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Site-directed mutagenesis of active-site-related residues in *Torpedo* acetylcholinesterase

Presence of a glutamic acid in the catalytic triad

Nathalie Duval[a], Suzanne Bon[a], Israel Silman[b], Joel Sussman[b] and Jean Massoulié[a]

[a]*Laboratoire de Neurobiologie, CNRS UA 295, Ecole Normale Supérieure, 46 rue d'Ulm, 75005 Paris, France* and [b]*The Weizmann Institute of Science, Rehovot 76100, Israel*

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Site-directed mutagenesis was used to investigate the role of acidic amino acid residues close to the active site of *Torpedo* acetylcholinesterase. The recently determined atomic structure of this enzyme shows the conserved Glu-327, together with His-440 and Ser-200 as forming a catalytic triad, while the adjacent conserved Asp-326 points away from the active site. Transfection of appropriately mutated DNA into COS cells showed that the mutation of Asp-326→Asn had little effect on catalytic activity or the molecular forms expressed, suggesting no crucial structural or functional role for this residue. Mutation of Glu-327 to Gln or to Asp led to an inactive product. These results support the conclusions of the structural analysis for the two acidic residues.

Acetylcholinesterase; Catalytic triad; Site-directed mutagenesis; COS cell

1. INTRODUCTION

The principal biological role of acetylcholinesterase (AChE, EC 3.1.1.7) is the termination of impulse transmission at cholinergic synapses by hydrolysis of the neurotransmitter, acetylcholine [1]. In keeping with this functional requirement, AChE is an extremely rapid enzyme, operating, under optimal conditions, at a rate approaching that at which substrate diffusion becomes rate-limiting [2].

Kinetic studies suggest that the catalytic mechanism of AChE is rather complex. The chemical steps of acylation and deacylation of the active-site serine are preceded by a conformational step [3]. In addition, the influence of the isotopic ratio in D_2O/H_2O solvents indicates the transfer of a single proton, suggesting that a charge relay system, such as that believed to operate in the serine proteases [4], which involves several protons, is not functional in this case [2,5].

The recent determination of the three-dimensional atomic structure of AChE from *Torpedo californica* permits, for the first time, direct visualization of its active site [6]. In particular, this structure shows that the active-site serine (Ser-200) forms a catalytic triad similar to that observed in chymotrypsin [4]. The participation of His-440 in such a triad had previously been inferred, since it is the only histidine residue conserved within the family of cholinesterases and homologous enzymes [7], and its mutagenesis to glutamine totally abolished catalytic activity [8]. The three-dimensional structure indeed placed His-440 within hydrogen-bonding distance of Ser200O_{gamma} [6]. The acidic member of the triad, Glu-327, had not been identified previously. In all serine hydrolase structures solved hitherto, this residue was an aspartic acid. *Torpedo* AChE [6], and the structurally related lipase from *Geotrichum candidum*, the structure of which was also solved only recently [9], are the first cases reported in which Asp is replaced by Glu.

It is interesting that Glu-327 is replaced by an aspartic residue in some of those esterases which present a clear homology of their primary structure with that of the cholinesterases [7]. In addition, this residue is immediately preceded by an aspartic acid in all known primary structures of cholinesterases. In the present study we report the effect of site-directed mutagenesis of Asp-326 and Glu-327 on the catalytic activity of *Torpedo* AChE.

2. MATERIALS AND METHODS

In order to mutagenize aminoacids 326 and 327 of *Torpedo* AChE, we amplified a restriction fragment (nucleotides 1017–1695 of the coding sequence, numbered as in [10]) in the expression vector CDM8-$AChE_T$ [7,11], using a mutagenizing oligonucleotide primer and a common primer. The mutagenizing primers were based on the following sequence:

5′-GAAGACTAGATCTTACTGGGAGTCAACAAGG*ACG*A-G*GGCTCGT-3′.

Correspondence address: J. Massoulié, Laboratoire de Neurobiologie, CNRS UA 295, Ecole Normale Supérieure, 46 rue d'Ulm, 75005 Paris, France.

This sequence corresponds to nucleotides 1017–1060, in the coding orientation; it contains a *BalI*I restriction site (underlined). Either one or two of the G nucleotides marked by asterisks (1048, 1051, and 1053) were replaced by other nucleotides: G^{1053}-C for Glu-327→Asp, G^{1051}-C for Glu-327→Gln, G^{1048}-A for Asp-326→Asn, and a combination of the last two changes for the double mutation Asp-326→Asn/Glu-327→Gln. The common primer was in the antisense orientation, and was complementary to nucleotides 1671–1695 of λAChE 15-2: 5′-TCGTTTGGGTTTCCAGTCTTTGCG-3′.

The amplification (PCR) reaction mixture contained 500 ng of the common and mutagenizing primer, 5 ng CDM8-$AChE_T$ DNA, 200 mM dNTP, one unit of Taq polymerase (Bioprobe Systems, Montreuil-sous-Bois, France), 5 μl of 10× concentrated Taq polymerase buffer, and sterile water to 50 μl. Amplification was performed as follows: 40 cycles of 1 min at 92°C; 1 min at 55°C; 3 min at 72°C, in an Hybaid heating block. The amplification mixture was electrophoresed in an agarose gel, and the amplification product was extracted, digested by *BglII* and *NsiI*, and ligated to CDM8-$AChE_T$, from which the corresponding fragment had been removed by digestion with *BglII* and *NsiI*, as described previously [11]. Competent bacteria were transformed with the ligation product, and plasmidic DNA from selected transformants was fully sequenced and used for transfecting COS cells as described [11].

After four days at 27°C, the transfected cells were extracted in 1% Triton X-100, 10 mM Tris-HCl pH 7, and the extract was assayed for AChE activity colorimetrically, according to Ellman et al. [12] using acetylthiocholine as the substrate, or radiometrically, according to Johnson and Russell [13], using [^{3}H]acetylcholine as substrate. Active-site titration, using the organophosphate inhibitor, *O*-ethyl-*S*^{2}-diisopropylaminoethyl methylphosphonothionate, was performed according to Vigny et al. [14]. Western blots were performed with the rabbit anti-*Torpedo* AChE antiserum, Tor-152, as described [11]. Sucrose gradient analysis of molecular forms was carried out as described previously [11].

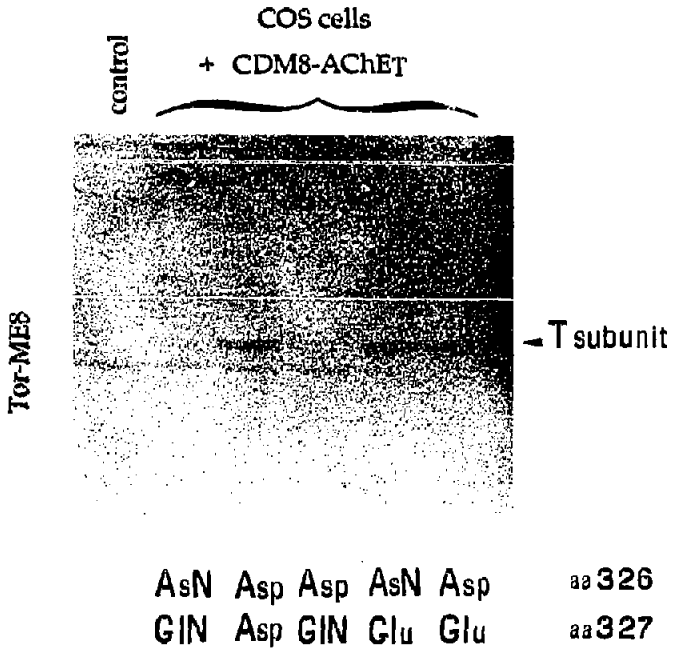

Fig. 1. Western blots showing the production of *Torpedo* AChE protein in transfected COS cells. Extracts from cells transfected with the wild-type and mutated constructions were analyzed in Western blots, using the monoclonal antibody Tor-ME8 (1/200). The amino acids at positions 326 and 327 are indicated below each lane; the right hand lane corresponds to the wild-type sequence. In all cases, the T subunit appears at the same position in the gel, with a similar intensity. It does not correspond to any Tor-ME8 reactive protein in non-transfected COS cells.

3. RESULTS AND DISCUSSION

We showed earlier that transfected COS cells produce active *Torpedo* AChE when transferred from 37°C to 27°C [11]. In the present experiments, we transfected COS cells with CDM8 vectors carrying the coding sequence of the T catalytic subunit (which generates collagen-tailed AChE in *Torpedo* electric organs), either in its wild-type form or after mutagenesis of either Asp-326 or Glu-327.

The mutagenized sequences were constructed by exchanging restriction fragments in the wild-type vector, CDM8-$AChE_T$. Since the vectors differ only at the mutagenized codons, expression is unlikely to be affected by the mutations. We checked, however, that all transfections produced similar amounts of *Torpedo* AChE protein, as recognized by the monoclonal antibody, Tor-ME8 [15] (Fig. 1).

The product of the mutation Asp-326→Asn was expressed at a level of catalytic activity similar to that of the wild-type enzyme. Under the conditions of the radiometric assay, active-site titration showed it to have a specific activity ca. 50% of that of the wild-type. Moreover, the mutation did not modify the pattern of molecular forms of AChE produced in the transfected COS cells, yielding G_4^{as}, G_2^{a} and a low level of G_1^{a} forms [11]. It is thus clear that Asp-326 is not essential either for the catalytic machinery of AChE or for the establishment and maintenance of the correct conformation. It should be remembered that, even at 27°C, COS cells produce mostly inactive, most probably misfolded, *Torpedo* AChE. A perturbation in the acquisition of the correct folding pattern would, therefore, be expected to decrease the yield of active enzyme markedly. Although Asp-326 is conserved in cholinesterases [7], the lack of a substantial effect upon its replacement by Asn is consistent with the fact that it is substituted by other amino acids in a number of cholinesterase-like proteins: Glu in some cases, but also His, Ser, Met or Gly [7].

In contrast, mutations Glu-327→Gln, Glu-327→Asp, and the double mutation, Asp-236→Asn/Glu-327→Gln, resulted in almost complete loss of enzymic activity, in agreement with the involvement of Glu-327 in a catalytic-site triad, whether as part of a charge-relay system or in a different functional or structural capacity [5]. Similar findings have been reported recently for human AChE [16].

It is particularly interesting that aspartate cannot substitute for glutamate in AChE, even though some cholinesterase-like enzymes (viz. *Drosophila* esterase P and esterase 6, rat lysophospholipase) possess an aspartic acid at the homologous position [7]. It is also worth noting that the non-catalytic cholinesterase-like proteins also present acidic residues at this position, either

Glu (*Drosophila* neurotactin) or Asp (*Drosophila* glutactin, mammalian thyroglobulin). In addition, these proteins contain a histidine residue at the position expected for that element of the catalytic triad, but do not possess a serine at the predicted position. The presence of both the acidic residue and the histidine in non-catalytic proteins may represent a non-functional inheritance from catalytically active ancestors; alternatively, an interaction between these two residues may be a requirement for maintaining the three-dimensional structure of the protein.

In the three-dimensional structure of *Torpedo* AChE, as determined by Sussman et al. [6], the spatial arrangement of the three segments of the polypeptide backbone which bear the residues of the catalytic triad is such that an aspartic residue at position 327 would be too short to interact with His-440. The fact that we do not find any activity in the Glu-327→Asp mutant shows that the structure is not flexible enough to compensate for the 3-Å distance. This rigidity contrasts with the apparent existence of an induced-fit step preceding the acylation-decylation reactions [3].

The present results confirm the existence of a catalytic triad, Glu-327/His-440/Ser-200, in *Torpedo* AChE. This is quite consistent with kinetic evidence indicating the presence of a charge-relay system in the homologous enzymes, butyrylcholinesterase and cholesterol esterase. In these latter enzymes, proton inventory analyses of the influence of the isotope ratio in D_2O/H_2O media indicate the transfer of several protons during catalysis (D. Quinn, personal communication). The fact that similar analyses show that a single proton is transferred in AChE illustrates the originality of this exceptionally rapid enzyme. Its detailed mechanism remains a puzzle, and a fascinating subject for future research.

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