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Molecular Forms of Acetylcholinesterase

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Several molecular forms of acetylcholinesterase are obtained from Electrophorus or Torpedo electric organs. They have been characterized by physico-chemical methods and observed by electron microscopy. The most complex D form is made up of a globular »head« containing probably twelve subunits, or three tetrameric groups of subunits, attached to a rod like tail. Two other asymmetric forms, C and A, may be derived from it by removal of one or two tetramers from the »head«. These forms can ultimately be degraded by proteolytic digestion or sonication into tetrameric and dimeric active enzymes, G and G'. No striking difference in the catalytic properties of these forms could be demonstrated. An analysis of their thermal denaturation suggests that internal breaks may exist in the polypeptide chains without being revealed in catalytic or sedimentation properties of the molecules. $\Delta H^{\ddagger}$ values demonstrate stabilizing interactions in the more complex molecules.

Analysis of subunits by SDS polyacrylamide gel electrophoresis shows that one main 90 000 subunit is progressively split into a 60 000 DFP-labelled chain together with smaller peptides in the 30 000 range. No difference could be found between D and G subunit patterns which could be identified to the tail component. Comparing the molecular weight of A (one tetramer plus tail) and G (tetramer), one finds that the mass of the tail should be in the 60 000—80 000 range. Recent micrographs suggest that it consists of three strands linked to the three tetramers in the head of D. We therefore propose a three stranded collagen like structure for the tail.

We discuss the possible physiological role of the asymmetric structure of acetylcholinesterase and its implication with the membrane association of the enzyme. Multiple forms of acetylcholinesterase are not genetically determined isozymes but rather represent different states of association of the active monomers. The significance of multiple forms of acetylcholinesterase, especially in mammals, is considered.

INTRODUCTION: MULTIPLICITY OF ACETYLCHOLINESTERASE FORMS

A multiplicity of acetylcholinesterase molecular forms has been found throughout the animal kingdom, from *Coelenterates* and worms[1], insects (flies)[2], to fishes[3,4] and mammals. It has been observed both in the mammalian nervous tissue and erythrocytes[5–11].

Heterogeneity of the enzyme has been demonstrated by chromatography, ultracentrifugation, or by starch or polyacrylamide gel electrophoresis. The matter is sometimes complicated by reversible aggregation of the enzyme in

low salt conditions. This is particularly well documented for acetylcholinesterase of fish electric organs[4,12–15].

One of the most commonly used methods for demonstrating a multiplicity of acetylcholinesterase species is polyacrylamide gel electrophoresis. Although this technique is rapid, it presents, in our opinion, severe limitations for study of acetylcholinesterase, as for other membrane bound enzymes. It has been shown[16] that the mobility of Triton X-100 solubilized acetylcholinesterase vary with the non ionic detergent concentration. In the case of monoamine oxidase, the Triton X-100 solubilized enzyme displays a number of active bands in polyacrylamide gel electrophoresis, which are all converted into a single band after treatment with the chaotropic salt sodium perchlorate[17].

I — ACETYLCHOLINESTERASE OF FISH ELECTRIC ORGANS.

A) Existence of Distinct Molecular Forms

Since 1969, we have been studying the multiple molecular forms of acetylcholinesterase from the electric organs of *Electrophorus* and *Torpedo,* mainly by means of sucrose gradient centrifugation[4].

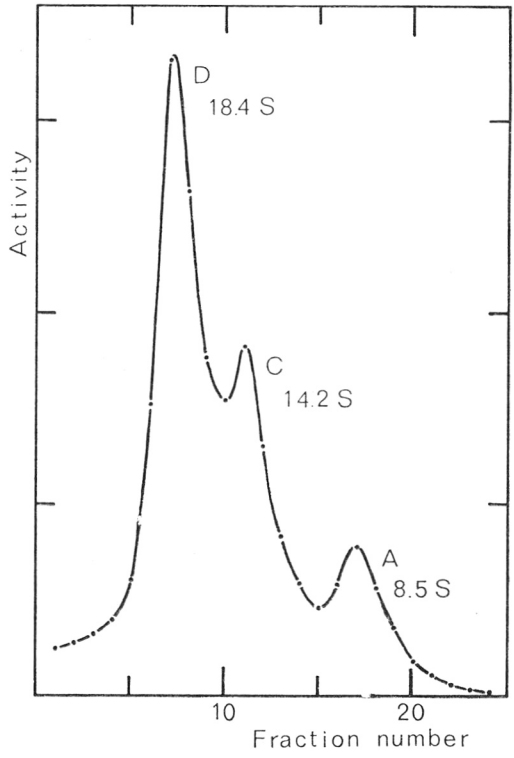

Fig. 1. Acetylcholinesterase activity distribution in a sucrose gradient. A freshly prepared homogenate of *Electrophorus electricus* has been centrifuged in a 5—20% sucrose gradient containing NaCl 1 M, $MgCl_2$ $5 \cdot 10^{-2}$ M Tris pH = 7, 10^{-2} M (Beckman LII 65 centrifuge equipped with an SW 56 rotor 42 500 r.p.m., during $7^1/_2$ hours, at 3 °C). Three peaks are clearly separated. The heavy D molecular form being the most abundant one.

Sedimentation constants are determined with internal markers (yeast alcohol dehydrogenase 7.4 S, and *E. Coli* β galactosidase 16 S).

The acetylcholinesterase activity profile obtained with total extracts of electric organs is a complex one, containing three different peaks, in both electric fishes (Fig. 1). In order to avoid complicating factors, we decided not to use detergent for solubilizing the enzyme, although the use of Triton X-100 allows a complete solubilization while not modifying the sedimentation profile. Acetylcholinesterase is thus extracted from electric tissue by homogenization in a high salt buffer (1 M sodium chloride).

We have been able to separate each peak from the others, without any rearrangement, so that they may really be considered as distinct molecular entities, and studied as such.

B) Globular and Asymmetric Acetylcholinesterase from Electrophorus Electric Organs

We soon discovered that these molecular forms (called A, C and D) behave as highly asymmetrical particles[18]:

— their Stokes radius, determined from molecular sieve chromatography, is far too large when compared to their sedimentation constant (Table I).

TABLE I

Physicochemical Characteristics of Electrophorus Acetylcholinesterase Molecular Forms

		Sedimentation constant	Stokes Radius nm	Molecular weight
Asymmetric forms	D	18.4 S	15.0	1 100 000
	C	14.2 S	14.4	780 000
	A	9 S	12.4	430 000
Globular forms	G	11.8 S	8.3	370 000
	G′	7.7 S	6.1	180 000

— they are sensitive to ultrasonic vibrations, which degrade them into more globular, but still active molecules. One of these molucules, G_s, is indistinguishable by sedimentation from a minor component already present in electric organ extracts, G. The other, G_s', is smaller.

Another convenient way of degrading the asymmetrical molecules A, C and D is proteolysis: the *Electrophorus* enzymes are easily converted by trypsin into a G_t form[4,18]. Like G_s obtained by sonication, and the naturally occurring G, G_t sediments at 11.8 S. (We will later refer to these 11.8 S forms as G). It is to be noted that *Electrophorus* acetylcholinesterase chromatographically purified according to Leuzinger and Baker[19] sediments at 11.1 S[20], a value close to that of G, but very reproducibly distinct from it.[21]. This purified enzyme is therefore a different molecule, G_p.

We have then identified six molecular forms or groups of molecular forms, each sedimenting as a homogeneous species. They fall into two classes: asym-

metrical forms (A, C, D) and »globular« forms (G, G_p, G′). The asymmetrical forms, especially D, seem to be more intact structures. Apart from their peculiar hydrodynamic properties, they share the ability to aggregate at low ionic strength[4]. For the *Electrophorus*[12] and *Torpedo*[15] acetylcholinesterase, this phenomenon has been known for a long time. It has been studied by Grafius and Millar[13,14], for the *Electrophorus* enzyme, and seems to involve mainly electrostatic interactions.

II — A SEARCH FOR DIFFERENT SUBUNIT INTERACTIONS IN ELECTROPHORUS ACETYLCHOLINESTERASE MULTIPLE FORMS

All forms of *Electrophorus* acetylcholinesterase may be derived by gradual degradative procedures[22] from the complex D form, as schematically shown in Fig. 2. They must therefore possess identical active sites, but their enzymic properties might be modified, due to homologous or perhaps heterologous subunit interactions.

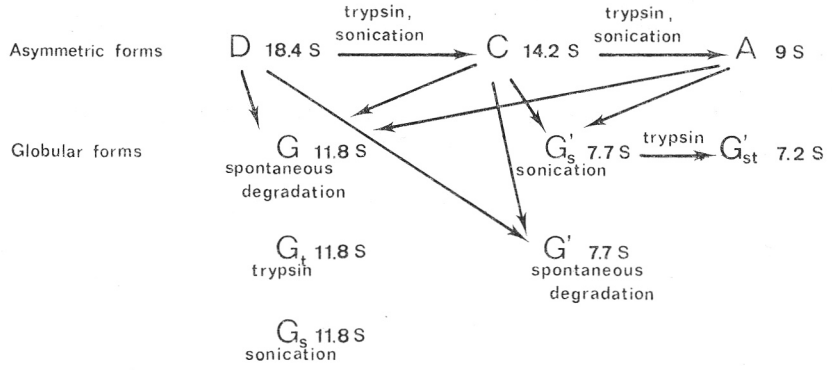

Fig. 2. Scheme of observed degradative conversion between the molecular forms of *Electrophorus* acetylcholinesterase.[22] The chromatographically purified form G_p (11.1 S) is not shown because we have never obtained it in our preparative procedures.

A priori this is rather likely in view of the well attested existence of interactions between catalytic and secondary sites of acetylcholinesterase, which might involve more than one subunit and even possibly regulatory subunits.

Although acetylcholinesterase kinetics cannot be formalized in the orthodox allosteric model[23], it is well established that it possesses peripheral sites binding Ca^{++} ions, quaternary ammonium ligands, and particularly hydrophobic molecules such as gallamine, d-tubocurarine and atropine, so that allostery, and also induced fit have been invoked (*cf.* ref.[11]).

Doubts may be raised about some of the elaborate models sometimes presented, especially when kinetic experiments have been performed at very low ionic strength: it has been known for a long time that salts increase both K_m and V_{max} of acetylcholinesterase[24] and that it reduces drastically its affinity for cationic ligands. Crone[25] found that complex effects of gallamine, as described by Changeux[15], Kitz *et al.*[26], Roufogalis and Quist[27] could no longer be demonstrated in 0.15 M ionic strength. Moreover, the physiological significance of peripheral sites involved in the binding of such foreign or artificial molecules as those mentioned above is far from obvious.

A) Kinetics of Electrophorus molecular forms

In view of possible regulatory interactions between subunits, which, in complex asymmetric molecules, might be different from those observed in the more widely studied G form, we have compared the kinetic behaviour of the various molecular forms, limiting ourselves to substrate hydrolysis studies. In our experimental conditions, substrate hydrolysis follows Michaelian kinetics and we have not been able to discover any major differences between the molecular forms. Their K_m's for acetylcholine are identical (Fig. 3).

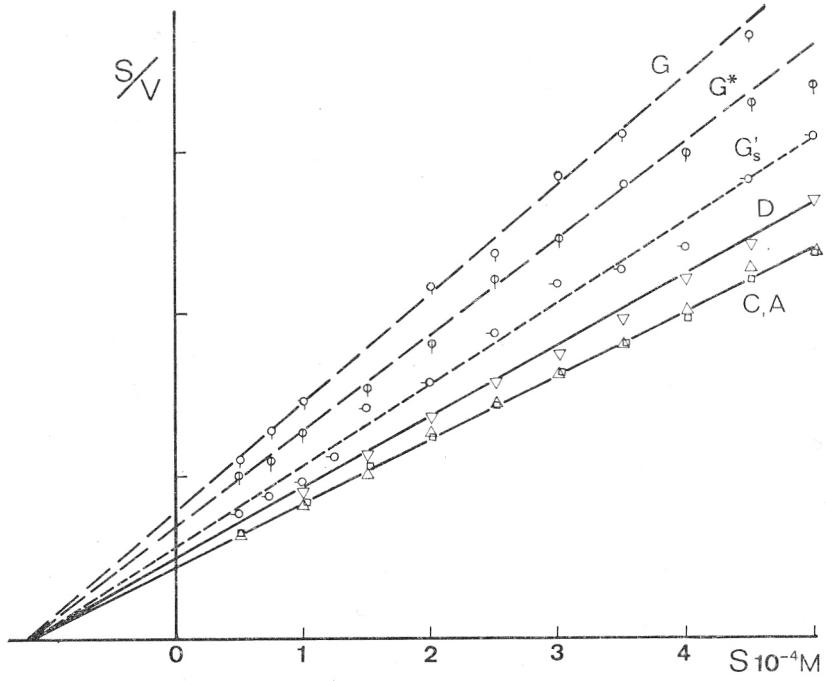

Fig. 3. Dixon plot for different molecular forms of *Electrophorus* acetylcholinesterase. Substrate: acetylthiocholine-Ellman's reaction medium: dithiobis nitrobenzoic acid (DTNB) 10^{-2} M; Tris pH = 7 $5 \cdot 10^{-2}$ M. Reduction of DTNB by thiocholine is monitored at 412 nm.
G* is 98% heat inactivated G form.
K_m values for all molecular forms are identical.

Excess substrate inhibition has been interpreted as involving a secondary site of the enzyme[27,28]. Millar *et al.*[29] did not observe this effect for their 7.4 S (»half monomer«) from (obtained by Triton X-100 treatment). However, we find that all forms, including the smaller G_s' form, are subject to excess substrate inhibition.

B) Thermal inactivation studies of Electrophorus acetylcholinesterase

We have compared the thermal inactivation curves of the various molecular forms of acetylcholinesterase, hoping that differential stabilities might reveal different subunit interactions in the molecules (unpublished results). Inactivation has been studied at pH = 7, since this corresponds to maximal

stability of the enzyme (Fig. 4), in presence of salts (NaCl 1 M, $MgCl_2$ 0.05 M) and serum albumin (0.5 mg/ml). Stabilization of acetylcholinesterase by albumin has been reported and is often used in titration media. We know that purified dilute solutions are rapidly inactivated by adsorption to the tube walls, and this can be prevented in the presence of nonspecific protein.

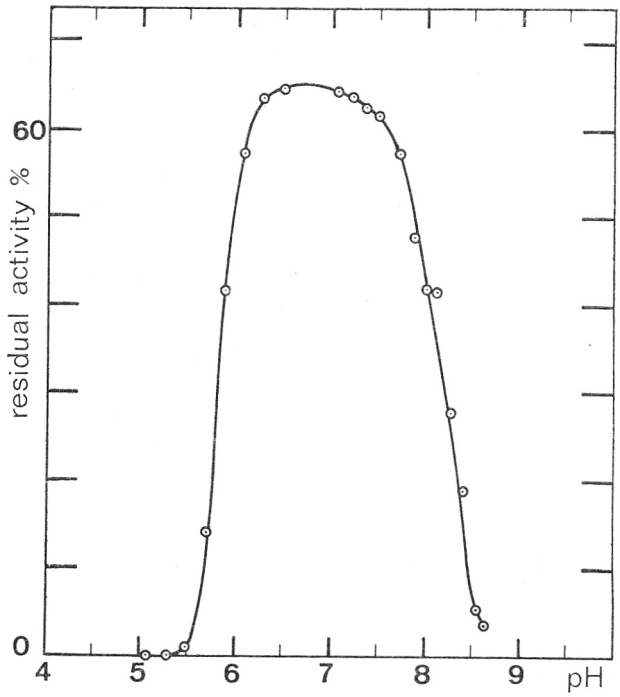

Fig. 4. Thermal stability of *Electrophorus* acetylcholinesterase as a function of pH. Saline buffer NaCl 1 M; $MgCl_2$ $5 \cdot 10^{-2}$ M; sodium acetate, cacodylate or borate 10^{-2} M. Ordinates are % residual activity after exposure at 40 °C for 112 hours. Maximum stability is at pH = 6.9. The peak is sharper and inactivation faster in lower salt media.

Two points can be made from the results shown in Fig. 5: thermal stability seems to increase with complexity of the molecule, and the inactivation curves apparently do not obey first order kinetics. We have found however that a hundred-fold dilution of the enzyme does not modify the inactivation curve (Fig. 6). In spite of the nonlinearity of the denaturation curves, (Figs. 5 and 6), linear van't Hoff plots can be constructed, and the apparent $\Delta H^{\ddagger}$ determined (Fig. 7). It must be noted that exposure to high temperatures does not cause any conversion from one form to another.

The somewhat greater stability of the asymmetric forms, especially D, may well be due to stronger interactions between subunits in these more complex structures. $\Delta H^{\ddagger}$ values are however rather close to each other, in view of the large differences in denaturation rates (Fig. 5).

On the other hand, the results shown in Figs. 5 and 6 may be explained if we assume that the degradative processes leading in sequence from D to G′ are liable to produce intra-subunit cuts, which, while not modifying the actual

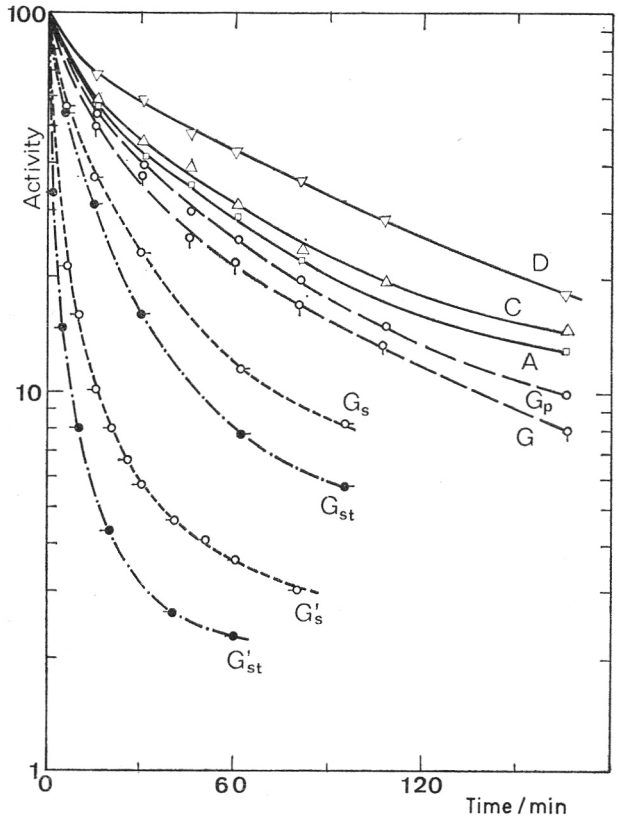

Fig. 5. Thermal denaturation curves for *Electrophorus* acetylcholinesterase. Saline buffer NaCl 1 M; $MgCl_2$ $5 \cdot 10^{-2}$ M; Na cacocylate pH 6.9 10^{-1} M; bovine serum albumin 0.5 mg/ml. Temperature 50 °C. Residual activity is plotted on a logarithmic scale. Notations for the different molecular forms are defined in Fig. 2. G_{st} is trypsin treated G_s, it sediments at 11.8 S). Similar curves are obtained at pH = 6 or pH = 8.7.

structure or altering markedly the interactions of each subunit at low temperature, facilitate its denaturation for entropic reasons. (The rate limiting step for denaturation being assumed to require a free enthalpy $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T \cdot \Delta S^{\ddagger}$, breaks in the polypeptide chains may be thought to leave $\Delta H^{\ddagger}$ unchanged while increasing the number of configurations in the unfolded state, and therefore $\Delta S^{\ddagger}$). This hypothesis, where a variable number of breaks in the polypeptide chains favours thermal denaturation, while not affecting $\Delta H^{\ddagger}$ nor the low temperature state of the enzyme, is consistent with the comparison we have made of G_P acetylcholinesterase before and after extensive thermal denaturation (99%): although its relative rate of inactivation is reduced, in agreement with the nonlinearity of isothermal inactivation curves, the K_m and $\Delta H^{\ddagger}$ values for inactivated G_P (G_P*) are identical to the original values (Figs. 3 and 7).

We are then led to think of each form, characterized by a well defined sedimentation constant, as of a collection of partially split molecules rather than a homogeneous population.

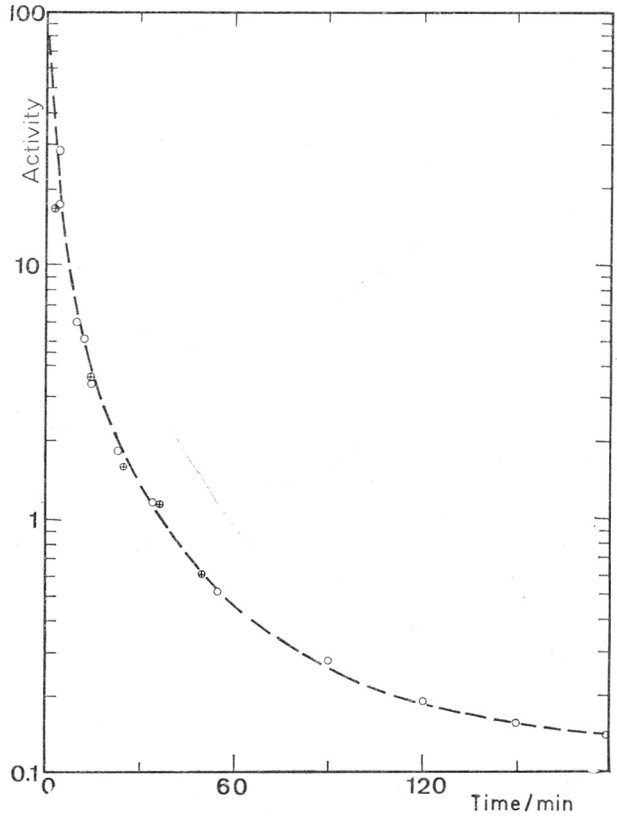

Fig. 6. Effect of enzyme concentration on the thermal inactivation curve. Experimental conditions as in Fig. 5.
○ Chromatographically purified acetylcholinesterase;
Gp original acticity $\Delta D_{412mn}/(\text{min } \mu l) = 4$ (Ellman's reaction medium); hundredfold dilution of the same enzyme.

The closeness of $\Delta H^{\ddagger}$ values show that enzymatically active subunits are not engaged in dramatically different interactions in the different species, thereby conforting the kinetic observations (Fig. 3).

III — MOLECULAR STRUCTURE OF ELECTROPHORUS ACETYLCHOLINESTERASE

A) Electron Microscopy and Molecular Weights: Grapelike Model

As we have already mentioned, acetylcholinesterase forms fall into two distinct classes, asymmetric (A, C and D) and globular (G, G′).

It has been possible to purify and observe these five kinds of molecules in the electron microscope[30] (Fig. 8): G′ and G appear composed of respectively two and four globules (the diameter of which is about 6.7 nm). On the other hand, A, C and D are built of two parts: a globular »head« made up of globules similar to those found in G and G′, and a rod-like »tail«. This element is about 50 nm long and 2—3 nm thick.

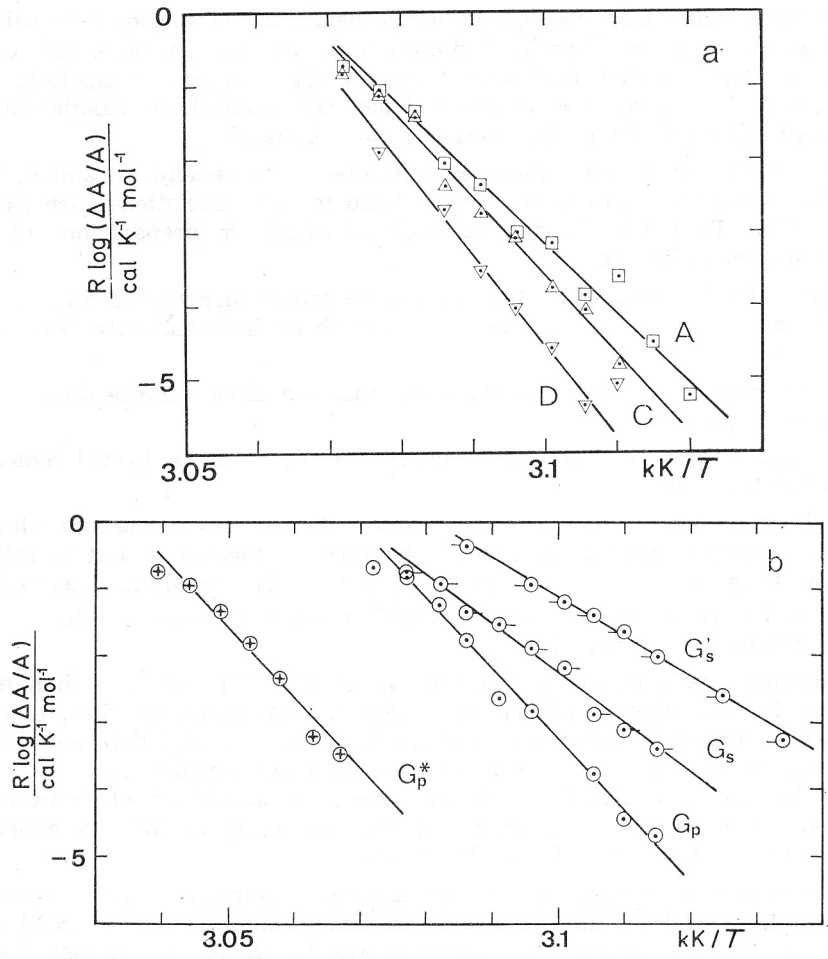

Fig. 7. Van't Hoff plots constructed from thermal inactivation rates. Ordinates: $R \log \Delta A'/A$, where R is the gas constant, $\Delta A/A$ is the relative activity variation in 4 minutes, at pH = 6.9 in saline buffer (*cf.* Figs. 4, 5, 6).

a) asymmetric forms b) globular forms.

G_p* is 99% heat inactivated G_p (*cf.* Fig. 6).

Apparent $\Delta H^{\ddagger}$ values, calculated from the slopes of these curves, are as follows:

$\Delta H^{\ddagger}$ /kcal mol^{-1}:	95	107	126	106	74	59
Form:	A	C	D	G_p and G_p*	G_s	G_s'

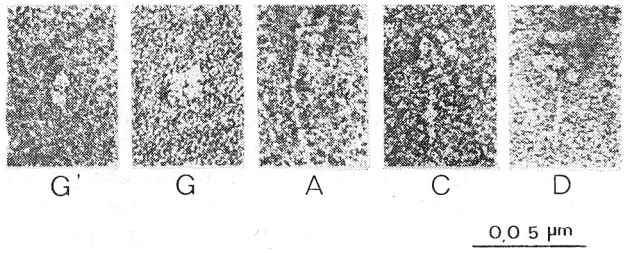

Fig. 8. Electron microscopic appearance of *Electrophorus* acetylcholinesterase (from ref.[59])

We have found that the hydrodynamic behaviour of species A in solution is that of an elongated ellipsoid of similar molecular weight, mass and overall dimension. The electron microscopic images are therefore compatible with the hydrodynamic properties of these structures in solution[31]. Similar images have been published by Dudai, Herzberg and Silman[32].

In addition, they have observed dumb-bell like structures, and so have Wermuth *et al.*[33] who interpret them as head to tail associations such as D.D, D.C or C.C. We have seldom seen such structures in preparations of well defined molecular forms.

The molecular weights of the various molecular forms have been derived from a combination of values obtained for three independently determined parameters[31]:

— *Sedimentation constants (S)* were obtained from sucrose density gradient centrifugations.

— *Stokes radii* (R_e) were graphically derived from calibrated molecular sieve elution profiles.

— *Partial specific volume,* $\bar{v}$: buoyant density in a caesium chloride gradient isopycnic centrifugation may be directly measured, but it may be different from that in water, because of differential solvation; alternatively, we derived $\bar{v}$ from comparative data with marker proteins in H_2O *vs.* D_2O sucrose gradient centrifugation[31].

Molecular weights, being proportional to $R_e \cdot S/(1 - \bar{v})$, are then easily obtained by the above method, as is the determination of the molecular The values thus determined are reported in Table I. Direct determination of molecular weights by low speed centrifugation equilibrium have been performed for the D, C and G forms, and are in good agreement, with values obtained by the above method, as in the determination of the molecular weight of G by Dudai and Silman[34] (350 000).

The molecular weight of acetylcholinesterase subunits after dissociation has been measured by numerous authors using centrifugation or SDS polyacrylamide gel electrophoresis: subunits are found in the 60 000—100 000 daltons range[20,34-36] and this corresponds to the molecular weight expected for each globule, given its electron microscopic dimensions (diameter 6—7 nm). We believe therefore that each globule consists of one subunit. The globular forms G′ and G are thus considered as a dimer and a tetramer. We will elaborate on this point later.

Our results on the molecular weights of acetylcholinesterase molecular forms, and their electron microscopic appearance are explained by the following hypothesis: the globular heads of A, C and D are built up of respectively one, two, and three tetrameric units[37].

In effect, the tetramer appears as a particularly stable unit in the sense that different degradative processes (sonication, proteolysis) convert the more complex asymmetric forms into active tetramers. These may be already individualized in the intact D molecule: electron microscopic observation of D molecules in which the globules are sufficiently spread out to be counted show that it is indeed possible to identify three groups of four globules[38] (Fig. 9).

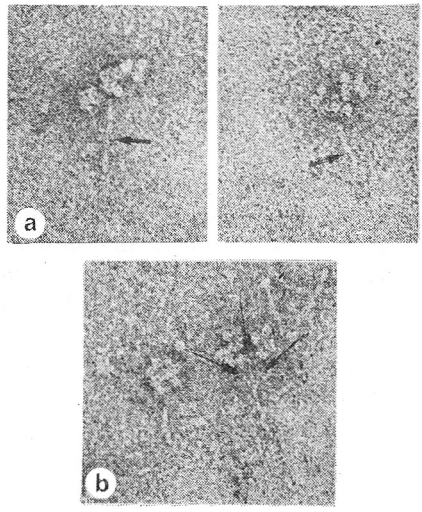

Fig. 9. Electron microscopic image of *Electrophorus* acetylcholinesterase D (from refs.[38,49]).
a: arrows point to the tails of the D molecules.
b: three strands, shown by the arrows seem to emerge from three tetramers, and join to form the distal part of the tail.

B) Subunits of Acetylcholinesterase: SDS Polyacrylamide Gel Analysis

We now come to the most puzzling question about the odd grape-like structure of asymmetric acetylcholinesterase: the nature of the tail moiety.

An obvious approach to its investigation is the subunit analysis of the asymmetrical molecules. We have studied the subunits in SDS polyacrylamide electrophoresis. In a series of experiments after SDS denaturation and disulfide reduction, we found[39] two major bands in A, C and D as well as in G.

In agreement with other authors, we find that both of these bands can be labelled[34–36,40] by radioactive DFP. Although they stain positively for carbohydrate, so that their glycoproteic character[39,41] renders an estimation of molecular weights from electrophoretic migration rather doubtful, they are close to 60 000 and 90 000 (manuscript in preparation).

Further investigation has revealed that the relative distribution of these two bands depends upon the enzyme preparation: in fresh, rapidly purified enzyme preparations, the heavier band is largely predominant, whereas older preparations show a larger amount of the lighter one, and of a smaller, more diffuse, non- DFP-labelled band in the 35 000 daltons range, as well as still smaller peptides. It appears that the heavy 90 000 band represents the original polypeptide chain, which is easily split during purification and/or storage into a 60 000 and a 35 000 component. We have already seen that partial degradation of the polypeptide chains, without disruption of the enzyme or loss of activity might explain anomalies in the thermal denaturation curves. Since there are four active sites per tetramer[42], it seems very likely that all 90 000 subunits are identical in the intact enzyme.

When no reduction is performed, dimer bands are found, which indicate the occurrence of inter-subunit disulfide bridges, as already found by Wilson and collaborators[43,44]. Moreover, the 90 000 band is also present, but no 60 000 nor 35 000 band can be detected. Reduction allows these two parts of the original subunit to separate: they must therefore be held together by intra subunits disulfide bonds.

Rosenberry and collaborators[35,36], studying the »11 S« (G_p?) form, have come to similar conclusions. The basic 75 000 subunit is thought to split into a 50 000 chain containing the active serine, and a 27 000 chain which is then further degraded. Dudai and Silman[34] made similar observations for the »11 S« form. Our observations have been made on the asymmetric, especially D, forms, as well.

Such an instability of acetylcholinesterase subunits render an identification of any specific tail subunits extremely difficult.

Two facts are quite clear, however:

— the DFP labelled subunits of all forms, asymmetrical or globular, are identical. From our work as well as that of others[34–36], they appear to be derived from one type of basic subunit. Although differences may exist while not being revealed by SDS gel electrophoresis, this justifies considering G′ and G as dimer and tetramer.

— this casts doubt on models which assume two different kinds of subunits such the one we had tentatively suggested in a previous study[39] as well as those of Leuzinger[45] or Brodbeck and collaborators[33,46,47].

— no major polypeptide chain, absent in the globular forms, can be identified in the complex asymmetric form D (or in C or A). Some minor bands can be seen in the high molecular weight region of the gel ($>$ 100 000)[39], but we can dismiss them as potential candidates for the tail for two reasons: none is intense enough (such a mass would amount to at least one tenth of whole protein if one such chain were present in every D molecule) and, besides, the total mass of the tail must be much smaller, as deduced from comparisons between the molecular masses of tetramers and asymmetric forms.

We have noted that conversion of D to C and of C to A seems to be achieved by removal of one tetramer unit (350 000 daltons or four 90 000 chains) at each step, so that the tail itself must account only for the difference between A (430 000) and the tetramer G. Assuming a molecular weight 350 000 for G (ref.[34]), we find a maximum difference of 80 000. The figures given in Table I yield a value of 60 000. Such a mass is sufficient to account for the electron microscopically observed »tail«. The mass of an α helix 50 nm long would be about 40 000, that of an extended chain, 14 000 (ref.[48]). It is difficult, for reasons of structural symmetry, to imagine how three tetramers might bind to a single polypeptide chain and, if it were so, how the release of one, then two of the tetramers would leave the attachment site sufficiently intact for this chain to remain bound to the last tetramer, as in the A molecule.

Alternatively, a more attractive hypothesis would be that the tail is made up of several chains, probably three, each of them initially attached to one of the tetramers in D and perhaps wound together in a collagen like helix: some of our recent micrographs suggest such a structure[49] (Fig. 9b). Such chains, in the 20 000 molecular weight range (60 000/3), would be quite hard

to identify, since they would fall in the same region as other partially degraded chains.

It must be kept in mind that the mass difference between the A and G forms does not necessarily represent the whole mass of the tail: it is quite possible that a structural change of the basic subunits occurs when asymmetric forms are converted into G; this »globular« molecule is in fact rather asymmetric itself (f/f_o : 1,8). A major rearrangement however would be expected to alter markedly the catalytic properties of acetylcholinesterase (Fig. 3).

C) *Sucrose Gradient Sedimentation, Amino Acid Composition, and Immunological Approach to the Tail Problem*

We have tried to analyze the protein obtained after conversion of D to G by proteolysis, without denaturation: in sucrose gradient centrifugations, although some 280 nm absorbing material remained on top of the gradient, no clear peak was found. It is quite possible that proteolysis degrades the tail moiety into very small unrecognizable fragments.

We have looked for another clue to the nature of the tail in amino acid analyses of the different molecular forms. No significant differences have been found, and the results for all forms are very close to those already reported for the tetrameric acetylcholinesterase[19,50,51]. The tail element, therefore, does not change significantly the amino acid composition.

Another attempt to identify it has been tried by immunological methods: we have raised antibodies in rabbits against all forms of acetylcholinesterase[52,53]. Although some antigen-antibody reactions, as studied by complement fraction, are somewhat different with asymmetric and globular forms, all antibodies react with all forms; analogous results have been obtained by Gurari *et al.*[54]. Moreover, exhausting an anti-D anti-serum with G acetylcholinesterase yielded no antibodies directed specifically against the asymmetric molecule.

It is impossible to conclude this discussion without emphasizing the extreme elusiveness of the tail element and the difficulty of its characterization, in the absence of any specific label.

IV — SIGNIFICANCE OF MULTIPLE MOLECULAR FORMS OF ACETYLCHOLINESTERASE

We have described the molecular forms of acetylcholinesterase obtained from *Electrophorus electricus* electric organs. The same forms have been identified in other tissues of the fish: muscle, brain and spinal cord; the complex forms are more labile in nervous tissue because of endogenous hydrolytic enzymes.[55,56]

Exactly homologous acetylcholinesterase forms, have been found in *Torpedo marmorata* electric organs. Their physical chemical parameters (sedimentation constants, molecular weights) are slightly different, but their structure is very similar. Micrographs of the Torpedo D form shows a thin tail connected to a globular head made up of a dozen globules[49].

It must be stressed that *Electrophorus* being a fresh water Teleost, and *Torpedo* a marine Elasmobranch, are widely separated on the evolutionary scale. The common properties of *Electrophorus* and *Torpedo* acetylcholinesterase must therefore be quite general among fishes.

The very odd asymmetrical structure observed for the more intact and complex molecules must somehow play a well defined functional part. The

activity itself seems totally independent of this structural peculiarity. It is therefore likely that the tail is involved in the positioning of the enzyme. Treatment with phospholipase C induces a conformational transition of form D into a globular particle, without any measurable loss of mass, and low salt aggregation, a property shared by the asymmetric molecules is then abolished[57]. It is thus possible that some phospholipid residues play a determinant role in maintaining the asymmetric structure. This of course suggests that the tail, being rather a stem or a stalk, anchors the enzyme into the membrane, perhaps at a specific membrane site.

Examination of such a hypothesis cannot be done by a study of solubilized acetylcholinesterase alone and we are currently studying membrane bound enzyme.

Are multiple forms of acetylcholinesterase isozymes?

It is not obvious whether the multiple forms encountered in many different animals, *e. g.* mammals, are at all similar to those described above. Such multiple forms are often referred to as isozymes. Strictly speaking, this term should apply to enzymes of equivalent activity, coded by distinct genes, and thus differing in their primary polypeptide structure. Thus electric organs acetylcholinesterase multiple forms cannot be correctly called isozymes, since they rather represent different stages of disruption of an original complex structure and apparently are built up of one main type of subunit (which may be secondarily cleaved into smaller polypeptides).

It may be that in some cases acetylcholinesterase multiplicity could arise from the existence of real isozymes. One of the best indications for this seems to be the occurrence of two chromatographically separable, and electrophoretically different forms, obtained by Shafai and Cortner[8,9] from human erythrocytes. These two forms can be rearranged under certain conditions, and this process could be interpreted as

$$\alpha_2 + \beta_2 \rightleftarrows 2\alpha\beta$$

In this model, α and β might be isozymes *stricto sensu*.

A direct comparison of mammalian and fish acetylcholinesterase may be attempted more favorably for the rat diaphragm enzyme, since Hall[58] has examined it with methods very similar to ours. In this case three main forms can be characterized; the faster sedimenting molecule (16 S) being restricted to the end-plate region. This suggests a specific structure for this enzyme, perhaps a complex between acetylcholinesteratic subunits and a synaptic component. In our laboratory we have confirmed and extended these observations and we are currently studying the properties of rat acetylcholinesterase multiple forms. Apart from the superficial analogy arising from the very multiplicity of acetylcholinesterase forms, marked differences seem to emerge, at the moment, between their molecular properties in rat and *Electrophorus*. However, closer understanding of these various structures will probably reveal more profound homologies: the grape-like structures observed for fishes may be representative of enzyme-membrane association in general.

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DISCUSSION

M. R. Pavlič:

I think that the activation enthalpies are different for the different enzyme forms, and this would indicate that the forms are different thermodynamically.

J. Massoulié:

You are right. The $\Delta H^{\ddagger}$ values decrease from the more complex to the more degraded molecules (with the exception of G_p* which is obtained by a different procedure). These variations probably arise from stabilizing interactions between neighbouring subunits. Assuming a linear relationship between the measured catalytic activity, and the number of nondenatured active centers, I would expect that if each molecule were to be denatured as a whole, then the $\Delta H^{\ddagger}$ value for D, which possesses 12 subunits, would be approximately 6 times that of G′, which has only two subunits. However, the observed values are far from this ratio, and I think this means that the denaturation unit is not the whole molecule, but the monomer, or perhaps the dimer. In that sense, these units may be somewhat independent within the molecule, both structurally and catalytically.

SAŽETAK

Molekularni oblici acetilkolinesteraze

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Dobiveno je nekoliko molekularnih oblika acetilkolinesteraze iz električnog organa *Torpeda* odn. *Electrophorusa.* Ti su oblici definirani na osnovu fizikalno-kemijskih metoda i mikroskopskih analiza. Najkompleksniji oblik, D, složen je od okrugle »glave«, koja vjerojatno sadrži dvanaest osnovnih jedinica ili tri tetramerne skupine osnovnih jedinica, a pripojena je na štapićasti »rep«. Dva ostala asimetrična oblika, C i A, moguće je izvesti iz oblika D odvajanjem jednog odn. dva tetramera »glave«. Ti se oblici mogu do kraja razgraditi proteolizom ili metodom sonikacije, u tetramerne i dimerne aktivne enzime G i G′. Nije uspjelo utvrditi nikakvu značajnu razliku u katalitičkim svojstvima tih oblika. Analiza termalne denaturacije ukazuje na to da se raskidi polipeptidnih lanaca ne moraju očitovati promjenom katalitičkih i sedimentacijskih svojstava molekula. Vrijednosti $\Delta H^{\ddagger}$ upućuju na stabilizirajuće interakcije u kompleksnijim molekulama.

Analiza osnovnih jedinica metodom elektroforeze na SDS-poliakrilamidnom gelu, pokazala je, da se glavna podjedinica molekularne težine 90 000 progresivno razlaže u lanac označen DFP-om, molekularne težine 60 000, te manje peptide, koji

se nalaze u području molekularnih težina od oko 30 000. Između modela D i G, koje je bilo moguće identificirati sve do komponenata »repa«, nije nađena razlika. Usporedbom molekularne težine oblika A (tetramer s »repom«) i oblika G (tetramer), može se zaključiti da molekularna težina »repa« leži u rasponu između 60 000 do 80 000. Na osnovi istraživanja elektronskim mikroskopom postulirano je da se »rep« sastoji od tri lanca, vezana na tri tetramera »glave« i da je struktura slična kolagenu.

Prodiskutirana je moguća fiziološka uloga asimetrične strukture acetilkolinesteraze i njena povezanost s vezivanjem enzima na membranu. Višestruki oblici acetilkolinesteraze nisu genetski određeni izozimi, već je vjerojatnije da su to različita stanja međusobnog vezivanja monomera. Razmatrano je značenje višestrukih oblika acetilkolinesteraze, osobito u sisavaca.

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