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Differential Effects of "Peripheral" Site Ligands on *Torpedo* and Chicken Acetylcholinesterase

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SUMMARY

Comparison of the effect of three 'peripheral' site ligands, propidium, *d*-tubocurarine, and gallamine, on acetylcholinesterase (acetylcholine hydrolase; EC 3.1.1.7) of *Torpedo* and chicken shows that all three are substantially more effective inhibitors of the *Torpedo* enzyme than of the chicken enzyme. In contrast, edrophonium, which is directed to the "anionic" subsite of the active site, inhibits the chicken and *Torpedo* enzymes equally effectively. Two bisquaternary ligands, decamethonium and 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide, which are believed to bridge the anionic subsite of the active site and the "peripheral" anionic site, are much weaker inhibitors of the chicken enzyme than of *Torpedo* acetylcholinesterase, whereas the shorter bisquaternary ligand hexamethonium inhibits the two enzymes similarly. The concentration dependence of activity towards the natural substrate acetylcholine is almost identical for the two enzymes, whereas substrate inhibition of chicken acetylcholinesterase is somewhat weaker than that of the *Torpedo* enzyme. The experimental data can be rationalized on the basis of the three-dimensional structure of the *Torpedo* enzyme and alignment of the chicken and *Torpedo* sequences; it is suggested that the absence, in the chicken enzyme, of two aromatic residues, Tyr-70 and Trp-279, that contribute to the peripheral site of *Torpedo* acetylcholinesterase is responsible for the differential effects of peripheral site ligands on the two enzymes.

The main biological role of AChE is to terminate impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter ACh (1). ACh also serves as a substrate for butyrylcholinesterase, a closely related enzyme of unknown biological function (2).

Early kinetic studies indicated that the active site of AChE consists of two subsites, the "esteratic" and "anionic" subsites, corresponding to the catalytic machinery and the choline-binding pocket, respectively (3). The recent elucidation of the three-dimensional structure of *Torpedo* AChE (4) has served to confirm these earlier studies and has shown that AChE contains a catalytic triad similar to that present in other serine hydrolases (5). Unexpectedly, it has also revealed that this triad is located near the bottom of a deep and narrow cavity, which has been named the "active-site gorge." The cavity is lined by the rings of 14 aromatic residues that are conserved in the AChE sequences published so far (6). Various lines of evidence, including computer docking (4), affinity and photoaffinity labeling (7–9), structural studies of AChE-ligand complexes (9, 10), and site-directed mutagenesis (11–13), implicate several aromatic rings in the binding pocket for ACh. In particular, Trp-84 and Phe-330 seem to be directly involved in binding the quaternary moiety of ACh (4, 7–10). Thus, aromatic rings make an important contribution to the so-called anionic site, and theoretical considerations, as well as studies with model host-guest systems (14), suggest that charge-charge interactions involving the π electrons of the aromatic rings are involved (see also Ref. 15).

In addition to the anionic subsite of the active site, AChE possesses a "peripheral" anionic site, topographically distant from the choline-binding subsite of the active site (16–20). Certain compounds, such as propidium and gallamine, bind selectively to this site, serving as noncompetitive or uncompetitive inhibitors of AChE (17, 18, 20), and various bisquaternary ligands are believed to derive their enhanced inhibitory potency, relative to homologous quaternary ligands, from their ability to span the two anionic sites (16, 19). Several lines of evidence suggest that the peripheral site is located near the top of the active-site gorge. Thus, both affinity and photoaffinity labeling (7, 21) have identified peptide sequences located near

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ABBREVIATIONS: AChE, acetylcholinesterase; ACh, acetylcholine; ATCh, acetylthiocholine; BW284c51, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide.

the mouth of the gorge in the three-dimensional structure of *Torpedo* AChE. Structural studies of complexes of the native enzyme with the bisquaternary inhibitors decamethonium (9) and BW284c51[1] showed that both ligands lie along the gorge. One quaternary group is near Trp-84; this residue was already identified as an important constituent of the anionic subsite of the active site on the basis of affinity labeling (7), computer docking of ACh (4), site-directed mutagenesis (12), and structural studies of complexes of AChE with quaternary ligands (9, 10). The other quaternary group could be visualized close to the top of the gorge and also appeared to be near several aromatic rings, specifically Trp-279, Tyr-70, and Tyr-121 (9). Photoaffinity labeling (8, 9), as well as site-directed mutagenesis (11, 12), also implicates Trp-279 in the peripheral site.

The recent sequencing of cDNA corresponding to a major portion of the catalytic subunit of chicken AChE permits alignment of the *Torpedo* and chicken AChE sequences.[2] This alignment reveals that two of the aromatic residues implicated in the peripheral site, i.e., Tyr-70 and Trp-279, are absent in the chicken enzyme, being replaced by methionine and glycine, respectively. All of the other aromatic residues in the gorge are conserved, although the residue equivalent to Phe-330 in *Torpedo* AChE is replaced by tyrosine, as is the case in mammalian AChE. The residues of the catalytic triad are in the same positions as in the *Torpedo* enzyme, and two acid residues in the gorge, those corresponding to Asp-72 and Glu-199 in *Torpedo* AChE, are also conserved. In this report, we compare the effect of peripheral site ligands on the catalytic activity of chicken and *Torpedo* AChE, to assess the contribution of these residues to the peripheral site.

Experimental Procedures

Materials. ATCh iodide, 5,5′-dithiobis(2-nitrobenzoic acid), propidium iodide, gallamine triethiodide, *d*-tubocurarine, BW284c51, hexamethonium chloride, and decamethonium bromide were purchased from Sigma Chemical Co. (St. Louis, MO). Edrophonium chloride was from Hoffman-LaRoche (Basel, Switzerland), and [^{3}H]ACh iodide from NEN (Paris, France). Structures of the ligands used in this study are shown in Fig. 1.

AChE preparations. Detergent-soluble AChE fractions were obtained as described previously, from electric organ of *Torpedo marmorata* (22) or from chicken brain and muscle (23). The dimeric (G_2) form of AChE from *Torpedo californica* was purified by affinity chromatography, subsequent to solubilization with phosphatidylinositol-specific phospholipase C, as described previously (24).

Determination of AChE activity. AChE activity was determined either colorimetrically, with ATCh (25), or radiometrically, with [^{3}H]ACh (26). The reaction mixture for the colorimetric Ellman determination contained 1 mM 5,5′-dithiobis(2-nitrobenzoic acid), 0.01% gelatin, 100 mM Tris·HCl, pH 7.6, and the appropriate concentration of ATCh. The reaction mixture for the radiometric assay contained 3 mM [^{3}H]ACh, 1 mM $MgCl_2$, 100 mM NaCl, and 50 mM Tris·HCl, pH 7.4.

Inhibition of AChE activity. For determination of IC_{50} values, samples were preincubated with appropriate concentrations of the various inhibitors for 30 min at room temperature. AChE activity of a 25-μl aliquot was then determined radiometrically in a final reaction volume of 100 μl, being assayed for 20 min at room temperature. For determination of K_i values, samples were preincubated with appropriate concentrations of the various inhibitors for 5 min at room temperature. Suitable aliquots were added to 1 ml of the Ellman reagent reaction mixture containing the desired concentration of ATCh (usually 0.1–1 mM), and the change in absorption was monitored for 2 min in a Kontron Uvikon 940 spectrophotometer. Dixon plots of $1/V$ versus inhibitor concentration or Lineweaver-Burke plots were used to evaluate K_i values (27).

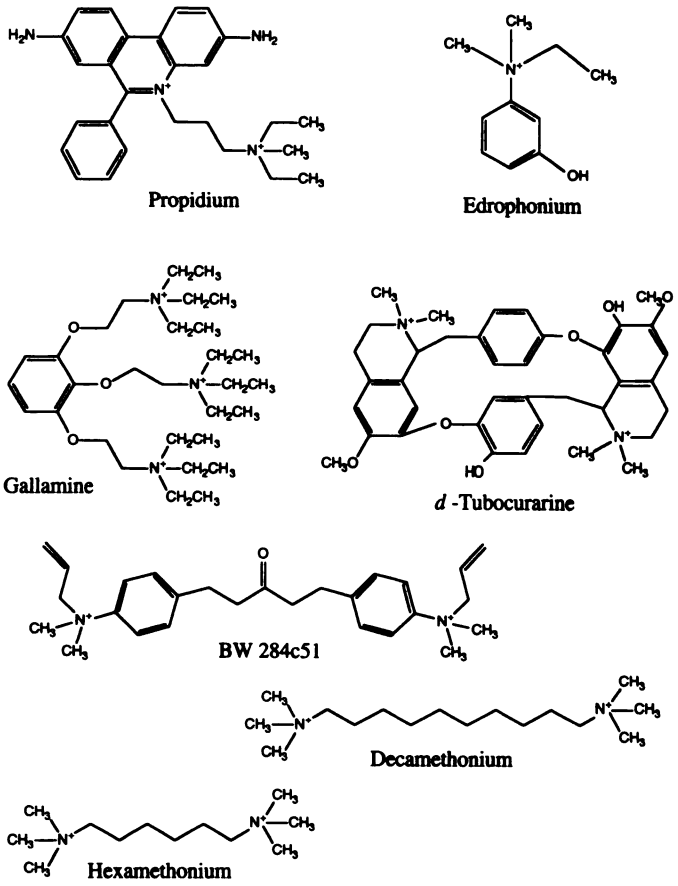

Fig. 1. Chemical structures of various reversible inhibitors of AChE used in this study.

Results and Discussion

Action of peripheral site inhibitors. As outlined in the introduction, the rationale for this study was to see whether the absence from chicken AChE of two aromatic residues that lie near the top of the active-site gorge in *Torpedo* AChE and are believed to contribute to the peripheral anionic site might affect the interaction of chicken AChE with peripheral site inhibitors. K_i values obtained for the various ligands, using a colorimetric assay with ATCh, are summarized in Table 1.

Propidium is an effective and well characterized peripheral site inhibitor of *Torpedo* AChE (20). Fig. 2A shows that propidium inhibited *Torpedo* AChE with an IC_{50} of 13 μM under the assay conditions used, whereas even with 2.5 mM propidium chicken brain AChE was inhibited by <10%. Propidium inhibited *Torpedo* AChE with a K_i of 2.8 μM (Table 1), whereas in attempts to estimate a K_i for the chicken brain enzyme no significant inhibition could be detected, even with substrate concentrations as low as 3 μM and propidium concentrations up to 1 mM. Thus, the inhibition constant for propidium must be substantially above 1 mM. In contrast, Fig. 2B shows no significant difference in the IC_{50} values for inhibition of chicken and *Torpedo* AChE by edrophonium, a potent competitive inhibitor directed to the anionic subsite of the active site (28),

[1] M. Harel, I. Silman, and J. L. Sussman, unpublished observations.
[2] A. Anselmet and J. Massoulié, unpublished results.

TABLE 1

K_i values for the various inhibitors of *Torpedo* and chicken AChE

Inhibitor	K_i[a]	
	Torpedo G_2 AChE	Chicken brain AChE
	μM	μM
Propidium	2.8	>1000
Edrophonium	0.15	0.23
Gallamine	15	740
d-Tubocurarine	12	>500
BW284c51	0.002	0.24
Decamethonium	0.34	23.6
Hexamethonium	84	242

[a] Γ/2 = 0.07.

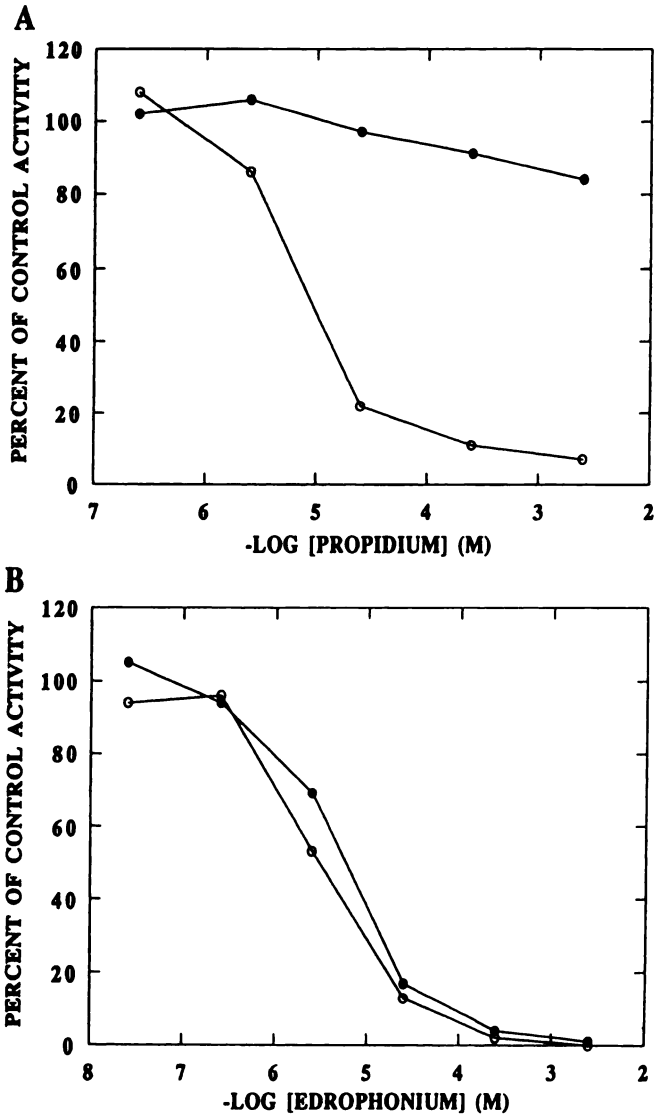

Fig. 2. Inhibition of *Torpedo* and chicken AChE by propidium and edrophonium. Aliquots of tissue extracts from *Torpedo* electric organ or chicken brain were assayed in the presence of propidium (A) or edrophonium (B), and the AChE activity was determined radiometrically in the presence of 3 mM substrate. Activities are expressed as percentages of the activity of control samples. ○, *Torpedo* electric organ; ●, chicken brain.

and the K_i values for edrophonium differed by a factor of <2 (Table 1). Similar results were obtained using chicken muscle AChE (data not shown).

To examine whether the low affinity of propidium for chicken AChE, relative to *Torpedo* AChE, is a property shared with other peripheral site inhibitors, we compared the effects on *Torpedo* and chicken AChE of two other peripheral site inhibitors, i.e., *d*-tubocurarine (17, 18) and gallamine (also known as flaxedil) (17). *Torpedo* AChE was inhibited well by *d*-tubocurarine (Table 1), whereas no inhibition of chicken AChE could be detected, even with substrate concentrations as low as 3 μM and inhibitor concentrations up to 0.5 mM. Gallamine, too, inhibited chicken AChE much more poorly than it inhibited the *Torpedo* enzyme, with its inhibition constant being 50-fold higher (Table 1).

Thus, the data obtained for these three peripheral site inhibitors support the supposition that the peripheral site of chicken AChE displays a reduced affinity for peripheral ligands, relative to *Torpedo* AChE, due to its lacking two aromatic residues. Our data are consistent with site-directed mutagenesis studies from three different laboratories. It was shown that the mutation Trp-279-Ala in *Torpedo* AChE reduced affinity for propidium at least 10-fold (11), whereas a similar mutation of the corresponding tryptophan residue, Trp-286, in human AChE reduced affinity for propidium by 12-fold (12). A recent detailed study from the laboratory of Palmer Taylor (29), on mouse AChE, showed that mutation of Tyr-72 and/or Trp-286, the residues corresponding to Tyr-70 and Trp-279 in *Torpedo* AChE, to nonaromatic residues markedly reduced affinity for propidium. Mutation of Trp-286 had an especially strong effect, reducing affinity by >2 orders of magnitude, whereas mutation of Tyr-72 reduced affinity by 8-fold. The double mutation Tyr-72-Asn/Trp-286-Arg had a similar effect on the affinity for propidium as did the single Trp-286-Arg mutation.

It was suggested by Kreienkamp *et al.* (30) that the AChE purified from the venom of the cobra, *Naja naja oxiana*, possesses a modified peripheral site, because substrate inhibition is weaker than that observed for *Torpedo* AChE and inhibition by propidium displays different characteristics. Cobra AChE has not yet been cloned or fully sequenced, and it will be interesting to see whether it too lacks any of the aromatic residues associated with the peripheral site.

Substrate inhibition. Kinetic and spectroscopic data presented earlier suggested that the peripheral anionic site of *Torpedo* AChE is identical to, or overlaps with, the ACh binding site involved in the substrate inhibition characteristic of AChE (31). One might thus predict substantially weaker substrate inhibition for the chicken enzyme, relative to *Torpedo* AChE. In fact, as can be seen from Fig. 3, which compares the dependence on substrate concentration of the activity of *Torpedo* and chicken AChE towards ATCh, the descending limb, which reflects substrate inhibition, is only slightly shifted to the right for chicken AChE, relative to the *Torpedo* enzyme. This very small effect, relative to those seen for the peripheral site ligands (see above), does not point to a direct involvement of the peripheral site in substrate inhibition. This is also in agreement with the recent site-directed mutagenesis data on mouse AChE that were already mentioned (29). Those authors showed little effect of mutation of either Tyr-72 or Trp-286, or both, on substrate inhibition. Even a triple mutation that included, in addition, Tyr-124, corresponding to Tyr-121 in *Torpedo* AChE,

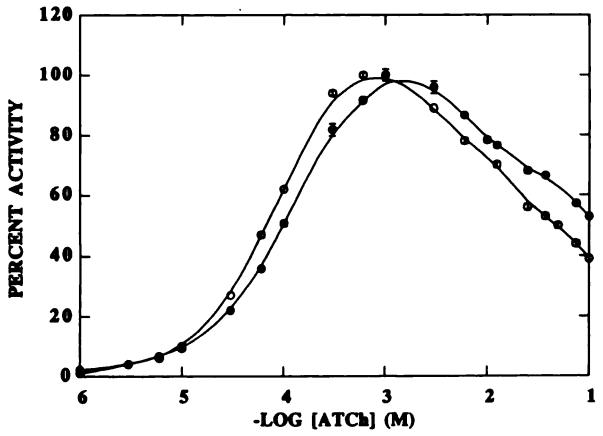

Fig. 3. Activity of *Torpedo* and chicken AChE as a function of substrate concentration. Enzymic activity of tissue aliquots was measured colorimetrically, using ATCh as substrate, according to the method of Ellman *et al.* (25). The data are expressed as a percentage of the maximal activity observed for each enzyme species. Each *point* represents the mean ± standard error of triplicate samples. ○, *Torpedo* electric organ; ●, chicken brain.

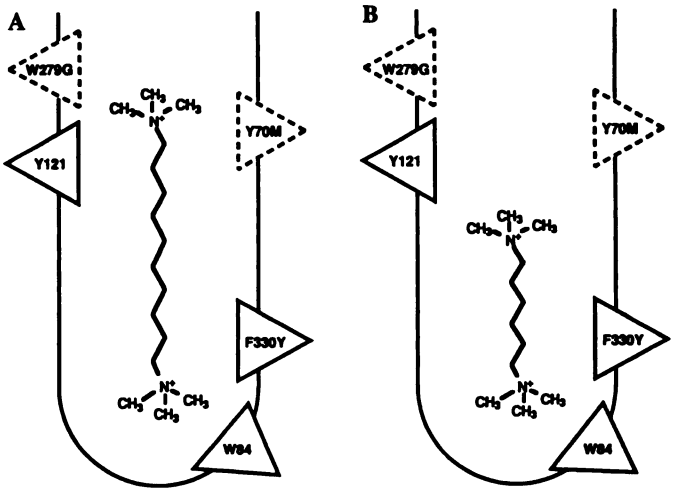

Fig. 4. Schematic representation of the interactions of decamethonium and hexamethonium with aromatic residues in the active-site gorges of *Torpedo* and chicken AChE. A, Decamethonium; B, hexamethonium. The active site is near the *bottom* and the mouth of the gorge is at the *top*. *Solid triangles*, aromatic residues conserved in AChE from the two species; *dashed triangles*, aromatic residues found only in *Torpedo* AChE. The residue numbers, as in the text, are for the *Torpedo* enzyme. The single-letter amino acid code is used; *letters to the left of the residue numbers* are for *Torpedo* and those to the *right*, where different, are for chicken.

had essentially no effect on substrate inhibition. Thus, both the comparison of chicken and *Torpedo* AChE and the data on the mouse AChE mutants argue against the involvement of the peripheral site, or at least the aromatic residues that contribute substantially to it, in substrate inhibition.

Effect of bisquaternary agents. Bisquaternary agents are known to be more potent inhibitors of AChE than are their corresponding monoquaternary counterparts (16, 32). This enhanced inhibitory capacity has been ascribed to their ability to span the peripheral site and the anionic subsite of the active site (33). Table 1 shows that chicken brain AChE was inhibited approximately 100-fold less effectively than *Torpedo* AChE by the bisquaternary compound BW284c51, which is used as a selective inhibitor of AChE in the presence of butyrylcholinesterase (34). Reduced sensitivity of chicken AChE, relative to mammalian AChEs, was reported earlier for related bisquaternary compounds (35). A similar change in K_i was also observed for decamethonium, another bisquaternary compound (in which the two quaternary groups are separated by 10 methylene groups), on comparison of the chicken and *Torpedo* enzymes. In contrast, hexamethonium, which is homologous to decamethonium but contains only six methylene groups in the spacer between the two quaternary groups, inhibited *Torpedo* and chicken brain AChE very similarly (Table 1). For both BW284c51 and decamethonium, the structural data obtained from X-ray crystallography of the corresponding *Torpedo* AChE-ligand complexes (9, 10)[3] clearly show that the ligands, as expected, bridge the two anionic sites, being in proximity to the aromatic rings of Trp-84 and Phe-330, at the bottom of the gorge, and to those of Tyr-70 and Trp-279, near the top of the gorge. This is depicted schematically for decamethonium in Fig. 4A, whereas Fig. 5A shows the actual crystal structure of the ligand-AChE complex in the vicinity of the bound ligand (9). The quantitative study of Bergmann and Segal (33) showed that a critical separation of the quaternary groups was necessary to obtain enhanced inhibition, relative to the corresponding monoquaternary compounds, and that hexamethonium was a much less effective inhibitor of human erythrocyte AChE than was decamethonium. Although we do not, as yet, have X-ray data for the complex of hexamethonium with *Torpedo* AChE, manual docking based on the X-ray structure of the decamethonium complex clearly reveals that hexamethonium is too short to span the two anionic sites and thus to benefit from this possibility. Most likely, it binds only at the anionic subsite of the active site, as shown schematically in Figs. 4B and 5B. This also explains why the absence of two aromatic residues in the peripheral site of chicken AChE has little effect on the K_i of hexamethonium.

Analysis of Lineweaver-Burke plots for inhibition of *Torpedo* AChE by BW284c51 and decamethonium indicated that inhibition was of a noncompetitive nature, whereas inhibition of the chicken enzyme was, in both cases, of a mixed type (data not shown). This difference may be ascribed tentatively to the lack of a strong binding site for the distal quaternary groups of these bisquaternary ligands at the top of the active-site gorge of chicken AChE.

A biological role for the peripheral anionic site of AChE has yet to be determined. The turnover number of the chick enzyme with ATCh is only slightly lower than that of the *Torpedo* enzyme (36). Thus, substantial impairment of the peripheral site has no direct effect on catalytic activity under physiological conditions.

Bisquaternary compounds such as decamethonium serve as potent neuromuscular agents whose pharmacology has, accordingly, been investigated extensively (37). In this system, also, their efficacy varies strongly as a function of the number of methylene groups in the spacer. Thus, a distance of approximately 12 Å between the quaternary groups, corresponding to 10–12 methylene groups, seems optimal for agonist action (38), whereas shorter compounds, such as hexamethonium, act as antagonists (39). In the electroplax of *Electrophorus electricus* too, decamethonium is an activator of the nicotinic receptor,

[3] M. Harel, I. Silman, and J. L. Sussman, unpublished obeservations.

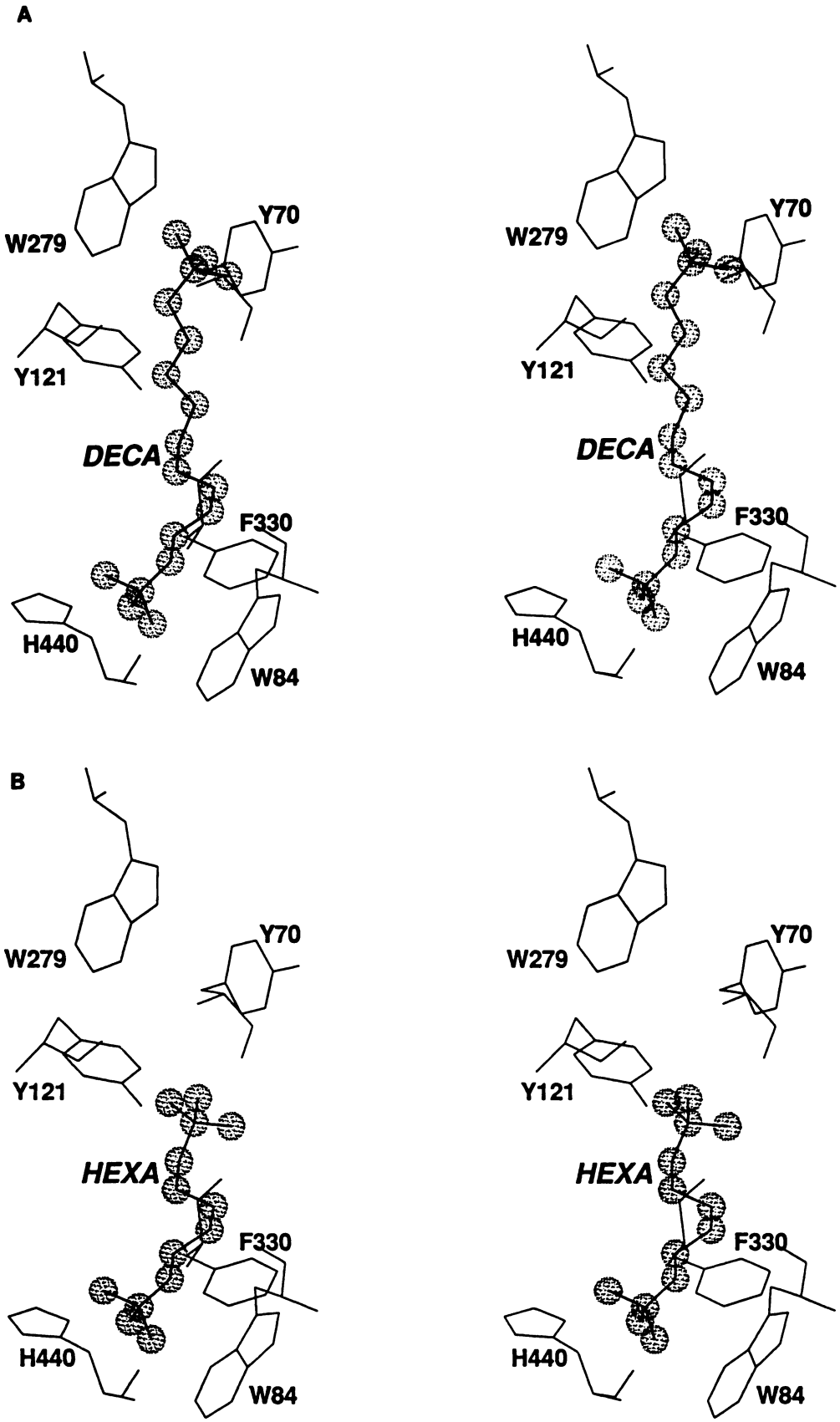

Fig. 5. Stereo representations of decamethonium and hexamethonium in the active-site gorge of *T. californica* AChE. A, The decamethonium-AChE structure represents a portion of the crystal structure of this complex, including the decamethonium-binding region (9). B, The hexamethonium model was constructed by deleting the upper four methylene groups of the decamethonium structure (A) and docking the upper quaternary group of decamethonium to the remainder of the structure, using the program FRODO (44). The aromatic groups displayed are the same as those shown in the schematic diagram (Fig. 4); in addition, the active site histidine, His-440, is shown as a reference point. Decamethonium (*DECA*) and hexamethonium (*HEXA*) are displayed as ball and stick models.

whereas hexamethonium is a blocker (40). Although there is no obvious sequence homology or structural similarity between AChE and the nicotinic ACh receptor, it is tempting to speculate that their ACh binding sites may display some similarity (41). Photoaffinity labeling studies have shown the presence of several aromatic rings close to the ACh binding site of the ACh receptor of *Torpedo* (42), and changes in labeling patterns observed subsequent to desensitization suggest that these rings may play a functional role in the regulation of ligand-gated channels (43). Thus, the apparent ability of decamethonium, but not hexamethonium, to span two functional sites in both AChE and the ACh receptor may hint at underlying structural and functional similarities between these two cholinergic proteins.

Acknowledgments

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