Site-directed mutants designed to test back-door hypotheses of acetylcholinesterase function

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Received 25 February 1996; revised version received 31 March 1996

Abstract The location of the active site of the rapid enzyme, acetylcholinesterase, near the bottom of a deep and narrow gorge indicates that alternative routes may exist for traffic of substrate, products or solute into and out of the gorge. Molecular dynamics suggest the existence of a shutter-like back door near Trp^{84}, a key residue in the binding site for acetylcholine, in the *Torpedo californica* enzyme. The homology of the Ω loop, bearing Trp^{84}, with the lid which sequesters the substrate in neutral lipases displaying structural homology with acetylcholinesterase, suggests a flap-like back door. Both possibilities were examined by site-directed mutagenesis. The shutter-like back door was tested by generating a salt bridge which might impede opening of the shutter. The flap-like back door was tested by de novo insertion of a disulfide bridge which tethered the Ω loop to the body of the enzyme. Neither type of mutation produced significant changes in catalytic activity, thus failing to provide experimental support for either back door model. Molecular dynamics revealed, however, substantial mobility of the Ω loop in the immediate vicinity of Trp^{84}, even when the loop was tethered, supporting the possibility that access to the active site, involving limited movement of a segment of the loop, is indeed possible.

Key words: Torpedo californica; Ω loop; Site-directed mutagenesis; Molecular dynamics; Disulfide

1. Introduction

The principal biological role of acetylcholinesterase (acetylcholine acetyl hydrolase; EC 3.1.1.7, AChE) is termination of impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter, acetylcholine (ACh) [1]. In accordance with this role, AChE is characterized by a high turnover number, especially for a serine hydrolase, functioning at a rate close to limitation by diffusion control [2,3]. Solution of the three-dimensional structure of AChE revealed that its active site is located at the bottom of a deep and narrow cavity, which was named the 'aromatic gorge', since its surface is lined predominantly by the rings of 14 conserved aromatic residues [4,5]. It was subsequently shown that, due to an asymmetric charge distribution, AChE displays a large dipole moment aligned along the aromatic gorge, which would attract a positively charged ion into the gorge towards the active site [6]. The location of the active site of a particularly rapid enzyme at the bottom of a narrow gorge, rather than on the enzyme surface, taken together with this dipole moment, prompted consideration of the traffic of the charged substrate (ACh) and products (acetate and choline) through the gorge [7]. The possibility was raised that a back door to the gorge might exist, which could provide an alternative route for exit of products or for movement of water. Indeed, molecular dynamics suggested that a shutter-like in-plane motion of Trp^{84}, Val^{129} and Gly^{441} would result in the transient opening of an aperture near the bottom of the gorge, large enough to pass a water molecule.

Another possible model for a back door arose from consideration of the structural homology of AChE with certain neutral lipases which, like AChE itself, are members of the α/β hydrolase-fold family of enzymes [8,9]. Such lipases contain a flap lying over the active site [10] which may be involved in their interfacial activation [11]. This flap corresponds to an Ω loop [12] in AChE, stretching from Cys^{67} to Cys^{94}, which includes Trp^{84}, with the indole ring of which the quaternary group of ACh is believed to interact [4,13]. Barak et al. [14] have suggested that binding of 'peripheral' site ligands, at the top of the active site gorge, might produce a conformational transition within this loop. The possibility that catalytic activity may be coupled to its movement, thus permitting concomitant escape of products, thus merits consideration. Furthermore, the recent solution of the crystal structures of complexes of *Torpedo* [15] and mouse recombinant [16] AChE with the snake venom toxin, fasciculin, revealed that the toxin completely blocks the entrance to the active site gorge. Yet kinetic studies have shown that the complex retains small but significant catalytic activity [17-19], suggesting that an alternative route for entry of substrate may also exist. Additional support for such a possibility comes from the observation that in trigonal crystals of *Torpedo* AChE the top of the gorge is blocked by a symmetry-related AChE molecule [5]. Despite this steric restriction, ligands can freely penetrate to the active site of the enzyme in such crystals [13].

The structural features of these putative back doors permitted the use of site-directed mutagenesis to design and generate mutants which might provide evidence for their existence. Thus, Gilson et al. [7] suggested that conversion of the conserved valine residue, Val^{129}, to an arginine might create a salt bridge with Glu^{445}, which would prevent opening of the shutter-like back door which they had proposed. The possible coupling of the Ω loop to catalytic activity might be

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Abbreviations: AChE, acetylcholinesterase; ACh, acetylcholine; ATCh, acetylthiocholine; MPT, *O*-ethyl-*S*2-diisopropylaminoethyl methylphosphothionate; DMEM, Dulbecco's modified Eagle's medium

PII S0014-5793(96)00374-2

Table 1
Kinetic characteristics of mutant acetylcholinesterases designed to test 'back door' hypotheses[a]

Mutant	K_m (mM)	k_{cat} (%)[b]
WT	0.04	100
C231S	0.10	100
V129R	0.05	86
V129W	0.16	71
V129K/C231S	0.08	50
V129R/C231S	0.09	64
G80C/C231S	0.11	93
V431C/C231S	0.06	93
G80C/V431C/C231S	0.08	93
V431C	0.07	50
G80C/V431C	0.1–0.2	100

[a]Expression and assay of WT and mutant enzymes were performed as described in section 2.
[b]Percentage relative to that of WT, taken as 100. The value of k_{cat} for the wild-type enzyme, expressed in COS cells, was indistinguishable from the value of 4.8×10^5 min^{-1} reported for *Torpedo* AChE purified from electric organ tissue [28].

similarly tested, either by generation of a salt bridge or by formation of a novel disulfide bridge which would restrict movement of the flap.

In the following, we present a theoretical examination of the feasibility of the 'flap' model for the back door, and describe the generation and characterization of a number of mutants designed to examine the possible existence of back doors to the active site of both the 'shutter' and 'flap' types.

2. Materials and methods

2.1. Materials

Acetylthiocholine iodide (ATCh), 5,5′-dithiobis(2-nitrobenzoic acid) and eserine sulfate were from Sigma (St. Louis, MO), and [^{3}H]acetylcholine (90 mCi/mM) was from NEN (Paris). The organophosphate, *O*-ethyl-*S*2-diisopropylaminoethyl methylphosphothionate (MPT) was a gift from Dr. I. Delamanche (Centre d'Etudes du Bouchet, Vert-le-Petit, France). Thiopropyl Sepharose 6B was purchased from Pharmacia (Uppsala, Sweden).

2.2. Simulated annealing

Simulated annealing [20] was used to sample many low energy conformations without carrying out a thorough conformational search on the protein. The protocol to carry out these simulations was provided as part of the computer modelling package SYBYL (Tripos Associates, Inc.), and they were executed on a Silicon Graphics Indigo 2 workstation.

2.3. Site-directed mutagenesis

A pEF-BOS vector, containing an insert coding the H catalytic subunit of *Torpedo marmorata* AChE [21], was introduced into *E. coli* strain RZ 1032 to obtain uracil-containing template DNA [22]. Single-stranded DNA was produced from the phagemid [23]. The following mutagenic oligonucleotides were used:

5′-CCCATTGTACTTGTCCAACGTCGAGG-3′ for V129K,
5′-CCCATTGTACCTGTCCAACGTCGAGG-3′ for V129R,
5′-CCGGCCAGCACAGGTTTGAGGC-3′ for V431C,
5′GCCCACGGGGAATTGGGCGAG-3′ for C231S
5′-CTCCGAGCATGGAAATCC-3′ for V80C.

The oligonucleotides are in the anti-sense orientation, to hybridize with the single-stranded DNA; the triplets corresponding to the mutated residues are underlined. Synthesis and ligation were achieved with T4 DNA polymerase and T4 DNA ligase in the same tube, after hybridizing 1.5 pmol of 5′-phosphorylated oligonucleotide with 0.05 pmol of template. The *E. coli* strain DH5-α was transformed with the resulting double-stranded DNA. Clones that presented the correct mutation were selected by sequencing. To obtain DNA, they were grown overnight in 500 ml cultures. The resulting DNA was purified by isopycnic equilibration in CsCl gradients. Double and triple mutants were obtained by successive mutagenesis steps.

Table 2
Inhibition by propidium of mutant acetylcholinesterases

Mutant	IC_{50}[a] (μm)
WT	1.3
C231S	1.3
G80C/C231S	2.5
V431C/C231S	1.0
G80C/V431C/C231S	1.6
V431C	1.1
G80C/V431C	1.8

[a]IC_{50} values were determined under the same assay conditions as employed in Table 1 (see section 2).

2.4. Transfections

COS-7 cells were transfected by the DEAE-dextran method [24]. The cells were seeded at a concentration of around 10^6 cells per 10 cm plate, in 10 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Before transfection, the medium was removed and the cells washed in PBS, and 3 ml of DMEM supplemented with 10% Nu-serum (Gibco-BRL) was added to each plate. For each plate, a solution of 10 μg DNA in 80 μl of TBS (25 mM Tris-HCl, pH 7.4/150 mM NaCl) was added dropwise to 160 μl of 10 mg/ml DEAE-dextran in TBS, at 37°C. This mixture was distributed over the surface of the plate, and chloroquine was added to a final concentration of 100 mM. After 3 h at 37°C, the cells were washed in PBS as above, and fed with the DMEM/Nu-serum culture medium. The Nu-serum was previously treated with soman (5×10^{-7} M) to block irreversibly any cholinesterase activity; this treatment was performed at least three days before use, so as to ensure that excess soman would be hydrolyzed during storage at 4°C, and thus would not inhibit AChE produced by the cultured cells. After incubation for 2 days at 37°C, the medium was changed, and the cells were transferred to 27°C for 3–4 days. The cells were scraped from 10 cm dishes, containing ca. 5×10^6 cells, and extracted, by Potter homogenization, in 300 μl of a buffer containing 50 mM Tris-HCl, pH 7.0, 40 mM $MgCl_2$, 1% Triton X-100, and 1/20 of a stock solution of protease inhibitors (20 mM EDTA; 2 mg/ml bacitracin; 50 U/ml Zymofren; 100 μg/ml pepstatin A; 100 μg/ml leupeptin). As before, extracts contained 2–6 Ellman units/mg protein of AChE activity [21]. Extracts were stored at −80°C until used.

2.5. Cholinesterase assays

Cholinesterase activity was determined either radiometrically, with [^{3}H]acetylcholine [25], or spectrophotometrically, using ATCh as substrate [26].

K_m and V_{max} were estimated at 20°C by the spectrophotometric method. The assay medium was 0.33 mM 5,5′-dithiobis-2-nitrobenzoic acid/1% Triton X-100/0.02 M sodium phosphate, pH 7.0, containing ATCh at a final concentration of 0.01–5 mM. Usually, 10 μl of the COS cell extract was added per ml of assay medium, except in the case of the G80C mutant, for which 40 μl was taken. The reaction was recorded continuously, and was practically linear. K_m values were estimated from Lineweaver-Burk plots, and those of k_{cat} were calculated from the V_{max} values on the basis of the concentration of active sites determined as described below. IC_{50} values for inhibition by propidium were determined essentially as described previously [27].

2.6. Active site titration

The concentration of AChE active sites in the COS cell extracts was determined using the organophosphate, MTP, as titrant, as described previously [28]. In brief, a stock solution of MTP, stored at 10^{-2} M in methylethylketone at −80°C, was diluted to 10^{-4} M in water, then diluted to an appropriate concentration in extraction buffer, viz. 10 mM $MgCl_2$/0.1% Triton X-100/0.01% bacitracin/10 mM Tris-HCl, pH 7.0. Aliquots of cell extracts (15 or 20 μl) were incubated overnight with an equal volume of extraction buffer containing 0–10 nM MTP. The residual rate of hydrolysis was determined by direct addition of 1 ml of assay medium, containing 1 or 2 mM ATCh, to the same tube.

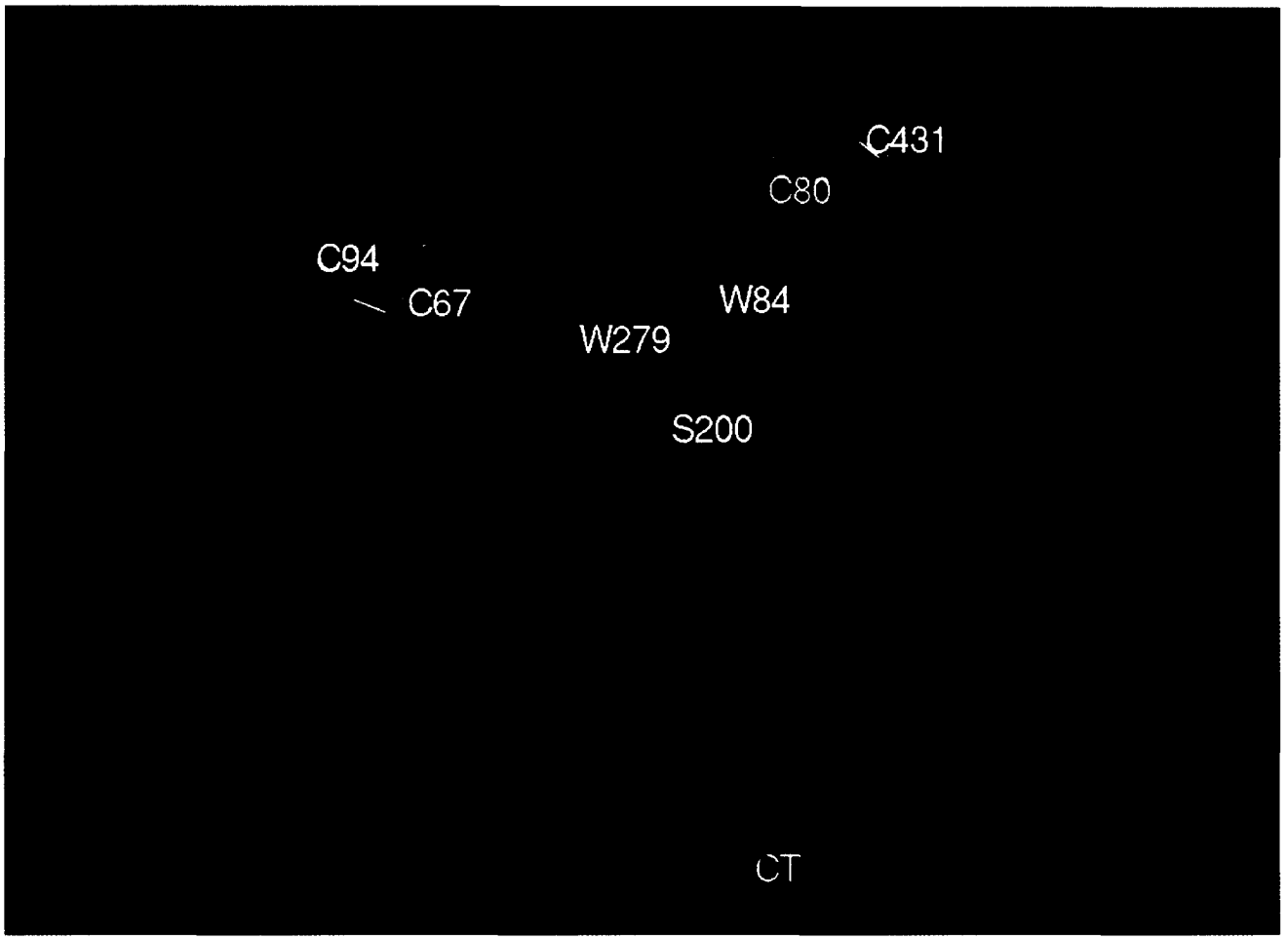

Fig. 1. Backbone Cα trace of the structure of AChE from *Torpedo californica* into which an additional disulfide has been engineered and energy-minimized. The backbone is shown in green; the engineered disulfide, Cys^{80}-Cys^{431}, and the preexisting disulfide, Cys^{67}-Cys^{94}, are shown in yellow; the Ω loop, from Cys^{67} to Cys^{94}, is shown as a red ribbon. Side chains of Trp^{84} (red), Ser^{200} (cyan) and Trp^{84} (cyan), within the active site gorge, are shown for orientation. NT and CT denote the amino and carboxyl termini, respectively.

2.7. Covalent chromatography

Covalent chromatography on thiopropyl Sepharose 6B [29] was used to distinguish between mutant AChEs containing or devoid of reactive free thiol groups. A batch procedure was employed as follows: COS cell extracts were prepared as described above, after 48 h at 37°C, followed by 5 days at 27°C. The extracts were diluted in the extraction buffer so as to yield approximately equal enzymic activities (ca. 3 mOD units/μl/min). The thiopropyl Sepharose was washed several times with 10 mM $MgCl_2$/50 mM Tris-HCl, pH 7.0 (TM buffer). Aliquots of 40 μl of each of the diluted extracts were added to 1.5 ml Eppendorf tubes containing 160 μl of 1% Triton X-100/10 mM $MgCl_2$/50 mM Tris-HCl, pH 7.0 (TMT buffer) and either 100 μl of TM buffer, in control tubes, or 100 μl of thiopropyl Sepharose 6B suspended in an equal volume of TM buffer. The tubes were then rotated for 18–20 h at 4°C. After centrifugation, aliquots of the supernatants were assayed by the Ellman procedure. In one experiment, rotation was carried out for a further 75 min at 25°C, and aliquots again taken for assay. Identical results were obtained.

2.8. Thermal inactivation

In the thermal inactivation studies, the same extracts employed above in the covalent chromatography procedure were diluted as follows: 5 μl aliquots of the cell extracts, together with 120 μl of 1 M Tris-HCl, pH 7.0, and 30 μl of 20% Triton X-100, were brought to a volume of 6 ml with H_2O, to yield final concentrations of 20 mM Tris-HCl and 0.1% Triton X-100. Aliquots (100 μl) were incubated at 34°C for the indicated times before being assayed for residual enzymic activity.

[(1)] For the human recombinant AChE mutants, the numbers in parentheses refer to the corresponding amino acid residues in *T. californica* AChE.

3. Results and discussion

Gilson et al. [7] predicted that replacement of Val^{129} in *Torpedo* AChE by an Arg residue might impede the transient opening of the putative channel for water visualized by molecular dynamics, thereby affecting enzymic activity. It can be seen from Table 1 that this mutation, viz. V129R, had little effect on either K_m or k_{cat}. Nor was any significant effect on these kinetic constants observed in a double mutant, V129R/C231S, or on a similar mutant, V129K/C231S, in which the Val residue had been replaced by Lys rather than by Arg. Even replacement of Val^{129} by a bulky Trp residue, in the V129W mutant, had little or no effect on k_{cat} and increased K_m 4-fold at most. Thus, site-directed mutagenesis yields no positive experimental evidence in support of the functional significance of the back door predicted by molecular dynamics. Similar conclusions were reached by Kronman et al. [30], who analyzed mutants of human AChE: the mutants D131(128)N[(1)] and E84(82)Q, which could alter the local electric field near the back door, and V132(129)A, which could

Table 3
Binding of wild type and mutant acetylcholinesterases to thiopropyl Sepharose 6B

Mutant	Unbound catalytic activity[a] (%)
WT	101, 97
G80C	61, 60
V431C	47, 45
G80C/V431C	97, 96
C231S	81, 99
C231S/G80C	52, 62
C231S/V431C	47, 57
C231S/G80C/V431C	99, 97

[a]The figures given represent the percentage of initial AChE activity retained in the supernatant, and the two values for each mutant were taken from two independent experiments. For experimental details see section 2.

enlarge the opening and facilitate the transit of products through the back door, had no significant effect on K_m and k_{cat} for ATCh hydrolysis. The mutant V132(129)K increased 6-fold the K_m for ATCh, but not for the uncharged isosteric substrate, *S*-3,3-dimethylbutyl thioacetate, and also decreased the affinity for the active site inhibitor edrophonium, showing that it does not act by blocking the exit from the active site.

As mentioned in Section 1, a macroscopic movement has been reported in a loop in *Candida rugosa* lipase which is equivalent to the Cys^{67}-Cys^{94} Ω loop of *Torpedo* AChE [10,11]. This led us to examine the mobility of this loop in AChE both by site-directed mutagenesis and by computer simulation.

A test of the hypothesis that movement of the Ω loop around Trp^{84} would occur during catalysis could be provided by a mutation which would tether this loop to the main body of the enzyme, e.g. by double mutation of two suitable residues to cysteines, which could result in formation of a disulfide bridge. Accordingly, we conducted a search for all possible short distances (i.e. less than 6 Å) between C_α atoms of residues in the Ω loop and C_α atoms in the rest of the protein. We found only three pairs which satisfied this distance criterion: (i) Pro^{86}-Ser^{124}; (ii) Gln^{69}-Ser^{122}; (iii) Gly^{80}-Val^{431}.

The first possibility, viz. Pro^{86}-Ser^{124}, was considered unappealing because it involves a proline residue which is partially conserved amongst the cholinesterases [31,32]. The second possibility, Gln^{69}-Ser^{122}, would produce a putative disulfide bridge which would be close to the preexisting one, and would not, therefore, appear to provide a means of 'locking-in' the position of the loop. The third possibility seemed the most attractive, since Val^{431} is not conserved, and Gly^{80} is only partially conserved among cholinesterases. Inspection of the three-dimensional structure of AChE suggested that the thiol groups of these two cysteines should be no more than 2 Å apart, and thus capable of forming a disulfide bridge, as has been demonstrated to occur for a number of proteins (see, for example, [33,34]). Furthermore, a computer-generated double mutant, G80C/V431C, in which the two cysteines were linked by a disulfide bridge, led to a reasonable structure after a few cycles of energy minimization (Fig. 1).

Based on the above considerations, a double mutant was

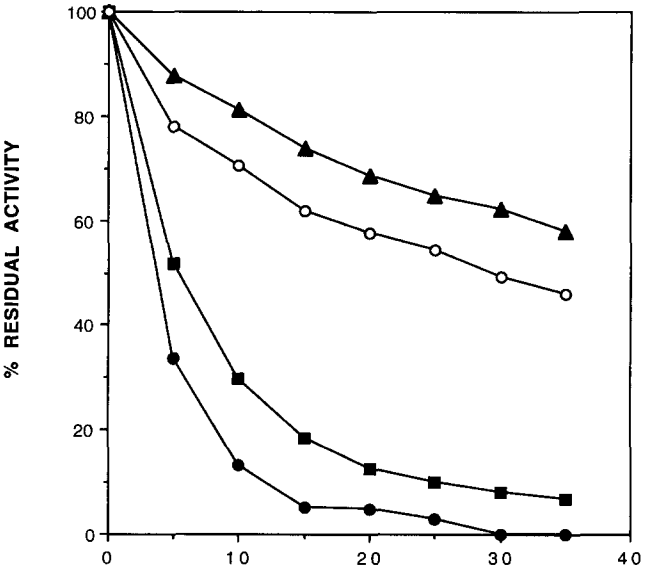

Fig. 2. Thermal inactivation of wild-type AChE and of G80C and V431C mutants. Samples were incubated at 34°C, and aliquots withdrawn at appropriate times for assay of enzymic activity, as described in Section 2. ▲, WT; ●, G80C; ■, V431C; ○, G80C/V431C.

generated in which Gly^{80} and Val^{431} were both replaced by cysteine residues, G80C/V431C, as well as the corresponding single mutants, G80C and V431C. Mutants were also constructed in which the nonconserved Cys^{231} residue of *Torpedo* AChE was mutated to Ser concomitantly with the introduction of the new Cys residues. The C231S mutation alone does not affect the catalytic activity of *Torpedo* AChE (see Table 1), although it abolishes the inhibition of enzymic activity by organomercurials [35], and we wished to investigate the possibility that it might affect folding of the mutant enzymes into which the additional cysteine residues had been engineered. On the basis of catalytic activity, all the mutants generated were expressed at adequate levels in COS cells. Most of them displayed activity equivalent to 40% or more of that of the wild type. Substantially lower activities were displayed by the G80C mutant (ca. 4% of wild-type levels), the G80C/C231S double mutant (ca. 10%) and the G80C/V431C double mutant (ca. 20%), and those in which the C231S mutation was included were expressed as well or better than those in which it was omitted. The differences observed may well be due to misfolding resulting from formation of 'non-native' disulfides, but more extensive and quantitative studies will be needed to clarify this issue.

Table 1 shows that all the mutants in which either one or two additional cysteine residues had been inserted, with or without the C231S mutation, displayed K_m and k_{cat} values not very different from those displayed by the wild-type enzyme or by the C231S mutant itself.

It has been suggested that binding of a 'peripheral' site ligand, at the top of the active-site gorge, might produce a conformational transition within the 'anionic' subsite of the catalytic site, specifically in the Ω loop containing Trp^{84} [14].

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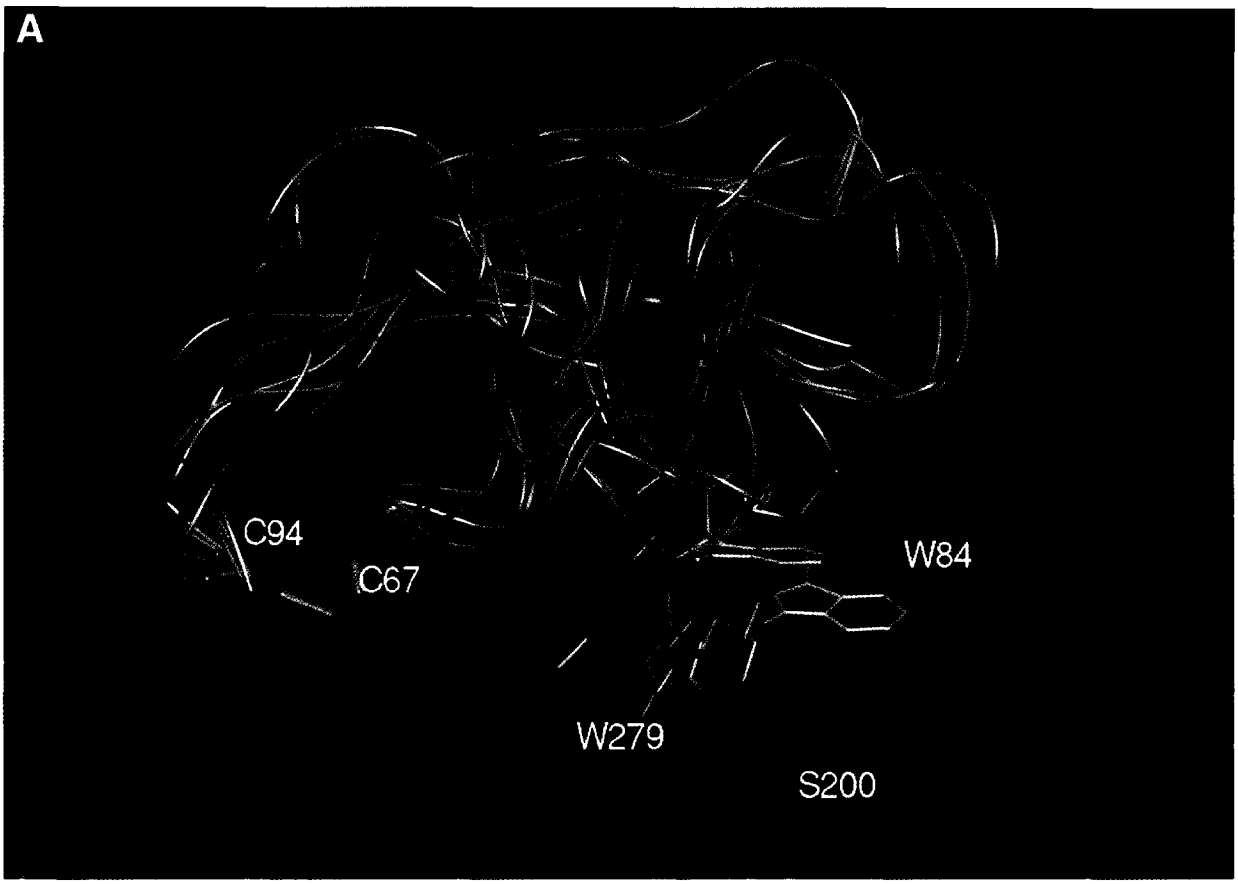
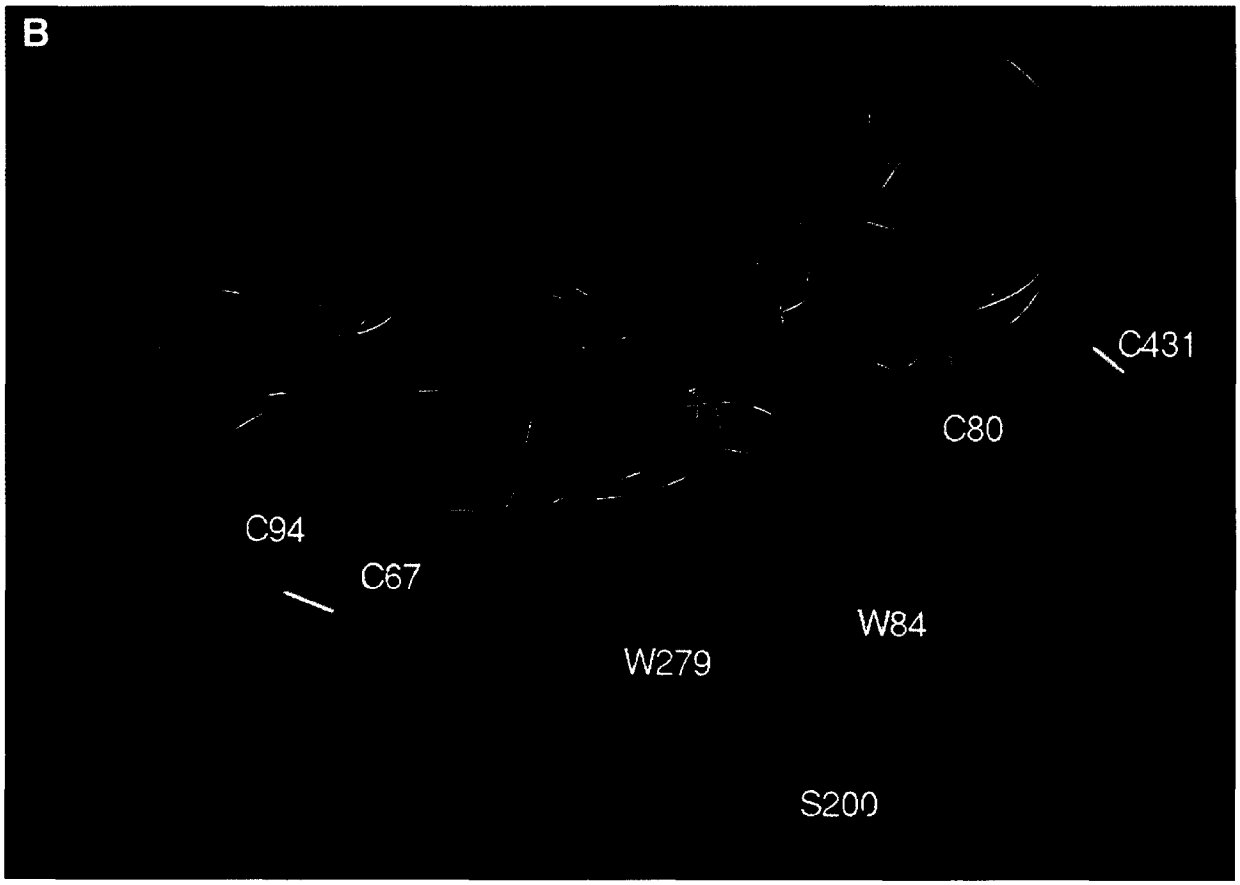
Fig. 3. Superposition of nine energy-minimized conformations of the Ω loop, Cys^{67}-Cys^{94}, in native *T. californica* AChE (A) and in the engineered energy-minimized mutant with the additional disulfide, Cys^{80}-Cys^{431} (B). Color coding of the two 3-D structures is as in Fig. 1. The nine representative conformations of the Ω loop, obtained by molecular dynamics, are shown in white, except for the conformation which deviates most from the native one, which is shown in pink. Three representative conformations of the side chain of Trp^{84} are shown in white.


A
C94
C67
W84
W279
S200


B
C431
C80
C94
C67
W84
W279
S200

It was, therefore, of interest to examine the susceptibility to a characteristic 'peripheral' site ligand, namely propidium [36], of the mutant enzymes that we had generated by conversion of Gly^{80} and/or of Val^{431} to Cys. The data presented in Table 2 show no appreciable effect of any of these mutations on inhibition by propidium.

Since expression of *Torpedo* AChE in COS cells produces only small amounts of enzyme protein, it was not possible to resort to direct estimation of thiol groups so as to verify that the Cys^{80}-Cys^{431} disulfide bridge had indeed been formed. We adopted a covalent chromatography procedure to resolve this issue. This procedure was originally developed as a specific purification procedure in which proteins containing free thiol groups are selectively bound to a column containing activated thiol groups by a thiol-disulfide exchange reaction, and subsequently eluted by use of a low molecular weight thiol reagent [37]. Thiopropyl Sepharose 6B [29] serves as a particularly suitable resin for covalent chromatography (see, for example, [38]). We reasoned that if the disulfide bridge had formed, the triple mutant, G80C/V431C/C231S, should not bind to the column, being devoid of any free thiol group, thus behaving similarly to the control mutant, C231S. In contrast, the two double mutants, G80C/C231S and V431C/C231S, should be bound. Table 3 shows that, as predicted, neither the single mutant, C231S, nor the triple mutant, G80C/C231S/V431C, was bound significantly to the thiopropyl Sepharose column, whereas about half of the catalytic activity of the two double mutants, G80C/C231S and V431C/C231S, was retained. This provides strong evidence that, as predicted, the Cys^{80}-Cys^{431} disulfide bond had indeed been formed. Although wild-type *Torpedo* AChE contains a free sulfhydryl group on Cys^{231}, this group is buried in the native enzyme [4] and, being inaccessible, reacts very sluggishly with disulfides [39]. Correspondingly, the double mutant, G80C/V431C, is not bound, whereas the two single mutants, G80C and V431C are bound ca. 60 and 45%, respectively, in good agreement with the results for the mutants in which Cys^{231} had also been mutated (Table 2).

Thermal inactivation experiments also supported the conclusion that the Cys^{80}-Cys^{431} disulfide had indeed been formed. As already mentioned, engineering of de novo disulfide bridges between suitably juxtaposed amino acid residues, by mutagenesis of both residues to cysteines, frequently leads to stabilization [33,34]. Fig. 2 shows clearly that although the double mutant, G80C/V431C, does not differ greatly in its thermal stability from wild-type AChE, it is much more stable than either of the single mutants, G80C and V431C.

We considered the possibility that if a Cys^{80}-Cys^{431} disulfide bond were indeed formed in the triple mutant, it might be reduced by the thiocholine generated by hydrolysis of ATCh in the spectrophotometric assay, since the thiocholine would be generated adjacent to this bond. Accordingly, we examined the catalytic activity of the various mutants by the radiometric assay, employing [^{3}H]acetylcholine as substrate. No significant difference was noticed between the activity of the triple mutant and of the other mutants on ATCh and on [^{3}H]acetylcholine (not shown).

Thus, overall, the catalytic properties of the triple mutant do not lend support to the 'flap model' for the back door.

In addition to site-directed mutagenesis, we also examined the conformational freedom of the Ω loop by a molecular dynamics approach. Thus simulated annealing runs were performed, employing a protocol provided with the program SYBYL, for both the native structure and for the computer-generated double mutant. Of the 5098 atoms in the AChE catalytic subunit, only 376 atoms contained in the Cys^{67}-Cys^{94} loop, and a few side-chains forming part of the walls of the active-site gorge, were included. The total time of the run was 40 ps, during which period 10 heating and annealing cycles were generated. During each cycle, the system was heated to 500 K, followed by molecular dynamics for 3200 fs. This was followed by a cooling schedule in which the system was cooled to 50 K over a period of 80 ps. The resulting conformations obtained after these cooling cycles were saved, and subsequently subjected to 100 steps of energy minimization, using a conjugate gradient method available as part of the program SYBYL. Fig. 3A,B shows the superpositions of nine of these structures, on both the native and computer-generated double mutant, revealing substantial movement in the whole loop, especially near Trp^{84}, for both the native and the mutant structures. However, movement of the loop in the mutant structure is, not surprisingly, somewhat more restricted.

These theoretical findings are in agreement with the biochemical studies described and discussed above. Our data thus suggest that if, in fact, the Ω loop serves as a 'flap'-type back door, involved in traffic of substrate and products, it cannot undergo a major conformational movement of a type which might be inferred from the open and closed lipase structures determined by Cygler and coworkers [10,11].

In summary, the various *Torpedo* AChE mutants generated in COS cells, which were designed to provide experimental evidence for either the 'shutter' model or the 'flap' model of the putative back door to the aromatic gorge of AChE, have failed to provide support for either model. They do not, however, rule out either model conclusively, and it will be necessary to seek other lines of evidence so as to address the real dilemma raised by the experimental data mentioned in Section 1, which clearly demonstrate that even when the entrance to the active site gorge appears to be completely blocked, both substrate and inhibitors have access to the active site [5,13,15-19]. We intend to use time-resolved crystallography [40] as a direct experimental approach to studying the traffic of substrate and products within the gorge, using photolabile precursors of choline and carbamylcholine as experimental tools ([41], L. Peng, I. Silman, J.L. Sussman and M. Goeldner, submitted).

Acknowledgements: The expert technical assistance of Anne le Goff is gratefully acknowledged. This research was supported by grants from the U.S. Army Medical Research and Development Command, under Contract No. DAMD17-93-C-3070, NIH Grant P41RR-04293, L'Association Française-Israelienne pour la Recherche Scientifique et Technologique (AFIRST), Le Centre National de la Recherche Scientifique, La Direction des Recherches et Etudes Techniques, L'Association Française contre les Myopathies, the Human Capital and Mobility Programme of the European Community, the Kimmelman Center for Biomolecular Structure and Assembly, and funding to the Cornell Theory Center from the NSF, New York State, the Advanced Research Project Agency and IBM. I.S. is the Bernstein-Mason Professor of Neurochemistry.

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