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An electrostatic mechanism for substrate guidance down the aromatic gorge of acetylcholinesterase

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ABSTRACT **Electrostatic calculations based on the recently solved crystal structure of acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) indicate that this enzyme has a strong electrostatic dipole. The dipole is aligned with the gorge leading to its active site, so that a positively charged substrate will be drawn to the active site by its electrostatic field. Within the gorge, aromatic side chains appear to shield the substrate from direct interaction with most of the negatively charged residues that give rise to the dipole. The affinity of quaternary ammonium compounds for aromatic rings, coupled with this electrostatic force, may work in concert to create a selective and efficient substrate-binding site in acetylcholinesterase and explain why the active site is situated at the bottom of a deep gorge lined with aromatic residues.**

Acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7; AcChoEase) is a serine hydrolase that serves principally to terminate signal transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine (AcCho) in the synaptic gap (1). In accordance with its biological role, AcChoEase is a very rapid-acting enzyme (2), operating at nearly diffusion-limited rates (3–5). The x-ray structure of AcChoEase from *Torpedo californica* has now been solved (6), showing that its active site lies near the base of a deep and narrow gorge lined predominantly by aromatic groups. Sussman *et al.* (6) suggested that movement of AcCho down the gorge toward the active site might be facilitated by an "aromatic guidance" mechanism, in which the positively charged quaternary group of AcCho could interact sequentially, through charge–charge interactions (7), with π electrons belonging to these aromatic rings.

Despite a conspicuous paucity of negatively charged residues in the active-site gorge, the enzyme has an overall net charge of −11e (3). Coupled with the strong ionic-strength dependence of the on-rate for the cationic substrate, this fact suggests that the spatial distribution of charged residues may be a structural feature of importance to enzyme function. In the present study, therefore, we used computational techniques (8) to examine the electrostatic properties of AcChoEase.

METHODS

The evaluation of the electrostatic potential and field was done by using the finite-difference algorithm DELPHI (9–11), designed to solve the Poisson–Boltzmann equation numerically. A cubic grid of 65 × 65 × 65 points that includes the protein molecule and the solvent was defined. The solvent and the protein molecule were treated as homogeneous dielectric media. The former was represented, using the Debye–Huckel model, by a high-dielectric medium, ε_{solv} = 78.3, containing counterions (ionic strength of 0.01 M), whereas the latter was approximated by a cavity of low dielectric constant ε_{prot}. Values of ε_{prot} of 4 and 10 led to very similar results for the electrostatic-field vectors. The shape of this cavity was computed from the x-ray structure of AcChoEase (6). The missing atoms and polar hydrogens were added by using the program SYBYL (Tripos, St. Louis). The focusing feature available within DELPHI was also used. A set of point charges from the program AMBER (12) was assigned to the protein atoms. An initial calculation, consisting of 200 iterations using the linear approximation of the Poisson–Boltzmann equation and 50 iterations of the nonlinear approximation, was done with the protein cavity occupying 33% of the grid volume. This calculation was followed by a second one, where 66% of the grid volume was filled by the protein. This second calculation consisted of 300 iterations of the linear approximation to the Poisson–Boltzmann equation and 150 iterations of the nonlinear one. The net charge for the x-ray-resolved structure of AcChoEase was assumed to be −8 electrons (see below). This value compares well with those reported (3). We have omitted three acidic residues (Asp-1, Asp-2, and Glu-489) from our calculations because they are not resolved in the x-ray map. However, these residues are on the protein surface and distant from the gorge, giving them a minor quantitative and negligible qualitative impact on the results.

RESULTS AND DISCUSSION

Electrostatic potential and field calculations using the program DELPHI (9–11) demonstrate that AcChoEase has a remarkably strong electrostatic dipole, as illustrated in Figs. 1 *Left* and 4. Contours of electrostatic potential drawn at values of $\pm 1\ kT/e$ reveal a negative potential that extends roughly over half the protein surface and a positive potential contour that covers the other half. The electrostatic dipole is aligned directly along the axis defining the center of the gorge, and field lines indicate that positively charged molecules, such as the natural substrate of the enzyme AcCho will be drawn toward and down the aromatic gorge leading to the active site (Figs. 1 *Left*, 4 and 5). Thus, our results are in accord with the suggestion that a part of the AcChoEase surface, larger than the ligand-binding site itself, is operational in "trapping" cationic ligands (3) and with other computational studies which suggest the particular charge distribution in AcChoEase enhances the rate of substrate binding (13).

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Abbreviations: AcChoEase, acetylcholinesterase; AcCho, acetylcholine; GLIP, *Geotrichum candidum* lipase; SOD, bovine Cu-Zn superoxide dismutase.

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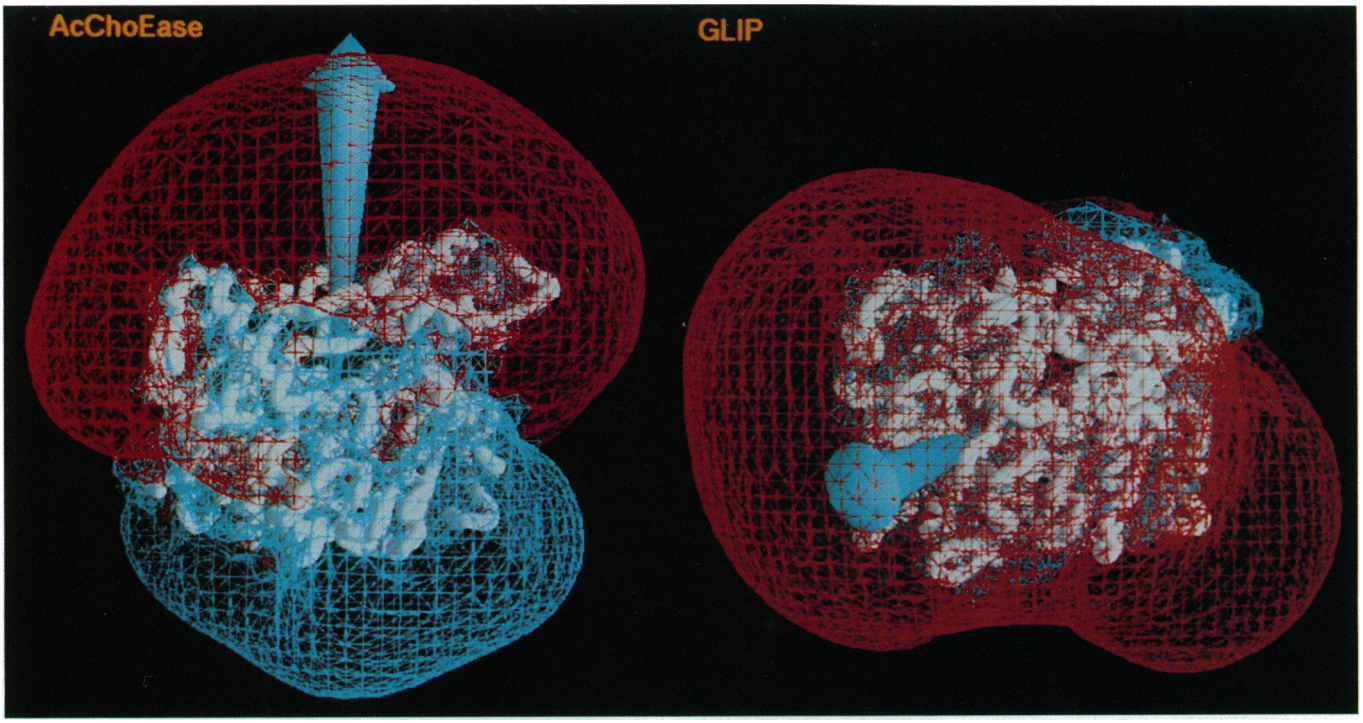

FIG. 1. Backbone drawings of AcChoEase (*Left*) and *Geotrichum candidum* lipase (GLIP) (*Right*) with electrostatic isopotential surfaces superimposed as generated with the program GRASP (A. Nicholls and B. Honig, College of Physicians and Surgeons, Columbia University, New York). Orientations of the proteins are the same in Fig. 2. Backbones of the proteins are represented by white "worms." The red surface corresponds to the isopotential contour $-1\ kT/e$, and the blue surface corresponds to the isopotential contour $+1\ kT/e$, where k = the Boltzmann constant, T = temperature, and e = electronic charge. Arrows indicate directions of the dipoles in each protein.

To put the above observation into perspective, we have similarly calculated data on bovine Cu-Zn superoxide dismutase (SOD) and found that the electrostatic dipole of AcChoEase is approximately one order of magnitude greater than that of SOD. Getzoff *et al.* (14, 15) have provided intriguing evidence through protein engineering that electrostatic guidance is important in attracting the negatively charged superoxide ion into the active site of SOD, and Sines *et al.* (16) have supported this hypothesis with theoretical calculations. However, it should be noted that higher moments than the dipole—e.g., quadrupole—could also be operating to guide a substrate. Thus, our comparison of SOD and AcChoEase must be regarded as largely qualitative at this point, although the electrostatic field of AcChoEase clearly outlines an effective target for positively charged substrates, which is much larger than the gorge orifice (Figs. 1, 4, and 5).

An interesting control calculation can be performed by using the crystal structure of GLIP. GLIP and AcChoEase are of similar size, display significant sequence homology, and have a remarkably similar fold in which an identical and novel catalytic triad (Ser-His-Glu) (6, 17) is situated in a virtually isomorphous spatial conformation, as can be seen in Figs. 1 and 2 (19, 20). Despite an overall charge of −16 e (i.e., twice that of AcChoEase), GLIP exhibits a dipole moment only ≈35% of that of AcChoEase (183 Debye vs. 505 Debye for AcChoEase). Furthermore, the orientation of the dipole for GLIP is markedly different and shows no obvious relationship to the presumed entrance trajectory of the substrate to the active site, which is thought to be positioned similar to that in AcChoEase (Figs. 1 *Right* and 2 *Lower*; see ref. 20). Presumably, the absence of electrostatic forces guiding substrates into the active site of GLIP is of no consequence because its natural substrate is uncharged.

As mentioned above, the number of charges in the active-site gorge of AcChoEase is very small—i.e., there are only four charged residues, all acidic, directly associated with this highly aromatic structure (ref. 21; Fig. 3). This immediately raises the question of the origin of the unusually high dipole moment. The overall dipole moment due to the peptide backbone alone is negligible, as is that associated with aromatic side chains because a united atom topological model has been used for these calculations. Histidine residues are all assigned zero net charge. However, there is a marked preponderance of acidic amino acids in the "northern" hemisphere—i.e., that lying above the active-site triad—and a somewhat smaller preponderance of basic amino acids in the "southern" hemisphere (Fig. 3). Within the gorge there are four acidic residues (Glu-285, Asp-72, Glu-199, and Glu-327, the latter is a member of the catalytic triad). To assess their importance, we calculated the dipole moment after neutralizing the charges on their side chains. This neutralization resulted in only a slightly reduced dipole moment (471 Debye), showing that the dipole is generated by the overall charge distribution throughout the enzyme and is not due merely to acidic residues within the gorge.

Most of the acidic residues in the northern hemisphere are much more than 5 Å from the gorge axis (see Fig. 3) and, thus, lie behind the aromatic residues that line the gorge (Figs. 4 and 5). This result suggests a simple analogy—namely, that they function like an "affinity electrophoresis" column in which the electrostatic dipole drives the cationic AcCho down the gorge, and the aromatic groups provide a series of low-affinity binding sites for the quaternary choline headgroup. At the same time, the aromatic groups may fulfil a shielding role, inasmuch as a gorge lined with acidic residues might bind AcCho too avidly *en route* to the catalytic triad. While facilitating movement of AcCho into the gorge, the aromatic groups might play an as-yet-undefined role in preventing the entrance of hydrated metal cations. This hypothesis could provide a rationale for location of the active site at the bottom of a deep gorge and explain why neither heavy atom used in isomorphous replacement (Hg, U) was found within the gorge (6). It is of interest that the arrangement of negatively charged residues behind a layer of aromatic rings

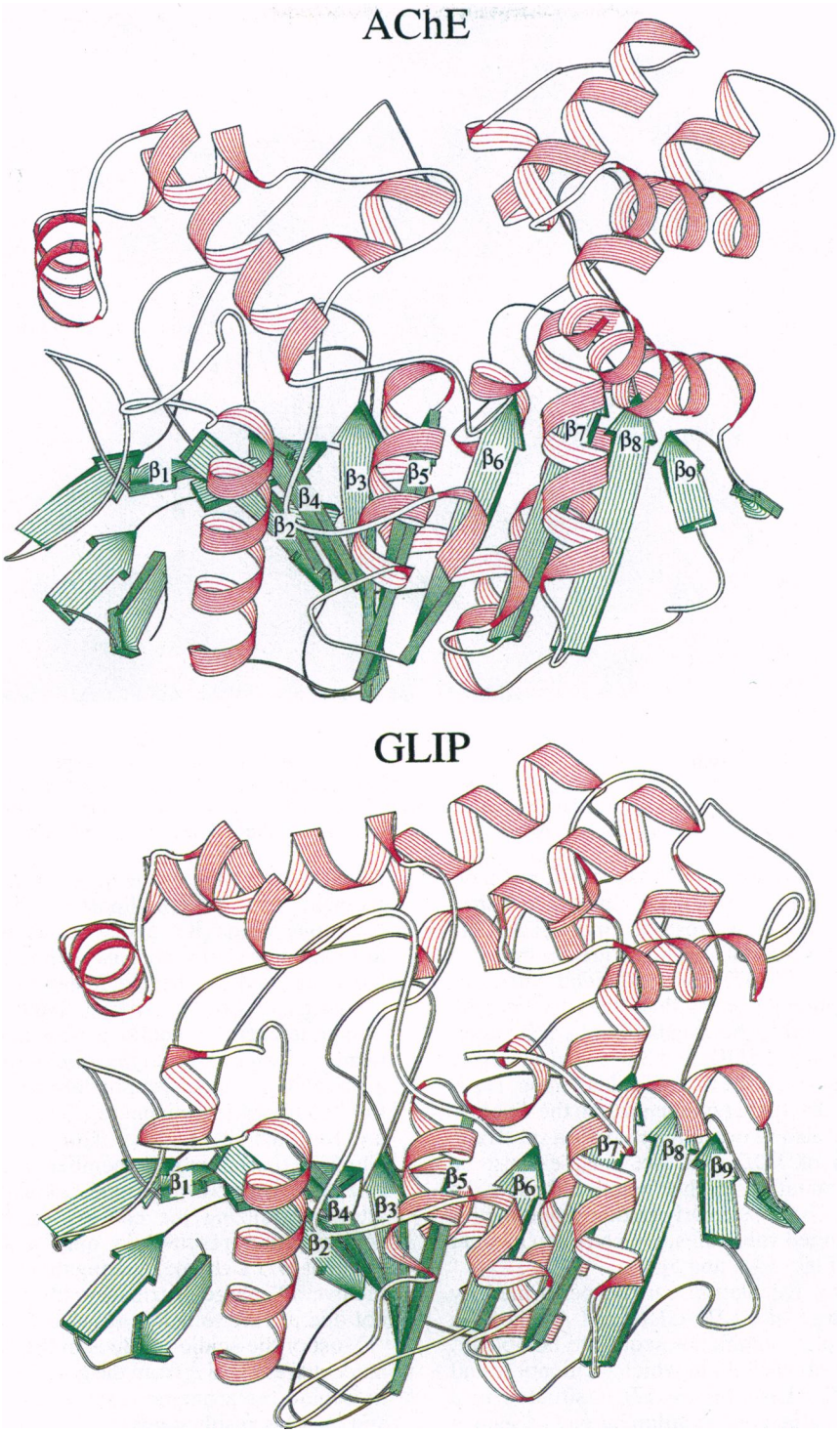

FIG. 2. Ribbon diagrams (18) of AcChoEase (*Upper*) and GLIP (*Lower*). α-Helices are color-coded in red, and β-strands are in green. Both enzymes are in the same orientation. Strands of the central β-sheet are numbered sequentially (β_0–β_{10}) in both enzymes following the scheme of the α/β-hydrolase fold (19, 20). The active-site gorge of AcChoEase is located in the crevice between the α-helices above strand β_5.

resembles the binding pocket of McPC603 for the choline moiety of phosphocholine (7, 22, 23). Recent calculations also support an important energetic role for buried ionized groups in the ligand-binding process of this system (24).

The case of AcChoEase differs from that of SOD in several important respects: (*i*) The dipole moment of AcChoEase is ≈10 times that of SOD. (*ii*) The AcChoEase substrate is a cation, suggesting that nature uses both negative and positive electrostatic guidance mechanisms in its repertoire. (*iii*) AcChoEase is not a metalloenzyme; hence, the dipole must be generated entirely by its residue composition and its fold. (*iv*) The active site of AcChoEase is buried at the bottom of a relatively deep and narrow gorge compared with the one in SOD. (*v*) Two products of the reaction (negatively charged acetate and a positively charged choline) are substantially larger than those of SOD. Thus, understanding how the products of the reaction catalyzed by AcChoEase are cleared from the gorge is a vexing problem; because choline retains a net positive charge, diffusion out of the gorge is opposed by the electric field.

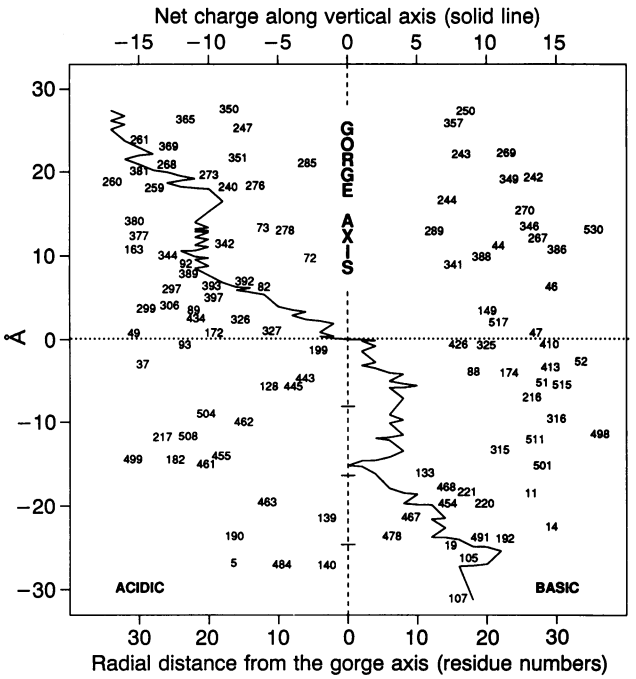

FIG. 3. Distribution of charged groups relative to the gorge axis in AcChoEase. Position of the gorge axis (i.e., the line approximating the center of the gorge) was determined visually with computer graphics and is represented by the middle vertical line (---). The horizontal axis (· · · ·) represents a reference plane perpendicular to the gorge axis, at the level of the gorge bottom. This level is defined as the center of a 1.4-Å probe sphere at the point of its deepest possible penetration. Residue numbers indicate the relative positions of charged groups along the gorge axis (vertical scale) and their radial distance from the gorge axis (bottom horizontal scale). Acidic residues (aspartate, glutamate) are left of the gorge axis, and basic residues (lysine, arginine) are right of the axis. At each level along the gorge axis, the solid line (—) indicates the total cumulative charge between a plane perpendicular to the gorge axis at that level and the reference plane. The overall net charge on the enzyme, −8 electronic charges (e), is an algebraic sum of the net charge above (−17 e) and below (+9 e) the reference plane. The three acidic residues that are not resolved in the crystal structure would likely be located near the gorge axis at the bottom of the figure. There is clearly an unequal distribution of positive and negative charges in the two halves of the enzyme, giving rise to an overall net dipole. Note that only three negatively charged groups (residues 72, 199, and 285) are close enough to the gorge axis to interact directly with substrates in the gorge. The positions of several residue numbers were adjusted slightly for clarity.

This last point has led us to speculate about alternative mechanisms for the clearance of the reaction products away from the active site to explain the high turnover rate of AcChoEase. Fig. 5 shows that the electric-field vectors point down the active-site gorge towards Glu-199, Trp-84, and Glu-443. Trp-84 has already been implicated as a key element in the "anionic" binding site of AcChoEase (refs. 6, 21, and 25; M. Harel, I. Schalk, L. Ehret-Sabatier, F. Bouet, M. Goeldner, C. Hirth, P.H.A., I.S., and J.L.S., unpublished work). Although the indole ring of Trp-84, indeed, points into the gorge, the residue as a whole is on the protein surface. Visual examination showed that Trp-84 and the adjacent Met-83 are, in fact, free to undergo a conformational change, thus creating a putative *exit* for choline from the active site. This "back door" for choline release might involve a larger movement of residues 67–94. These residues, which form one wall of the gorge, have the conformation of a disulfide-linked Ω loop (26).

The results presented here highlight a remarkable structural feature of AcChoEase, which may have considerable bearing on its catalytic behavior and which must be considered in any detailed description of its mode of action.

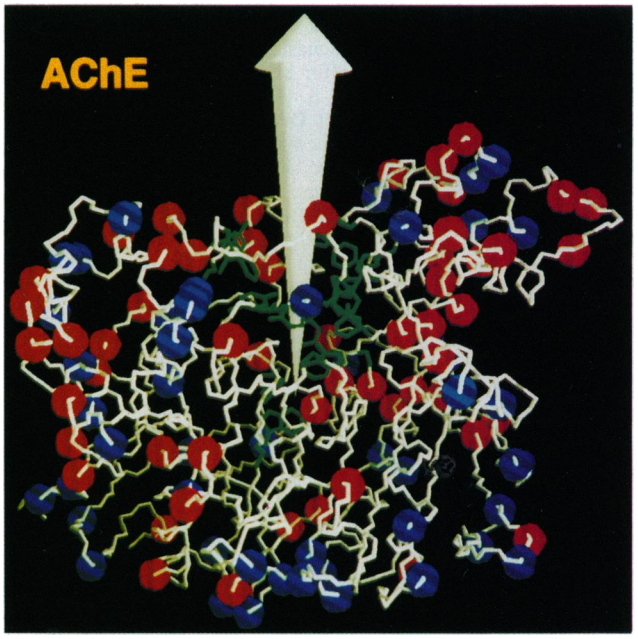

FIG. 4. Backbone trace of AcChoEase in white, generated with the program GRASP, showing the aromatic side chains lining the gorge (in green), positively charged residues (blue), and negatively charged residues (red). As in Fig. 1, the arrow indicates direction of the dipole.

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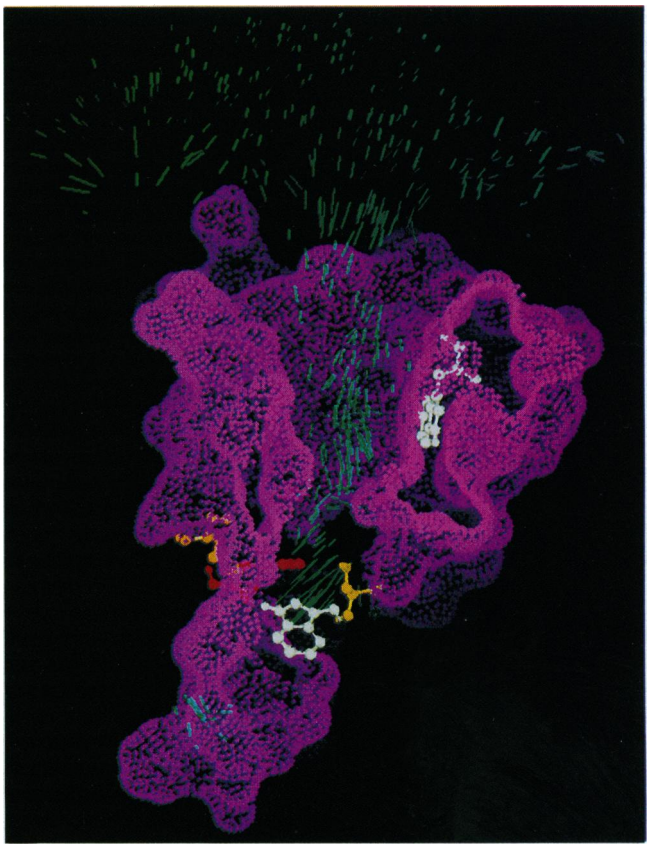

FIG. 5. Cross section through the active-site gorge of AcChoEase. The molecular surface is shown in lavender, and electrostatic field vectors are shown in green. The catalytic triad Ser-200, Glu-327, and His-440 are shown in yellow, orange, and red, respectively. Trp-279, at top of the gorge, and Trp-84, adjacent to the triad, are shown in white.

copy of the program GRASP; Mirek Cygler for providing the GLIP coordinates and for valuable discussion; John DiMaio for helpful discussions; and Herve Hogues for his help in generating figures. This work was supported by U.S. Army Medical Research and Development Command Contract DAMD17-89-C-9063, The Minerva Foundation, Association Franco–Israelienne pour la Recherche Scientifique et Technologique, The United States–Israel Binational Science Foundation, and the Kimmelman Center for Biomolecular Structure and Assembly. I.S. is Bernstein–Mason Professor of Neurochemistry; P.H.A. is supported by the L.P. Markey Charitable Trust. Part of this research was conducted with the resources of the Cornell Theory Center, which receives major funding from the National Science Foundation and International Business Machines Corporation, with additional support from New York State Science and Technology Foundation and members of the Corporate Research Institute.

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