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CHOLINESTERASES AND CHOLINERGIC RECEPTORS

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GENERAL DISCUSSION

ON MODELS FOR THE LIGAND BINDING SITES OF CHOLINESTERASES

E. Reiner:

During this meeting several models for the binding sites of cholinesterases have been postulated, and some of the older models discussed again. At the end of this meeting it might therefore be of interest to summarize the characteristics of these models trying to explain whether the models account for the difference between acetylcholinesterase and cholinesterase, for the mechanism of substrate inhibition, for regions peripheral to the active site, for the polarity of the binding sites and their cooperativity.

W. N. Aldridge:

I have presented in my paper a rather simple and perhaps superficial model for the way substrates and inhibitors orientate with the catalytic centre of acetylcholinesterase. My reasons for doing this are twofold. First, it is obvious that the surface presented by the flexible tertiary structure of a protein is bound to be heterogeneous and somewhat variable. Only the major contributing factors to binding and activity may be evaluated. My second reason is that the present model involving an esteratic and an anionic site is in some ways unsatisfactory.

I have stated in my paper that I have attempted to reconcile in one model results obtained from rather different approaches and these have been listed. The ground rules for the development of this model have been: (1) Acetylcholinesterase is a very unspecific enzyme. (2) The esteratic area can operate on its own — i. e. without ligands binding to sites outside the area. This view is derived from the catalysis of hydrolysis of small substrates and inhibition by small inhibitors. (3) The esteratic area in eel and erythrocyte cholinesterase is restricted in size, e. g. substrates containing acyl groups larger than propionyl are poorly hydrolysed. (4) Acceleration of the reactions of small substrates or inhibitors is achieved by change in their distances for reacting groups in the esteratic area and/or by the extrusion of water from it. (5) Many of the substances which react readily with acetylcholinesterase are uncharged, e. g. many organophosphorus compounds, carbamates, sulphonates, substrates and reversible inhibitors. (6) The main determinants for reaction are the esteratic site and the hydrophobic area (*cf. Aldridge, these Proceedings,* p. 229). The hydrophobic area contains determinants for the structural specificity of binding. (7) The contribution of electrostatic forces to binding may be quite small and I consider that the emphasis on such binding implied by the terminology »the anionic site« to describe the substrate and inhibitor specificity of acetylcholinesterase should be much reduced. (8) The hydrophobic area is involved in substrate inhibition.

W. H. Hopff:

Our model is fundamentally based on the original Wilson model. The approach for the characterization of the active site was a chemical structure/activity approach. The »crevices« at the anionic and esteratic centres were evaluated by sterically modified acetylcholine homologues and represent limitations which are exact measurements of atomic distances with the aid of Dreiding models.

Additional to the anionic and esteratic centre of the Wilson model, there is strong evidence, that the esteratic oxygen of acetylcholine is bound by a δ^-/δ^+ bond to the enzyme as we have already pointed out. As I have mentioned earlier, some features of our model are still under investigation.

For substrate inhibition we consider two possibilities: The first assumes an acetylcholine molecule to place its »head« in the anionic crevice of an acetylated enzyme thus disturbing adequate hydrolysis of the acetyl group. Another possibility is, that two acetylcholine molecules approach the anionic centre at the same time.

They both might be in equilibrium with respect to charge and distance with the negative charge of the anionic centre. One of these two molecules could eventually get in the right position for hydrolysis, but would be hampered by the second molecule.

The major advantage of our model(s) is the differentiation between acetylcholinesterase and cholinesterase. To our knowledge there is no evidence of any phenomenon that could not be explained with our model(s) in this respect. In view of the fact, that we are dealing with two completely different enzymes, there have to be two different models. Although the two enzymes have some characteristics in common, one model in our view can never explain the described different structure/activity relations.

The most important feature of our model(s) (*cf.* Hopff *et al., These Proceedings,* p. 316) however deals with space limitations at the active centres. The fact that the hydrolysis of acetoxyethyl-*N,N,N*-tripropylammonium iodide is no longer catalyzed by both enzymes, but in the presence of acetylcholine is a fairly good inhibitor, may be explained only with our model(s).

P. W. Taylor:

While models offer a useful framework for designing experimental protocols, they often prove deficient in providing a precise description of three dimensional structure of a macromolecule's active site. An obvious means of obviating this difficulty is to have available the crystal structure of the enzyme. Short of this, structures of the active centers of related enzymes may provide a basis from which the site can be modeled with more fidelity. It is evident that substantial similarities in catalytic mechanism prevail when acetylcholinesterase is compared with other serine hydrolases and the behavior of the serine hydrolases begin to diverge mainly when substrate specificity is considered. For example, in these enzymes similar sequences exist around the catalytic serine (Schaffer *et al., Biochemistry* **12** (1973) 2946), a charge relay system confers greater nucleophilicity to the catalytic serine (Blow *et al., Nature* **221** (1969) 337), and an oxyanion hole tends to stabilize the tetrahedral transition state of the carbonyl esters (Robertus *et al., Biochemistry* **11** (1972) 4293). Chemical modification and transition state analogue studies also illustrate that these mechanistic features are intrinsic to acetylcholinesterase (Froede and Wilson, in: P. Boyer (Ed.), *The Enzymes,* Vol. 5, Academic Press, New York 1971, pp. 87—114; Lienhard, *Science* **180** (1973) 149). This information, when coupled with data on substrate or inhibitor specificity, enables one to add dimensional properties to a description of the active center of acetylcholinesterase.

For ester hydrolysis, the transesterification step involving formation of the acyl enzyme proceeds through a tetrahedral transition state or adduct. In this state the carbonyl bond is stabilized through hydrogen bond donation from two amide bonds which tends to increase the electrophilic character of the carbonyl carbon (Robertus, *et al. Biochemistry* **11** (1972) 4293). The high reactivity of the alkylphosphates must, in part, be a consequence of their tetrahedral geometry and, hence, similarity to a true transition state analogue. Thus, the phosphoryl oxygen would be directed towards the oxyanion hole and would serve as a hydrogen bond acceptor. Accordingly, one of the two alkoxy or alkane groups bonded to the tetrahedral phosphorus would be directed towards the substrate acyl pocket while the other would be oriented in the direction of the anionic site stabilizing the quaternary group of choline (*cf.* Taylor *et al., these Proceedings* p. 256). An orientation similar to phosphoryl enzyme should also prevail with the alkanesulfonyl enzymes since the two sulfone oxygens and the alkane group are tetrahedrally disposed around the sulfur. However, the alkane group has two potential orientations since either of the two oxygens could be directed towards the oxyanion hole. Steric considerations would govern the preferred orientation. Constraints on fit in the acyl pocket are evident from substrate specificity experiments. Substrate affinity of butyrylcholine is considerably reduced from acetyl or propionylcholine (Augustinsson, *Arch. Biochem. Biophys.* **23** (1949) 111). Sulfonylation rates by *N*-methylpyridinium esters of alkanesulfonic acids are reduced at the ethane to propane substitution (Taylor and Jacobs, *Mol. Pharmacol.* **10** (1974) 93). Finally, with bulky asymmetric alkylphosphates ste-

reospecificity in the phosphorylation reaction is seen (A a r o n *et al.*, *J. Amer. Chem. Soc.* **80** (1958) 456).

Thus, a preferred orientation of the acyl moiety is adopted in formation of the sulfonyl and phosphoryl enzymes and this feature needs to be accounted for in the model of the active site. Our fluorescence studies with bisquaternary ligand binding to the active center in the native, sulfonyl and phosphoryl enzymes (T a y l o r and J a c o b s, *Mol. Pharmacol.* **10** (1974) 93) support earlier postulates of an orientation of the second quaternary nitrogen directed away from the catalytic serine (K i t z and W i l s o n, *J. Biol. Chem.* **237** (1962) 3245). With the *bis*-methonium and *bis*-ethonium ligands, a reduction in binding energy is absent when the native acetylcholinesterase is compared with the methane and ethane sulfonyl enzymes. However, it becomes appreciable with the propanesulfonyl enzyme (T a y l o r and J a c o b s, *Mol. Pharmacol.* **10** (1974) 93). This strongly suggests that at the ethane to propane juncture the alkyl group can no longer be accommodated in the acyl pocket and bond rotation occurs where the other sulfone oxygen is directed into the oxyanion hole and the alkyl group is now oriented towards the binding site for the quaternary group. These observations are internally consistent with findings of others that show steric limitations of the acyl pocket and point to the importance of geometric considerations in the development of an active site model.

R. M. Krupka:

The main characteristic of the very simple model I have discussed (cf. K r u p k a, *these Proceedings*, p. 303) is an active center crevice which limits access of molecules larger than acetylcholine and which marks a boundary between the active center proper and adjacent regions. Numerous observations have shown the latter to be capable of adsorbing non-polar molecules, but so far as we can tell they are not involved in binding or catalysis with acetylcholine. The peripheral non-polar regions are therefore to be distinguished from non-polar residues in the crevice itself, such as those bonding with the *N*-methyl groups of the substrate, or those making up the presumptive phenyl adsorption region near the esteratic site.

At least one set of observations, involving butyrylcholine and two different types of acetylcholinesterase, has suggested that if a substrate is large enough to interact strongly with the peripheral region it does not undergo hydrolysis. Butyrylcholine is a fairly good substrate of fly acetylcholinesterase, with a V_{max} and an affinity somewhat lower than for acetylcholinesterase (both are about half), in these respects it behaves in accordance with the pattern seen in bulky acetylcholine analogs, with both fly and bovine acetylcholinesterase. On the other hand butyrylcholine is an extremely poor substrate of bovine acetylcholinesterase, which has more stringent specificity requirements for both the acyl and quaternary ammonium groups (owing probably to the crevice being smaller). Hydrolysis is now practically undetectable, but the affinity is increased, to about 5 times that for acetylcholine. Judging by the experiments with methanesulphonyl fluoride and other cationic substrates, it seems likely that the trimethylammonium group is bound in the anionic site but that the acyl chain is too long to swing down onto the esteratic site; it reaches, instead, peripheral non-polar residues near the esteratic site, where it becomes anchored. We can be reasonably sure of this location because butyrylcholine is reported to protect the enzyme against methanesulphonyl fluoride. Affinity is therefore increased, but the catalytic rate is necessarily very low.

In reaction with organophosphorus and carbamate inhibitors, however, the peripheral regions probably play quite a different role. There are many examples in the literature where addition of non-polar substituents to such compounds increases the overall reaction rate with the enzyme, either because of enhanced affinity or increased rates of reaction of the enzyme-inhibitor complex, or both. Indophenyl acetate seems to provide a model for this kind of behavior. Being too large to enter the active center, it appears to be adsorbed nearby, in a manner that for several reasons may be termed »non-specific«: (1) the attractive forces are probably hydrophobic, which are unspecific with respect to size and shape; (2) the hydrolysis rate is very low; (3) the esteratic site is not involved in the binding process; and (4) chemical modification of the enzyme in the region of the anionic site increases catalytic activity towards this substrate. The latter observation suggests that the indophenyl acetate molecule is so unfavourably positioned for catalysis that this

structural alteration, which is disastrous for other substrates, chances to improve the orientation. Coming back now to the inhibitors, these are also very poor substrates, in fact much worse than indophenyl acetate, and their normally unfavourable location in the complex is not necessarily worsened by formation of additional hydrophobic bonds in the peripheral non-polar region, and may even be improved.

The reason for distinguishing these peripheral sites from the active center proper seems self-evident, since the latter is the seat of catalysis, and is likely to be conserved during evolution, whereas the former may only play a role in enzyme tertiary or quaternary structure or possibly in interaction with the membrane, and is likely to be more mutable. Studies of the peripheral sites are of unquestionable importance with regard to the specificity and design of pesticides, but unless these regions are directly involved in the normal catalytic mechanism a knowledge of their structure may not help us to understand enzyme catalysis or even the normal function of acetylcholinesterase. It is to be hoped in any case that these proposals may soon be subjected to further experimental test.

The above discussion touches on only two of the five suggested topics: hydrophobic bonding and peripheral sites. My comments on the others are brief. (1) Regarding substrate inhibition, the model does not allow for simultaneous binding of two acetylcholine molecules, and inhibition of eel or bovine enzyme is ascribed to addition of substrate to an acetyl-enzyme intermediate. The mechanism in which two substrate molecules are bound at a single anionic site predicts strong substrate inhibition with *N,N*-dimethylaminoethyl acetate, but to the best of my knowledge this is not found. To explain inhibition of fly acetylcholinesterase the model must be expanded to include two centers of negative charge. (2) Differences in substrate specificity between cholinesterase and acetylcholinesterase indicate that the crevice, in the region of the esteratic site, is larger in the former than in the latter. The absence of substrate inhibition could possibly be due to rate-limiting acetylation. (3) The model makes no attempt to explain cooperativity, but the reality of this phenomenon in acetylcholinesterase may still be in doubt. At any rate we found no sign of cooperativity in substrate kinetics down to about 2 μM with eel or bovine enzyme, at normally high or very low salt concentrations. The preparations do show anomalous behavior with many (unnatural) inhibitors, but personally I hesitate to call this true cooperativity if substrate alone behaves in a perfectly simple fashion and if non-specific addition to peripheral sites and binding to a second anionic site (as in the insect enzyme) could possibly account for the behavior. However enzyme preparations of higher specific activity than ours (which were obtained commercially) may indeed exhibit real substrate cooperativity, as suggested at this meeting. Perhaps agreement on this point will soon be reached.

E. Reiner:

Aldridge and myself have postulated a model for acetylcholinesterase (*Biochem. J.* **115** (1969) 147) and assumed that the enzyme has an active site and an allosteric site, the latter not being catalytically active. Each site can bind substrates and inhibitors, and the sites are independent as far as binding is concerned. However, binding to the allosteric site will prevent hydrolysis of the substrate at the active site. Consequently, substrate hydrolysis would be an allosteric reaction, and inhibition by compounds other than substrates could be brought about by the same mechanism. That model explained the kinetics of reversible inhibition of acetylcholinesterase by several coumarin derivatives, and also substrate inhibition by acetylcholine and acetylthiocholine. However, the model does not apply when the substrate is phenylacetate and when the enzyme is cholinesterase (*cf.* Reiner and Simeon, *these Proceedings*, p. 326—330).

T. L. Rosenberry:

Enzyme catalysis may involve dynamic changes of the enzyme structure as an intrinsic part of each substrate turnover. Such conformational changes may be proposed by analogy, from equilibrium studies in which inhibitor binding results in an observable change in enzyme structure, or they may be inferred directly from kinetic studies with the substrate in which a unimolecular reaction involving either enzyme alone or an enzyme—substrate complex may be discerned as part of the reaction pathway. From steady-state kinetics one obtains information about only

the rate-limiting step in overall substrate turnover; but despite this restriction, my report presents evidence which suggests that such a unimolecular reaction preceding general acid-base catalysis may be rate-limiting for certain acetylcholinesterase substrates at low concentration. This unimolecular reaction has been called an »induced-fit« step virtually by definition.

An induced-fit mechanism for acetylcholinesterase has been proposed previously by others (*cf.* T. L. Rosenberry, *Acetylcholinesterase*, in: A. Meister (Ed.), *Advances in Enzymology* **43**, John Wiley and Sons, New York 1975), and the present proposal simply emphasizes that induced-fit may limit the overall reaction rate in some circumstances. The general concept of induced-fit together with the substantial evidence that the acetylcholinesterase catalytic site consists of esteratic and anionic sub-sites which may be simultaneously occupied by different ligands allow one to account for several characteristics of acetylcholinesterase. The esteratic site appears surrounded by non-polar residues while the anionic site is in a more polar area. Mono-quaternary ligands with the highest affinity for the catalytic site, *e. g.*, tensilon, 1-methyl-acridinium, appear to interact with both sub-sites simultaneously. Substrate inhibition appears to arise from the binding of a substrate molecule to the anionic site in the acetylenzyme with a consequent block of deacetylation. Acetylcholinesterase differs perhaps most strikingly from serum cholinesterase in having a smaller esteratic site which excludes carboxylic acid ester acyl groups larger than propionyl.

Acetylcholinesterase has peripheral anionic sites which are becoming better defined through the work of Taylor, Mooser and others at this meeting. Peripheral sites may exert allosteric effects on the catalytic sites in certain circumstances. Although we have looked carefully, we have obtained no evidence of allosteric cooperativity between catalytic sites in the native eel 11 S acetylcholinesterase tetramer.

K.-B. Augustinsson:

The classical model for cholinesterases, first proposed by Zeller and Bissegger (*Helv. Chim. Acta* **26** (1943) 1619), has been used as a tentative model ever since. It has been improved recently by the ideas of one or more hydrophobic areas at which the interaction (sorption, van der Waals forces) of hydrophobic alkyl radicals of substrates or inhibitors can occur. Studies with organophosphate inhibitors and with active-site-directed equilibrium fluorescent probes have established (Bracha and O'Brien, *Biochemistry* **7** (1968) 1545, 1555; Kabachnik *et al.*, *Pharmacol. Rev.* **22** (1970) 355) marked peculiarities in the structure and length of different hydrophobic patches on the active surface of the two types of cholinesterases, peculiarities which seem to be responsible for the different properties of the two enzymes. Another type of peculiarity which differentiates acetylcholinesterases from serum cholinesterases (Augustinsson, *Biochim. Biophys. Acta* **128** (1966) 351) is the charge availability of the quaternary nitrogen group being more important than the shape for acetylcholinesterase; for the serum enzyme the charge plays a less important role in comparison with the shape of the quaternary group. Van der Waals forces seem to be the more dominant type of force involved in complex formation in case of the latter cholinesterase type. This model is still a useful tool in the discussion as a basis for more detailed features presented at this symposium on the mechanism and structure of these enzymes.

G. Mooser:

The ligand binding site topography of acetylcholinesterase from *Electrophorus electricus* must incorporate two anionic centers: one associated with the active site which imparts specificity and orientation to substrate binding, and the other, located about 14 Å from the active site, which interacts with the active site to modify ligand binding and possibly catalysis.

The two sites are bridged by the two cationic functions of decamethonium and structurally related bis-quaternary ammonium compounds (cf. Mooser and Sigman, *these Proceedings*, p. 273). The specificities of the two sites differ significantly. Compounds which bind to the peripheral site are generally polycationic with aromatic or rigid hydrophobic centers such as d-tubocurarine, gallamine,

atropine and propidium (cf. Mooser and Sigman; Taylor *et al.; these Proceedings,* pp. 273 and 256).

Based on the *d*-tubocurarine binding pattern, the enzyme can exist in two ionic strength dependent forms. Under physiological ionic strength conditions, *d*-tubocurarine binds exclusively at the peripheral site while under low ionic strength conditions there is significant binding at both the peripheral site and the active site. These ionic strength effects cannot be accounted for by Coulombic considerations alone and must reflect ionic strength dependent structural alterations.

Functionally, ligand binding at the peripheral site can markedly influence ligand affinity at the active site. Stopped flow experiments have demonstrated that ligand induced conformational interactions between the two sites are partly responsible for the destabilization of active site complexes by peripheral site ligands. However, the distance between the two sites is small enough to suggest that steric factors may be operative as well.

R. D. O'Brien:

I disagree with Dr. Krupka about a need to determine which binding sites are physiologically »functional«. Only one substrate (acetylcholine) of the numerous substrates and inhibitors which we all study can be called physiological. Nor should we worry about a modest proliferation of proposed binding sites; the esteratic site is surrounded by amino acids, and each one is a potential binding site, the nearby ones for isosteric and probably some far distant ones for allosteric agents.

I think that minimally we must postulate the existence of four binding sites: hydrophobic, anionic, indophenyl and charge-transfer (or π-π). The first and last could perhaps be identical. Unfortunately the traditional approach (*i. e.* studies of inhibitions of enzymic activity with various pairs of substrates and/or inhibitors has not been very profitable in plotting out these sites, in part because many agents which enjoy quite different binding sites all attack the same catalytic site. The fluorescent displacement techniques we have heard about provide an extremely promising new approach. But it is surely surprising that we have heard nothing about enzyme modification, in which effective deletion of particular sites can abolish activity towards specific agents. An interesting variant will be the study of mutant enzymes, which in the case of house fly enzymes can have almost total loss of binding for some inhibitors, with little change in acetylcholine binding. The development of a series of mutants (probably only possible for invertebrates, because of practical considerations) offers the promise of quite extensive site plotting.

U. Brodbeck:

In his comments Dr. Taylor referred to chymotrypsin not only as a well characterized serine hydrolase but also as an exemplary model for acetylcholinesterase action. I would like to extend his thoughts by pointing at an important factor contributing to the high efficiency observed in enzyme catalysis. As shown by Henderson et al. (*Cold Spring Harbor Symp.* **36** (1972) 63) the tosyl group attached to Ser-195 of α-chymotrypsin displaces several water molecules from the active site. In analogy, Krieger *et al.* (*J. Mol. Biol.* **83** (1974) 209—230) described a similar situation for trypsin in which solvent molecules are displaced from the active site upon binding of benzamidine, a potent competitive inhibitor. These two observations support the hypothesis that substrate induced increases in hydrophobicity enforce the interactions among the charge relay system which is thought to be an essential part in the catalytic mechanism of serine hydrolases (Hunkapiller *et al., Biochemistry* **12** (1973) 4732—4743).

Another aspect, I would like to comment on, concerns the multitudes of different binding sites imposed on just one subunit. If these »binding sites« are nothing else but numerous negative charges that are influenced to varying degrees by poly-cations like spermine and spermidine, then we would call these interactions quite unspecific. In this case similar effects should be observable with poly-cations acting on negatively charged proteins of different structure, function and origin. On the other hand let us assume for the moment that there are specific binding sites for such a variety of different ligands as proposed in the model of Dr. Rosenberry. Are we not in this case overextending the capabilities of one subunit which would have to accommodate all these different sites? If this were real then to my know-

ledge, acetylcholinesterase would constitute the first enzyme carrying on a single subunit ligand binding sites distinctly aparat from the active center crevice.

J. Massoulié:

It has been shown by Myers in 1952 that erythrocyte acetylcholinesterase has its K_m and V_{max} both increased by salts, and we have found the same effect with the Torpedo enzyme either with monovalent or divalent ions. Excess substrate inhibition is also shifted to higher values of substrate concentration. These effects which appear as inhibitory at low substrate concentration and activating at high concentration, resemble some of the effects that are seen with ligands, for example those described by Dr. Kossorotow with polyamines. I wonder whether salt effects must be explained by cation binding to specific sites of the enzymes in a similar way to that proposed for polyamines. I see for instance that cations appear in one of Dr. Taylor's graph as low affinity ligands, competitive with the fluorescent probe propidium diiodide. This implies that there are discrete states of the enzyme, the ratios of which vary with cation concentration. Another model would be that variations in ionic strength modify the protein conformation in a continuous way. Which is the best way to describe the acetylcholinesterase behaviour?

W. N. Aldridge:

There are obviously many similarities between the models. One of the weak aspects of my model is that it does not specify that substrate inhibition may occur by reaction with the acetyl enzyme. However, our results with haloxon and the coumarin leaving group in our view did not support this concept (Aldridge & Reiner, 1969) and I consider that other work is required on the effects of quaternary ammonium and other compounds on the rates of deacylation of acyl-acetylcholinesterases — it might be useful to examine those acyl-acetylcholinesterases where the rate of deacylation may be measured directly.

The models presented by Drs. Taylor and Hopff have some points in common with mine. However I do not think they adequately deal with acceleration by quaternary ammonium compounds or inhibition by small inhibitors or hydrolysis of small substrates. I find it difficult to avoid the conclusion that quaternary ammonium compounds must change, by a »knitting together« process, the size of the esteratic area.

S. Maričić:

In connection with Dr. Taylor's discussion on ionic strength effects and use of fluorescence methods, may we regard Dr. Eldefrawi's fluorescent experiments for the displacement reactions with ytterbium as evidence in favour of specific cation binding?

M. E. Eldefrawi:

Yes, I think ytterbium and other rare earth fluorescent elements hold great promise for studies on specific cation binding. We are using them presently to study calcium binding to the acetylcholine-receptor protein.

I. Silman:

I would like to comment that although most of the discutants have not taken into account cooperativity in their model building considerations, acetylcholinesterase differs from the serum proteases in its subunit molecular weight and multisubunit structure. Furthermore, the results of Drs. Wilson, Massoulié, Rosenberry and ourselves suggest that pairs of subunits are linked by disulfide bonds, a finding which is unique for a catalytic protein. Although these structural characteristics may be perhaps related to enzyme-membrane interaction, it is hard to believe that they have no influence on catalytic activity.