL PROCEDURES**

**Materials**—Acetylthiocholine iodide, 5,5'-dithio-bis(2-nitrobenzoic acid) (Ellman’s reagent), 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromoide (BW284c51), and dithiothreitol were products of Sigma. Substituted methanethiosulfonates (sodium (2-sulfonato-ethyl) methanethiosulfonate (MTSSEI), benzyl methanethiosulfonate (MTSBN)), 2-(trimethylammoniumethyl)methanethiosulfonate bromide (MTSET), and 2-aminoethyl methanethiosulfonate hydrobromide (MTSEA), and acrylodan were purchased from Toronto Research Chemicals, Inc. (Toronto, Canada) or Molecular Probes (Eugene, OR), respectively. Fasciculin 2 (purified from the venom of Dendroaspis angusticeps (m)-bis(2-nitrobenzyl)trifluoroacethonone and m-tert-butyl trifluoroacetyl acetone (TFK and TFFC, respectively) were synthesized as described (23) and kindly provided by Dr. Daniel M. Quinn (University of Iowa). T-[(Methylethoxy)phosphinyl]-1-methylquinolinium iodide (MEPQ) (24) was a gift of Drs. Yacov Ashani and Bhupendra P. Doctor (Walter Reed Army Research Center, Washington, D.C.). All other chemicals were of at least reagent grade.

**Production of Enzymes**—A cDNA encoding mouse AChE truncated at position 548 and yielding a monomeric form of the enzyme has been characterized previously (25). Mutant mouse AChE cDNAs encoding the monomeric form of the enzyme were generated either by Kunkel (26) or polymerase chain reaction-mediated (Stratagene Quik Change Kit) standard mutagenesis procedures. The presence of the mutation was detected by restriction enzyme digestion, and cassettes containing the mutation were subcloned into the mammalian expression vector, pCDNA3 (Invitrogen, San Diego, CA). The nucleotide sequences of the cassettes were confirmed by double stranded sequencing to ensure that spurious mutations were not inadvertently introduced into the coding sequence. The plasmids were purified by standard protocols involving alkaline phenol-chloroform extraction, polyethylene glycol precipitation, and CsCl gradients.

Human embryonic kidney (HEK 293) cells were purchased from American Type Culture Collection (Atlanta, GA) and, 24 h before transfection, plated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at a density of 1.5 × 10⁶ cells per 10-cm dish. Ten µg of plasmid DNA per plate were added to the cells using standard calcium phosphate transfection methods. After approximately 16 h after transfection, the cells were rinsed with phosphate-buffered saline. For transient expression of mutant enzymes, cells were supplied with serum-free media for 48 to 72 h. The media containing the secreted monomeric enzyme was periodically collected and the cells were resupplied with fresh serum-free media. This process continued until at least three harvests of enzyme were obtained. In some cases, enzymes were concentrated for kinetic experiments by use of Centriprep or Centricon 30 concentrators (Millipore Corp., Bedford, MA).

For large scale productions of enzyme, stable transfectants were selected by G418 resistance following co-transfection with a neomycin resistance gene. After transfection and rinsing with phosphate-buffered saline, cells were allowed to recover in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum for 3–5 days after which G418 was set at 355 nm and emission was detected between 420 nm and 500 nm. Emission and excitation slits were set at 5 nm. Rate constants (kobs) for fasciculin association with acrylodan-labeled mutant AChE was assessed by utilization of an Applied Photophysics SX.18MV Fluorometer (Leatherhead, UK) stopped-flow reaction analyzer and observation of the time-dependent increase in fluorescence above 420 nm upon excitation at 355 nm by means of a 420-nm emission cut-off filter. Data were fit according to a single exponential rise to maximum fluorescence. Since competition experiments between radioactively labeled fasciculin and BW284c51 show that binding of these ligands is mutually exclusive (32), fasciculin dissociation rate constants (kobs) were determined by measuring the time dependence of the decrease in fluorescence for the fasciculin-AChE-acrylodan conjugate upon addition excess BW284c51 at several concentrations. Here, data were fit to a single exponential decay to a minimal value. For the Y124C, but not the H287C, fasciculin-AChE-acrylodan conjugate, this observed rate was dependent on the concentration of BW284c51. In this case, linear regression of a reciprocal plot of the observed rate and the concentration of BW284c51 yielded the desired fasciculin dissociation rate constant. Equilibrium dissociation constants (Kd) were calculated from the ratios of kobs to kuns.

**RESULTS**

**Analysis of Substrate Hydrolysis**—All of the mutant enzymes show kinetics of acetylthiocholine hydrolysis similar to wild type enzyme, indicating that catalytic activity is preserved and the enzymes containing the newly introduced cysteine residues
Substituted Cysteines on Acetylcholinesterase

TABLE I

Constants for acetylthiocholine hydrolysis by wild type and mutant mouse AChEs unconjugated or conjugated with aromatic (benzyl-MTS), negatively charged (MTSES, SO₃⁻), positively charged (MTSET, N(CH₃)₃⁺; MTSEA, NH₂⁻) or fluorescent (acrylodan) cysteine labeling compounds

Data are shown as means ± S.D. typically from three measurements. Data were fit to the following scheme,

\[ \frac{k_{\text{cat}}}{K_m} = \frac{K_{\text{cat}}}{b} \]

S + E \rightarrow ES \rightarrow E + P

where S is substrate, E is enzyme, and P is product. In this scheme, S can combine at two discrete sites to form two binary complexes, ES and SE. 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 (25).

| Enzyme        | \( K_m \) | \( K_{ss} \) | b     | \( k_{\text{cat}} \) | \( k_{\text{cat}}/K_m \) |
|---------------|----------|-------------|-------|-----------------|-------------------|
| Wild type     | 46 ± 3   | 15 ± 2      | 0.23 ± 0.01 | 1.40 ± 0.1       | 3.0               |
| Y124C        | 65 ± 17  | 20 ± 14     | 0.22 ± 0.08 | 1.42 ± 0.31      | 2.2               |
| Y124C-benzyl  | 59 ± 13  | 5.9 ± 2.4   | 0.53 ± 0.04 | 0.80 ± 0.03      | 1.4               |
| Y124C-SO₃⁻    | 58 ± 7.5 | 0.57 ± 0.17 | 0.39 ± 0.06 | 1.12 ± 0.18      | 2.2               |
| Y124C-N(CH₃)₃⁺ | 1130 ± 410 | 13 ± 2.5     | 0.21 ± 0.04 | 0.31 ± 0.06      | 0.098             |
| Y124C-NH₂⁻    | 2360 ± 720 | 20 ± 10     | 0.10 ± 0.04 | 0.21 ± 0.04      | 0.11              |
| Y124C-acrylodan | 280 ± 30    | 42 ± 44     | 0.32 ± 0.29 | 0.057 ± 0.004    | 1.1               |
| A382C        | 59 ± 3.7 | 11 ± 3.4    | 0.19 ± 0.04 | 0.14 ± 0.091     | 2.8               |
| H329C        | 58 ± 7.5 | 12 ± 5.5    | 0.21 ± 0.06 | 1.80 ± 0.20      | 3.1               |

a Data from Ref. 25. Kinetic values of acetylthiocholine hydrolysis for wild type enzyme exposed to MTS labeling compounds are within experimental error of the values listed here.

fold properly (Table I). The Michaelis constant (\( K_m \)) appears unaffected by the mutations, while the secondary site binding constant (\( K_{ss} \)) is only marginally increased for the mutant Y124C, as observed previously for the mutant Y124Q (25). The turnover rate, \( k_{\text{cat}} \), and the ratio of \( k_{\text{cat}} \) to \( K_m \), as a measure of catalytic efficiency, and the relative activity of the enzyme with two substrate molecules simultaneously bound, as reflected by the b factor, are all within experimental error of the values for wild type enzyme.

Modifications of the cysteine at position 124 by the MTS compounds (Fig. 1) alter the kinetics of acetylthiocholine hydrolysis (Table I and Fig. 2A). Labeling of Y124C with a benzylthiol elicits a minimal influence on the kinetic constants, whereas conjugation with a negatively charged sulfonate moiety affects \( k_{\text{cat}} \) but not \( K_m \). The largest variation of \( K_m \) is observed with positively charged MTS compounds. In comparison to the mutant enzyme, derivatization with a quaternary amine (MTSET) or a primary amine (MTSEA) increases \( K_m \) 17- and 36-fold, respectively, while having little effect on \( K_{ss} \) or b values. Only small changes in \( k_{\text{cat}} \) are observed for the MTS-modified enzymes. The inability of certain conjugates to induce significant alterations in the kinetics of acetylthiocholine hydrolysis required sequential modification to detect completeness of labeling. Kinetic measurements in which Y124C was first exposed to either benzene or sulfonate MTS reagents then to MTSEA confirmed that reaction with the initial compounds proceeded to near completion (Fig. 2B). Acrylodan labeling of Y124C affects both \( K_m \) and \( k_{\text{cat}} \), while \( K_{ss} \) and b values remain similar to unreacted Y124C. \( K_m \) is increased approximately 4-fold, while \( k_{\text{cat}} \) is reduced 25-fold, resulting in a 100-fold loss in enzyme efficiency as measured by the ratio of \( k_{\text{cat}} \) to \( K_m \).

Analysis of Trifluoroacetophenone Inhibition—Rate constants for the diffusion-limited TFK\(^+\) association to and its dissociation from Y124C-labeled enzymes are shown in Table II. The bimolecular association rate of the cationic inhibitor TFK\(^+\) with Y124C is slightly slower than that for wild type enzyme. Derivatization of Y124C with either neutral (benzyl) or negatively charged (sulfonate) reagents further decreases this rate, but modestly. In contrast, placement of a positive charge at the Y124C position decreases the association rate constant substantially. The primary amine moiety reduces \( k_{\text{cat}} \) 52-fold while the quaternary ammonium group reduces it over 200-fold. In contrast, the bimolecular rate constants for TFK\(^+\) with the same set of Y124C-labeled enzymes are nearly unal-
Substituted Cysteines on Acetylcholinesterase

**Analysis of Fasciculin Inhibition and Binding**—Mutant enzymes, both free and conjugated with acrylodan, were examined with respect to fasciculin binding (Table III). For free enzymes, equilibrium inhibition constants ($K_i$) were obtained from measurements of enzyme activity. Increases in $K_i$ for Y124C and H287C were due to increases in the dissociation rates ($k_{on}$), resulting in a 12- and 2-fold greater $K_i$, respectively, in comparison to wild type enzyme. As expected, since the substituted residue is removed from the fasciculin-binding site, A262C showed no differences in kinetic constants of fasciculin binding. For acrylodan-labeled mutants (Fig. 3), equilibrium dissociation constants ($K_i$) were measured from the fluorescence signals (Fig. 4). Interestingly, derivatization of Y124C with acrylodan does not substantially change the rate constant of fasciculin association ($k_{on}$) and alters the dissociation rate ($k_{off}$) only 5-fold, when compared with unreacted Y124C (Figs. 5 and 6). However, in comparison to the mutant enzyme, the H287C-acrylodan conjugate shows alterations in both the association and dissociation rates, which increase by 3- and 45-fold, respectively, resulting in a 15-fold loss in affinity. Kinetic constants of fasciculin binding were not obtained for the A262C-acrylodan conjugate due to the absence of a detectable change in fluorescence signal upon combining the enzyme and toxin, but measurements of inhibition of catalysis by fasciculin indicate that the $K_i$ of fasciculin is unaltered by this modification.

**Acrylodan Fluorescence Emission Spectra**—Studies of acrylodan in solvents of increasing polarity show large bathochromic shifts (to longer wavelengths) and decreases in quantum yield associated with a greater dipole moment of the solvent [22, 33]. The fluorescence emission maxima of the acrylodan-modified enzymes likely reflect the degree of solvent exposure and polarity of the immediate environment around the substituted acrylodan. Thus, rank ordering of the $\lambda_{max}$ for emission shows the acrylodan bound to Y124C to be the most buried, followed by A262C and H287C (Table IV). This result is to be expected in that Tyr$^{224}$ is situated approximately one-third of the distance toward the gorge base, whereas Ala$^{292}$ and His$^{287}$ appear as surface residues in the crystallographic structures (Fig. 7).

Addition of fasciculin is likely to further occlude the acrylodan molecules from the solvent and perhaps also decrease the polarity of the immediate environment surrounding acrylodan. The spectrum and quantum yield of acrylodan conjugated at position A262C are not altered by fasciculin titration, consistent with its position removed from the active site gorge. By contrast, the two acrylodans that are located near the fasciculin-binding site and likely to be shielded from solvent or in direct contact with fasciculin show the greatest hypsochromic shifts and increases in quantum yield (Fig. 4). Y124C shows the largest shift of 23 nm to 477 nm for an emission maximum. Its position within the gorge and the subsequent capping by fasciculin explain why this wavelength is the shortest value achieved (Table IV). The shift relative to the enhancement of fluorescence yields a clear isosbestic point. Loomis points only arise when two discrete species or states are present in the titrations. Thus, neither the free enzyme nor the fasciculin complex reveal a composite of conformational states in terms of acrylodan signal. Nevertheless, the fasciculin complex and free enzyme differ substantially in acrylodan environment (Fig. 4A).

Acrylodan conjugated at H287C also shows a hypsochromic shift, but of smaller magnitude, from 524 to 507 nm (Fig. 4B). This shift likely reflects its position near loops II and III of fasciculin, where, by examination of the crystal structure [7], there are limited van der Waals contacts between fasciculin and AChE. Hence, occlusion here would be expected to be only partial, perhaps still allowing solvent access at this locus.

**DISCUSSION**

Large scale production and purification of three cysteine substitution mutants used in this study illustrate the feasibility and potential afforded by the coupled techniques of cysteine

---

**Fig. 2.** Concentration dependence of acetylthiocholine hydrolysis for Y124C and the cysteine substituted derivatives of Y124C AChE. Only relative values are shown here. Absolute activity values may be calculated from this figure and Table I. **Panel A,** Y124C and its derivatives: ○, underivatized Y124C; ■, Y124C-benzyl; ▲, Y124C-SO$_3$; ○ Y124C-CH$_3$; □, Y124C-NH$_3$; △, Y124C-acrylodan. **Panel B,** Y124C derivatives that lacked a clear alteration of acetylthiocholine hydrolysis in comparison to unmodified Y124C were subsequently reacted with MTSEA to verify that the first derivatization reaction approached completion. Failure to react would have generated a fraction of the enzyme yielding a reaction profile similar to Y124C-CH$_3$. ○, Y124C-benzyl; ○, Y124C-benzyl followed by reaction with MTSEA; ▲, Y124C-acrylodan. **Panel C,** Y124C derivatives that lack a clear alteration of acetylthiocholine hydrolysis in comparison to unmodified Y124C were subsequently reacted with MTSEA to verify that the first derivatization reaction approached completion. Failure to react would have generated a fraction of the enzyme yielding a reaction profile similar to Y124C-CH$_3$. ○, Y124C-benzyl; ○, Y124C-benzyl followed by reaction with MTSEA; ▲, Y124C-acrylodan. **Panel D,** Y124C derivatives that lacked a clear alteration of acetylthiocholine hydrolysis in comparison to unmodified Y124C were subsequently reacted with MTSEA to verify that the first derivatization reaction approached completion. Failure to react would have generated a fraction of the enzyme yielding a reaction profile similar to Y124C-CH$_3$. ○, Y124C-benzyl; ○, Y124C-benzyl followed by reaction with MTSEA; ▲, Y124C-acrylodan.
substitution mutagenesis and labeling at discrete positions on the AChE molecule. Selected sites can be specifically and efficiently mutated and then modified to a diverse set of unnatural amino acid side chains, including fluorophores and spin labels, both of which can reveal structural characteristics through spectroscopic analysis. Here, we combine characterization of catalytic and inhibition parameters with an analysis of fluorescence signals in an effort to understand ligand accessibility to the active site and the local environment at the mutated positions. The Y124C enzyme, in which the mutation is situated inside the active center gorge and at a point of constriction, was utilized most extensively to address questions of ligand access. To date, we have been unable to modify substituted cysteines at positions deeper than this constriction point but still within the active center gorge.

Insertion of Cationic, Neutral, and Anionic Side Chains within the Active Center Gorge—Charged and neutral cysteine labeling agents of differing sizes allowed us to dissect the influence of electrostatic and steric hindrance within the gorge. The presence of a positive charge inserted near the constriction point in the gorge substantially reduced the rate of TFK\textsuperscript{+}, but not TFK\textsuperscript{0}, conjugation while rates of dissociation of the conjugated TFKs remained essentially unchanged (Table II). Taken together, these data argue against a mechanism of purely steric hindrance resulting from the presence of the unique side chain.

**TABLE II**

| Enzyme                  | \(k_{\text{on}}\) \(10^9 \text{M}^{-1} \text{min}^{-1}\) | \(k_{\text{on WT}}\) \(k_{\text{on mutant}}\) | \(k_{\text{off}}\) \(10^{-3} \text{min}^{-1}\) | \(k_{\text{on}}\) \(10^9 \text{M}^{-1} \text{min}^{-1}\) | \(k_{\text{on WT}}\) \(k_{\text{on mutant}}\) | \(k_{\text{off}}\) \(10^{-3} \text{min}^{-1}\) |
|-------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Wild type               | 160 ± 13                                       | 1                                             | 5 ± 2                                         | 2.3 ± 0.56                                    | 1                                             | 16 ± 4                                        |
| Y124C                   | 110 ± 4                                        | 1.5                                           | 14 ± 6                                        | 1.3 ± 0.02                                    | 1.8                                           | 19 ± 2                                        |
| Y124C-benzyl            | 63 ± 7                                         | 2.5                                           | 7 ± 3                                         | 1.6 ± 0.04                                    | 1.4                                           | 31 ± 12                                       |
| Y124C-SO\textsubscript{2}\textsuperscript{−} | 84 ± 3                                         | 1.9                                           | 8 ± 2                                         | 0.75 ± 0.04                                   | 3.1                                           | 14 ± 5                                        |
| Y124C-N(CH\textsubscript{3})\textsubscript{3}\textsuperscript{+} | 3.1 ± 0.4                                      | 52                                            | 16 ± 12                                       | 0.70 ± 0.04                                   | 3.3                                           | 12 ± 2                                        |
| Y124C-acrylodan         | 0.72 ± 0.03                                    | 220                                           | 14 ± 4                                        | 0.40 ± 0.02                                   | 5.8                                           | 6 ± 3                                         |
| Y124C-acrylodan         | 2.5 ± 0.2                                      | 64                                            | 38 ± 10                                       | 0.12 ± 0.02                                   | 19                                            | 20 ± 2                                        |

**FIG. 3.** Polyacrylamide gel electrophoresis in the presence of SDS for wild type (WT) and Y124C substituted mouse AChE. – and + denote incubations with acrylodan as described under “Experimental Procedures.” Lanes 1–4 and 6–9 are the reaction mixtures prior to and after size exclusion chromatography, respectively. The double banding pattern reflects the heterogeneity in AChE due to differential glycosylation (cf. Ref. 29). Panel A, acrylodan fluorescence visualized by UV illumination. Panel B, protein visualized by silver staining. Lane 5, molecular weight standards.

**FIG. 4.** Fluorescence emission spectra of acrylodan-labeled Y124C (A) and H287C (B) AChE following titration with fasciculin 2. In both cases, fasciculin produces a hypsochromic shift and enhancement of fluorescence quantum yield. The larger shift for Y124C reveals a clear isosbestic point indicative of the two (free and fasciculin bound) species. Panel A, initial enzyme concentration determined by absorbance at 280 nm was 95 nM and changed less than 10% during the course of the titration. Fasciculin concentrations were as follows: 0, 12, 25, 37, 50, 70, 95, and 140 nM. Panel B, initial enzyme concentration determined by absorbance at 280 nm was 250 nM and changed less than 5% during the course of the titration. Fasciculin concentrations were as follows: 0, 25, 50, 75, 100, 120, 150, 170, and 190 nM.
serine forming a hemiketal conjugate without dissociation of
ters affecting gorge entry. TFK reacts with the active center
become manifest until the side chain bulk greatly exceeds that
(38) factors. Hence, the steric influence does not
insertions of the cationic side chains likely arise from altering
points to residues near Asp\textsuperscript{74}, Tyr\textsuperscript{124}, and Phe\textsuperscript{338} forming a
the gorge has the most dramatic influence on ligand rates. A
substrate binding, acylation, and deacylation
terms of serial substrate binding, acylation, and deacylation
steps, then constants may be applied as follows,

\[
\begin{align*}
E-\text{SER-OH} + \text{AcSCh} & \xrightleftharpoons[k_1]{k_2} \text{[E-\text{SER-OH}--AcSCh]} \\
& \xrightarrow{k_{\text{cat}}} \text{E-\text{SER-OAc} + ChSH} \\
& + \text{AcO}^- + \text{H}^+ 
\end{align*}
\]

\textbf{SCHEM E 1}

where \text{E-SER-OH} is the active enzyme, \text{E-SER-OAc} the acetyl
intermediate, \text{ChSH thioc holine}, and \text{AcSCh acetylthiocholine.}

For this scheme we can derive the following equations.

\[
\begin{align*}
K_m &= \frac{k_{-1} + k_2}{k_1} \\
k_{\text{cat}} &= \frac{k_2 k_3}{k_2 + k_3} \\
K_m &= \frac{k_1 k_2}{k_{-1} + k_2}
\end{align*}
\]

(Eq. 1)

(Eq. 2)

(Eq. 3)

In Scheme 1 and the sequence of equations, \(K_m\) is the only term
containing the rate constants for the ligand encounter and
dissociation of the initial complex denoted in brackets. As dis-


**Substituted Cysteines on Acetylcholinesterase**

**Fig. 7.** The active center gorge of mouse AChE with bound fasciculin 2 as viewed from the side of the gorge. Shown are the active center serine at the base of the gorge (Ser-203), the three positions of the cysteine substitutions: Tyr-124, His-287, and Ala-262, and the An-37 which marks a position on loop III of fasciculin. Loop II is the portion of the fasciculin structure buried in the mouth of the active center gorge. Since the region between residues 258 and 264 is unresolved in the fasciculin complex (7), its fold from the tetrameric crystal in the apoenzyme (8) has been superimposed over this region.

cussed previously, the initial encounter for cationic ligands appears diffusion limited. By contrast, $k_{cat}$ encompasses the two subsequent steps. $k_{cat}$ may be thought of as the geometric mean of the rates of acylation and deacylation and is dominated by the greater of the two constants.

The data in Table I reveal a clear distinction between the cationic and acrylodan modifications of the inserted cysteines. The amine substitutions influence $K_m$ only and therefore are selective for the first encounter. With a positive charge on the amine, it seems likely that electrostatic repulsion adds to the diffusional barrier imposed by geometric constraints of the gorge. In affecting primarily $k_{cat}$, acrylodan, perhaps because of its larger steric influence and possible perturbation of the dynamics of the gorge during the catalytic cycle, diminishes the efficiency of the acylation and/or deacylation steps. The rigidity of the inserted acrylodan aromatic group may preclude small changes in gorge conformation required for catalysis. Early studies of Wilson and colleagues (37), in which the acetyl intermediate of Electrophorus AChE was trapped by quench-flow techniques, revealed comparable rates for acylation and deacylation. Should a similar situation apply for mouse AChE, acylation and deacylation are both likely to be affected by acrylodan conjugation.

The data in Table I also reveal that substitution of the sulfonate anion at the 124 position selectively decreases the value of $K_m$. This finding is consistent with the region of the 124 side chain encompassing the substrate inhibition site, which by competition studies is thought to reside at the peripheral site (38–40). In accordance with this scheme, insertion of the negative charge selectively enhances the binding of a second substrate molecule in the vicinity of the peripheral site. A similar $b$ value for the modified and the wild type enzyme indicates that the ternary complexes of both enzymes show diminished catalytic efficiency compared with the Michaelis complex. Interestingly, a putative pre-Michaelis complex (41) that may show an increase in affinity with the addition of a negative charge near aspartate 74 does not affect catalytic efficiency.

The anionic side chain of aspartate 74 opposes residue 124 in the gorge at a similar depth from the surface. Hence, it becomes of interest to compare the influence of these proximal charges in the gorge on TFK association and steady state kinetic parameters of substrate hydrolysis. The D74N mutation, which eliminates the anion in the gorge passage, selectively increases $K_m$ some 30-fold for acetylthiocholine (25) and slows the rate of TFK$^+$ association by 35-fold (42). Substrate and ligand affinity are also affected by the D74K mutation (39). These values are comparable to those for the Y124C mutation upon modification with the positively charged methanethiosulfonates (Tables I and II), showing that negation or reversal of charge at position Asp$^{124}$ can be mimicked by alteration of residue 124.

It is also of interest that the primary amine, a modification of smaller size than the trimethylammonium moiety, but possessing the capacity to coordinate water molecules, causes the greatest increase in $K_m$ for acetylthiocholine. By contrast, the rate of association of TFK$^+$ is reduced the most by the presence of the quaternary ammonium derivative. This disparity may arise from differences between TFK$^+$ and acetylthiocholine in structural rigidity and hydrophobicity. In addition, the environment of the gorge can be expected to be differentially affected by a hydrophobic quaternary ion and a hydrated cation. The distinct effects of modifications by cationic side chains on $K_m$ and TFK$^+$ association may also indicate an involvement of rate constants encompassing deacylation and substrate or product dissociation in the $K_m$ term.

**Influence of Residue Modification on Fasciculin Binding—**

Comparison of the influence of cysteine modification on fasciculin binding shows the tyrosine 124 modification (Y124C) to effect a 12-fold decrease in fasciculin affinity, whereas the H287C and the A262C have little or no effect on fasciculin affinity (Table III). These findings are consistent with previous work showing that an aromatic cluster consisting of Trp-226, Tyr$^{124}$ and Tyr$^{72}$ constitutes the residues responsible for the nearly 8 orders of magnitude difference in the fasciculin 2 $K_d$ between AChE and butyrylcholinesterase (40). Moreover, the Y124Q substitution, which reflects the AChE to butyrylcholinesterase residue difference at this position, results in a 100-fold reduction in affinity. In contrast to the aromatic triplet, which is in contact with the tip of loop II, His$^{287}$ is found at the enzyme surface and interacts only weakly through van der

| **TABLE III**
| Kinetic and equilibrium constants for inhibition or binding of fasciculin to AChE prior to and after modification of the free cysteine by acrylodan |
| Data are shown as means ± S.D. typically of three measurements. |
| Enzyme | $k_{cat}$ | $k_{cat}$ | $K_I$ or $K_D$ |
|---|---|---|---|
| Wild type$^a$ | $3.9 ± 0.01$ | $6.5 ± 0.6$ | $17 ± 2$ |
| Y124C$^b$ | $2.9 ± 0.4$ | $59 ± 3$ | $210 ± 20$ |
| Y124C-acrylodan$^b$ | $3.4 ± 0.4$ | $302 ± 68$ | $890 ± 225$ |
| H287C$^b$ | $3.7 ± 0.1$ | $14 ± 0.3$ | $37 ± 2$ |
| H287C-acrylodan$^b$ | $11 ± 2$ | $630 ± 56$ | $570 ± 115$ |
| A262C$^b$ | $4.1 ± 0.2$ | $6.5 ± 0.3$ | $16 ± 1$ |

$^a$ Kinetic constants derived from measurements of inhibition of acetylthiocholine hydrolysis.

$^b$ Kinetic constants derived from intensity of the fluorescence signals.

| **TABLE IV**
| Emission maxima of mouse AChE mutants labeled with acrylodan |
| Data are shown as mean values of at least three determinations. |
| Enzyme | No fasciculin | Saturating fasciculin | Hypsochromic shift |
|---|---|---|---|
| Y124C | 500 | 477 | 23 |
| A262C | 517 | 517 | 0 |
| H287C | 524 | 507 | 17 |

Acrylodan emission maxima (nm)
Waals forces at the perimeter of loops II and III (7). Hence, a smaller reduction in binding energy would be anticipated. Ala\textsuperscript{262} is on the surface, but positioned some 28 Å from the gorge entry along a circumferential surface. As such, Ala\textsuperscript{262} would not be expected to affect fasciculin binding kinetics through direct contact.

Addition of acrylodan to Y124C forms a conjugate, but one in which the fasciculin affinity is only 4-fold lower than that of Y124C AChE unmodified by acrylodan. Since the native residue tyrosine confers high affinity, it is possible that the binding energy lost through steric hindrance by substitution of the large aromatic residue is partially compensated by the \( \sigma \)-cation interaction between toxin loop II and the aromatic cluster to which the acrylodan residue contributes. Acrylodan conjugation at the 287 position diminishes the binding affinity, suggesting partial contact and occlusion of a complementary binding surface.

The differing kinetic basis for the loss of affinity with the two high affinity inhibitors, fasciculin and TFK\textsuperscript{+}, is also of interest. Acrylodan reduces the ability of TFK\textsuperscript{+} to form a hemiketal at the base of the gorge, consistent with a restriction of diffusional access. Fasciculin, despite binding at the surface of the enzyme, shows a slower rate of association than the TFK derivatives and modification of the enzyme surface does not affect its association rate. Rather rates of dissociation of fasciculin are affected with the modifications. Thus, the stability of the complex correlates with the capacity of fasciculin to dissociate from its binding site, a finding consistent with acrylodan residing at a contact surface in the complex.

**Fluorescence Properties of the Acrylodan-conjugated Enzymes**—Acrylodan fluorescence emission, similar to other substituted aminonaphthalenes, is sensitive to the dielectric constant of the solvent, where a reduction in dielectric constant gives rise to a hypochromic shift and enhancement of quantum yield (21, 22). The sensitivity to solvent environment arises from excitation of the fluorophore effecting a change in its dipole moment. The difference between excitation and emission wavelengths depends on the solvent dipoles reorienting in response to the excitation. In solvents of decreasing polarity or dipole moment, reorientation effects of the solvent are diminished. Although correlations can be made in various solvents of differing dielectric constant, the enzyme surface will not behave as an isotropic, uniform solvent. Nevertheless, the emission wavelength should reflect the apparent solvent polarity or dielectric constant around the fluorophore. Clearly, the 124 position in the free enzyme appears to be the most buried and/or most closely associated with hydrophobic residues (Table IV). Acrylodan conjugated at the 262 and 287 positions, both of which should be on the enzyme surface, show longer maximal emission wavelengths.

Addition of fasciculin causes the largest hypochromic shift with acrylodan substituted at the 124 position, consistent with a sandwiching between the residues from the tip of loop II and adjacent aromatic residues on the enzyme. A shift of smaller magnitude is seen for the 287 substitution, in accord with the expectation from the crystal structure of the complex that it may be partially occluded, while acrylodan at the 262 position exhibits no change in emission maximum or quantum yield upon binding of fasciculin. Hence, shifts in the emission spectra of acrylodan or other aminonaphthalenes are predictive of the immediate environment of the fluorophore and the influence exerted by an associating ligand.

By mapping other positions on the AChE surface, a more complete analysis of the polarity of the enzyme surface is possible. Moreover, other fluorophores with longer lifetimes than acrylodan should be well suited for examining solvent exposure through collisional quenching and for ascertaining segmental motion from the decay of fluorescence anisotropy. While the analysis can be based on the crystal structure template, solution based approaches to structure may reveal details of conformation and molecular motion not discernable in a static crystal structure.

**Acknowledgment**—We thank Dr. Zoran Radic for advice and many valuable discussions related to this study.

**REFERENCES**

1. Cygler, M., Schrag, J. D., Sussman, J. L., Harel, M., Silman, I., Gentry, M. K., and Doctor, B. P. (1993) Protein Sci. 2, 366–382.
2. Massoulié, J., Pezzementi, L., Bon, S., Krejci, E., and Valette, F.-M. (1993) Proc. Neurobiol. 41, 31–91.
3. Taylor, P., and Radic, Z. (1994) Annu. Rev. Pharmacol. Toxicol. 34, 281–329.
4. Rosenhenn, T. L. (1975) Adv. Enzymol. 43, 103–218.
5. Quinn, D. M. (1987) Chem. Rev. 87, 955–979.
6. Sussman, J. L., Harel, M., Frolow, F., Oenfen, C., Goldman, A., Toker, L., and Silman, I. (1991) Science 253, 872–879.
7. Bourne, Y., Taylor, P., and Marchot, P. (1995) Cell 83, 503–512.
8. Bourne, Y., Taylor, P., Bougis, P. E., and Marchot, P. (1999) J. Biol. Chem. 274, 2963–2970.
9. Ripoll, D. R., Faerman, C. H., Axelsen, P. H., Silman, I., and Sussman, J. L. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5129–5132.
10. Gilson, M. K., Straatsma, T. P., McCammon, J. A., Ripoll, D. R., Faerman, C. H., Axelsen, P. H., Silman, I., and Sussman, J. L. (1994) Science 263, 1276–1278.
11. Kronman, C., Ordentlich, A., Barak, D., Velan, B., and Shafferan, A. (1994) J. Biol. Chem. 269, 27819–27822.
12. Velan, B., Barak, D., Ariel, N., Leitner, M., Bino, T., Ordentlich, A., and Shafferan, A. (1996) FEBS Lett. 395, 22–26.
13. Harel, M., Kleywegt, G. J., Raveli, B. R. G., Silman, I., and Sussman, J. L. (1995) Structure 3, 1355–1366.
14. Eastman, J., Wilson, E. J., Cerjevansky, C., and Rosenhenn, T. L. (1995) J. Biol. Chem. 270, 19694–19701.
15. Radic, Z., Quinn, D. M., Velom, D. C., Camp, S., and Taylor, P. (1995) J. Biol. Chem. 270, 20391–20399.
16. Taylor, P., and Lappi, S. (1975) Biochemistry 14, 1989–1997.
17. Berman, H. A., and Taylor, P. (1994) Biochemistry 33, 8566–8576.
18. Levy, D., and Ashani, Y. (1986) Biochem. Pharmacol. 35, 1079–1085.
19. Radic, Z., Pickering, N. A., Velom, D. C., Camp, S., and Taylor, P. (1993) Biochemistry 32, 307–310.
20. Lew, J., Coruh, N., Tsigeiy, I., Garrod, S., and Taylor, S. S. (1997) J. Biol. Chem. 272, 1507–1513.
21. Prendergast, F. G., Meyer, M., Carlson, G. L., Iida, S., and Potter, J. D. (1983) J. Biol. Chem. 258, 7541–7544.
22. Nair, H. K., Seravalli, J., Arbuckle, T., and Quinn, D. M. (1994) Biochemistry 33, 8566–8576.
23. Marchot, P., Khe ́lif, A., Ji, Y.-H., Mansuelle, P., and Bougis, P. E. (1993) J. Biol. Chem. 268, 12458–12467.
24. Lakowicz, J. R. (1983) Principles of Fluorescence Spectroscopy, Plenum Press, New York.
25. Harel, M., Quinn, D. M., Nair, H. K., Silman, I., and Sussman, J. L. (1996) J. Am. Chem. Soc. 118, 2340–2346.
26. Zhou, H.-X., Wlodok, S. T., and McCammon, J. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9280–9283.
27. Bazylinski, M., Roehy, E., and Kirsch, J. F. (1986) Biochemistry 25, 125–130.
28. Freeo, H. C., and Wilson, I. B. (1984) J. Biol. Chem. 259, 1110–1113.
29. Radic, Z., Reiner, E., and Taylor, P. (1991) Mol. Pharmacol. 39, 98–104.
30. Shafferan, A., Velan, B., Ordentlich, A., Kronman, C., Grussfeld, H., Leitner, M., Flaschner, Y., Cohen, S., Barak, D., and Ariel, N. (1992) EMBO J. 11, 3561–3568.
31. Radic, Z., Duran, B., Velom, D. C., Li, Y., Cerjevansky, C., and Taylor, P. (1994) J. Biol. Chem. 269, 11223–11229.
32. Szegletes, T., Mallender, W. D., Thomas, P. J., and Rosenhenn, T. L. (1999) Biochemistry 38, 122–133.
33. Hosen, N. A., Radic, Z., Tsegelay, I., Berman, H. A., Quinn, D. M., and Taylor, P. (1996) Biochemistry 35, 10995–11004.