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Homology Model of Juvenile Hormone Esterase From the Crop Pest, *Heliothis virescens*

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ABSTRACT **Juvenile Hormone Esterase (JHE) plays an essential role in the development of insects since it is partially responsible for clearing juvenile hormone (JH), one of the hormones that is responsible for insect metamorphosis. JHE is a 60 kDa enzyme that selectively hydrolyzes the α/β unsaturated ester of JH. Because of its pivotal role in insect development, we have targeted JHE for use as a biopesticide. In this study, we have constructed a homology-based molecular model of JHE from the agricultural crop pest, *Heliothis virescens*. JHE is a member of the α/β hydrolase fold family of enzymes and was built according to two structures in the same family: acetylcholinesterase from *Torpedo californica* and lipase from *Geotrichum candidum*. Analysis of the active site region reveals extensive conservation between JHE and its templates. A surprise was the presence of a conserved Ser near the catalytic triad. Docking of JH III into the active site has provided insight into protein-substrate interactions that are corroborated by experimental observation. The model is being used as a predictive basis to design biopesticides. In this regard, we have identified a site on the protein surface that is suggestive of a recognition site for the putative JHE receptor. Proteins 1999; 34:184–196.**

Key words: biologically-based insecticide; *Heliothis virescens;* insects; hormones; homology modeling; α/β hydrolase fold

INTRODUCTION

Insect metamorphosis is a tightly regulated process in that many cellular events must occur in a precisely timed manner to result in the maturation of a larva to an adult form. One of these events includes a precipitous decrease in JH titers during the last larval instar that is necessary for pupation to occur. This decrease in JH titer, in at least some species, is modulated by Juvenile Hormone Esterase (JHE) in the insect hemolymph.[1] Because of its role in regulating JH titers and thus insect development, JHE has been a target for development as a biologically-based insecticide.

Development of biologically-based insecticides is an important technical goal because the agricultural industry faces the challenge of maintaining high yields in food production coupled with less reliance on chemically-based insecticides.[2] The approach of using genetically engineered baculoviruses and other vector systems to introduce cDNA sequences encoding for insecticidal proteins represents a promising approach for insect control.[3] Three cDNA clones of JHE have been isolated from the agricultural pest, *Heliothis virescens*.[4] Recombinant JHE produced in a baculoviral expression system has been shown to reduce caterpillar feeding and increase speed of kill over the natural baculoviruses.[3] Both the natural and recombinant JHE forms cause anti-JH effects upon injection into the caterpillar, *Manduca sexta*.[5,6] Modified forms of JHE that are produced recombinantly in baculovirus have been shown to be more highly insecticidal than the wild-type recombinant JHE in *Trichoplusia ni* and *H. virescens*.[8] It is thus one of our goals to understand the structure/function relationships of JHE so that improved forms of JHE can be rationally designed as biologically-based insecticides.

JHE (EC 3.1.1.1), a member of the carboxylesterase family, is an enzyme that quickly decreases JH titers by hydrolyzing the highly stable α/β unsaturated methyl ester of JH to the metabolically inactive carboxylic acid. Figure 1 shows the reaction catalyzed by JHE. JHE is a 60 kDa protein that possesses a k_{cat}/K_m of approximately 7×10^6 $M^{-1}sec^{-1}$ making it suitable for scavenging trace quantities of JH prior to pupation.[9]

Sequence alignments have shown that JHE is a member of the α/β hydrolase fold family of proteins which includes many esterases and lipases.[10] This newly characterized family contains considerable structural similarities despite minimal apparent sequence homology. The α/β hydrolase fold consists of a catalytic triad of nucleophile-His-acid and a structural core of 8 α/β sheets, 8 β sheets connected by α helices. The catalytic triad of JHE is predicted to be composed of Ser_{203}-His_{448}-Glu_{334} based on site-directed mutagenesis and amino acid homology.[11] Of the X-ray crystal structures known to be members of the α/β hydrolase fold family, JHE displays the highest se-

Abbreviations: JHE, juvenile hormone esterase; JH, juvenile hormone; SCR, structurally conserved region; rms, root mean square deviation; PDB, Protein Data Bank.

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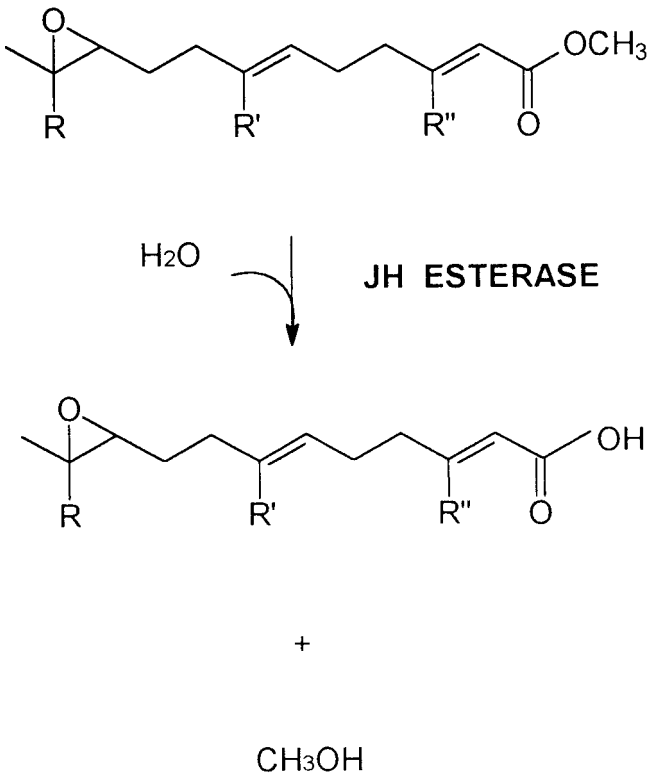

Fig. 1. Enzymatic reaction catalyzed by JHE. JH0: R=R'+R"=ethyl; JH I: R=R'=ethyl, R"=methyl; JHII: R=ethyl, R'=R"=methyl; JHIII: R=R'=R"=methyl. Note that the ester is conjugated with the 2,3-olefin making it planar, highly resistant to nucleophilic attack and stable to base. In contrast to acetylcholinesterase JHE is an ester of a large acid and small alcohol.

quence homology to acetylcholinesterase. Interestingly, both JHE and acetylcholinesterase have been established as key regulatory enzymes that are involved in the catabolism of chemical mediators, juvenile hormone, and acetylcholine, respectively. However, the two enzymes display intriguing differences in catalytic function in terms of k_{cat} and K_m. Both enzymes have similar k_{cat}/K_m, but JHE possesses a very low K_m while acetylcholinesterase has a high k_{cat} for their respective substrates. These kinetic constants are consistent with their physiological roles. Acetylcholinesterase must rapidly degrade a locally high concentration of substrate whereas JHE must efficiently degrade small quantities of JH systemically. Acetylcholinesterase and most esterases hydrolyze esters of large alcohols and small acids. In contrast, JHE selectively hydrolyzes esters with small alcohols and large acids.

Not only are the catalytic properties of the enzyme interesting, but the enzyme must appear and disappear very rapidly from the hemolymph of the insect during the last larval instar if normal development is to occur. The enzyme is very stable to many chemical reagents but is rapidly taken up and degraded by pericardial cells lining the insect heart by receptor-mediated endocytosis.[12, 13] It has recently been shown that JHE associates with a putative heat shock cognate protein (Hsp) that is located in the trans Golgi network of pericardial cells where it is believed to sort proteins destined for the lysosomes.[14] With this background, an understanding of JHE structure will allow us to use a knowledge-based approach to alter JHE by site-directed mutagenesis for insect control.

An important step in the development of structure/function relationships in proteins is the comparison of the enzymatic and chemical properties with the three-dimensional structure. While JHE is believed to be a member of the α/β hydrolase fold family, only limited structural information is available for esterases. In fact, acetylcholinesterase is currently the only esterase whose crystal structure is known. Recently, models for pancreatic cholesterol esterase (sterol ester hydrolase, EC 3.1.1.13) from rat and human were determined based on the X-ray structure of acetylcholinesterase and two lipases.[15] While knowledge of the structure of acetylcholinesterase has provided insight into its catalytic properties, a three-dimensional structure of JHE might give some clues about the opposing catalytic properties of the two enzymes. In the absence of an X-ray crystal structure, we have constructed a three-dimensional model of JHE by comparison to related proteins in the α/β hydrolase fold family by the method of homology-based molecular modeling.[16] In this communication, we present the deduced three-dimensional structure of JHE from the crop pest, *H. virescens,* and discuss implications of its structure as applied to catalytic activity, uptake and degradation and the development of biologically-based insecticides.

MATERIALS AND METHODS

All modeling was performed on a Silicon Graphics INDY (Mountain View, CA) using the visualization software, Insight II (Version 95.0) from BIOSYM/MSI, Inc. (San Diego, CA). Homology[17] was used for model generation and evaluation and Discover[18] was used for energy minimization. All minimizations utilized the CVFF forcefield. Profiles-3D[19] interfaced to Insight II was used to evaluate both sequence alignments and the final model.

Primary Sequence Alignment and Model Generation

Two high resolution X-ray crystal structures were used for model generation: acetylcholinesterase from *Torpedo californica* (PDB entry 1ace[20]) and lipase from *Geotrichum candidum* (PDB entry 1thg[21]). These structures have been refined at resolutions of 2.8 and 1.8 Å, respectively. Sequence alignment and model generation were performed as described in Thomas et al., 1997.[22] Briefly, the alignment of Cygler et al., 1993[23] was used for modeling with minor modifications at the C-terminal end of the protein. The α-carbon coordinates of both acetylcholinesterase and lipase have been categorized based on their structural homology into groups that include "low variable" and "high variable." [23] α-Carbons in the "low variable" set were chosen for model building of the SCRs. These "low variable" regions are composed of 170 α-carbon atoms that when superimposed for model building, yield a rms of 1.83

Å. Amino acids of acetylcholinesterase in this region include the following: 30–68, 91–102, 110–124, 141–155, 166–211, 218–227, 318–331, 397–403, 437–448, 475–483. The model was refined and analyzed for integrity as described in Thomas et al., 1997.[22]

Two nucleic acid sequencing errors were discovered in the original published clone of the plasmid, 3hv21. The revised sequence is available in Genbank (accession number AF037196). While modifying the model it was concluded to revise the model to match the sequence of the plasmid, 3hv16, since this clone has been used for most subsequent mutagenesis and expression studies (accession number AF037197). In the work reported here, the correction was made to the original model and this was achieved in a straightforward manner without consequence to the overall model. The rebuilt portions consist of seven single substitutions as follows: Val 10 Leu, Gly 303 Glu, Val 324 Ile, Val 446 Ala, Ala 464 Val, Phe 540 Leu and Thr 541 Ser. The model was further energy minimized as described for the original model though no major effect on the model was anticipated. These substitutions did not produce any major or minor steric clashes in the model; six out of seven of the substitutions are located on the surface of the protein. No conformational differences were observed due to the required correction in sequence and no steric clashes were introduced into the backbone or the sidechains. The rms between the 4,368 backbone atoms of the previous model and the new one is 0.59 Å. For the 110 atoms of the catalytic triad and the additional active site Ser, the rms between the previous model and the new one is 0.35 Å.

Electrostatic potential maps and dipole moments for the JHE model were generated using the software package, GRASP.[24] GRASP uses a simplified Delphi algorithm to compute the electrostatic potential on the surface of a protein molecule. Both the in-built full charge option and AMBER were used to assign charge and atomic radius data. The accessible surface was generated using the default sphere of radius 1.4 Å. The ESFF forcefield in Insight II was used for the dipole moment calculation.

JH III Docking Calculations

Juvenile Hormone III was docked into the active site of JHE based on the original 3hv21 sequence using a Monte Carlo method. Torsion angles in the small flexible substrate were included in the Monte Carlo algorithm such that the terminal two atoms were not altered in their position during generation of the conformers. The JH was placed to ensure that there were no steric clashes with the protein, and the energy used in the docking procedure would not be dominated by such contacts in the initial calculations. The $CO(OCH_3)$ group of JH was positioned so that it was in proximity to His 448 and Ser 203. This position placed Ser Oγ 203 4.18 Å from the ester carbonyl carbon and the His Nϵ2 448 3.40 Å from the same carbon. This distance would be representative of the approach of the carbonyl carbon of the esterolytic moiety of JH as it approaches the hydroxyl group of Ser 203 for catalysis. The docking calculations would not have been expected to predict an approach any closer than a close non-bonded contact. Prior to any automated docking, energy minimization was performed on side chains in close proximity to $CO(OCH_3)$. This excluded the catalytic residues Ser 203, Glu 334, and His 448. Hence, the catalytic residues maintained the conformation observed in acetylcholinesterase and lipase. The Monte Carlo docking in Insight II was performed allowing eight active torsion angles in JH. The initial JH conformer and side-chains identified in the vicinity of the active site were energy minimized by 300 steps of conjugate gradient minimization using the CVFF forcefield in Insight II. Side chains included in the calculation were Phe 119-Ser 129, Leu 154, Gln 202, Ala 204, Gly 205, Met 228-Gly 230, Ser 234, Phe 236, Phe 237, Leu 290-Val 297, Thr 331, Ser 333, Cys 335-Leu 342, Phe 393, Val 394, Cys 397, Ser 398, Phe 401-Glu 403, Tyr 428, Met 439-Gly 447 and Ile 449-Thr 453. All side chains in the calculation were within 5 Å of JH in its starting conformation. These conformers were saved to use as initial criteria for subsequent steps. Subsequently generated and minimized structures were saved if they were within 10 kcal of the lowest energy conformer, and the saved substrate had to be greater than 0.5 Å in rms from previously stored conformations. The method of docking used is implemented by Molecular Simulations, Inc. (San Diego, CA) in Biosym Tool Command Language and the scripts were extended to perform the calculations as described.

RESULTS AND DISCUSSION

Structural Description of JHE Model

The homology-based model of JHE was constructed using the templates of two high-resolution crystal structures: acetylcholinesterase from *T. californica* and lipase from *G. candidum* in the α/β hydrolase fold family to which JHE displays 27.9 and 27.2% identity, respectively. Thomas et al., 1997 describe the preliminary building of this model in which the amino acid sequence from the clone, 3hv21, was used.[4, 22] Subsequently, the model was revised to match the sequence of the clone, 3hv16. All modeling was done on the sequence representing the processed form of the protein without its 19 amino acid leader sequence. This form accounts for 70% of the processed enzyme in insect blood.[4] The sequence alignment of Cygler et al., 1993 was used for modeling JHE with minor modification in the C-terminal-most helix, α14 (Figure 2).[23] A three residue-gap was introduced into the JHE sequence at position 529. This new alignment generates a third salt bridge in JHE (Asp 399-Arg 529) that is conserved in both acetylcholinesterase (Asp 397-Arg 517) and lipase (Asp 425-Arg 529). Furthermore, the addition of the gaps improved the sequence homology as measured in Insight II using the mutation matrix between JHE and lipase to which it was aligned. Not only was the alignment of JHE Arg 529 and lipase Arg 529 improved, but this new alignment of JHE 529–546 with the corresponding amino acids of lipase yielded 19% identity versus 13% for the previous alignment. Therefore, we conclude that this salt bridge is likely to exist in JHE. There are two additional conserved salt bridges in JHE, Arg 47-Glu 90 and Arg

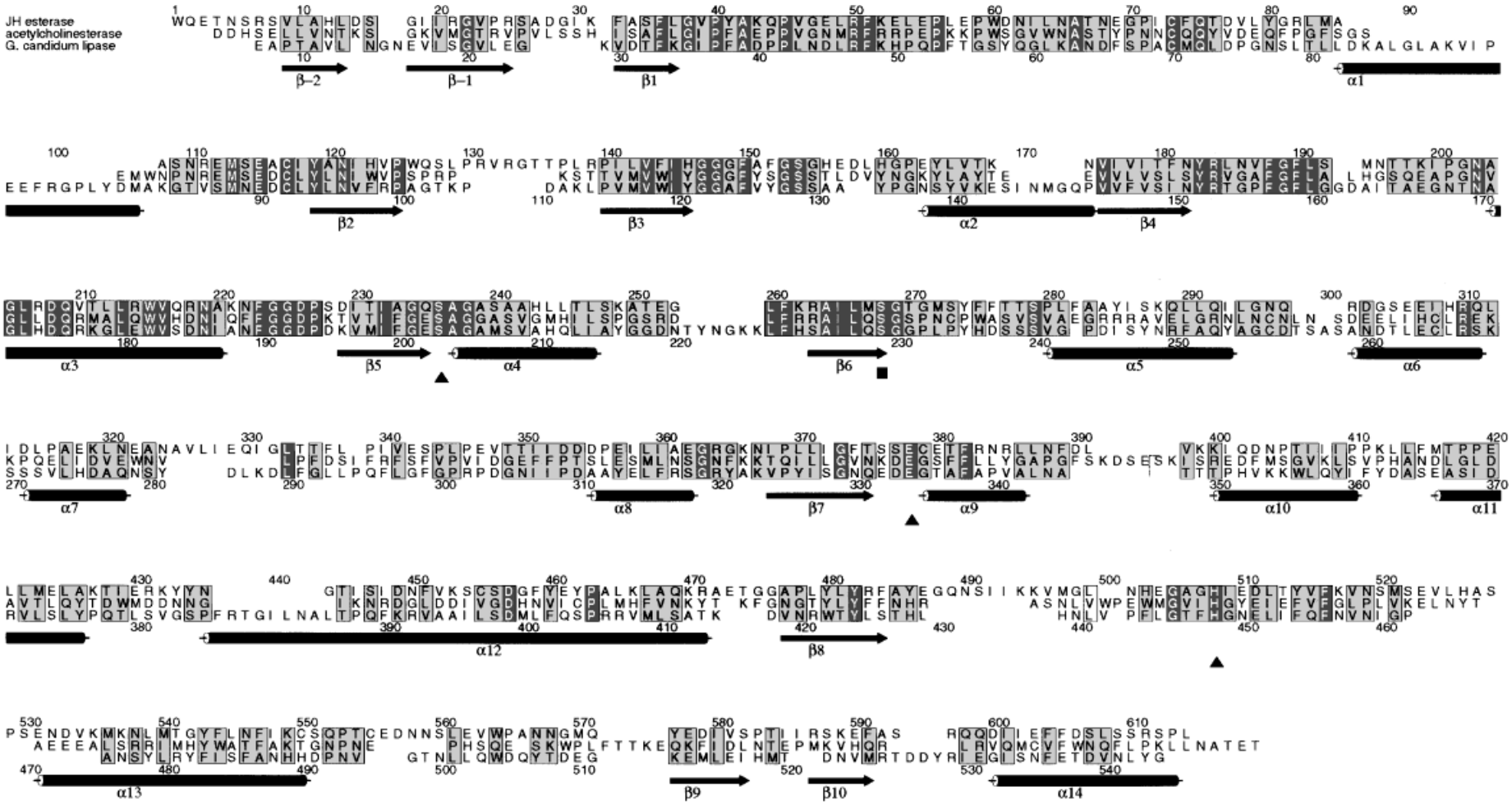

Fig. 2. Sequence alignment of JHE from *Heliothis virescens* with acetylcholinesterase and lipase from *G. candidum* as illustrated by the program, ALSCRIPT.[39] The sequence of JHE derived from *H. virescens* (clone 3hv16) [9] was aligned with acetylcholinesterase from *T. californica* (ACE), lipase from *G. candidum* (THG) according to the method of Cygler et al., 1993 [23] with minor modification in the C-terminal 20 amino acids. Regions of identity and homology among the amino acid sequences are shown in black and gray, respectively. α Helices and β sheets are shown as cylinders and arrows, respectively, and are numbered according to Sussman et al., 1991.[20] Numbering below the sequence alignment is that of JHE amino acids. Numbering above the sequence is that of JHE amino acids including the gaps. Triangles refer to active site amino acids, square refers to Ser 229.

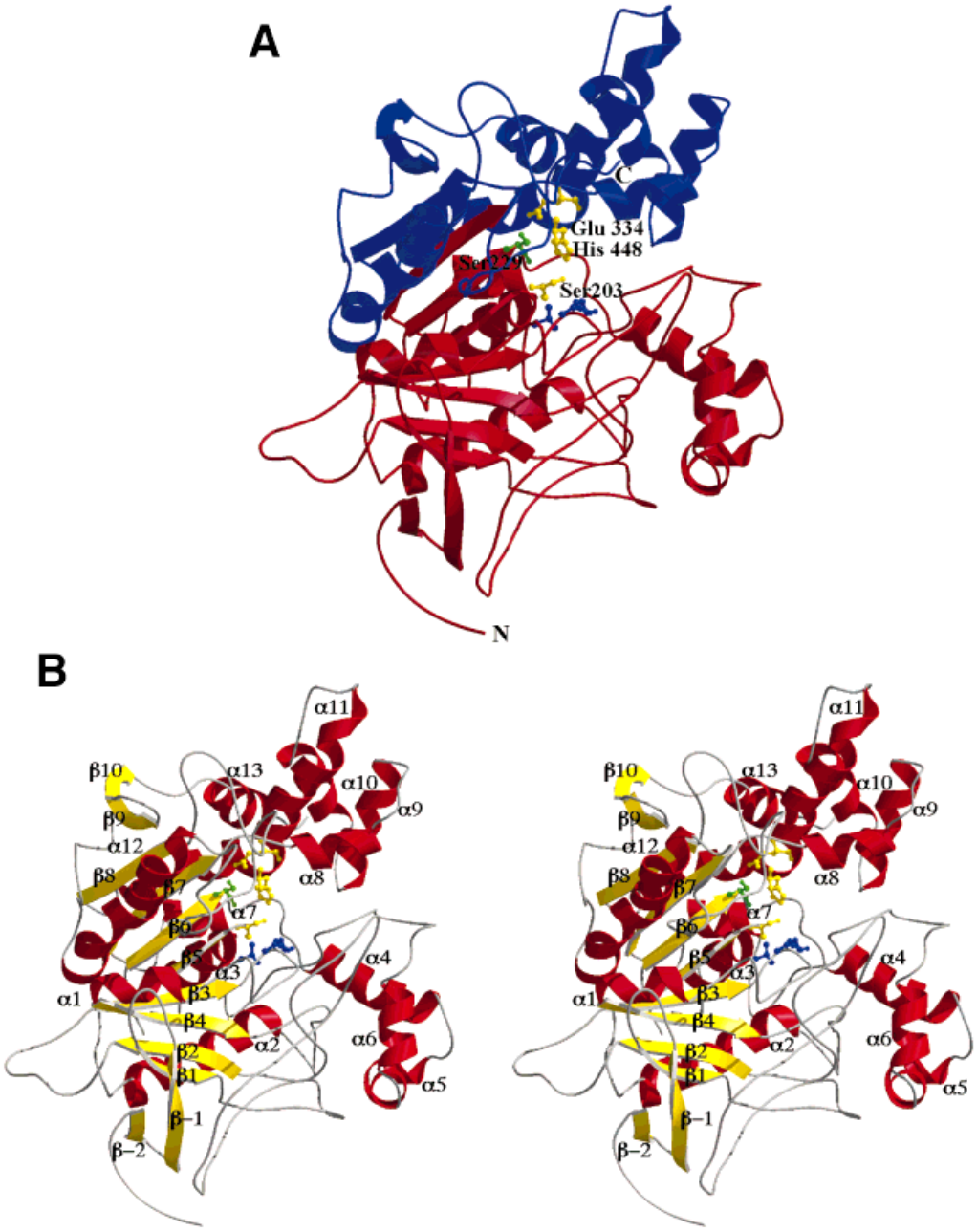

Fig. 3. Topology of JHE as illustrated by *Raster 3D*.[40] **A.** Topology displaying the N- and C-terminal domains of JHE in red and blue, respectively. The axis between the two domains is approximately perpendicular to the plane of the page. Catalytic amino acids (Ser 203, Glu 334, His 448), Ser 229 and the oxyanion hole residues (Gly 123, Gly 124, and Ala 204) are colored in yellow, green, and blue, respectively. **B.** Stereogram of the topology of JHE showing secondary structures as labeled in Figure 2. Catalytic amino acids, Ser 229 and oxyanion hole residues are colored as in Figure 3A. The α helices (red) and β sheets (yellow) are numbered according to Sussman et al., 1991[20] as shown in Figure 2.

153-Asp 175. The effects of salt bridge, Arg 47-Glu 90, have been tested experimentally by mutating Arg 47 to His.[11] This mutation decreased activity by about 50-fold and the observed K_m increased from 34 to 67 nM.

Analysis of model validity was examined using several different algorithms all of which indicated overall good model quality with the only regions of marginal quality being those classified in the templates as "highly variable" by Cygler et al.[22, 23]

Figure 3 shows the topology of the JHE model which is typical of proteins in the α/β hydrolase fold family. This family of proteins, which is believed to have evolved by divergent evolution, adopts a characteristic core of 8 β sheets connected by α helices despite a lack of significant sequence similarity. The most distinct differences between the templates and the JHE model include a difference in length of helix α2 (Fig. 2). In JHE helix α2 is shortest while it is longest in lipase. The helix, α1, is present only in

TABLE I. Percent Sequence Identity Between JHE, Acetylcholinesterase and Lipase Domains

	Percent sequence identity[a]			
	ACE (1–323)	Lipase (1–350)	ACE (324–end)	Lipase (351–end)
JHE (1–330)	33.4	34.4		
JHE (331–end)			21.4	17.1

[a]Sequence identities were determined using pairwise alignment in Insight II. A Dayhoff mutation matrix was used with a gap penalty and gap length of 6 and 1.7, respectively. Domains are defined by amino acid sequence number and are defined according to Cygler et al., 1993.[23]

lipase and composes the "lid" that covers the active site that is involved in interfacial activation. The loop region between sheets β8 and β9 containing the active site His is longer in JHE than in the templates. The helix, α12, is longest in lipase and is shortened by 8 amino acids at the N-terminal end as compared to JHE. The three proteins of major interest in this study, acetylcholinesterase, lipase and JHE, consist of both N- and C-terminal domains although there is much greater identity among the three in the N-terminal domain. Table I shows the sequence identities for the domains between JHE and each of its templates. Figure 3 displays the topology of each individual domain showing the active site channel that appears to be at the interface of the two domains. This view of the protein illustrates the possibility that "breathing" between the N- and C-terminal domains could vastly change the size of the gorge leading to the active site which might be important for the substrate movement within the enzyme.

Proteins of the α/β hydrolase fold family typically possess several well-conserved residues at the active site, a hydrophobic core, disulfide bridge, and salt bridges.[23] The JHE model includes one disulfide bond (Cys 70-Cys 92) that is conserved in both the acetylcholinesterase and lipase templates. This disulfide connects a loop of variable length between β1 and β2 in the α/β hydrolase fold proteins to the rest of the protein. In lipase, this disulfide bond tethers a 27 amino acid insertion (helix α1 in Figure 2) that is believed to act as the "lid" covering the active site channel that is involved in "interfacial activation."[21] JHE is not predicted to display "interfacial activation" and, as such, is missing this helix in the sequence. There are two other Cys in JHE (Cys 335-Cys 397) that are not known to be conserved in α/β hydrolase fold proteins. These could potentially form a disulfide bond in the model. Cys 335 is located in the sequence SECET where the Glu immediately prior to the Cys is part of the catalytic triad. In other esterases, lipases and related sequences this position is occupied by a Gly in most cases and by a Phe in some esterase sequences. JHE is the single known example in which Cys immediately follows the catalytic Glu. Cys 397 is located in the middle of helix α12 (Fig. 2) and is a Val and Leu in acetylcholinesterase and lipase, respectively. The side-chain S to S distance in the refined model is ~4.5 Å. A disulfide at 335–397 in JHE would anchor the same helix, α12, that is anchored by the disulfide bond in acetylcholinesterase, Cys 402-Cys 521.

Characteristics of the Active Site/Catalytic Tetrad

The spatial arrangement of the catalytic triad is well-conserved among α/β hydrolase fold proteins and JHE appears to be no exception. This is not surprising due to the high sequence and structural similarity in all three regions; these regions are classified as "low variable."[23] The side chains of the three active site residues of JHE are superimposable with the corresponding amino acids in acetylcholinesterase and lipase to a rms of 0.55 and 0.53 Å, respectively. Figure 4A shows the superimpositions of amino acids involved in the catalytic triad of JHE with its templates. The amino acids that compose the catalytic triad of JHE have been confirmed experimentally by site-directed mutagenesis.[11] These authors individually mutated Ser 203 to Gly, His 448 to Lys and Glu 334 to Gln and found in each case no detectable activity using either JH III or $C_6H_{13}OCH_2C(O)SCH_3$ as substrates.

Wallace et al., 1996 have derived a three-dimensional template for Ser-His-Asp-containing proteins present in the Brookhaven Protein Data Bank based on α-lytic protease (LPR), a well-defined and representative Ser protease.[25] They have developed a scheme that allows the prediction of whether a protein can be classified as catalytic by examination of root mean square distances of Ser hydroxyl oxygens and the Asp carboxyl oxygen.[25] JHE displays high sequence homology with its template proteins of the α/β hydrolase fold family, acetylcholinesterase, and lipase. Thus, we expected that a fit similar to the criteria of Wallace et al., 1996 would yield good superimpositions.[25] Indeed, we find that the distances between JHE Glu Oϵ1 334 and acetylcholinesterase Glu Oϵ1 327, after a structural superimposition of the active site imidazole rings of His is 0.43 Å (Figure 4B, 4C). Likewise, the distance between JHE Ser Oγ 203 and acetylcholinesterase Ser Oγ 200 is 2.1 Å (Figure 4B, 4C). The comparable distances from both JHE and acetylcholinesterase to α-lytic protease, on which the consensus template is based, are 0.54 (JHE Ser Oγ 203 to LPR Ser Oγ 195) and 2.31 Å (acetylcholinesterase Ser Oγ 200 to LPR Ser Oγ 195) for Ser Oγ and 1.00 Å (JHE Glu Oϵ1 334 to LPR Asp Oδ1 102) and 0.87 Å (acetylcholinesterase Glu Oϵ1 327 to LPR Asp Oδ1 102) for Glu Oϵ1 to Asp Oδ1 in both JHE and acetylcholinesterase, respectively, when superimposed by fitting all atoms of the imidazoles to that of α-lytic protease (Figure 4B, 4C). The cutoff for significance as defined by Wallace et al., 1996 is 2.0 Å.[25] Therefore, despite the fact that the acid moiety is different in these three esterases, the active site geometry appears to be well conserved. Furthermore, the template-derived triad containing Asp appears to be applicable to triads containing Glu for the purposes of predicting catalytic potential based on steric factors.

An additional conserved Ser (229 in JHE) is present in the active site of Ser proteases and appears to be as well conserved as the nucleophilic Ser in the catalytic site. Wallace et al., 1996 found in their comparisons of Ser proteases that while some triads had root mean square

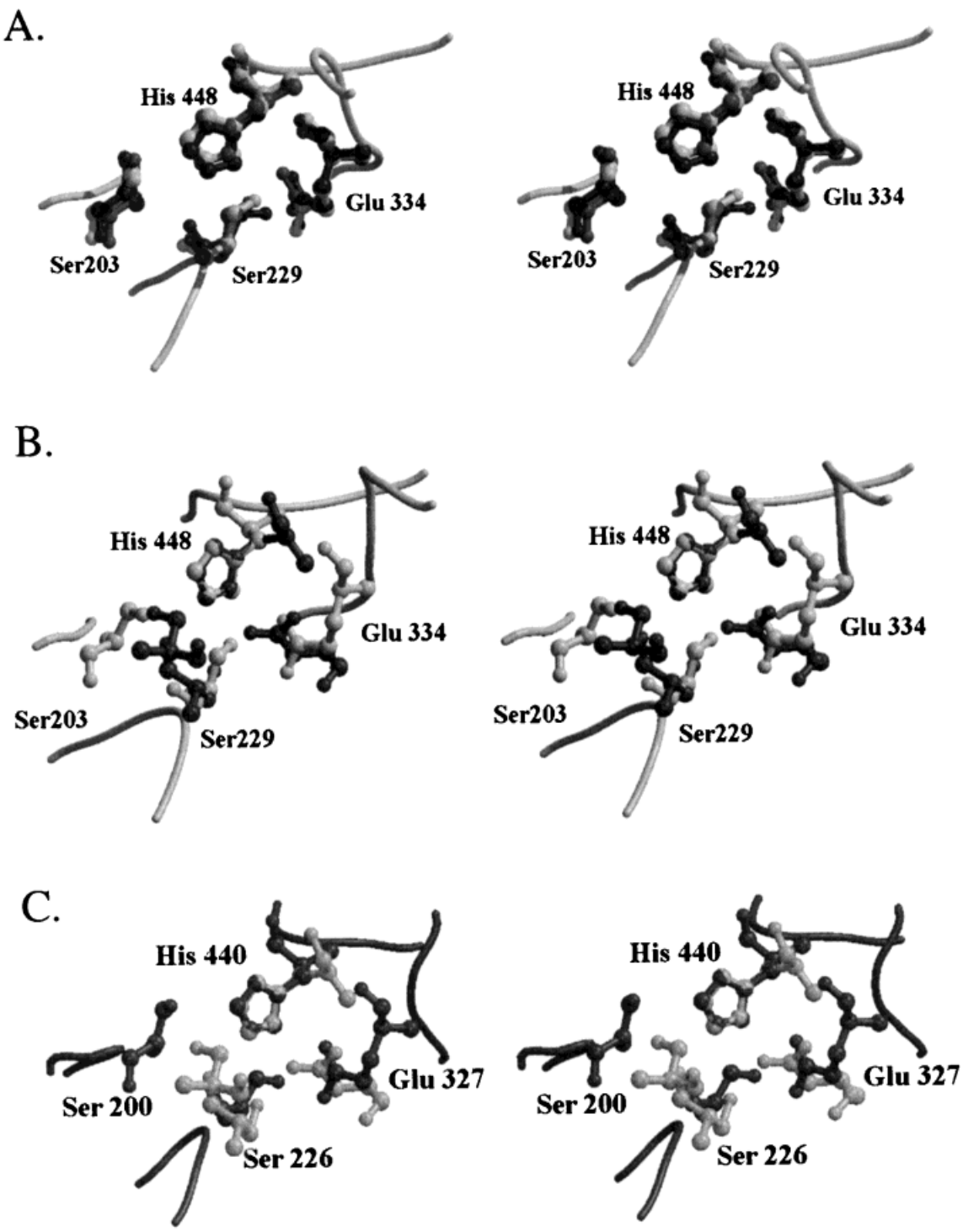

Fig. 4. **A.** Stereogram displaying the superimposition of amino acids of interest in the vicinity of the catalytic tetrad as calculated by Insight II and illustrated by *Raster 3D* for JHE, acetylcholinesterase and lipase.[40] The numbering corresponds to that of JHE. The side chains of the catalytic residues of the equally minimized structures were superimposed to a rms of 0.55 and 0.53 Å for acetylcholinesterase and lipase, respectively. Side chains of JHE, acetylcholinesterase and lipase are shown in light gray, black and medium gray, respectively. JHE Ser 203 is thought to be the nucleophilic Ser while JHE Ser 229 appears to be highly conserved in esterases. **B.** Superimposition of α-lytic protease (dark gray) and JHE (light gray)using the numbering system of JHE. Superimposition of amino acids in the vicinity of the active site similar to the specification of Wallace et al., 1996[25] where the side chain active site imidazoles of the respective catalytic His were superimposed with that of α-lytic protease. The catalytic triad of α-lytic protease contains Asp instead of Glu. **C.** Superimposition of α-lytic protease (light gray) and acetylcholinesterase (dark gray). Numbering is that of acetylcholinesterase. α-Lytic protease is not a member of the α/β hydrolase fold family of proteins. Thus, the similar spatial arrangement of the catalytic amino acids must be convergent.

distances of ~0.5 Å from the functional template there was a second peak of proteins at approximately 5 Å that suggested an alternative conserved catalytic triad.[25] A systematic study of the steric arrangement of active site residues in Ser proteases has shown that there is an additional Ser in the active site of these enzymes that is well conserved and has been termed the catalytic "tetrad."[26] This additional serine that completes the tetrad is present in JHE, acetylcholinesterase and lipase at positions 229, 226, and 249, respectively (Figure 4A). A survey of aligned esterase and lipase sequences reveals that this non-catalytic Ser is well-conserved with the exception of 2 sequences out of a total of 54: bovine thyroglobulin and a lipase. Bovine thyroglobulin is not catalytically active. Of 200 Ser proteases of bacterial and mammalian origin, all appear to have the additional Ser with the exception of three.[27] A comparison of the non-catalytic Ser in JHE and acetylcholinesterase to α-lytic protease shows that these Sers superimpose well. Acetylcholinesterase (Ser Oγ 226) and JHE (Ser Oγ 229), were superimposed with α-lytic protease by fitting the side chain atoms of the imidazoles of the respective catalytic His. The resulting distances between JHE Ser Oγ 203 and acetylcholinesterase Ser Oγ 200 to Ser Oγ 214 of α-lytic protease were 1.47 and 2.10 Å, respectively. These distances are near the cutoff value of 2.0 Å for significance for the group I and II Ser proteases as calculated by Wallace et al., 1996.[25] Therefore, we propose that in the α/β hydrolase fold enzymes that we have studied, this Ser is functionally significant. The catalytic triad in the Ser protease family is near the N-terminus of the protein and opposite in orientation from the more C-terminal triad in the esterase-lipase family. The presence of the second Ser in esterases in an identical three-dimensional orientation to the same in the Ser protease family argues strongly for an essential role for this amino acid in the structure and/or catalytic activity of both families of enzymes.

Several site-specific mutants have been produced to address the functional significance of the non-nucleophilic Ser in Ser proteases. McGrath et al., 1992 found that replacement of this extra Ser with amino acids that alter the local electrostatic environment caused a decrease in catalytic activity and the free energy of catalysis.[27] Most recently, site-directed mutagenesis of two Sers in *Escherichia coli* outer membrane phospholipase A_2 has shown that Ser 152, a non-nucleophilic Ser is likely to be involved in hydrogen bonding to the triad residues.[28]

The high conservation of Ser at position 229 in JHE and in apparently homologous positions in other members of the esterase/lipase family strongly suggests an essential role. Furthermore, it is even more compelling that this Ser is conserved among families of proteins that are evolutionarily divergent, i.e. Ser proteases and α/β hydrolase fold proteins. We have examined the active site in the vicinity of Ser 229 in JHE. The model of JHE was built according to acetylcholinesterase in this region. Although acetylcholinesterase is a lower resolution structure than the lipase template, the geometry of the atoms in this regions is very similar (Figure 4A).

Several possibilities for the role of this extra Ser have been examined. We examined potential hydrogen bond formation between Ser 229 and the catalytic triad amino acids and alterations in electrostatic potential as a result of mutation of Ser 229. Both hydrogen bonding and changes in electrostatic potential were found to be unlikely for JHE (data not shown). However, a conserved solvent water molecule has been identified in both acetylcholinesterase and lipase X-ray crystal structures that is within hydrogen bonding distance (2.6 and 2.7 Å oxygen to oxygen distance, for acetylcholinesterase and lipase, respectively) to the extra Ser. Thus it could be postulated that this Ser orients water for the hydrolytic part of the reaction. Examination of a representative set of several Ser proteases reveals that a water is present within hydrogen bonding distance of the extra Ser in these cases as well. Chymotrypsin (PDB = 1acb), thrombin (PDB = 1hag), pancreatic elastase (PDB = 1ela) and leukocyte elastase (PDB = 1hne) were each superimposed according to the side chains of the catalytic triad members including Cα. The putative hydrogen bonding distance from the oxygen of water to Ser Oγ ranged from 2.7–3.3 Å. In fact, conserved water molecules were found in 9 out of the 14 structures examined. The nearest potential hydrogen bond donor is, in fact, this conserved Ser in both Ser proteases and α/β hydrolase fold family proteins that we examined. Thus, we postulate, based on our calculations, that the additional Ser present in the active site might orient water, possibly for hydrolysis of the acyl enzyme. For serine proteases, an analogous Ser has been postulated to orient the acid of the catalytic triad while the in lipases it was proposed to hydrogen bond to the oxyanion hole.[27, 28]

In most esterases and lipases, the sequence in the vicinity of the active site Ser is G*E*SAG. However, the corresponding sequence in JHE is G*Q*SAG. The Glu immediately prior to the catalytic serine has been mutated to a Gln in acetylcholinesterase; the mutant demonstrated an approximate 6-fold increase in K_m and a 2-fold decrease in activity compared to the wild-type enzyme.[29] Figure 4 shows the catalytic triad residues of JHE and other amino acids of interest in the active site. In JHE the Gln, which is isosteric to Glu, appears to be closer in proximity to the active site Ser than the corresponding Glus in the templates. This variation in JHE from the esterase norm may partially explain its very low K_m for its substrates. Comparison of the JHE structure containing a docked substrate, JH III, versus the same structure without reveals that in the presence of JH III Gln 202 is closer to Ser 203 due to a change of approx. 25° in the dihedral angle, psi, of Gln 202. This ultimately brings the side chain in closer proximity to His 448 which, in the presence of ligand, has an imidazole plane that is slightly changed. This change in plane shortens the hydrogen bonding distance between His Nδ1 448 and Glu Oϵ2 334 by approx. 0.5 Å which would yield a stronger hydrogen bond.

Docking of the Substrate, JH III, to the Active Site of JHE

One of the purposes of constructing a model of JHE is to develop a structural understanding of the catalytic properties of the enzyme. We are especially interested in the comparison of catalytic properties and structure of JHE and acetylcholinesterase. Both enzymes are involved in the catabolism of chemical mediators: JH and acetylcholine in JHE and acetylcholinesterase, respectively. However, in order for JH and acetylcholine to approach the catalytic Ser at the base of the active site channel and have the subsequent transition state stabilized by the conserved oxyanion hole residues, they must approach in the opposite orientation. In JHE the leaving group is the methoxy group which will be located at the base of the channel. On the other hand, the leaving group in acetylcholine is the choline moiety which is distal to the base of the channel and can easily be envisioned exiting the channel. It has long been known that the preferred substrates of JHE are esters of small alcohols and large acids.[1] This is in contrast to acetylcholinesterase and most general esterases where common substrates are large alcohol esters of acetic acid. Based on our experimental data and this model, we believe that, in order for JHE to have a k_{cat}/K_m that approaches the diffusion limit, a large-scale motion of the enzyme such as "breathing" between the two domains, as mentioned earlier, or an as yet unidentified exit channel is plausible.

JH III has been docked into the active site of the JHE model using automated docking procedures to examine interactions between protein and substrate. 83 conformations (i.e. a 15 kcal energy range) were found with energies of 4,412–4,427 kcal. In all solutions the JH kept the essential hydrogen-bonding pattern that was the basis for the starting model even though energy minimization had been performed on all saved conformations. No other specific hydrogen bonds were found. The ester moiety did not dock such that the ester carbonyl was significantly closer to Ser 203, though a movement of up to ~1 Å might have been possible before the methoxy group clashed with Tyr 237. Figure 5 shows the lowest energy conformer docked into the active site of JHE. JH is in a relatively contracted conformation unlike acetylcholine in acetylcholinesterase. Several modeling and X-ray studies have shown that acetylcholine is present in its fully extended conformation with the quaternary ammonium group interacting with Trp 84 at the base of the active site gorge.[20, 30, 31] A number of hydrophobic amino acids line the active site channel in JHE: Leu 134, Phe 119, Leu 80, Pro 295, Phe 338, Leu 343, Ile 390, Val 394, Ile 449 and Leu 452. The conformations of these residues, in general, seem to have maximized the hydrophobic associations possible at the sides of this cavity. This is not surprising considering the lipophilicity of the substrate. At the opposite end of the channel near the orifice is a Tyr 77 that is in close proximity to the epoxide moiety of JH III. In the minimized structure this Tyr appears to orient the epoxide moiety (Fig. 5). In this conformation the distance between the epoxide oxygen and the Tyr hydroxyl oxygen is too distant

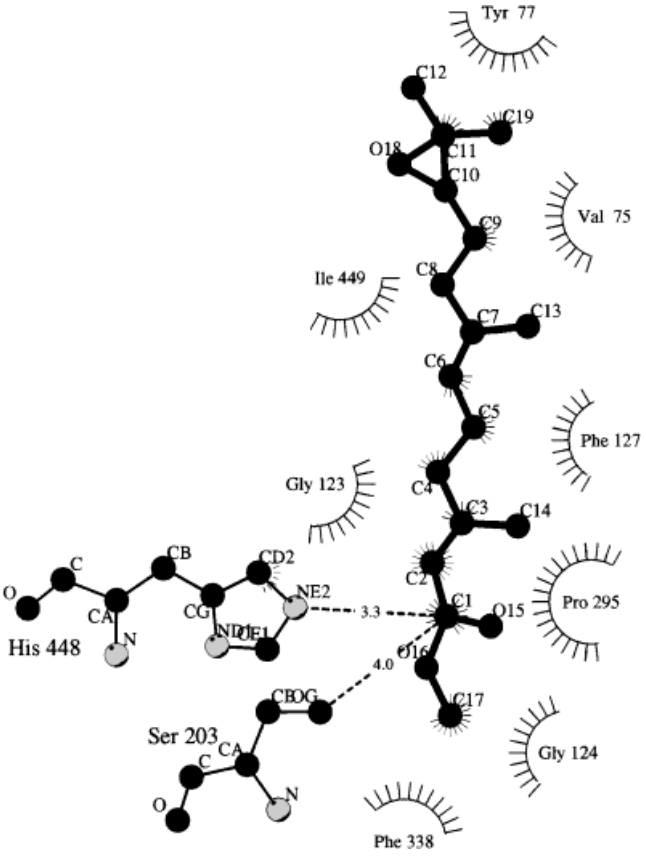

Fig. 5. Lowest energy conformer of JH III docked into the active site of JHE and visualized by LIGPLOT.[41] Amino acids in closest proximity to the substrate are displayed in "eyelash" presentation. Distance monitors in Å are represented by dashed lines. Two of the oxyanion hole residues (Gly 123, Gly 124) are shown. Note that the orientation of JH III in the catalytic site is proposed to be opposite in orientation to acetylcholine in acetylcholinesterase.

for a hydrogen bond. However, one could envision that Tyr 77 could orient JH once it is in the active site channel. A closer examination of the electrostatic potential in the region of the channel shows a concentration of negative charges at the base of the active site near the catalytic amino acids (Figure 6B). Despite the fact that JH III is highly hydrophobic and uncharged, we found that it has a dipole moment of 10.3 Debye as calculated in GRASP using the ESFF forcefield in Insight. This dipole moment is aligned with its negative end in the direction of the epoxide moiety. This is consistent with the positive end being in closest proximity to the negatively charged potential surface at the base of the channel (Figure 6A).

JHE contains a deep narrow gorge of approximately 23 Å in length, the base of which contains the catalytic tetrad. The gorge appears at the interface of the two domains of the protein (Figure 3A). Hence, it appears that the less conserved C-terminal domain makes a significant contribution to the amino acids that line the channel, especially at the orifice where the substrate appears to enter the channel. Thus, it could be presumed that the less conserved C-terminal domain lends at least some substrate

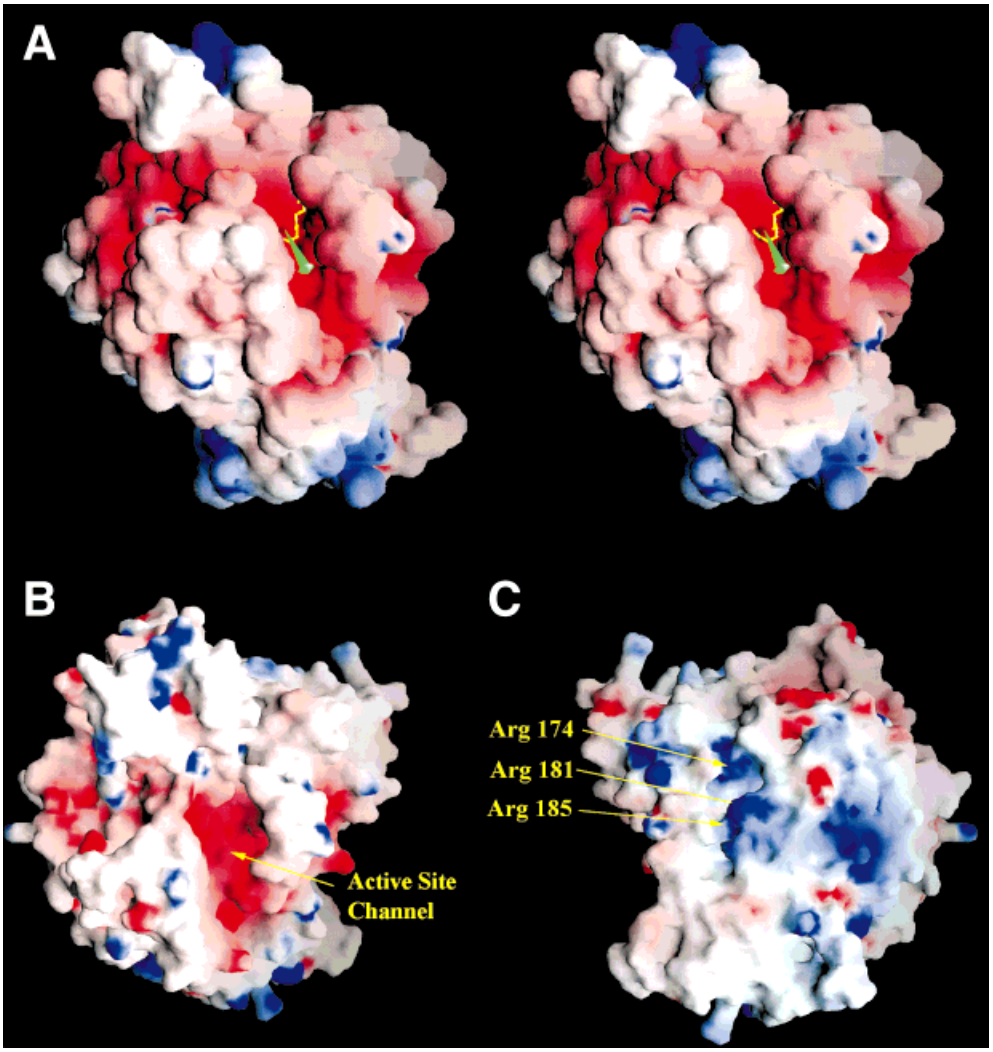

Fig. 6. **A.** Stereogram depicting the accessible surface of JHE colored by electrostatic potential as calculated by GRASP using the in-built full charge set to calculate charges.[24] Positively charged regions are shown in blue while negatively charged regions are shown in red. Lowest energy conformer of JH III (yellow) is shown docked into the active site as in Figure 5. Dipole moment of JH III was calculated in GRASP using the ESFF forcefield in Insight II and is depicted by the green arrow. Positive end is at the tip of the arrow and is facing outward from the channel. **B.** Electrostatic potential surface of one face of JHE showing the active site channel. **C.** Electrostatic potential surface (opposite face-rotation about y by 180 degrees) of JHE showing an α-helix containing three Args that are believed to play a role in receptor recognition.

selectivity by governing interactions between protein and substrate at the opening of the channel. The channel itself is lined primarily by hydrophobic residues, 20% of which are aromatic in nature. In acetylcholinesterase, approximately 40% of the amino acids that line the gorge are aromatic in nature.[20] Trp 84 in acetylcholinesterase has been well-studied experimentally and has been found to be an essential component of the anionic (choline) binding site and interacts with the quaternary ammonium group.[20] The term, "anionic site," as it applies to acetylcholinesterase does not imply ionic interactions and is used in a historical context. This Trp is not conserved in JHE and, in fact, is present as a gap in our preferred sequence alignment (Fig. 2). The putative oxyanion hole in acetylcholinesterase is composed of the residues, Gly 118, Gly 119, and Ala 201, which through their main chain amide nitrogens, stabilize the transition state.[20] In JHE these residues are conserved and are Gly 123, Gly 124, and Ala 204, thus implying that they serve to form the oxyanion hole in JHE as well (Fig. 3A, 3B).

The aromatic amino acids lining the active site channel in acetylcholinesterase, as mentioned previously, have been hypothesized to function in an aromatic guidance mechanism which directs the positively charged choline substrate to the active site.[20] In fact, there exists a strong electrostatic dipole that is aligned with the active site channel and is believed to effectively guide acetylcholine to the active site.[32] Calculation of the dipole moment of JHE by GRASP reveals that while the direction is similar to that in acetylcholinesterase, the magnitude of the dipole is 28% of that for acetylcholinesterase.[33] A closer examination of the N- and C-terminal domains of each protein and

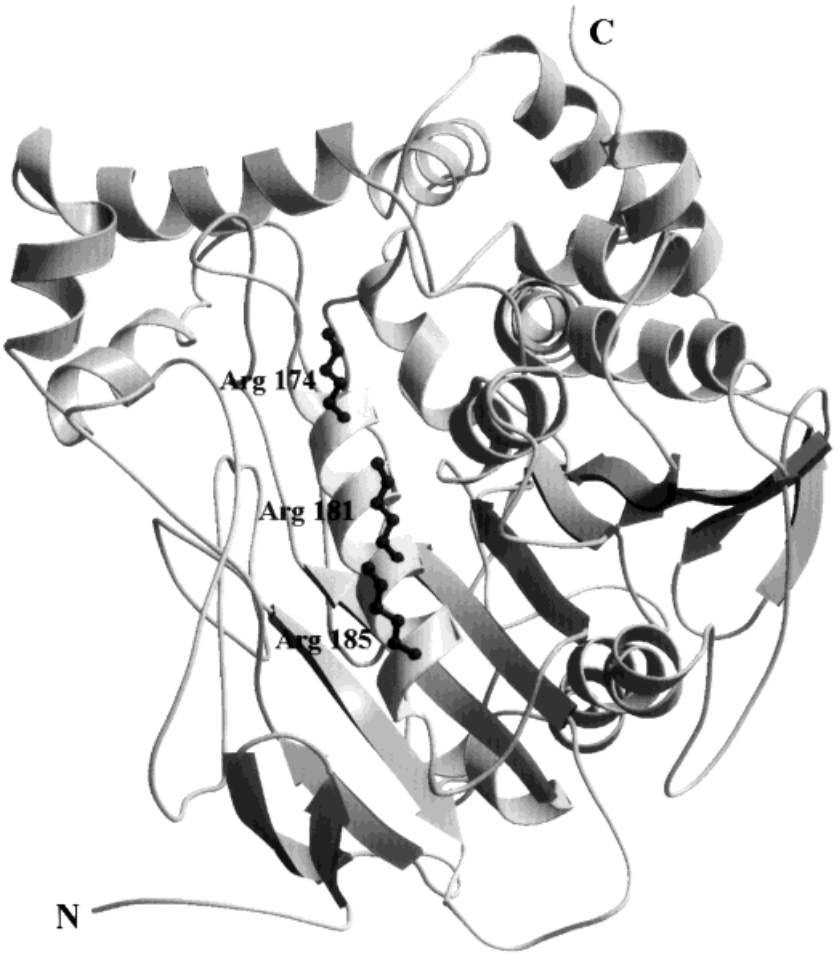

Fig. 7. Topology of JHE as illustrated by *Raster 3D* showing the Args of interest believed to play a role in receptor recognition.[40] Orientation of JHE is exactly as in Figure 6C. The Args shown in this amphipathic α-helix are illustrated in blue in Figure 6C.

their respective dipole moments shows that the dipole moments of the N- and C-terminal domains of JHE are 63 and 54%, respectively, of the magnitude of the equivalent domain in acetylcholinesterase. Although there is a comparable number of charged residues in the acetylcholinesterase and JHE molecules, JHE has not retained the large dipole considered to be important for guidance of a charged substrate to the active site. This observation is not surprising, but is consistent with the dipole being important for the guidance of the positively charged acetylcholine but not for the hydrophobic JH.[32] Such electronic interactions fall off much more slowly with distance than other weak molecular interactions. We anticipated this effect in the C-terminus on the basis of lower homology between the two enzymes. However, the electrostatic character of the more similar N-terminal domain has not been retained. The biological role of acetylcholinesterase requires a very high k_{cat} while JHE has a much lower k_{cat} commensurate with its role as a scavenger enzyme operating at extremely low substrate concentrations. Thus, the electronic attraction of acetylcholinesterase for its substrate is likely much more biologically important than similar electronic effects in JHE.

Pericardial Cell Receptor Recognition Sites and Design of Biologically-Based Insecticides

The major impetus for building this model from an applied point of view is to apply the structural knowledge obtained from the model and the experimental studies in insect biochemistry and physiology to effectively engineer improved insecticides based on JHE. Our major focus, in this respect, is to design altered forms of JHE that display impaired uptake and degradation.

Previous studies of uptake and degradation of JHE have shown that it is removed from the insect hemolymph by a receptor-mediated process.[13] The uptake studies done in the insect, *M. sexta*, demonstrate that JHE is removed from hemolymph by pericardial cells that surround the insect heart.[13, 34] These pericardial cells serve both secretory and degradative functions in insects and serve as liver analogues.[35] Pharmacokinetic analysis of recombinant JHE revealed that it has a half-life of 1.2 hours in the hemolymph while several foreign proteins displayed half-lives of several days.[13] Removal of JHE was shown to proceed via a first-order saturable process that is strongly suggestive of receptor-mediated endocytosis.

We are currently focusing on the elucidation of amino acids on the surface of JHE involved in recognition by the pericardial cells. The goal is to alter these residues to effectively interfere with JHE uptake as an approach to develop biologically-based insecticides. It is plausible that the receptor that removes JHE from insect hemolymph is related to the LDL (Low Density Lipoprotein) receptor superfamily due to the good sequence homology between JHE and some lipases known to be internalized by these receptors. Several lipoprotein receptor genes have recently been isolated in insect systems: the vitellogenin receptor gene in *Drosophila melanogaster* and mosquito.[36, 37] The X-ray crystal structure of the LDL receptor binding domain of human apo-lipoprotein E has been elucidated and from this a structural basis for binding of the apolipoprotein to the LDL receptor has been proposed.[38] The amino acids that are important for receptor binding were determined to be several basic amino acids that are clustered along one patch of an amphipathic helix.[38]

We examined the electrostatic potential surface of JHE for a similar motif and indeed, found some similarity on the face opposite that of the active site channel as previously discussed. Figure 6B, 6C shows the electrostatic potential map of both faces of JHE using GRASP.[24] Charges were calculated using both the in-built full charge option and the AMBER charge set. No significant differences in the calculated accessible surfaces were observed with either the full charge option or with AMBER. The outstanding feature of this map consists of a concentrated region of positively charged amino acids that are part of an amphipathic helix similar to what is found in apolipoprotein E. This helix is highly conserved in acetylcholinesterase and lipase (50% identical to JHE) but does not appear to be amphipathic in either case. In contrast, JHE has 3 Args (Arg 174, 181, 185) in this helix such that at each of three turns in the helix, an Arg faces the surface (Fig. 7). These Args are not conserved in either acetylcholinesterase and lipase (Fig. 2).

CONCLUSIONS

In this communication we have shown that despite a relatively low sequence homology, one can confidently build a homology model of α/β hydrolase fold enzymes due to their relatively good structural homology. Our results together with the previous experimental data lend insight into the structure/function relationships of JHE, making it valuable as a tool to predict physical properties of the protein such as its degradation and stability.

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