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Anionic subsites of the catalytic center of acetylcholinesterase from *Torpedo* and from cobra venom

(serine esterase family/anionic binding site/affinity labeling/aziridinium/peripheral binding site)

HANS-JÜRGEN KREIENKAMP*, CHRISTOPH WEISE*, RAIVO RABA†, AAVO AAVIKSAAR†, AND FERDINAND HUCHO*‡

*Institut für Biochemie, Freie Universität Berlin, Thielallee 63, 1000 Berlin 33, Federal Republic of Germany; and †Biochemistry Department, Institute of Chemical Physics and Biophysics of the Academy of Sciences of the Estonian Soviet Socialist Republic, Tallinn, Estonia, U.S.S.R.

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ABSTRACT **A peptide of acetylcholinesterase (AcChoEase; acetylcholine acetylhydrolase, EC 3.1.1.7) from the venom of the cobra *Naja naja oxiana* labeled by the affinity reagent *N,N*-dimethyl-2-phenylaziridinium (DPA) has been identified. The sequence is Gly-Ala-Glu-Met-Trp-Asn-Pro-Asn. In AcChoEase from *Torpedo californica*, a homologous peptide was labeled and isolated. Its sequence is Ser-Gly-Ser-Glu-Met-Trp-Asn-Pro-Asn, representing positions 79 through 87. In both cases labeling can be prevented by 0.1 mM edrophonium, indicating that the respective peptides form part of the anionic subsite of the catalytic center. The modified residue was tryptophan (Trp-84 in *Torpedo* AcChoEase) in both enzymes. In contrast to AcChoEase from *Torpedo*, the enzyme from cobra venom does not contain a peripheral anionic binding site.**

Based on sequence comparisons, the cholinesterases (1, 2) have recently been defined as members of a superfamily of related enzymes (3). This group includes hepatic microsomal carboxylesterase (4), cholesterolesterase (5), lysophospholipase (6), the *Drosophila* esterase-6 (7), juvenile hormone esterase (8), and two esterases found in inclusion bodies in *Dictyostelium* (9). Nonenzymatic members of the family are the carboxyl-terminal domain of thyroglobulin (10) and the recently identified cell adhesion protein neurotactin (11).

The catalytic mechanism of all enzymes of this family is believed to be similar; it is widely assumed that it involves a catalytic triad similar to the one present in the active site of the serine proteases and of some phospholipases; this view, however, is still controversial (12). In many cases the physiological substrate is unclear. The best-understood member of the cholinesterase family is acetylcholinesterase [AcChoEase; acetylcholine (AcCho) acetylhydrolase, E.C. 3.1.1.7], one of the key proteins in chemical nerve impulse transmission at cholinergic synapses. The primary structure of AcChoEase from different organisms has been derived from cloned cDNAs (1, 13–17). Most investigations so far have focused on the enzyme from the electric organ of *Torpedo*. Another rich source is the venom of a variety of elapid snakes (18). Preliminary sequence analysis has shown that the primary structure of AcChoEase from the venom of the cobra *Naja naja oxiana* is highly homologous to the enzyme from *Torpedo* (19).

The tertiary structure of AcChoEase based on x-ray analysis of the crystalline protein (20) is not known yet. The active-site serine has been mapped to position 200 in the enzyme from *Torpedo* electric organ (21). The histidine residue involved in catalysis has been shown to be His-440 (22). Especially the sequence around the active-site serine is highly conserved in all enzymes mentioned above.

Comparatively little is known about those domains of the protein that are involved in substrate binding and thus confer substrate specificity to the different enzymes of the esterase family. In AcChoEase the anionic subsite in the catalytic center is involved in binding the positively charged cholinium moiety of the substrate acetylcholine (23). In addition to the anionic subsite in the catalytic center, so-called peripheral binding sites have been postulated (24).

To date, these ligand binding sites have been characterized by enzyme kinetics and binding studies. Recently we used the affinity reagent *N,N*-dimethyl-2-phenylaziridinium (DPA) to localize regions in the primary structure of *Torpedo* AcChoEase that are involved in the binding of positively charged ligands (25). DPA combines the features of a quaternary ammonium ion with high electrophilic reactivity (26). It is an irreversible inhibitor of cholinesterases (27). Two labeled AcChoEase sequences have been identified, one apparently being a component of the peripheral anionic subsite, and the other, of the catalytic-center anionic subsite (25).

Here we report on further elucidation of the active site of the *Torpedo* enzyme by isolating and sequencing a peptide of the anionic subsite labeled by DPA. The homologous peptide from AcChoEase from venom of the cobra *Naja naja oxiana* was identified by the same method. These results allow conclusions as to the structure of the substrate binding site and the postulated "anionic" nature of this site.

The physiological function of the peripheral AcCho binding sites is not clear. Since it can be shown to exist only at low ionic strength, the possibility of a regulatory function has been questioned (28). Here we compare the DPA-labeling pattern of anionic subsites of AcChoEase from *Torpedo* and cobra venom and examine whether differences can be correlated with catalytic parameters.

MATERIALS AND METHODS

Preparation of AcChoEase. The membrane-bound G2 form of AcChoEase was released from *Torpedo* electroplax membrane by cleavage of the phospholipid anchor with phosphatidylinositol-specific phospholipase C from *Bacillus thuringiensis* (29), kindly supplied by F. Götz (Tübingen, F.R.G.). The enzyme was further purified by affinity chromatography on *N*-methylacridinium-Sepharose (30). The protein was eluted with 5 mM decamethonium and dialyzed against 50 mM sodium phosphate buffer (pH 7.0). This procedure yielded ≈2 mg of homogeneous G2 form from 100 g of electric tissue.

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Abbreviations: AcCho, acetylcholine; AcChoEase, acetylcholinesterase; DPA, *N,N*-dimethyl-2-phenylaziridinium; >PhNCS, phenylthiohydantoin.

‡To whom reprint requests should be addressed.

Cobra venom AcChoEase was prepared from *Naja naja oxiana* venom (Tashkent Integrated Zoo Plant, U.S.S.R.) by affinity chromatography as described (31).

Measurement of AcChoEase Activity. AcChoEase activity was measured photometrically with acetylthiocholine as substrate as described by Ellman *et al.* (32). Substrate inhibition was investigated by varying concentrations of acetylthiocholine. Inhibition by propidium was measured with a radiometer pH-stat and AcCho as substrate.

Synthesis of [^{3}H]DPA. Tritiated *N,N*-dimethyl-2-hydroxy-2-phenylethylamine was synthesized in the Institute of Molecular Genetics (Academy of Sciences of the USSR, Moscow) by palladium-catalyzed tritium exchange from the *p*-bromophenyl compound. Treatment with $SOCl_2$ led to *N,N*-dimethyl-2-chloro-2-phenylethylamine. This substance was stored in dry ethanol at a concentration of 0.23 mM. The radiochemical purity was 95.7% as determined by TLC. From this compound, the aziridinium compound DPA forms spontaneously in aqueous medium.

Affinity Labeling of AcChoEase with [^{3}H]DPA. Affinity labeling was performed in 50 mM sodium phosphate (pH 7.0). The protein concentration was usually in the range of 1–1.5 mg/ml in a final reaction volume of 1 ml. [^{3}H]DPA was added directly from the stock solution so that the final ethanol concentration was below 5%. The sample was adjusted to 1 mM DPA with nontritiated compound. The final specific radioactivity was 127,000 cpm/nmol. The reaction was allowed to proceed for 6 hr; after this time all reagent should be decomposed (33). The incorporated radioactivity and residual enzyme activity were determined after removal of free label by gel filtration on Sephadex G-25. For control experiments with various AcChoEase inhibitors, the protein concentration was 0.32 mg/ml in a final reaction volume of 200 μl.

Proteolytic Cleavage. Reduction, carboxymethylation, and proteolytic cleavage with trypsin and chymotrypsin were performed as described (19).

Peptide Separation. AcChoEase digests were loaded directly onto a reverse-phase HPLC column (Vydac C_{18}, 250 × 4.6 mm). Peptides were eluted with a linear gradient from 0 to 50% organic phase (acetonitrile containing 0.1% CF_3COOH) in 150 min. Radioactive peaks were rechromatographed on a shallow gradient (slope of 1% organic phase in 4 min).

Protein Sequencing. Protein sequencing was performed on a Knauer model 810 modular sequencer, equipped with an on-line HPLC system for detection of phenylthiohydantoin (>PhNCS)-conjugated amino acids. One-third of the degradation products of each cycle was monitored for radioactivity by scintillation counting.

RESULTS

Inactivation of AcChoEase by DPA. DPA was shown to be a potent inhibitor of AcChoEase from *Torpedo* electric organ (25) and cobra venom, both of which were irreversibly inactivated by treatment with 1 mM DPA. The activity was reduced to 25% of the initial value for *Torpedo* AcChoEase (25) and to 1% in the case of cobra AcChoEase (Table 1).

Incorporation of [^{3}H]DPA and inactivation of cobra AcChoEase can be partially prevented by 0.1 mM edrophonium. Decamethonium (1 mM) was much less effective, while the peripheral-site ligand propidium did not protect the enzyme against inactivation (see Table 1). This is in contrast to the results observed with the *Torpedo* enzyme, where decamethonium had the strongest protecting effect (25).

Identification of Radiolabeled Peptides in *Torpedo* AcChoEase. As reported (25), reverse-phase HPLC separation of a tryptic/chymotryptic digest of [^{3}H]DPA-modified *Torpedo* AcChoEase yielded four radioactive peaks on a rather high radioactive background representing unspecific labeling. We describe here further characterization of peptide II (according to the numbering in ref. 25).

Table 1. Protection of DPA incorporation and enzyme inactivation of AcChoEase from Cobra venom by specific AcChoEase inhibitors

Inhibitor	Inhibitor, mM	Activity*	DPA incorp.†
None (control)	—	1	2.12
Decamethonium	1.0	38	1.89
Edrophonium	0.1	54	1.46
Propidium	0.1	3	1.85

*Percent of untreated enzyme.
†Moles of DPA incorporated per mole of AcChoEase catalytic subunit.

Fractions containing peak II were individually rechromatographed; in each case radioactivity was eluted in one fraction, part of which was sequenced. The sequence was Ser-Gly-Ser-Glu-Met-Trp-Asn-Pro-Asn in each case, corresponding to positions 79–87 in the published *Torpedo* AcChoEase sequence (1). In the sixth step—Trp-84 in the primary structure—no >PhNCS signal could be obtained, whereas the major portion of the applied radioactivity was eluted from the sequencer (see Table 2). The amount of radioactivity released in the sixth degradation cycle corresponded to the amount of >PhNCS-amino acid in the other steps. This indicates that Trp-84 is the labeled amino acid. As reported (25), modification of this peptide (*Torpedo* peptide II) could be prevented by decamethonium and edrophonium, but not by propidium.

Identification of Radiolabeled Peptides in Cobra Venom AcChoEase. With AcChoEase from cobra venom, the pattern of labeled peptides was different from the one observed with

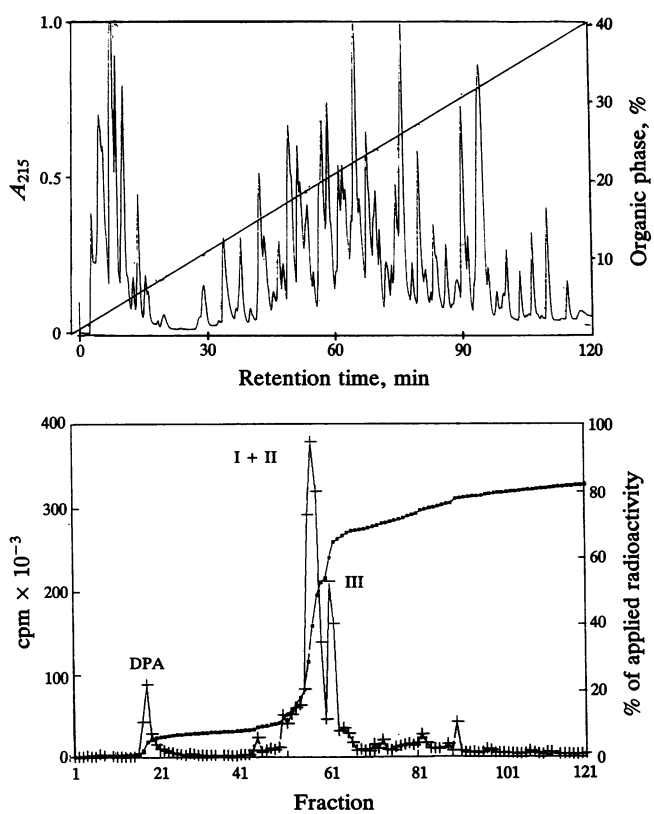

FIG. 1. HPLC separation of fragments of [^{3}H]DPA-labeled AcChoEase from cobra venom generated by combined tryptic and chymotryptic cleavage; the UV (*Upper*) and the radioactivity (*Lower*) profiles are shown.

Table 2. Results of Edman degradation of [^{3}H]DPA-labeled peptides of AcChoEase from *Torpedo californica* and cobra venom

	Torpedo peptide II peak			Cobra peptide peaks								
				I			II			III		
Step	Amino acid	pmol	cpm	Amino acid	pmol	cpm	Amino acid	pmol	cpm	Amino acid	pmol	cpm
1	Ser	ND	831	Glu	Out	572	Many	ND	893	Glu	60	828
2	Gly	ND	398	Met	135	342	Gly	ND	720	Met	47	466
3	Ser	ND	322	—	[147]	19163	Ala	99	560	—	[54]	7014
4	Glu	25	355	Asn	142	3566	Glu	82	457	Asn	37	1415
5	Met	13	289	Pro	91	131	Met	55	387	Pro	29	573
6	—	[8.4]	1102	Asn	53	653	—	[64]	8323	Asn	37	426
7	Asn	10	397	—	—	436	Asn	81	2014	—	—	290
8	Pro	6.4	200	—	—	382	Pro	32	950	—	—	222
9	Asn	3.2	152	—	—	—	Asn	45	630	—	—	—

The amount of >PhNCS-amino acid (pmol) and of radioactivity (cpm) released in each cycle is indicated. Some amino acids including serine and glycine are not calibrated (ND, not determined). "Out" means out of calibration range. The picomole values given in brackets were calculated from the radioactivity. The numbering of cobra AcChoEase peptides corresponds to that in Fig. 1 and of *Torpedo* AcChoEase peptides corresponds to that in ref. 25.

Torpedo AcChoEase. Here combined tryptic and chymotryptic cleavage of the [^{3}H]DPA-modified enzyme revealed two major peaks of peptide-bound radioactivity (Fig. 1). The first, rather broad peak could be resolved into two peaks by rechromatography. The resulting three peaks (peptides I–III) contained 20%, 10%, and 10% of the total protein-bound radioactivity, respectively. Labeling of all three peptides was prevented by 1 mM edrophonium (data not shown). Edman degradation of proteins I and III yielded the sequence Glu-Met-Xaa-Asn-Pro-Asn, where Xaa is an unknown amino acid. With the third step radioactivity was released, while no >PhNCS signal could be observed. The sequence of protein II was Xaa-Gly-Ala-Glu-Met-Xaa-Asn-Pro-Asn. The first amino acid could not be unambiguously identified because of high background; in the sixth step there was no >PhNCS signal, and radioactivity was released with this step (Table 2). The data indicate that peptide II overlaps peptides I and III. The appearance of peak II is obviously due to incomplete chymotryptic cleavage of the Ala-Glu bond. The labeled sequence Glu-Met-Xaa-Asn-Pro-Asn is identical in both esterases; therefore, it is very likely that the labeled residue is also a tryptophan in cobra venom AcChoEase.

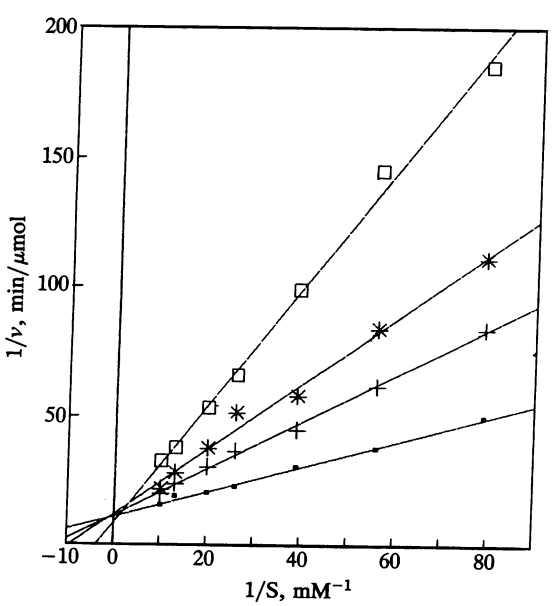

FIG. 2. Lineweaver–Burk plot of inhibition of AcChoEase from cobra venom by propidium. ■, Without inhibitor; +, with 1.05 μM propidium; *, with 2.1 μM propidium; □, with 4.2 μM propidium. S, substrate concentration; v, velocity.

Propidium Inhibition of Cobra Venom AcChoEase. With the *Torpedo* enzyme, one labeled peptide was identified as part of the peripheral anionic site by protection experiments with propidium. The complete absence of the corresponding labeled peptide in cobra venom AcChoEase led us to ask whether there is any peripheral site at all in this enzyme. Inhibition experiments with propidium and AcCho as substrate at low ionic strength gave Lineweaver–Burk plots that are typical for pure competitive inhibition (Fig. 2); the K_i was 4×10^{-6} M. In contrast, Taylor and Lappi (34) showed that with the *Torpedo* enzyme, propidium is a noncompetitive inhibitor and binds to the peripheral anionic site with a K_i of 3.2×10^{-7} M at low ionic strength. This may indicate that there is no peripheral anionic site in cobra venom AcChoEase.

Substrate Inhibition of AcChoEase. To substantiate this conclusion, we investigated the substrate dependence of the catalytic activity with both the *Torpedo* and the cobra enzyme. We compared the degree of substrate inhibition, which is well documented for *Torpedo* AcChoEase and for mammalian AcChoEases (32). There was clear substrate inhibition in the case of the *Torpedo* enzyme (Fig. 3), beginning at an acetylthiocholine concentration of 1 mM. On the other hand, cobra venom AcChoEase showed such an effect, albeit less pronounced, only at 10 mM acetylthiocholine.

DISCUSSION

The catalytic center of AcChoEase is believed to be composed of an esteratic (the site of ester hydrolysis) and an anionic subsite (23). We recently reported that AcChoEase

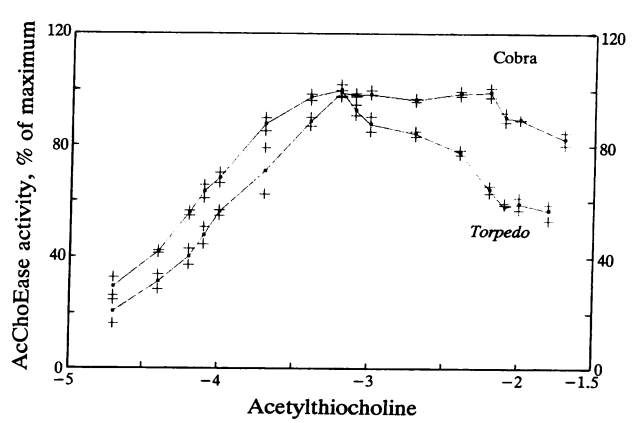

FIG. 3. Dependence of enzymatic activity on substrate concentration for cobra venom and *Torpedo* AcChoEase.

from *Torpedo californica* can be specifically labeled by tritiated cationic affinity label DPA (25). Two modified radioactive peptides were isolated and sequenced. The labeling of the first peptide, Asp-Leu-Phe-Arg (positions 217–220), could be prevented by edrophonium. The peptide is therefore assumed to be part of the anionic subsite of the catalytic center. A second radioactive peptide had the sequence Lys-Pro-Gln-Glu-Leu-Ile-Asp-Val-Glu (positions 270–278). Modification of this peptide could be prevented by propidium, which is a specific ligand of the peripheral anionic site.

In this study we show that a third labeled peptide from *Torpedo* AcChoEase has the sequence Ser-Gly-Ser-Glu-Met-Xaa-Asn-Pro-Asn (positions 79–87). The labeled amino acid was identified as Trp-84. Labeling of this peptide was sensitive to edrophonium, identifying it as a second peptide (along with Asp-Leu-Phe-Arg) forming part of the anionic site in the catalytic center of *Torpedo* AcChoEase; we call this *Torpedo* peptide II in Table 2.

One mole of AcChoEase from cobra venom incorporates 2.12 mol of DPA. About 40% of the peptide-associated radioactivity is sensitive to edrophonium. The remaining 60% can be considered as unspecific binding of the highly electrophilic reagent; in HPLC separation, this gives a high background of radioactivity. The protective effect of decamethonium and propidium is much weaker with cobra venom AcChoEase than with *Torpedo* AcChoEase. This is in agreement with the reduced affinity of those substances for cobra AcChoEase. [We found a K_i of 1.4×10^{-5} M for decamethonium at 0.1 M NaCl/phosphate buffer (pH 7.8); data not shown.]

From digests of AcChoEase from cobra venom, we separated by HPLC three labeled peptides, labeling of which could be prevented by edrophonium, a compound binding to the anionic subsite of the catalytic center. Two of these peptides (peptides I and III) contained the same sequence, Glu-Met-Xaa-Asn-Pro-Asn, while a third peak (peptide II) contained the overlapping peptide Xaa-Gly-Ala-Glu-Met-Xaa-Asn-Pro-Asn. As in *Torpedo* AcChoEase, the labeled amino acid residue was probably tryptophan. We conclude that the peptide around Trp-84 is essentially contributing to the anionic binding site within the catalytic center in *Torpedo* AcChoEase as well as in cobra venom AcChoEase. The sequence Glu-Met-Xaa-Asn-Pro-Asn is completely conserved in all vertebrate cholinesterases sequenced so far. It is absent from the other members of the serine esterase family.

Recently replacement of Asp-70 by glycine in human butyrylcholinesterase (homologous to Asp-72 in *Torpedo* AcChoEase) was reported to disrupt anionic site interactions (35, 36). There is converging evidence that the region around this residue and Trp-84, both contained in the first disulfide loop of AcChoEase (positions 67–94), is mainly contributing to the cholinium binding site of cholinesterases.

In cobra AcChoEase, no peptide corresponding to the sequence Asp-Leu-Phe-Arg (positions 217–220 in Torpedo AcChoEase) was labeled by DPA. The precise reason for this difference is unclear, since the complete primary structure of the cobra enzyme is not known yet. This part of the sequence is not conserved throughout the cholinesterase family. The difference in the labeling pattern might reflect a modification in the architecture of the catalytic center (Fig. 4).

The existence of a tryptophan residue at the active site was anticipated from fluorescence studies (38). This is especially interesting in the light of observations from "host–guest–chemistry" (39), where it could be shown that macrocyclic compounds composed of polar and aromatic fragments can bind quaternary ammonium ions with rather high affinity (40–42). In the nicotinic AcCho receptor from *Torpedo*, several aromatic amino acid residues have been identified by affinity labeling that are part of the AcCho-binding region

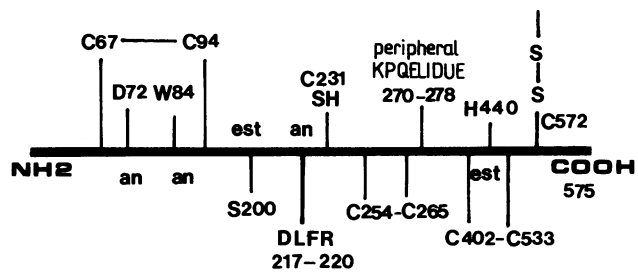

FIG. 4. Molecular organization of AcChoEase from *T. californica*; the scheme includes residues involved in binding at the esteratic site (est; refs. 21 and 22), at the anionic site of the catalytic center (an; refs. 25, 35, and 36, and this work), and at the peripheral anionic site (25). The disulfide bridges are shown as in ref. 37. Amino acids are indicated in single-letter code.

(43–45). In the case of the phosphocholine-binding antibody McPC603, the presence of tyrosine and tryptophan residues in the choline-binding pocket was shown directly by x-ray crystallography (46). The contribution of aromatic amino acid residues to the formation of "anionic" binding sites like the AcCho binding site of AcChoEase might turn out to be a more general feature.

It should be mentioned that DPA, as a very electrophilic reagent, could have reacted with a carboxylate side chain as well. Such an ester linkage could possibly be too labile to survive HPLC separation and Edman degradation. The early peak in the HPLC chromatogram identified as free DPA might result from the decomposition of such an unstable reaction product. In the case of the cobra venom Glu-Met-Xaa-Asn-Pro-Asn peptides I and III, it seems improbable from the amount of radioactivity released in the sequencer steps (Table 2) that an amino acid residue other than Trp-84 is modified to an appreciable extent.

In the case of AcChoEase from the electric organ of *T. californica*, the existence of an additional peripheral anionic binding site is well documented (24, 25). A variety of uncompetitive AcChoEase inhibitors of which propidium is typical are assumed to associate specifically with this binding site (34). The apparent binding affinity of the peripheral site is reduced at high ionic strength, possibly because of competition between the cationic ligand and the inorganic cations. On that ground, the physiological relevance of these findings was questioned (28).

The effect of propidium on AcChoEase from cobra venom is rather different. Here this compound acts as a purely competitive inhibitor, and the binding at low ionic strength is reduced by >1 order of magnitude as compared with the *Torpedo* enzyme. From this we assume that the peripheral site is absent from AcChoEase from cobra venom or has a very low affinity. Interestingly, the inhibitory constant for cobra venom AcChoEase at low ionic strength ($K_i = 4 \times 10^{-6}$ M) coincides with the value for *Torpedo* AcChoEase measured at high ionic strength ($\Gamma/2 = 0.225$, $K_i = 3.8 \times 10^{-6}$ M) (34). This low-affinity binding might be explained by binding of propidium to the active center in both enzymes.

Differences in DPA labeling of the peripheral site can also be seen at the peptide level. In *Torpedo* AcChoEase the labeling of a peptide with the sequence Lys-Pro-Gln-Glu-Leu-Ile-Asp-Val-Glu could be prevented by propidium (25); in accordance with the kinetic findings, such a peptide was totally missing in AcChoEase from cobra venom. The fact that decamethonium very poorly protected the enzyme from inactivation by DPA also supports the concept that there is only one anionic site in cobra venom AcChoEase. Decamethonium is supposed to bridge both anionic sites with its two quaternary ammonium groups in *Torpedo* AcChoEase (47). The absence of one of those sites results in weaker binding.

Recently it has been argued that the peripheral site is involved in substrate inhibition (22). Binding of ligands at the peripheral site might induce a conformational change so that the correct alignment of the amino acid residues of the catalytic triad is altered (48). Recent mutagenesis experiments indicated that Glu-199 adjacent to the active serine is also important in this conformational change (22). Preliminary sequence data indicate that AcChoEase from *Torpedo* electric organ and from cobra venom are very similar (19). Despite this similarity and identical substrate specificity, the two enzymes differ in both the occurrence of a peripheral binding site and substrate inhibition behavior. A similar coincidence is known for the butyrylcholinesterases, which (*i*) cannot be shown to contain a peripheral binding site and (*ii*) do not exhibit substrate inhibition (49). It is noteworthy that propidium binding can be demonstrated only at low ionic strength, whereas substrate inhibition is also observed at physiological ionic conditions. The physiological relevance of both phenomena remains to be shown.

From the labeling experiments reported here and previously (25), a picture of the anionic subsite of AcChoEase emerges that describes an aromatic contribution to this site in addition to its being negatively charged. While the active site of cobra AcChoEase seems to be rather similar to that of the *Torpedo* enzyme, no evidence could be obtained for the existence of a peripheral anionic site in the AcChoEase from the snake venom.

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