Structural insights into the putative bacterial acetylcholinesterase ChoE and its substrate inhibition mechanism

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**Mammalian acetylcholinesterase (AChE) is well-studied, being important in both cholinergic brain synapses and the peripheral nervous systems and also a key drug target for many diseases. In contrast, little is known about the structures and molecular mechanism of prokaryotic acetylcholinesterases. We report here the structural and biochemical characterization of ChoE, a putative bacterial acetylcholinesterase from *Pseudomonas aeruginosa*. Analysis of WT and mutant strains indicated that ChoE is indispensable for *P. aeruginosa* growth with acetylcholine as the sole carbon and nitrogen source. The crystal structure of ChoE at 1.35 Å resolution revealed that this enzyme adopts a typical fold of the SGNH hydrolase family. Although ChoE and eukaryotic AChEs catalyze the same reaction, their overall structures bear no similarities constituting an interesting example of convergent evolution. Among Ser-38, Asp-285, and His-288 of the catalytic triad residues, only Asp-285 was not essential for ChoE activity. Combined with kinetic analyses of WT and mutant proteins, multiple crystal structures of ChoE complexed with substrates, products, or reaction intermediate revealed the structural determinants for substrate recognition, snapshots of the various catalytic steps, and the molecular basis of substrate inhibition at high substrate concentrations. Our results indicate that substrate inhibition in ChoE is due to acetate release being blocked by the binding of a substrate molecule in a nonproductive mode. Because of the distinct overall folds and significant differences of the active site between ChoE and eukaryotic AChEs, these structures will serve as a prototype for other prokaryotic acetylcholinesterases.**

In mammals, acetylcholinesterase (AChE) plays a pivotal role in cholinergic brain synapses and neuromuscular junctions through the termination of impulse transmission via rapid hydrolysis of acetylcholine (ACh), an important cationic neurotransmitter in the nervous system (1). The rapid hydrolysis of ACh into acetic acid and choline by AChE is achieved at a rate close to that of a diffusion-controlled reaction and, therefore, AChE is recognized as one of the most efficient enzymes in nature (2). Having a catalytic triad consisting of Ser, His, and Glu residues, AChE follows the classical esterase mechanism (3) and two half-reactions are required to complete the full catalytic cycle.

In recent decades, the structure and function study of the AChEs from metazoans has been particularly important in the context of the development of inhibitors to block the activity of AChE for various purposes, e.g. the treatment of Alzheimer’s disease (4). The first crystal structure of an acetylcholinesterase from *Torpedo californica* (TcAChE) was published nearly 30 years ago (5). This was followed by the determination of ~250 structures of AChEs of eukaryotic sources, many of which are in complex with various inhibitors (6). Despite the long history of research on structure and inhibition of eukaryotic AChEs (7, 8), novel insights have been gained into these pivotal enzymes in recent years, e.g. the dynamics of back door opening (9), the steric and dynamic parameters implicated in the reaction within the active-site gorge (10), and the possible repurposing of AChE inhibitors as anti-cancer agents (11).

ACh hydrolyzing activity has also been recognized in many prokaryotic species from various bacterial classes (12) although they lack a nervous system. This was especially the case for the bacteria from the *Pseudomonas* genus, which are able to inhabit a large range of environments with many species such as *Pseudomonas aeruginosa* being pathogens for many different hosts (13). Acetylcholinesterase activity has been observed in *Pseudomonas fluorescens* after growth with ACh as the sole source of carbon (14, 15). The putative acetylcholinesterase from *P. aeruginosa* strain PAO1, named ChoE, has also been characterized (12, 16). Despite its small size (287 residues) relative to that of eukaryotic AChEs (~560 residues), ChoE is able to catalyze the hydrolysis of both acetylthiocholine (ATCh) and its analog propionylthiocholine (PTCh). The purified ChoE enzyme of *P. aeruginosa* strain PAO1, named ChoE, has also been characterized (12, 16). Despite its small size (287 residues) relative to that of eukaryotic AChEs (~560 residues), ChoE is able to catalyze the hydrolysis of both acetylthiocholine (ATCh) and its analog propionylthiocholine (PTCh). The purified ChoE enzyme of *P. aeruginosa* is inhibited by substrate at high substrate concentrations, exhibiting enzymatic properties similar to eukaryotic AChEs (12). In view of the enzymatic activity of ChoE as well as the abundance of ACh present in the corneal epithelium (17), it was proposed that ChoE could be a virulence factor involved in...
corneal infection by *P. aeruginosa* (18). Production of choline by ChoE for subsequent catabolic processes by other metabolic pathways was also suggested to be linked to the pathogenicity of *P. aeruginosa* and its survival during murine lung infection (19, 20).

In contrast to the eukaryotic AChEs, little is known about the structure and molecular mechanism of prokaryotic acetylcholinesterases. Due to the low sequence identity (<26%) between ChoE and the proteins whose structures are available in the Protein Data Bank (PDB), it remains elusive how this enzyme works at the molecular level. In this study, we have performed structural and biochemical characterization of ChoE. Multiple structures were determined at a resolution of 1.35-1.85 Å in the presence of substrates, products, or reaction intermediate using both WT and the catalytic triad mutants. These results have uncovered the first atomic details of a putative bacterial acetylcholinesterase and provided insights into the mechanism of substrate inhibition.

Results

**ChoE is required for *P. aeruginosa* to utilize ACh as the sole source of carbon and nitrogen**

Choline can serve as the sole source of carbon and nitrogen to support *P. aeruginosa* growth and can be acquired through an acetylcholinesterase activity of ChoE, encoded by the *choE* (*PA4921*) gene (12). Growth of the WT PAO1 strain and the deletion mutant strain PAO1::ΔPA4921 (PW9287) was assessed in a microplate reader at 37 °C and both strains were unable to grow in salt solution containing 0.66% KH$_2$PO$_4$, 0.3% K$_2$HPO$_4$, and 3% MgSO$_4$ unless choline was added as a source of carbon and nitrogen. The WT strain was able to use ACh as a substrate, but the mutant strain could not (Table 1). These results confirmed that ChoE is required for the bacteria to grow when ACh is the sole source of carbon and nitrogen.

**Characterization of WT and mutant ChoE**

Using both ATCh and PTCh substrates, we studied the enzyme kinetics of WT ChoE and mutants of the catalytic triad and substrate-binding residues. Our results showed that WT ChoE has similar activity on ATCh and PTCh (Table 2 and Fig. 1A). Based on the apparent catalytic efficiency (*k*_cat/,*K*_m) of ChoE exhibits slightly higher specificity on PTCh, consistent with the previous report (12). Using an elevated concentration (30 nM) of ChoE, we also detected its activity toward butyrylthiocholine (BTCh) (Table 2 and Fig. 1A) in contrast to the previous report in which a lower concentration of enzyme was used (12). In our assays, substrate inhibition was observed at high concentrations of the substrate with a *K*_s(app) of 0.9 mM for ATCh. Substrate inhibition was modeled upon the mechanism first described by Krupka (21) that considered substrate inhibition of AChE as arising from trapping an EIS (acyl–enzyme intermediate substrate complex) at high substrate concentrations. Such EIS complex of ChoE would be kinetically indistinguishable from an EPS complex (enzyme-product–substrate ternary complex) and is thus fully consistent with the EPS observed in the crystal structures of ChoE (see below). Although the kinetic data could be equally well fitted with a model describing inhibition as arising from the formation of an enzyme-substrate-substrate complex (ESS, Table 2), as assumed by Sánchez et al. (12), an ESS inhibitory complex of ChoE appears unlikely given the lack of evidence of a second substrate-binding site by crystallography.

The enzymatic assays carried out with the catalytic triad mutants (S38A, D285N, and H288N) showed that the catalytic activity was abolished in both S38A and H288N mutants, whereas the D285N mutation diminished the enzymatic activity severely, indicating that both Ser-38 and His-288 are indispensable for ChoE to fulfill its hydrolyase function (Table 2 and Fig. 1B). Notably, replacement of Asp-285 by an asparagine has led to a 40-fold reduction of *k*_cat(app) together with an increase of *K*_m(app) by 16-fold, reflecting a 640-fold drop of catalytic efficiency when ATCh is the substrate. The large impact on *k*_cat(app) upon mutation indicates that Asp-285 is, albeit not essential, an important component of catalytic triad in ChoE. The kinetic analysis of the mutants of substrate-binding residues (Y106A and W287A) (Table 2 and Fig. S1) using ATCh and PTCh as substrates indicated that the Y106A mutation has led to less than ~2-fold drop of *k*_cat(app) but to a 14-20-fold increase of *K*_m(app), whereas the W287A mutation has resulted in a slight increase in *k*_cat(app) but a dramatic increase in *K*_m(app). The results obtained from two technical replicates of two biological replicates.

### Table 1

*P. aeruginosa* growth in salt solution supplemented or not with choline or acetylcholine

| Salt solution | Salt solution with 20 mM choline | Salt solution with 20 mM acetylcholine |
|---------------|----------------------------------|--------------------------------------|
| WT strain (PAO1) | −−                             | −                                   |
| Mutant strain (PAO1::ΔPA4921) | ++                             | +                                   |
| ++                             | −                                 | −                                   |

*Growth is shown by a plus (+) or a double plus (++) according to the extent of growth, and no growth by a minus (−). The results were obtained from two technical replicates of two biological replicates.*

### Table 2

Kinetic data of WT ChoE and mutants

| Enzyme Substrate | *k*_cat(app) ($s^{-1}$) | *K*_m(app) (μM) | *K*_s(app) (μM) | *k*_cat/*K*_m (M$^{-1}s^{-1}$) |
|------------------|-------------------------|-----------------|----------------|--------------------------------|
| WT ATCh          | 200 ± 25                | 0.1 ± 0.02      | 0.9 ± 0.2      | 2.0 × 10$^6$                  |
| S38A ATCh        | −                       | −               | −              | −                              |
| H288N ATCh       | −                       | −               | −              | −                              |
| D285N ATCh       | 5.0 ± 0.3               | 1.2 ± 0.3       | −              | 3.1 × 10$^3$                  |
| Y106A ATCh       | 99 ± 4                  | 2.2 ± 0.5       | −              | 4.6 × 10$^3$                  |
| W287A ATCh       | 214 ± 9                | 1.6 ± 0.18      | −              | 1.3 × 10$^3$                  |
| WT PTCh          | 316 ± 43                | 0.07 ± 0.02     | 1.0 ± 0.3      | 4.5 × 10$^5$                  |
| S38A PTCh        | −                       | −               | −              | −                              |
| H288N PTCh       | −                       | −               | −              | −                              |
| D285N PTCh       | 6.2 ± 0.2               | 0.28 ± 0.05     | −              | 2.2 × 10$^4$                  |
| Y106A PTCh       | 179 ± 5                | 1.37 ± 0.11     | −              | 1.3 × 10$^4$                  |
| W287A PTCh       | 190 ± 5                | 1.60 ± 0.13     | −              | 1.2 × 10$^4$                  |
| WT BTCh          | 6.2 ± 0.2               | 0.03 ± 0.04     | 0.5 ± 0.3      | 1.0 × 10$^5$                  |
| WT pNPB          | 83 ± 13                 | 0.8 ± 0.3       | −              | 1.0 × 10$^5$                  |
| WT pNPB          | 439 ± 45               | 0.35 ± 0.10     | −              | 1.2 × 10$^4$                  |
| WT pNPB          | 5.8 ± 0.3               | 0.12 ± 0.01     | −              | 4.8 × 10$^4$                  |
| WT pNO           | ATCh + PI               | 10.2 ± 0.7      | 0.1 ± 0.02     | 11.9 ± 4.3                    |
| WT pNO           | ATCh + TEAC            | 57.6 ± 1.8      | 0.06 ± 0.01    | 175 ± 104                     |

*Fit to Michaelis–Menten equation (Equation 3).*
in a small or no change of $k_{\text{cat(app)}}$ accompanied by a 16-fold increase of $K_{m(app)}$. Intriguingly, substrate inhibition is abolished for all three mutants D285N, Y106A, and W287A.

The kinetic analysis of ChoE with the substrates 4-nitrophenyl acetate ($p$NPA), 4-nitrophenyl propionate ($p$NPP), 4-nitrophenyl butyrate ($p$NPB), and 4-nitrophenyl octanoate ($p$NPO) demonstrated that ChoE exhibits a lower specificity on these $p$-nitrophenyl esters as compared with the ATCh and PTCh substrates (Table 2 and Fig. 1C). Importantly, the esters bearing extra C-C bonds beyond the propionyl group, such as $p$NPB and $p$NPO, are either not substrates or very poor substrates, consistent with our molecular docking in which the esters with a long tail could not be accommodated in the active site pocket with a proper geometry required for catalysis (Fig. S2). Taken together with the data on ATCh, PTCh, and BTCh, these results suggest that the substrates of ChoE with high specificity are limited to the esters having an acetyl or a propionyl group. Notably, no substrate inhibition was observed with two of the $p$-nitrophenyl esters ($p$NPA and $p$NPP). This may well be the case of the third substrate ($p$NPB) but because it was not soluble at more than 1 mM in our assay condition, the absence of substrate inhibition could not be verified with this substrate.

Prompted by the docking results (Fig. S2), we have also investigated the inhibitory effects of tetraethylammonium chloride (TEAC) and propidium iodide (Pl), the latter of which is an inhibitor that can bind to either the peripheral anionic site (PAS) of mouse AChE (22) or the A-site of human butyrylcholinesterase (23). These two molecules contain a quaternary ammonium group, similar to ATCh and PTCh. Kinetic analysis has confirmed that the presence of either molecule at 1 mM could indeed inhibit the activity of ChoE leading to a 3.5-20-fold decrease of $k_{\text{cat(app)}}$ when ATCh was used as the substrate, whereas essentially not affecting $K_{m(app)}$. Interestingly, the $K_{SI(app)}$ values for substrate inhibition by ATCh were much higher (13-194–fold) in the presence of TEAC or PI (Table 2 and Fig. 1D).

**ChoE adopts a typical fold of the SGNH hydrolase family**

The DNA sequence encoding the signal peptide (residues 1-20) at the N-terminal was not included in our construct. All the residues (21-307) in the mature protein are built into the model because of the excellent quality of electron density maps. Having five $\beta$-strands with an order of $\beta2-\beta1-\beta3-\beta4-\beta5$ and 10 $\alpha$-helices, ChoE has the characteristic $\alpha/\beta/\alpha$-globular fold of the SGNH hydrolase family (24, 25) (Fig. 2A). The most similar structures of ChoE, obtained through the DALI search (26), include the esterase EstJ15 (PDB 5XTU) (27), the phospholipase A2 VvPlpA (PDB 6JKZ) (28) (Fig. 2B), and the esterase...
EstA (29). However, these enzymes share a relatively low sequence identity of 23–26% with ChoE. Notably, EstJ15 has the same Ser-His-Asp catalytic triad as that in ChoE, whereas VvPlpA lacks the Asp residue in the catalytic triad of ChoE and EstJ15 and instead utilizes an unusual Ser-His-chloride catalytic triad to fulfill the catalysis (28). Similar to EstJ15 and VvPlpA, ChoE is monomeric based on size exclusion chromatography and the crystal structures. It is worth mentioning that, for the 10 most similar proteins retrieved from the DALI search, none contained a substrate or a product molecule in the active site. Quite remarkably, despite the same reaction being catalyzed by ChoE and eukaryotic AChEs, their overall structures bear no similarities.

**The native ChoE structure**

All the structures reported here were obtained from the crystallization conditions containing 100 mM MES buffer. The native ChoE structure determined at a resolution of 1.35 Å shows unambiguously the presence of a MES molecule in the substrate-binding pocket located at the center of ChoE (Fig. 3A). Calculation of electrostatic potential indicated that this pocket is negatively charged (Fig. 3B), a structural feature facilitating the entrapment of the positively charged quaternary ammonium moiety of the ACh substrate.

As shown in Fig. 3C, the MES molecule is bound to the substrate-binding pocket lined by the aromatic residues Trp-73, Tyr-106, Tyr-107, Phe-150, and Trp-287. At the bottom of the pocket are the catalytic residues Ser-38 and His-288 as well as the oxyanion hole composed of Gly-98 and Asn-147. Asp-285, the third residue forming the catalytic triad, is next to His-288, such that His-288 is within the hydrogen bonding distance (2.6-2.9 Å) of both Asp-285 and Ser-38, the pattern of which is similar to that of eukaryotic AChEs (5) and resembles that of serine proteases as well (30, 31). In this structure, the ethanesulfonic moiety of MES is exposed to the solvent and interacts with Tyr-107 and Asp-154 via water-mediated hydrogen bonds, whereas its morpholine ring is buried inside the pocket with its oxygen atom close to a well-defined water molecule (Wat-459) (3.2 Å) at the bottom of the pocket. Located at the center of the oxyanion hole, this water molecule is stabilized by the hydrogen bonds with the main chain amide groups of Ser-38 and Gly-98, the hydroxyl group of Ser-38 as well as the side chain amide group of Asn-147.

Both Asp-285 and His-288 are located at a U-turn loop where Asp-285 forms hydrogen bonds with the hydroxyl of Tyr-68 and the main chain amides of Glu-286, Trp-287, and His-288 at this loop. These interactions contribute significantly to the stabilization of the local structure as evidenced by the fact that the D285A mutant is insoluble, presumably due to the perturbation or disruption of the overall folding of the protein. Similarly, the imidazole ring of His-288 is sandwiched between Trp-73 and Trp-287 and also establishes van der Waals contacts with Tyr-39, suggesting a structural role of the His-288 side chain. To minimize the detrimental effects on the local structure and increase our chance to obtain structures of relevant protein-ligand complexes as exemplified in our previous work (32, 33), we mutated both Asp-285 and His-288 to an asparagine. Indeed, both D285N and H288N mutants showed similar behavior to the WT protein.
To obtain the ChoE complex bound with ATCh, we tried both co-crystallization and soaking methods using the crystals of WT enzyme and all three mutants, S38A, D285N, and H288N. Among these, soaking the H288N mutant crystals with 100 mM ATCh for 5 min gave the best result. In this H288N-ATCh complex, the ATCh substrate is clearly bound to the substrate-binding pocket (Fig. 4A). The overall structure is almost the same as the native one, with an r.m.s.d. value of 0.38 Å for all the Ca atoms. In the substrate-binding pocket, a few aromatic residues, such as Tyr-107 and Trp-287, undergo a minor shift or tilting in response to the substrate-binding event. Notably, contrary to the MES molecule captured in the native structure, the ATCh substrate molecule binds deeper and reaches the bottom of the pocket. As a result, the water molecule Wat-459 observed in the MES complex is expelled and, expectedly, this position is instead filled by the carbonyl oxygen of ATCh. The quaternary ammonium moiety of ATCh makes π-cation interactions with Tyr-106 and Trp-287, with the distances between the two closest carbon atoms being 3.5 and 3.9 Å, respectively. This part is further stabilized by van der Waals contacts with Tyr-107 and Phe-150. The carbonyl oxygen is anchored by multiple hydrogen bonds with Ser-38, Gly-98, and Asn-147, which form the oxyanion hole. The methyl group points to the acyl-binding pocket and establishes van der Waals contacts with Tyr-39, Ala-146, and Phe-150. The carbonyl carbon is only 2.4 Å away from the OG1 atom of Ser-38, which is consistent with the role of Ser-38 as a nucleophilic attacking group once it is deprotonated by His-288 of the catalytic triad. In this mutant structure, Asn-288 is still within the hydrogen bonding distance of both Asp-285 and Ser-288, which is consistent with the role of Ser-38 as a nucleophilic attacking group once it is deprotonated by His-288 of the catalytic triad. In this mutant structure, Asn-288 is still within the hydrogen bonding distance of both Asp-285 and Ser-288, which is consistent with the role of Ser-38 as a nucleophilic attacking group once it is deprotonated by His-288 of the catalytic triad.

Structure of the ChoE-PTCh complex

After many trials, the ChoE-PTCh complex was obtained via a quick soaking (2 min) of the WT ChoE crystals with 50 mM PTCh. In the two ChoE molecules in the asymmetric unit, the PTCh substrate is found only in one molecule (Fig. 4B) as the other one likely contains products (propionate and thiocholine).
Structure of putative bacterial acetylcholinesterase ChoE

A
W287
D285
F150
N288
Y39
N147
Y107
S88

B
W287
D285
F150
A146
N147
Y107
S88

C
W287
F150
N285
TCh
Y106
H288

D
W287
F150
N285
PTCh
Y106
H288

(acyl-enzyme)

E
W287
F150
D285
N147
Y107
H288
S88

F
W287
F150
N285
N147
Y107
H288
S88

G
W287
F150
D285
N147
Y107
H288
W73
A38

H
W287
F150
D285
N147
Y107
H288
W73
A38
Structure of putative bacterial acetylcholinesterase ChoE

(TCh), similar to acetate (ACT) and TCh described below. As expected, the overall structure is almost the same as that of the H288N-ATCh complex. Because of the presence of one additional C-C bond and the intact His-288 residue in the binding pocket, PTCh displays some differences in its configuration as compared with the ATCh molecule in the H288N-ATCh structure, whereas the quaternary nitrogen and carbonyl oxygen of both PTCh and ATCh are kept at almost identical positions to maintain the π-cation interactions and hydrogen bonds described above for ATCh. This observation emphasizes the critical importance of the choline head and carbonyl moiety in ChoE-substrate recognition. The additional C-C bond in PTCh relative to ATCh is surrounded by Tyr-39, Ala-146, Phe-150, and Trp-287 with a short distance of 3.4 Å from Phe-150. The potential steric hindrance imposed by this hydrophobic acyl-binding pocket rationalizes the poor activity of ChoE on BTCh (one more carbon than PTCh) (Table 2).

A remarkable feature of the ChoE-PTCh complex is that the side chain of Ser-38 adopts two conformations with one hydrogen bonding to His-288, whereas the other one rotates away from His-288 and points toward Gly-97. Given the spatial arrangement of both conformations, only the first one would be compatible with deprotonation by His-288 in preparation for catalysis. Comparison of substrate recognition between ChoE and eukaryotic AChEs

With structures of both ATCh- and PTCh-bound ChoE available, we could better visualize the similarity and difference between ChoE and eukaryotic AChEs. Consistent with its small size of 287 residues, ChoE possesses a relatively shallow tunnel to anchor the ligands, e.g. the ethanesulfonic oxygen of the MES molecule located on the protein surface is ~11 Å away from the OG atom of Ser-38, as compared with TaChE (537 residues) whose active site is buried at the bottom of a narrow gorge of ~20 Å deep. As a result, the substrate molecule maintains its direction upon entry into the tunnel/pocket until it reaches the catalytic site in ChoE (Fig. 5A). This is in sharp contrast to TaChE in which the long axis of substrate at the catalytic anionic site (CAS) is perpendicular to the entry path (Fig. 5B). The functionally important peripheral anionic site (PAS) in eukaryotic AChEs (34, 35) is thus absent in ChoE, as the quaternary ammonium group of both ATCh and PTCh molecules bound in the substrate-binding pocket of ChoE is not far from the protein surface (Fig. 5A).

Another drastic difference between ChoE and eukaryotic AChEs lies in the spatial arrangement of the catalytic triad residues. In ChoE, the side chains of Asp-285, His-288, and Ser-38 are almost linearly arranged, whereas those of Glu-327, His-440, and Ser-200 in TaChE, albeit having a planar array, form a triangle (Fig. 5, C and D). This is due to the difference of overall folding and the distinct structural elements harboring the catalytic residues in these two enzymes. Importantly, Ser-200 in TaChE is located at the sharp turn of a strand-turn-helix motif, the so-called nucleophile elbow, commonly seen in the α/β-hydrolase-fold enzymes (36, 37). In contrast, such structural feature is absent in ChoE and other members of the SGNH hydrolase family (25). Moreover, the residues forming the oxyanion hole also differ in these enzymes: TaChE utilizes the backbone amide groups of three residues (Gly-118, Gly-119, and Ala-201), whereas the oxyanion hole of ChoE is formed by the backbone amides of both Gly-98 and the catalytic residue Ser-38 as well as the side chain amide of Asn-147 (Fig. 4, A and B).

Despite these differences, a striking resemblance between ChoE and eukaryotic AChEs was observed concerning the strategy employed by these enzymes to recognize the substrate: in both enzymes, the substrate-binding pocket is delineated by a series of aromatic residues, two of which (Tyr-106 and Trp-287 in ChoE or Trp-84 and Phe-330 in TaChE) play a pivotal role in stabilizing the quaternary ammonium group of substrates, i.e. the choline head, via π-cation interactions (Fig. 5, C and D). The significant increase of $k_{\text{app}}$ of both Y106A and W287A mutants (Table 2) have unequivocally confirmed the importance of both Tyr-106 and Trp-287 in anchoring the substrates. In addition to these two aromatic residues, the acyl-binding pocket of both enzymes is formed by several hydrophobic residues conferring substrate specificity.

Trapping of the reaction intermediate in the D285N mutant structure

Inspired by the kinetic results that the D285N mutant exhibits significantly lower activity, we crystallized the D285N mutant followed by soaking with either ATCh or PTCh molecule with the aim to capture a reaction intermediate. Indeed, a short soaking (2 min) of crystals in the presence of 50 mM PTCh has led to the visualization of an acyl-enzyme intermediate. In both ChoE molecules in the asymmetric unit, strong electron density continuously extends beyond the OG atom of Ser-38 and is best modeled as a covalently bound propionyl group at 100% occupancy (Fig. 4C). Comparison with the PTCh-bound ChoE structure indicated that the binding pocket does not have major changes except that the plane containing the propionyl moiety rotates ~60° from its reactant position to bring the carbonyl carbon atom into covalent bonding distance of the OG atom of Ser-38 (Fig. 4D). This way, the carbonyl oxygen maintains...
all H-bonds with the residues forming the oxyanion hole. Although Asn-285 adopts a conformation similar to that of Asp-285, its H-bonding interactions with His-288 are now weaker, as evidenced by the change of distance from 2.9 to 3.2 Å (or 3.5 Å in molecule B) due to a slight shift of its side chain. Located above the reaction intermediate is a TCh molecule whose quaternary ammonium group is clearly visible but the thiol moiety is weaker in the electron density maps, probably due to the existence of two or more conformations and/or lack of stabilization interactions from the binding pocket (Fig. 4C). Nevertheless, its quaternary ammonium group occupies almost the same position as that of PTCh and is also stabilized by the π-cation interactions with Tyr-106 and Trp-287. Notably, a round electron density is found above the covalently-linked intermediate and is best modelled as one water molecule, which is within the H-bonding distances of both His-288 (3.2 Å) and the carbonyl oxygen (3.0 Å) and is 3.2 Å away from the carbonyl carbon of reaction intermediate. Due to the replacement of Asp-285 by Asn, the pKa of His-288 is likely decreased and thus the deprotonation of this water by His-288 would be less efficient, retarding the deacylation step. Particularly in the current structure, and in contrast to the native enzyme, the NE2 atom of His-288 is presumably protonated given that it is merely 2.9 Å away from the OG atom of the acyl intermediate. This most likely scenario of His-288 being protonated would further impede the deacylation step leading to the entrapment of reaction intermediate in this mutant structure.

In the second molecule of ChoE acyl-enzyme in the asymmetric unit, the corresponding water molecule has weaker electron density and is 3.4 Å away from His-288.

**Product binding mode of ChoE**

As mentioned above, product molecules, TCh and propionic acid, were observed in one of the two ChoE molecules in the asymmetric unit upon soaking WT crystals with 50 mM PTCh. Similarly, through soaking crystals with ATCh, we have also obtained the structures of WT (Fig. 4E) and D285N mutant (Fig. 4F) complexed with acetate and TCh, the products of ATCh hydrolysis. Comparison of the immediate environments of Asp-285 and Asn-285 revealed that the imidazole ring of His-288 slightly tilted upon the D285N mutation. In all these structures, the quaternary ammonium group of TCh is found to be placed at a position similar to the ones described above. In addition to the π-cation interactions with Tyr-106 and Trp-287, the positively charged moiety of TCh is further stabilized by the acetate molecule through electrostatic interactions with a distance of 3.8-4.1 Å between its quaternary nitrogen and the oxygen atom of the latter. This moiety could be easily identified due to its pyramid shape (Fig. 4F and Fig. S3) but the thiol moiety is more mobile possibly due to its exposure to the solvent (protein surface) and the lack of stabilizing interactions with nearby protein residues. Thus, the position of the thiol moiety of TCh modeled in these product complexes is less reliable. In
contrast, the acetate molecule could be unambiguously located largely because of the stabilizing effects rendered by five hydrogen bonds with Ser-38 (both main chain amide and side chain), Gly-98, Asn-147, and His-288. A prominent feature of all these acetate-bound structures is that Ser-38 adopts the alternate conformation, i.e. the one rotating away (3.7 Å) from His-288. Most probably, this “resting” conformational state of Ser-38 is driven by its better geometry to form hydrogen bonding interactions with the acetate molecule trapped at the oxyanion hole.

Structure of the ChoE S38A-acetate-ATCh complex

Considering the fact that product molecules were routinely observed in the structure when WT proteins were used for crystallization or soaking experiments with the ATCh substrate, we resorted to the mutant proteins to gain insights into the structural basis of substrate inhibition. This approach has resulted in the structure of the S38A mutant bound with both acetate and ATCh when the mutant crystal was soaked 3 min with 100 mM ATCh. Both acetate and the substrate ATCh molecules were clearly visible in the electron density maps (Fig. 4, G and H). Because S38A has no detectable activity, the acetate molecule most likely came from the autohydrolysis of ATCh, in the stock solution before the soaking experiment or during the soaking step. No noticeable differences were observed in the immediate environment of Ala-38 when compared with the WT structures. Of note, despite the absence of hydroxyl group at Ala-38, the binding mode of acetate is almost identical to the one in the product complex of WT ChoE.

Because of the presence of an acetate molecule at the bottom of the pocket, the ATCh substrate binds differently from the one observed for the productive binding mode. This new non-productive position of ATCh causes a slight shift or tilting of both Tyr-106 and Trp-287. As a result, the π-cation interactions between its quaternary ammonium moiety and these two aromatic residues are well maintained. This positively charged moiety also establishes the electrostatic interactions with the acetate buried beneath. Moreover, the parallel packing of the acetyl thiol moiety against the Trp-287 indole ring further stabilizes the ATCh molecule in this new binding mode. Additional van der Waals contacts come from Trp-73, Tyr-107 and Phe-150. This new positioning of ATCh exposes its sulfur and carbonyl carbon atoms to the solvent.

Discussion

We report here the structural and biochemical characterization of ChoE, a putative bacterial acetylcholinesterase from P. aeruginosa. Our enzyme kinetic analysis and structural data indicated that ChoE mainly catalyzes the hydrolysis of esters bearing an acetyl or a propionyl group. Although future investigation is required to unambiguously define the bona fide or physiological substrate of ChoE being acetylcholine, our results showed that ChoE is indispensable for P. aeruginosa to grow when acetylcholine is provided as the sole carbon and nitrogen source. On the other hand, we cannot exclude the possibility that other known bacterial esterase(s) may have the acetylcholinesterase activity. Nevertheless, to the best of our knowledge, the structural features governing the binding of choline head (quaternary ammonium group) have not been previously documented in the literature on bacterial esterases and therefore the structures presented here are the first of this kind. Using both WT and mutant proteins, we have successfully determined a comprehensive set of ChoE structures in complex with substrates, products, or reaction intermediate. In combination with the kinetic analysis of WT and mutant proteins, these results have provided molecular insights into the active site architecture, catalytic mechanism, and structural basis of substrate inhibition in bacterial acetylcholinesterases.

As described above, ChoE and eukaryotic AChEs differ in many aspects, including their overall structure, active site architecture, spatial arrangement of catalytic triad as well as the composition of oxyanion hole, the last two of which are largely because ChoE and eukaryotic AChEs do not bear any similarity in their overall structures. Our kinetic studies of ChoE indicated that the kinetic turnover number (Kcat) and maximum activity (Vmax) of ChoE (5 x 10^-7 M) is lower than that (10^4 s^-1) of eukaryotic AChEs (38, 39). Overall, the apparent Kcat/Km values (2 x 10^5 versus 2 x 10^6 M^-1 s^-1) is about 2 orders of magnitude lower in ChoE, which may not be surprising as AChEs possess unique structural features and are highly efficient enzymes that approach the upper limit allowed by diffusion of the substrate (1, 2). It should be noted that a similar kinetic analysis of ChoE conducted previously at 37 °C revealed a higher Kcat/Km value of ~1 x 10^5 M^-1 s^-1 (12). The comparable Kcat/Km values between ChoE and AChEs may be explained by the fact that the structural determinants for substrate recognition in both ChoE and AChEs share significant similarity with the π-cation interactions being pivotal on anchoring the quaternary ammonium group of the substrate. This comes as no surprise from an evolutionary point of view. Search through the Protein Data Bank yielded a series of proteins that bind acetylcholine or choline, e.g. acetylcholine-binding protein AchBP (PDB 3WIP) (40), cell wall endolysin (PDB 1OBA) (41), and choline-binding protein OpuBC (PDB 3R6U) (42). Inspection of these structures revealed the widespread presence of π-cation interactions between the quaternary ammonium of choline head and its surrounding aromatic residues in these proteins despite their different foldings.

Different from human AChE (43) and some hydrolyses of the SGNH family, such as AlgX (44) and AXE2 (45) in which mutation of any catalytic triad residues abolishes detectable activity, Asp-285 in the catalytic triad of ChoE is important but not essential for catalysis. Indeed, for some other enzymes in the same SGNH family, replacement of catalytic residues by alanine does not necessarily lead to inactive enzymes, as evidenced by the cases of EstD1 (46), AlgI (47), and TAP (48). The similar arrangement of the catalytic triad in ChoE and other hydrolyses in the SGNH family indicates the conserved role of these catalytic residues as illustrated in Fig. 6. Ser-38, once deprotonated by His-288, functions as a nucleophile to attack the carbonyl carbon of ACh. His-288 is critical for both acylation and decylation steps as it deprotonates Ser-38 in the first half-reaction and one water molecule in the second half-reaction. The residues forming the oxyanion hole are critical to stabilize the negative charge built up on the tetrahedral intermediates. Asp-285...
hydrogen bonds to the imidazole ring of His-288 and significantly increases the $pK_a$ of His-288 facilitating the role of the latter. A 40-fold $k_{cat(app)}$ reduction for the D285N mutant is consistent with its role in initiating the catalysis. We have also observed a 16-fold increase of $K_m(app)$ for this mutation with ATCh and to a lesser extent with PTCh. Although Asp-285 is not directly involved in substrate binding, the D285N mutation makes His-288 less electronegative and consequently favors the protonation state of Ser-38. A shift to the protonated Ser-38 will be less desirable to anchor the partially positively-charged carbonyl carbon thus decreasing the binding. Moreover, Ser-38 was observed to adopt two conformations: the “productive” conformation, positioned within the H-bonding distance of His-288, is prevalent when the substrate molecule is bound while the alternate “unproductive” conformer dominates in the presence of the acetate product as shown in the crystal structures of both WT and the D285N mutant. Therefore, the increase of $K_m(app)$ might also be due to the shift of the equilibrium of these two conformers of Ser-38 upon mutation of Asp-285 leading toward a preference for the alternate conformer and thus decreasing the binding affinity for substrate.

Concerning the distinct rotamers adopted by Ser-38, it is worth mentioning that the alternate conformations of the serine residue in the catalytic triad are not rare, as evidenced by several other esterases or lipases including the acetylxylan esterase (49), the Bacillus subtilis lipase (50), the acetyl esterase
**Structure of putative bacterial acetylcholinesterase ChoE**

HerE (51), and the feruloyl esterase (52). Such conformational flexibility of serine in the esterases may be more common than observed and could play an important role in fine-tuning the spatial reorganization of the catalytic triad in concert with the various steps of catalysis.

It is important to stress that the catalytic mechanism and the role of each catalytic triad residue in ChoE resemble those of eukaryotic AChEs (43, 53). In this regard, structurally distinct ChoE and AChEs constitute an intriguing example of convergent evolution by which these two enzymes have evolved to catalyze the same hydrolysis of acetylcholine. Indeed, previous systematic investigation has identified many such nonhomologous isofunctional enzymes, exemplified by the well-studied superoxide dismutases and glycosyl hydrolases (54–56).

In particular, for the glycosyl hydrolases, enzymatic hydrolysis of the glycosidic bond is governed by the general acid catalysis and requires an Asp–Glu dyad of the same geometric arrangement although these enzymes have numerous different folds (56). The current study adds one more case to the growing list of hydrolases, which were more frequently present among nonhomologous isofunctional enzymes likely because one of their substrates is the ubiquitous water molecule and the catalysis typically requires no coenzyme (54).

An interesting observation in our study is that we have captured the acyl-enzyme intermediate of the D285N mutant at nearly neutral pH (pH 6.5) when PTCh was used in crystal soaking. Although this mutant is still active, its much lower efficiency provided us the time window to visualize this snapshot of catalysis. As previously pointed out, either the formation of acyl-enzyme or the deacylation step could be rate-limiting for canonical esterases (3).

Trapping of acyl-enzyme in the D285N mutant indicates that deacylation is more likely the rate-limiting step for the WT enzyme, which was more frequently present among nonhomologous isofunctional enzymes likely because one of their substrates is the ubiquitous water molecule and the catalysis typically requires no coenzyme (54).

Further support for the mechanism proposed above is lent by the fact that substrate inhibition is also abolished in the D285N mutant. We should bear in mind that the *bona fide* leaving group after deacylation is acetic acid (CH$_3$COOH), not acetate (CH$_3$COO$^-$) (Fig. 6). In the WT enzyme, this hydrolysis product could be easily deprotonated upon release in its immediate environment by His-288 as the pK$_a$ of the latter is increased in the presence of Asp-285. Notably, before the arrival of a new substrate, Ser-38 may adopt the unproductive alternate conformation as was observed, *i.e.* its hydroxyl being distant from His-288. During this short-time window, His-288 would be fully engaged for the deprotonation of acetic acid. Both events would strengthen the affinity for acetate in the oxyanion hole, thus slowing down its clearance/departure, and also promote the binding of the positively charged substrate in the nonproductive binding mode. With this reasoning, the D285N mutation is simply detrimental to this process, thus leading to the abolition of substrate inhibition. Of note, this substrate inhibition mechanism is partially analogous to that of eukaryotic AChEs. Indeed, a high concentration of substrate in AChEs results in the binding of two nonproductive substrates, one at CAS and the other at PAS, thus preventing the departure of acetate from the active site (34, 35). Also similarly, a negatively charged and conserved glutamate residue in AChEs plays an important role in anchoring one nonproductive substrate in the CAS pocket. Replacement of this glutamate by a smaller aspartate residue in both TcAChE and human AChE abolishes substrate inhibition (57–59), possibly because aspartate could somehow reposition the substrate molecule in the pocket thus allowing both deacylation and clearance of acetate to proceed (34).

Substrate inhibition is commonly observed in enzymes. Far from being an experimental oddity, it was shown to be physiologically important for some enzymes (60). Notably in eukaryotic AChEs, substrate inhibition is important for effective transmission of the neural signal (60). Considering that ChoE is likely involved in function(s) unrelated to neural signal transmission, it seems rather surprising that it is subjected to substrate inhibition with an apparent inhibition constant similar to that of eukaryotic AChE (~1 mM). It is possible that substrate inhibition is a natural consequence for this type of enzyme due to the properties of the catalytic triad and the oxyanion hole that stabilize the acetate product. Otherwise, it is possible that the physiological context, in which *P. aeruginosa* benefits from substrate inhibition of ChoE, remains to be...
uncovered. Nonetheless, these results have established an interesting case of apparent convergent evolution whereby a similar catalytic mechanism and regulation by substrate inhibition are achieved through unrelated protein folds.

Collectively, our current study of ChoE has brought novel insights into the structure and function of prokaryotic acetylcholinesterases. Through extensive efforts of soaking experiments, we have captured the snapshots of different catalytic steps in ChoE, providing mechanistic insights into this enzyme. These first structures of a putative bacterial acetylcholinesterase will serve as a prototype for other cholinesterases of prokaryotic sources and will illuminate future studies on this family of enzymes. Last, the strategy we employed here, particularly the quick (2-5 min) soaking of crystals of WT and mutant enzymes with different substrates, may also be applied to the investigation of other hydrolases/esterases.

Materials and methods

Bacterial growth of WT and mutant P. aeruginosa in the presence of choline or acetylcholine

Bacterial strains used in this study are listed in Table S1. Bacteria from a pre-culture on tryptic soy agar were suspended to cells (Novagen) for target gene overexpression. The verified plasmid was transformed into cells (Promega Biosciences). The sequence of the recombinant gene was confirmed by full-length Sanger DNA sequencing.

Cloning, protein expression, and purification

The choE (PA4921, NP_253608.1) gene was amplified from chromosomal DNA of PAO1 by PCR without the first 60 nucleotides coding for a signal peptide. The forward primer PA4921_F (5′-GGTTAACGGATCCAGCACAATCCCCGGTCTGCCG-3′) and the reverse primer PA4921_R (5′-GGTTAAGAATTCTTACGGCCGGTAGCGTGCCG-3′) were designed to introduce sites for digestion by the restriction enzymes BamHI and EcoRI, respectively. The PCR products were digested with BamHI and EcoRI and ligated into the pET28a (Novagen) vector encoding an N-terminal His tag. The constructed plasmid pET28a-choE was transformed into E. coli DH5α cells (Promega Biosciences). The sequence of the recombinant gene was confirmed by full-length Sanger DNA sequencing.

Enzyme kinetic analysis of WT ChoE and mutants

A standard protocol was followed to measure the activity of ChoE using ATCh, PTCh, or BTCh as substrates (12, 61, 62) with minor modifications. Assays were conducted at 22 °C in 100 mM sodium phosphate buffer (pH 7.0), 0.33 mM 5,5-dithiobis-(2-nitrobenzoic acid) for 3 min. The released thiocholine was monitored using a Cary spectrophotometer at 412 nm. The experiments were performed in triplicates from which the reported values of the kinetic parameters were obtained by fitting the WT data to Equation 1, originally obtained to describe inhibition studies of eukaryotic AChE in the context of substrate inhibition caused by the formation of an ES complex (21).

Construction of ChoE mutants

PCR with the primers listed in Table S1 was performed on the genomic DNA of PAO1 and generated mutant choE fragments of 0.86 kb. Ser-38, Tyr-106, Asp-285, Thr-287, and His-288 were mutated to Ala-38, Ala-106, Ala-285 or Asn-285, Ala-287, and Asn-288, respectively. The Y106A, D285A, and D285N mutations were carried out by overlap-extension PCR, which consisted of two stages (48). First, two fragments with overlapping sequences were amplified by PCR, then a standard program of 15 cycles was employed after mixing these fragments and the WT choE gene as a template at the ratio of 1:1 without adding primers. Second, two primers, PA4921_F and PA4921_R, were added to amplify the full-length mutant sequence of choE. As Ser-38, Thr-287, and His-288 are close to the N and C terminus of ChoE, the forward S38A_F1 (for S38A mutation), reverse W287A_R1 (for W287A mutation), and H288N_R1 (for H288N mutation) long primers were designed for amplification to allow subcloning. The mutant genes were then subcloned and sequenced in the same manner as the aforementioned WT gene. The presence of the desired nucleotide substitutions was confirmed by DNA sequencing. The mutant proteins were expressed and purified following the same protocol for the WT protein.

Equation 1, originally obtained to describe inhibition studies of eukaryotic AChE in the context of substrate inhibition caused by the formation of an ES complex (21), Equation 1 was obtained by simply setting the exogenous inhibitor concentration to zero. Equation 1 would explain substrate inhibition equally well as being caused by the formation of an EIS complex or an ESS complex where the acyl bond would have been hydrolyzed within the active site. This equation is similar to Equation 2 that is frequently used to model substrate inhibition caused by binding of a second substrate molecule to the ES complex thus forming an ESS complex (60). Equations 1 and 2 differ only in that apparent constants are obtained by fitting data to Equation 1. Thus despite reflecting different mechanisms for substrate inhibition, the values of the kinetic parameters obtained with Equations 1 and 2 were identical (Table 2). When there was no substrate inhibition, the enzymatic kinetic data were analyzed with Equation 3 (Michaelis-Menten equation) as indicated in Table 2. Nonlinear
Structure of putative bacterial acetylcholinesterase ChoE

least-squares regression analyses were performed using Kaleidagraph (Synergy Software).

$$v_0 = \frac{V_{\text{max}}[S]}{K_m + q[S] + \frac{[S]^2}{K_{m(app)}}} = \frac{V_{\text{max(app)}}[S]}{K_{m(app)} + [S] + \frac{[S]^2}{K_{m(app)}}} \quad \text{(Eq. 1)}$$

$$v_0 = \frac{V_{\text{max}}[S]}{K_m + [S] + \frac{[S]^2}{K_m}} \quad \text{(Eq. 2)}$$

$$v_0 = \frac{V_{\text{max}}[S]}{K_m + [S]} \quad \text{(Eq. 3)}$$

To measure the activity of ChoE on p-nitrophenyl esters, pNPA, pNPP, pNPB, and pNPO were all dissolved in DMSO. Following the protocol described previously (12), the assays were conducted at 22 °C in 100 mM sodium phosphate buffer (pH 7.0) for 3 min with a final volume of 1 ml of reaction mixture. The concentrations of ChoE were varied to optimize rates: 0.6 nm ChoE was used for the substrate pNPP, and 30 nm ChoE was used for the other substrates pNPA, pNPB, and pNPO. The released p-nitrophenol was monitored using a Cary spectrophotometer at 410 nm.

The inhibitory effects of TEAC and PI on the activity of ChoE were also studied. The concentration of each inhibitor was set at 1 mM. The assays were then conducted in the same manner as described above for substrate ATCh.

Protein crystallization, data collection, structure determination and refinement, and structure comparison

For crystallization, ChoE was concentrated to 12 mg/ml. Crystals were grown at 4 °C using the microbatch-under-oil method by mixing 0.7 μl of 12 mg/ml of protein and 0.7 μl of reservoir solutions containing either a high concentration of salt (3 mM NaCl, 100 mM HEPES, pH 7.5) or PEG molecules (15% (w/v) PEG 20000, 100 mM MES, pH 6.5; or 25% (w/v) PEG 8000, 100 mM MES, pH 6.5), the latter of which was obtained after optimization at the latter stage of this work. Typically, crystals appeared within 4 weeks in the condition containing 3 mM NaCl, and 1 week in the condition containing PEG 8000 or PEG 20000. Upon the data collection for the first crystal form (from 3 mM NaCl) in early 2018, we tried to determine the structure of ChoE by molecular replacement without success. For phasing purpose, crystals from 3 mM NaCl were soaked in the reservoir solution supplemented with 400 mM 13C (5-aminoo-2,4,6-triiodosalicylic acid) followed by the single-wavelength anomalous dispersion (SAD) data collection at the wavelength of 1.7712 Å at the 08ID-1 beamline, Canadian Light Source. To collect data for the other structures, crystals from the PEG conditions were soaked 2-5 min with 50-100 mM ATCh chloride or PTCh iodide and then cryoprotected using the reservoir solution supplemented with 12–16% ethylene glycol or 20% glycerol before flash freezing. The data were collected at the wavelength of 0.97931 Å at the LRL-CAT beamline at the Advanced Photon Source, Argonne National Laboratory.

Data processing and scaling were performed with iMosflm (63). The crystals from high salt conditions (3 mM NaCl) are in the P6122 space group with cell dimensions of $a = b = 140.7$ Å, $c = 334.0$ Å. Each asymmetric unit contains four protein molecules, corresponding to a Matthews coefficient of 3.46 Å³/Da and a solvent content of 64.5%. The crystals from the PEG 20000 conditions belong to the P21 space group and have unit cell parameters of $a = 45.6$, $b = 81.5$, $c = 81.4$ Å, $β = 100.1°$. There are two protein molecules per asymmetric unit correspond to a Matthews coefficient of 2.16 Å³/Da and have a solvent content of 43.0%.

The SAD data of 2.70 Å resolution were used to locate the iodide ions with SHELXD (64). Phasing and density modification were performed using Phaser (65) and Refmac5 (66) in CCP4 (67). The initial model built through Autobuild (68) in Phenix (69) was used as the template for molecular replacement through MolRep (70) using the data sets of higher resolution collected from the crystals in the PEG condition. It should be pointed out that, after we solved the structure of ChoE, two new structures (PDB 5XTU (27) and 6JKZ (28), see below for structure comparison) released by the Protein Data Bank turned out to be good templates for the structure solution of ChoE by the molecular replacement approach. For all the structures reported here, multiple cycles of refinement using Refmac5 followed by model rebuilding with Coot (71) were carried out. The ligand molecules were placed in the model based on the electron density maps and refined. For the new ligand PTCh, it was refined using the geometric restraints prepared using REEL (72) in Phenix. All the models have good stereochemistry as analyzed with PROCHECK (73). Data collection and refinement statistics are shown in Table 3. Notably, Ser-145 in all these structures accounts for the 0.4% outlier in the Ramachandran plot.

Upon structure determination, a search through the DALI server (26) has given several structures with significant similarity to ChoE, among which are the esterase EstJ15 (PDB 5XTU (27)) with a Z-score of 33.5 and an r.m.s.d. of 2.1 Å for the 268 aligned Ca atoms, the phospholipase A2 VvPlpA (PDB 6JKZ) (28) with a Z-score of 28.5 and an r.m.s.d. of 2.1 Å for the 247 aligned Ca atoms, and the esterase EstA (PDB 3KVN) (29) with a Z-score of 26.3 and an r.m.s.d. of 2.3 Å for the 231 aligned Ca atoms.

Molecular docking

The potential esterase substrates (commercially available from Sigma-Aldrich) were identified and their 3D coordinates were obtained from the ZINC database (zinc docking.org) (74) in the MOL2 file format. In addition, we have also prepared the files for propidium and tetraethylammonium, two molecules containing a quaternary ammonium group present in ATCh and PTCh. Docking simulations were performed with the LeDock V1.0 software (75). The protein part of the MES-bound ChoE structure (6UQV) from this work was used, and the receptor was prepared using the LePro software (75). The size of the binding pocket included any protein atom within 10 Å of any heavy atom of the MES molecule in the active site pocket. The number of binding poses and r.m.s.d. were set to 20 and 0.5 Å, respectively. Validation of the docking protocol was carried out by using self-docking of MES, leading to an r.m.s.d. of 0.79 Å between the docked pose and the crystallographic structure.
### Table 3

| Structure                  | Phasing data set | WT ChoE MES complex | WT ChoE Product complex (ACT + TCh) | WT ChoE PTC complex (or product) complex | H288N ChoE ATCh substrate complex | D285N ChoE product complex (ACT + TCh) | D285N ChoE Acyl-enzyme | S38A ChoE ACT and ATCh complex |
|----------------------------|-------------------|----------------------|-------------------------------------|----------------------------------------|-----------------------------------|----------------------------------------|-------------------------------|-------------------------------|
| Space group                | P6122             | C2221                | P21                                 | P21                                    | P21                               | P21                                    | P21                           | P21                           |
| a, b, c (Å)                | 140.7, 140.7, 334.0 | 82.2, 109.7, 84.2    | 45.8, 81.8, 81.4                    | 45.6, 81.5, 81.5                       | 45.6, 81.6, 81.4                   | 45.7, 81.7, 81.2                     | 45.5, 81.6, 81.3               | 45.5, 81.6, 81.3               |
| Wavelength (Å)             | 1.7712            | 0.97931              | 0.97931                             | 0.97931                                | 0.97931                           | 0.97931                               | 0.97931                       | 0.97931                       |
| Resolution (Å)             | 50.2-2.70 (2.80-2.70) | 50.1-1.35 (1.42-1.35) | 50.1-1.65 (1.74-1.65)               | 50.1-1.85 (1.95-1.85)                 | 50.1-1.57 (1.65-1.57)             | 50.1-1.43 (1.51-1.43)               | 50.1-1.80 (1.90-1.80)         | 50.1-1.42 (1.50-1.42) |
| Unique hkl                 | 1,167,343         | 526,567 (66,425)     | 220,070 (32,265)                    | 338,188 (49,627)                       | 79,982 (11,661)                   | 104,293 (13,769)                    | 164,957 (14,704)               | 365,050 (31,557)               |
| Redundancy                 | 21.4 (21.5)       | 6.3 (5.6)            | 3.2 (3.2)                           | 6.8 (6.9)                              | 6.7 (6.7)                         | 6.7 (6.7)                             | 6.7 (6.7)                     | 6.7 (6.7)                     |
| Completeness (%)           | 100 (100)         | 99.5 (98.0)          | 96.5 (97.9)                         | 99.2 (99.2)                            | 97.7 (98.2)                       | 96.4 (87.3)                          | 88.7 (60.2)                   | 97.0 (97.0)                   |
| Rmerge (%)                 | 0.198 (0.842)     | 0.055 (0.883)        | 0.123 (0.550)                       | 0.162 (0.945)                          | 0.067 (0.911)                     | 0.056 (0.643)                       | 0.071 (0.426)                 | 0.044 (0.633)                 |
| l (Å)                      | 25.7 (5.2)        | 17.2 (2.2)           | 7.6 (2.5)                           | 9.3 (2.1)                              | 16.9 (2.2)                         | 13.3 (2.2)                           | 13.7 (3.0)                   | 17.0 (2.5)                   |
| Wilson B (Å²)              | 29.5              | 15.0                 | 8.6                                 | 19.9                                   | 18.3                              | 13.8                                  | 14.1                          | 14.9                          |
| Rwork (%)                  | 0.154 (4.212)     | 0.126 (3.371)        | 0.239 (3.261)                       | 0.183 (3.990)                          | 0.198 (5.189)                     | 0.171 (2.316)                       | 0.194 (5.289)                 | 0.194 (5.289)                 |
| Rfree (%)                  | 0.177 (4.212)     | 0.226 (3.371)        | 0.239 (3.261)                       | 0.183 (3.990)                          | 0.198 (5.189)                     | 0.171 (2.316)                       | 0.194 (5.289)                 | 0.194 (5.289)                 |
| B-factors (Å²)             |                    |                     |                                     |                                        |                                   |                                       |                               |                               |
| — (Å)                      |                    |                     |                                     |                                        |                                   |                                       |                               |                               |
| — (# atoms)                |                    |                     |                                     |                                        |                                   |                                       |                               |                               |
| — Protein                  | 19.3 (2.310)      | 16.5 (4.453)         | 27.1 (4.489)                        | 25.5 (4.482)                           | 21.1 (4.511)                      | 23.8 (4.494)                        | 21.1 (4.496)                  | 21.1 (4.496)                  |
| — Ligand                   | 36.8 (44)         | 28.6 (35)            | 38.3 (23)                           | 39.7 (20)                              | 30.2 (22)                         | 36.9 (14)                           | 29.7 (28)                   | 33.8 (690)                   |
| — Water                    | 35.6 (403)        | 27.0 (593)           | 33.7 (344)                          | 37.3 (617)                             | 33.4 (650)                        | 34.7 (609)                          | 33.9 (690)                   | 33.8 (690)                   |
| Ramachandran               |                    |                     |                                     |                                        |                                   |                                       |                               |                               |
| — Favorable (%)            | 99.6              | 99.6                 | 99.6                                | 99.6                                   | 99.6                              | 99.6                                  | 99.4                         | 99.4                         |
| — Generally allowed (%)    | 0                 | 0                    | 0                                   | 0                                       | 0                                 | 0                                     | 0                            | 0.2                          |
| — Outlier (%)              | 0.4               | 0.4                  | 0.4                                 | 0.4                                     | 0.4                               | 0.4                                   | 0.4                          | 0.4                          |
| R.m.s.d.                   |                    |                     |                                     |                                        |                                   |                                       |                               |                               |
| — Bonds (Å)                | 0.0165            | 0.0115               | 0.0146                              | 0.0136                                  | 0.0145                            | 0.0140                               | 0.0152                       | 0.0152                       |
| — Angles (%)               | 2.01              | 1.53                 | 1.79                                | 1.72                                    | 1.81                              | 1.64                                  | 1.86                         | 1.86                         |
| PDB code                   | 6UQV              | 6UQW                 | 6UQX                                | 6UQY                                    | 6UQZ                              | 6UR0                                  | 6UR1                         | 6UR1                         |

Structure of putative bacterial acetylcholinesterase ChoE

**Structure of* J. Biol. Chem.* (2020) 295(26) 8708–8724**
Structure of putative bacterial acetylcholinesterase ChoE

Following a visual inspection of the docked poses of the potential substrates or inhibitors, we selected pNPA, pNPP, pNPB, pNPO, propidium, and tetraethylammonium for activity or inhibition assays.

Data availability

The coordinates and structure factors of the structures reported here have been deposited in the Protein Data Bank under the accession numbers 6UQV, 6UQW, 6UQX, 6UQY, 6UQZ, 6UR0, and 6UR1. All remaining data are contained within the article and supporting information.

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Abbreviations—The abbreviations used are: AChE, acetylcholinesterase; ACh, acetylcholine; ATH, acetylthiocholine; ATCh, butrylthiocholine; TCh, thiocholine; ACT, acetate; CAS, catalytic anionic site; PAP, peripheral anionic site; pNPA, 4-nitrophenyl acetate; pNPP, 4-nitrophenyl propionate; pNPB, 4-nitrophenyl butyrate; pNPO, 4-nitrophenyl octanoate; PI, propidium iodide; TEAC, tetraethylammonium chloride; PDB, Protein Data Bank; SAD, single-wavelength anomalous dispersion; r.m.s.d., root mean square deviation; EIS, acetyl-enzyme intermediate structure complex; EPS, enzyme product-substrate ternary complex; ESS, enzyme-substrate-substrate complex.

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