BIOCHEMICAL JOURNAL LETTERS

A self-consistent mechanism for dealkylation in soman-inhibited acetylcholinesterase

A recent article by Shafferman et al. [1] requires a number of factual and conceptual corrections. That paper reports the effects of substitutions of the active-site residues of human acetylcholinesterase (HuAChE) on the dealkylation reaction of soman-inhibited HuAChE mutants. The authors interpret kinetic results by using the currently popular cation–π effect and supposedly refute a model previously proposed by our group [2–9]. We object to the misrepresentation of our model and the adoption of major tenets and the vocabulary of our publications without accrediting their source where the arguments are raised. There are two references to our papers in the wrong context and thus these references are misleading.

The basic tenet of our model [8] for the aging of soman-inhibited cholinesterases is the electrostatic 'push-pull' mechanism of transition-state stabilization. Development of the incipient carbocation is promoted by the ionized Glu-199 residue [*Torpedo californica* acetylcholinesterase (AChE) numbering] and supplemented by aromatic residues. The accumulating negative charge on the oxyanion is stabilized by the protonated form of His-440 and possibly by the oxyanion hole. Several experimental facts are explained by this model: (1) The pH-dependence over the pH range 3.5–10 (the adduct is resilient to denaturation) of the reaction in a number of cholinesterases is definitely bell-shaped with a lower pK of ≈ 4.5 and consistent with the ionization of a carboxylic acid, most probably Glu-199. (2) The carboxylic acid preceding the catalytic serine residue is conserved in many serine proteases, but with a different conformation and role, and is always ionized. Glu-199 in AChE is very mobile and appears to be a surrogate general base catalyst [3,8]. (3) Other soft negative electrostatic interactions with polarized aromatic entities may also contribute. (4) His-57 in covalently modified adducts of serine proteases has an enhanced pK. (5) The mechanism is consistent with the product distribution of the reaction as reported by Michel et al. [10]

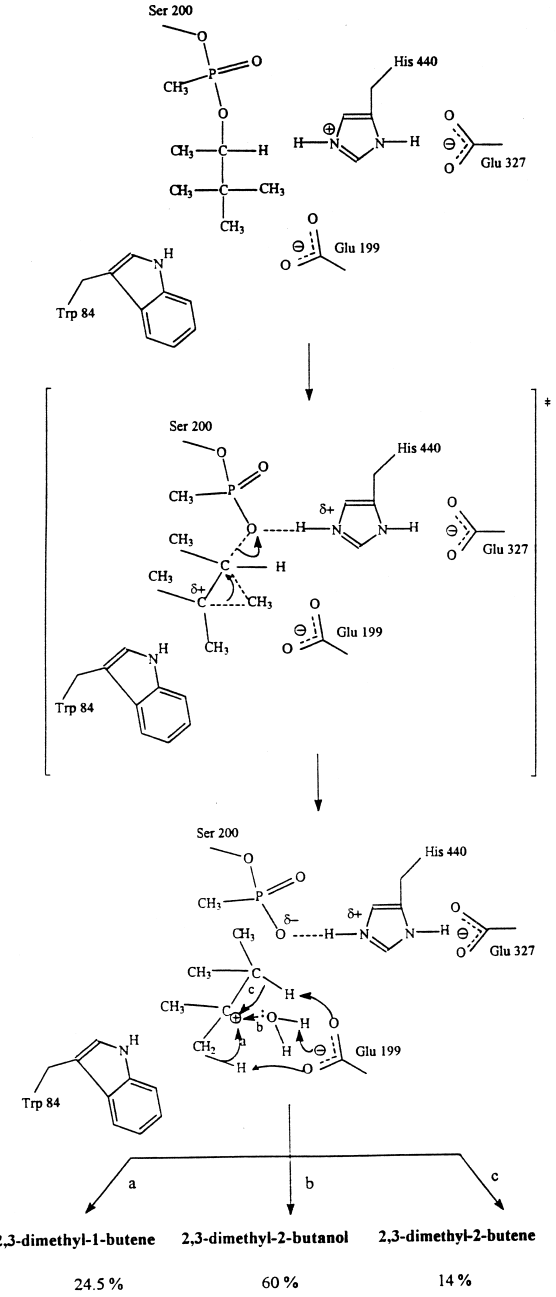
Shafferman et al. [1] state in the abstract that "... Glu-202 is not involved in the proton transfer to the phosphonyl moiety." Then, on page 837, the authors shed light on why the proposition was made in the first place: "... general acid catalysis has been previously suggested for the reactions of AChE with certain substrates [43]. A recent computational study has suggested that Glu-202, rather than His-447, might be involved in the protonation necessary to induce the aging process [14]." Both of these statements are incorrect. We have never suggested general acid catalysis by Glu-199 (residue 202 in HuAChE numbering). Instead, we have proposed participation of the protonated histidine residue; however, this is not credited to us. A detailed analysis of the H-bonding distances, exactly as presented in our paper [8], is also given in the paper by Shafferman et al. [1]. The section on pages 838–839 uses all the arguments we presented in a recent paper [8] on the molecular details of histidine catalysis of the aging reaction.

A strange suggestion by Shafferman et al. [1] is that, in addition to all the interactions the protonated His-447 is engaged in, it would also stabilize Glu-202 and enter an another cation–π interaction between it and a nearby phenylalanine residue. How could these interactions compete with the stabilizing effect of the catalytic glutamic acid residue (residue 327 in *T. californica* AChE)? If this proposal were correct, AChE would be a lame catalyst, since it would shut down its own function after the first catalytic cycle by engaging the protonated histidine residue in catalytically unproductive interactions. We have seen a danger of ionic interactions between $HisH^+$-440 and Glu-199 in our calculations and have alerted the reader to the problem as a potential artifact of the calculations.

Another baffling statement in the paper is "The proposed electrostatic barrier to a nucleophilic attack, due to the presence of additional negative charge [17], seems to be inconsistent with the reactivity properties of charged organophosphorus esters [18,19]." Why? There is a 1000-fold difference in the rate of hydrolysis of bis-4-nitrophenyl methylphosphonate and 4-nitrophenyl methylphosphonyl anion [11]. There are other and similar examples in the literature on organophosphorus compounds.

The oxonium-ion mechanism given in the paper is inconsistent with stabilization by hyperconjugation with H-C$^{\gamma}$ or C$^{\gamma}$-C$^{\beta}$ electrons or even with efficient electrostatic effects ≈ 5 Å away. It would be associated with an inverse solvent-isotope effect, whereas the observed values are between 1.1 and 1.3 for the maximal rate constant for aging in soman-inhibited AChEs [12]. The oxonium-ion mechanism is inadequate in explaining the product distribution. Our reaction scheme, Scheme 1 [12], can reconcile existing controversies and provide a self-consistent mechanism of the dealkylation in soman-inactivated AChE. This reaction is driven by the high electron density of Glu-199 (202) and aromatic residues, primarily Trp-84 (86), at the active site. Each of these residues contribute up to $\approx$ 100–200-fold acceleration of the reaction rate, and together they affect methyl migration (not hyperconjugation) from the overcrowded Cβ to Cα with nearly concerted bond fission to O. The accumulation of negative charge on the etheral oxygen at the transition state (in brackets) is ameliorated by the adjacent $HisH^+$-440 (447) and possibly by the positive electrostatic field of the oxyanion hole. A fleeting tertiary carbocation may develop that is rapidly trapped by three modes of proton removal by Glu-199 (202) [from (a) CγH_3, (b) an attacking water molecule and (c) CαH], leading to the product distribution reported by Michel et al. [10]. The anionic monoester product engaged in an ion-pair with the protonated catalytic histidine residue is also consistent with the neutron-diffraction data obtained by Kossiakoff and Spencer [13].

The kinetic data of Shafferman et al. [1] can also be explained by the model. The E202Q, E202D and E202A mutations (Glu-202 $\rightarrow$ Gln etc.) cause a 138–333-fold reduction of the rate of aging which can be dissected into a 3-fold effect due to size and 138-fold effect due to the lack of catalytic potential. The effect of E450A and Y133F mutations are on the conformation of the carboxylate of Glu-199 and thus disfavour catalysis. An over-1000-fold effect of the mutation W86A and a 15-fold effect of the mutation W86F can also be dissected into an approximate size effect and cation–π effect. These mutations also reduce the rate of phosphonylation by soman up to 15-fold, which can only be a size effect. Consequently, $\approx$ 100-fold of the effect on aging can



Scheme 1 The mechanism of dealkylation in soman-inhibited AChE

be attributed to the cation–π effect. Contrary to a statement made by Shafferman et al. [1], there is a tryptophan residue (position 215) in chymotrypsin and trypsin in the binding pocket only 1 Å farther from the pinacolyl group than in AChE, if one models the P_S soman-inactivated trypsin, yet the aging reaction (not a carbocation mechanism) is four orders of magnitude slower than in AChE [12]. An appropriate model reaction for dealkylation in the phosphonate diester of AChE would be more than ten orders of magnitude slower than aging at pH 5.5 and 37 °C. Four to five orders of magnitude of this transition state stabilization must come from the negative electrostatic effect, as described above, and another four to five orders of magnitude stabilization must originate from positive electrostatic stabilization or other elements [12]. In summary, general base catalysis and the cation–π effect each probably contribute about 20 % of the transition-state stabilization in the aging reaction in soman-inhibited cholinesterases.

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Aging of somanyl–acetylcholinesterase adducts: facts and models

The letter of Kovach et al. [1] requires some clarifications and corrections.

(a) The models for aging of somanyl–acetylcholinesterase (AChE) adducts, which both of our groups had already proposed in 1993 [2,3], attribute the same role for the catalytic His-447 (*440*) (upright and italic numbering refers to the amino acid position in human and *Torpedo californica* AChEs respectively). In both models the spacial arrangement of active-centre residues involved in aging rely on the three-dimensional structure of *T. californica* AChE [4] (or on the model for human AChE derived from it [5]), hence inter-atomic distances in the two models should be similar.

(b) While Kovach et al. suggest a certain role for Glu-202 (*199*) based on experimentation with wild-type enzyme [6] and on theoretical considerations [3,7], we have actually measured the effect of substitution of this glutamate (by glutamine, aspartic acid and alanine) on the rates of catalysis [8], phosphylation [2,9] and aging [2,10] and, on the basis of these results, we proposed a somewhat different role for Glu-202 (*199*).

(c) While Kovach et al. suggest that Glu-202 (*199*) plays a key role in the aging, we found that other amino acids contribute equally, or more, to the aging of somanyl–AChE adducts. Replacements of Glu-202 (*199*) or Phe-338 (*331*) both lower the rates of aging by over two orders of magnitude, while substitution of Trp-86 (*84*) by a non-aromatic residue, by three orders of magnitude. Neither Phe-338 (*331*) nor Trp-86 (*84*) are even mentioned in the model published by Bencsura et al. [7].

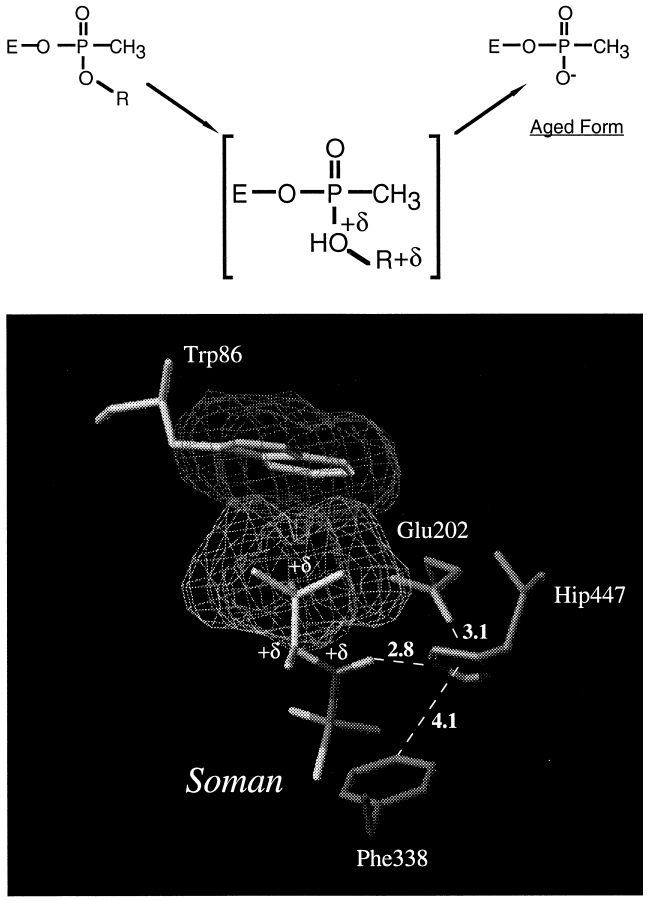

Figure 1 Transient intermediate in the 'aging' process of 1,2,2-trimethylpropylmethylphosphono-HuAChE

The phosphonyl moiety, some of the residues experimentally shown to be involved in the aging process, as well as the catalytic His-447, are depicted. The latter is presumably involved in maintaining a high oxonium-ion population on the phosphonyl moiety [2,10]. Both the charge and the positioning of the imidazolium moiety proximal to the alkoxy oxygen are stabilized by interactions with Glu-202 and with Phe-338 [10,17]. The interaction distances (Å) from the centroid of the protonated histidine (Hip447) imidazolium ring to $O^{\epsilon 1}$ (Glu202) and the C^{ζ} (Phe338) are shown by broken lines. The methylphosphonyl C_{β} methyl substituents and the indole group of residue Trp-86 exhibit a tight matching of their van der Waals surfaces (shown as grids) [10,17]. This interaction contributes to the stabilization of the evolving carbocation, in accordance with its role in interactions with positively charged active-centre ligands [14–16].

(d) We have made an honest attempt to give credit to those who contributed to the field of aging, including the Kovach group. Yet we now realize, regretfully, that we made a mistake in one sentence on page 837 of our paper [10] (see below), in referring to the work of Bencsura et al. [7].

(e) Contrary to the statements in the letter by Kovach et al. [1], we have not attempted to either present or refute their model of aging. Yet some of our results clearly do not conform with their model.

(f) We believe it is very inappropriate to use the letter [1] to introduce a new and yet unpublished model, especially since this model is quite different from the one we allegedly misrepresented. Worse still, the new model is not supported by new data and includes Trp-86 (*84*) without crediting us.

In the following we clarify briefly the similarities and differences in the role, assigned by each of our groups, to the various amino acids (underlined for emphasis) in the published mechanisms of aging.

His-447 (*440*): consistent with the commonly accepted function of histidine in the catalytic triads of serine hydrolases, both groups assign a role in proton transfer to His-447 (*440*). In fact we have proposed such involvement of His-447 (*440*) in the aging process several years ago [2] (see Figure 1).

Glu-202 (*199*): Bencsura et al. have stated (in the abstract of [7]) "Glu199 is a key residue in the electrostatic catalytic mechanism of AChE, in removal of the leaving group, and possibly by acting as an alternate general base catalyst." In our opinion, these views are difficult to reconcile with our experimental findings: (a) when the carboxylate at position 202 (*199*) is preserved [as in E202D (Glu-202 → Asp) HuAChE (human AChE)], the rate of aging decreases as much as for E202Q [and only about twice less than for the enzyme carrying alanine (E202A)] [2,10]; (b) the pH profiles of aging for the wild-type and the E202Q HuAChE adducts are equivalent [10]. These arguments are ignored in the BJ letter by Kovach et al. [1]. Our measurements of the pH profiles of aging indicate also that the phosphonyl protonation step [by His-447 (*440*)] is not affected by the presence or absence of carboxylate at position 202 (*199*). Regretfully our attempt to relate to this somewhat complex argument brought about the mistaken quotation in one sentence on page 837 [10] (the words "rather than His-447" should have not appeared).

Bencsura et al. seem to regard Glu-*199* as exerting a "strong electrostatic catalytic effect on most reactions" [7,11]. However, the results from different laboratories [2,8,12] demonstrated that replacement of Glu-202 (*199*) by a neutral amino acid, glutamine, has only a moderate effect on catalysis (a reduction of 3–6-fold in the values of k_{cat}). Thus the role of Glu-202 (*199*) in substrate hydrolysis and in aging appears to be different.

While Glu-202 (*199*) probably contributes to the electrostatic stabilization of the evolving carbocation, its other role in the aging process appears to involve specific interactions with elements of the H-bond network (see below) and with the protonated His-447 (*440*). Both interactions depend critically upon the precise positioning of its carboxylate (see Figure 1).

Kovach et al. [1] take advantage of the letter to speculate further that Glu-*199* is involved in determining the product distribution of aging (not mentioned in their previous publications). In view of the well-documented tendency of carbocations to rearrange in solution through 1,2-methyl shifts [13], one is required to support such a role of Glu-*199* by experimental evidence.

Glu-450(*443*) and Tyr-133(*130*): these residues are not mentioned in the model published by Bencsura et al. [7]. The role of these residues, in maintaining the precise positioning of Glu-202 (*199*) through an H-bond network [10], was first proposed by us in relation to the aging process [2] and subsequently to catalysis and interactions with covalent and non-covalent AChE ligands [9,14,15]. In the case of E202D, the different location of the aspartate side chain precludes formation of the H-bond network although a carboxylate group is still present at position 202 (*199*). This demonstrates that electrostatic interactions alone are not sufficient to account for the roles of either Glu-202 (*199*) or Glu-450 (*443*).

Phe-338 (*331*): involvement of this residue is not mentioned in the Bencsura et al. model [7]. This observation is ignored also in their present letter [1]. Replacement of this residue by alanine affects aging to the same extent as the substitutions of Glu-202 (*199*) [2,10]. We suggest that Phe-338 (*331*) stabilizes, through a cation–π interaction, a conformation of His-447 (*440*) in which the H-bond with the phosphonyl oxygen can be maintained (see Figure 1). Although such interaction should be possible during catalysis, our data show that replacement of Phe-338 (*331*) has

no effect on the rates of catalysis or of other interactions with AChE ligands [8,9,15,16]. These findings exemplify the flexibility of the active-centre residues and their potentially differential effects in aging as against catalysis. Consequently, as in the case of Glu-202 (*199*), one has to be very careful when drawing analogies between effects of structural changes on different processes.

Trp-86 (*84*): this residue, which is of a crucial importance in the aging process of somanyl–AChE, is again nowhere to be found in the Kovach et al. model [7]. The effect of its replacement by alanine on the rate of aging is 10-fold larger than those due to substitutions of either Glu-202 (*199*) or Phe-338 (*331*) [10]. The main role we attribute to Trp-86 (*84*) is in stabilization of the evolving carbocation through cation–π interactions (see Figure 1). The extent of such interaction should be determined by the degree of hyperconjugation in the alkoxy substituent and, consequently, by the branching at C_β. Recently, we have corroborated this prediction through usage of a series of phosphonates and by following aging rates with the different mutants at position 86 (*84*) [17]. We have shown that the values of $\Delta E^{\ddagger}$ exhibit (Figure 2 of [17]) a linear dependence on the degree of branching and that such dependence disappears when position 86 (*84*) is substituted by a non-aromatic residue (alanine). These findings also demonstrate that steric hindrance is not related to the rate of aging, since $\Delta\Delta E^{\ddagger}_{\mathrm{Trp-Ala}} = \Delta\Delta E^{\ddagger}_{\mathrm{Phe-Ala}}$ [17]. [$\Delta E^{\ddagger}$ is the difference in activation energies of aging for two different phosphonates in a given AChE enzyme, and $\Delta\Delta E^{\ddagger}$ is the difference in the $\Delta E^{\ddagger}$ of two AChE enzymes (Trp, wild-type; Phe, W86F; Ala, W86A) for the same phosphonate pair].

As stated in our paper [10], attempts to find residues analogous to Trp-86 (*84*) in other serine hydrolases participating in aging processes were unsuccessful (the indole of Trp-215 in trypsin cannot interact with the alkoxy group, as can be seen also in Figure 3 of the Bencsura et al. paper [7]).

Kovach et al. do not apply their stated standards regarding proper credit to work published by others. For example: (a) to date we are the only group to demonstrate the relevance of Trp-86 (*84*) to aging [10]; this contribution is not credited to us in the legend to their new model in the BJ letter [1]. (b) Kovach et al. provide in this letter [1] an explanation for the contribution of Glu-450 (*443*) and Tyr-133 (*130*) to the conformation of Glu-202 (*199*) using the arguments we have made both in 1993 [2] and in the recent paper [10], again without giving us credit. (c) In [11], page 8536, Kovach's group take credit for proposing, in 1993, the involvement of Glu-202 (*199*) in catalysis [3], which they claim was "subsequently" supported by us [2]. In fact, publication of [2] clearly preceded that of [3]. Furthermore, by 1992, Taylor's group [12] and ours [8] demonstrated by mutagenesis the involvement of Glu-202 (*199*) in catalysis.

Finally, models are valuable as aids in formulating hypotheses that could be tested experimentally. We are fortunate to live in an era where tools of modern molecular biology are readily available, and we believe that their use is imperative for resolving mechanistic issues involving enzymes, especially when one assumes particular roles for specific amino acids. In view of the progress made to date, we believe that the mechanism of aging will eventually be thoroughly understood.

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