Monomerization of tetrameric bovine caudate nucleus acetylcholinesterase

Implications for hydrophobic assembly and membrane anchor attachment site

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Tetrameric detergent-soluble bovine caudate nucleus acetylcholinesterase (AChE) was reduced and alkylated under conditions in which at least 95 % of initial activity is retained. This treatment alone did not result in monomerization of AChE, nor did it create a hydrophilic enzyme. However, in the presence of SDS the enzyme became monomerized. Incubation of AChE with trypsin in the presence of the reversible inhibitor edrophonium rendered the enzyme hydrophilic and led to catalytically active monomers being produced. SDS/PAGE of this preparation in non-reducing conditions revealed only a small decrease in the subunit molecular mass. *N*-Terminal sequencing of the enzyme, before and after trypsin treatment, yielded identical *N*-termini showing that the enzyme was monomerized subsequent to *C*-terminal tryptic cleavage. From our results, we conclude that the most *C*-terminal cysteine residue is involved in inter-subunit disulphide bonding as well as in the attachment of AChE to the membrane anchor. Furthermore, the *C*-terminal region in the primary structure provides an area for hydrophobic contacts between the different subunits and also between the subunits and the membrane anchor.

INTRODUCTION

The tetrameric form of brain acetylcholinesterase (AChE, EC 3.1.1.7) is an amphiphilic globular protein (G_4 form) anchored to the membrane via an as yet virtually undefined structure of approx. 20 kDa which is released from the subunits upon SDS/PAGE in reducing conditions (Gennari *et al.*, 1987; Inestrosa *et al.*, 1987). According to Inestrosa & Perelman (1990) and Roberts *et al.* (1991), two of the four catalytic subunits are interconnected via disulphide bonds, whereas the other two subunits are attached via disulphide bonds to the membrane-anchoring domain. The quaternary structure of bovine brain AChE resembles the tetrameric units of the A_{12} form of the asymmetric enzyme found in neuromuscular junctions of vertebrates, and that found in the electroplaques of *Torpedo* and the electric eel. Within these tetrameric units, two catalytic subunits are also attached via disulphide bonds to an anchoring domain (in these cases a collagenous tail; for a review see Massoulié & Toutant, 1988), and the other two subunits are also interconnected via disulphide bonds.

Whereas the complete primary structure of AChE from fetal bovine serum (FBS) is known (Doctor *et al.*, 1990), the enzyme from bovine caudate nucleus has only been partially sequenced (Heider *et al.*, 1991a; Roberts *et al.*, 1991). The brain enzyme shares more than 98 % identical residues with the FBS form throughout 130 amino acids identified to date. Since the last 40 amino acids of FBS AChE are identical to the *C*-terminal 40 amino acids encoded by a cDNA clone of human AChE (Doctor *et al.*, 1990; Soreq *et al.*, 1990), it is reasonable to assume that AChE forms from FBS and adult bovine brain are identical. From their primary structures it is seen that they both contain seven cysteine residues. The first six are located in conserved positions compared with all other known sequences of cholinesterases. From the sequence and the disulphide bonding pattern of AChE from *Torpedo californica* (McPhee-Quigley *et al.*, 1986), it can be concluded that the first six cysteine residues

of FBS AChE are also engaged in intrachain disulphide bonding. In FBS AChE (Doctor *et al.*, 1990), bovine brain AChE (Roberts *et al.*, 1991) and human AChE (Soreq *et al.*, 1990), the seventh cysteine residue is located at position 580 and is the one involved in inter-subunit disulphide bonding (Roberts *et al.*, 1991).

As shown by Gennari & Brodbeck (1985), limited treatment of amphiphilic G_4 AChE from human brain with proteinase K converted the detergent-soluble enzyme to a hydrophilic form. This result was confirmed by Fuentes *et al.* (1988) for the same enzyme form extracted from bovine caudate nucleus. Sucrose density gradient centrifugation revealed that the native enzyme from both sources remained tetrameric, whereas in the presence of SDS, i.e. in denaturing conditions, it was fully monomerized by proteinase K (Gennari & Brodbeck, 1985; Fuentes *et al.*, 1988). From these results, it may be concluded that the subunits of G_4 AChE from brain are held together by disulphide bonds as well as by hydrophobic interactions. In the present study, we further investigated the specific conditions which lead to monomerization of the brain enzyme in order to obtain information on the interactions involved in subunit assembly.

MATERIALS AND METHODS

All reagents used were of analytical or sequencing grade, from Fluka (Buchs, Switzerland), Sigma (St. Louis, MO, U.S.A.), Boehringer (Mannheim, Germany) or Applied Biosystems (Warrington, Cheshire, U.K.). Edrophonium (Tensilon) was a gift from Hoffmann-La Roche (Basel, Switzerland); 3-trifluoromethyl-3-(*m*-[125 I]iodophenyl)diazirine ([125 I]TID) was kindly donated by Dr. J. Brunner (ETH-Zürich, Switzerland).

Biological material

Bovine brain was purchased from the local slaughterhouse. Detergent-soluble AChE was extracted from caudate nuclei and purified essentially according to the procedure described for human brain AChE by Sørensen *et al.* (1982). In order to obtain

Abbreviations used: AChE, acetylcholinesterase; FBS, fetal bovine serum; [125 I]TID, 3-trifluoromethyl-3-(*m*-[125 I]iodophenyl)diazirine; DTT, dithiothreitol; G-PI, glycosyl-phosphatidylinositol; PVDF, poly(vinylidene difluoride).

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enzyme of highest purity, a second affinity chromatography step was routinely performed, yielding AChE with a specific activity of approx. 6000 units/mg of protein.

Enzyme assays

AChE activity was measured at room temperature by the method of Ellman *et al.* (1961). The assay solution contained 1 mM-acetylthiocholine iodide and 0.25 mM-5,5'-dithiobis(2-nitrobenzoic acid) in 100 mM-phosphate buffer, pH 7.4, with 0.1 % Triton X-100. Enzyme activity was expressed in units (μmol of substrate hydrolysed/min).

[^{125}I]TID labelling of AChE

[^{125}I]TID labelling was performed at room temperature with AChE samples containing about 3000 units/ml. The specific radioactivity of [^{125}I]TID was approx. 20 Ci/mmol. To 0.3 ml of enzyme solution, 10 μl of [^{125}I]TID in ethanol was added. Photocoupling was performed at room temperature within 1 min using an illumination device from Müller Elektronik (Baden-Dättwil, Switzerland). Power output to the lamp was limited to 240 W, and a CuSO_4 solution between lamp and probe was used as a cut-off filter unit. Excess [^{125}I]TID was removed by binding the labelled AChE to an affinity column, with subsequent washing of the bound enzyme with 10 mM-Tris, pH 7.4, containing 144 mM-NaCl and 1 % Triton X-100 until eluted radioactivity remained constant. Labelled enzyme was then eluted with the above buffer supplemented with 20 mM-ethoprophonium.

SDS/PAGE and autoradiography

SDS/PAGE was performed essentially according to Laemmli (1970) using 5–18 % polyacrylamide gradient gels. Samples were subjected to gel electrophoresis in non-reducing conditions [i.e. neither β -mercaptoethanol nor dithiothreitol (DTT) was included in the sample buffer].

Proteins were stained with Coomassie Brilliant Blue R-250. For autoradiography, gels containing [^{125}I]TID-labelled samples were dried and exposed to Fuji RX X-ray film at -70°C .

Electroblotting and amino acid sequencing

Electroblotting on to poly(vinylidene difluoride) (PVDF) membranes and subsequent staining of trypsin-treated bovine caudate nucleus AChE was performed essentially according to the procedure described by Heider *et al.* (1991a). Automated Edman degradation of proteins blotted on to PVDF membranes was done with an Applied Biosystems sequencer model 477A essentially according to the procedure described by Schaller *et al.* (1991).

Density gradient centrifugation

AChE samples (0.05–0.1 units) were layered on top of linear sucrose density gradients (5–30 %) in buffer (10 mM-Tris/HCl, pH 7.4, and 144 mM-NaCl, without or with 0.1 % Triton X-100). Centrifugation conditions, fractionation of the gradients and enzymic assay were carried out as described by Stieger *et al.* (1989).

Limited reduction of bovine caudate nucleus AChE

Limited reduction of bovine caudate nucleus AChE was carried out in the presence of 20 mM of the reversible inhibitor ethoprophonium. This compound protects AChE against inactivation by disulphide reducing agents (Rosenberry & Scoggin, 1984), and to a certain extent against inactivation by proteolytic attack (Dutta-Choudhury & Rosenberry, 1984).

Samples of [^{125}I]TID-labelled bovine caudate nucleus AChE, each containing 12 units (corresponding to approx. 6 pmol of tetramers) in Tris/HCl buffer (pH 8.0)/144 mM-NaCl/0.1 % Tri-

ton X-100, were incubated with 30 pmol, 60 pmol, 120 pmol, 1.2 nmol or 600 nmol of DTT at room temperature in the dark under nitrogen. After a 1 h incubation time, the reduced cysteine residues were alkylated by the addition of iodoacetic acid (in 100 mM-Tris/HCl, pH 8.0) at a 5-fold molar excess over DTT. Alkylation was allowed to proceed for 2 h. An aliquot of each sample was immediately layered on a sucrose density gradient; another aliquot was also applied on to a polyacrylamide gel and subjected to SDS/PAGE in non-reducing conditions.

Limited tryptic digestion

To AChE (350 units/ml, corresponding to approx. 60 $\mu\text{g}/\text{ml}$) in Tris/HCl buffer (pH 8.0)/20 mM-ethoprophonium/0.1 % Triton X-100, trypsin was added to a final concentration of 3 $\mu\text{g}/\text{ml}$ (corresponding to 5 %, w/w), and the mixture was incubated at 37°C . Aliquots were withdrawn at 0 min, 15 min, 30 min, 60 min, 2 h, 4 h and 16 h, and soybean trypsin inhibitor at a final concentration of 50 $\mu\text{g}/\text{ml}$ was added immediately. AChE activity of the samples was determined, and the samples were subjected to sucrose density gradient centrifugation in the absence or the presence of 0.1 % Triton X-100. Another aliquot was applied on to a polyacrylamide gel and subjected to SDS/PAGE in non-reducing conditions.

RESULTS

Reduction of brain AChE with DTT and subsequent alkylation by iodoacetic acid

AChE from bovine caudate nucleus was labelled with [^{125}I]TID, a reagent with a high affinity to hydrophobic phases which, upon u.v. irradiation, covalently binds to neighbouring molecules (Brunner & Semenza, 1981). In the present study, most of the

Fig. 1. SDS/PAGE under non-reducing conditions of DTT-treated and alkylated [^{125}I]TID-labelled bovine caudate nucleus AChE

[^{125}I]TID-labelled enzyme (6 pmol in each lane) was either mock-treated (lane 1) or treated with 60 pmol (lane 2), 120 pmol (lane 3) or 1.2 nmol (lane 4) of DTT for 1 h at room temperature in the dark under nitrogen. Subsequently, iodoacetic acid was added to each sample at a 5-fold molar excess over DTT. Alkylation was allowed to proceed for 2 h at room temperature, then samples were applied to a 5–18 % gradient gel. Lanes 5–8 represent the corresponding autoradiograms of lanes 1–4. The assignment of the bands at the left side of the gel corresponds to the nomenclature introduced by Inestrosa *et al.* (1987). Under these conditions, the anchor migrated as a broad band with an apparent molecular mass around 23 kDa.

Fig. 2. Sucrose density gradient centrifugation of reduced and alkylated AChE

Untreated enzyme (100 munits) was applied as a control on to gradients either containing 0.1% Triton X-100 (a, ●) or without Triton X-100 (○). An aliquot of the enzyme (100 munits), reduced with 1.2 nmol of DTT (same as shown in Fig. 1), was layered on gradients either with (b, ●) or without (○) 0.1% Triton X-100. The last seven fractions at the top of the gradient are not shown. The arrow indicates the position of catalase (11.4 S).

protein-bound [125 I]TID label was incorporated into the membrane anchor of brain AChE. However, significant amounts of label were also found in the catalytic subunit of the enzyme. This was seen on SDS/PAGE run in reducing conditions in which both the light and heavy monomers were [125 I]TID-labelled (result not shown). The light monomer is the catalytic subunit, whereas the heavy monomer consists of the catalytic subunit to

which the anchor is still attached (Inestrosa *et al.*, 1987). Under non-reducing conditions, SDS/PAGE showed that the subunits remained aggregated and migrated on the gel mainly as dimers and tetramers (Fig. 1, lanes 1 and 5). Treatment of [125 I]TID-labelled AChE with increasing amounts of DTT and subsequent alkylation with iodoacetic acid led to the formation of monomeric AChE (Fig. 1, lanes 2–4 and 6–8). After Coomassie Blue staining only the light monomer was visible, whereas in the autoradiogram the heavy monomer was also seen. Under these denaturing conditions monomerization was already observed when DTT was added at a 2.5-fold molar excess over catalytic subunits (Fig. 1, lanes 2 and 6), and it was practically complete at a 5-fold molar excess (Fig. 1, lanes 3 and 7). Incubation of bovine brain AChE with DTT at room temperature had virtually no effect on the catalytic activity of the enzyme up to a 50-fold molar excess.

Since the 50-fold molar excess of DTT led to monomerization of the enzyme on an SDS/polyacrylamide gel but preserved enzyme activity in the absence of SDS, we investigated the sedimentation behaviour of the catalytic activity of the DTT-treated enzyme on a sucrose density gradient in both the presence and the absence of Triton X-100. As shown in Fig. 2(b), the sedimentation profile of enzyme activity obtained in the presence of 0.1% Triton X-100 (●) revealed only small amounts of AChE migrating at a position corresponding to monomeric AChE (5.0 S) at a 50-fold molar excess of DTT compared with the non-treated enzyme (Fig. 2a). In the absence of Triton X-100 (○), most of the non-treated enzyme aggregated to forms of higher molecular mass (Fig. 2a). After treatment with DTT part of the enzyme was still aggregated, but virtually no activity was recovered in the position corresponding to the monomeric form (Fig. 2b). When the reduced and alkylated enzyme was kept at 37 °C for a prolonged period of time before subjecting it to the density gradient, the yield of monomeric catalytically active enzyme was increased, but the untreated control did not spontaneously disaggregate into monomers (results not shown). Again, virtually no activity was recovered in the position of the monomer when density gradient centrifugation was performed in the absence of Triton X-100.

Tryptic digestion of native brain AChE

As shown by Vigny *et al.* (1979), G_4 AChE from bovine superior cervical ganglia can be monomerized by combined

Fig. 3. SDS/PAGE under non-reducing conditions of trypsin-treated [125 I]TID-labelled enzyme

(a) Coomassie Blue stain of the trypsin-treated enzyme samples applied on to a 5–18% gradient gel; (b) corresponding autoradiogram. Digestion of the enzyme was conducted at a concentration of 5% (w/w) trypsin and was stopped by adding soybean trypsin inhibitor in a concentration of 50 μ g/ml after 0 min (lane 1), 15 min (lane 2), 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), 4 h (lane 6) or 16 h (lane 7). Lane 8 contains the 16 h control (trypsin and trypsin inhibitor present throughout incubation).

Fig. 4. Sucrose density gradient centrifugation of untreated AChE (a) or AChE treated at 37 °C for 4 h (b) or 16 h (c) with 5% (w/w) trypsin

As a control, the untreated enzyme was kept for 16 h at 37 °C. The trypsin-treated enzyme samples were applied either on to gradients with 0.1% Triton X-100 (●) or on to gradients without Triton X-100 (○). The last eight fractions at the top of the gradient are not shown. The arrow indicates the position of catalase (11.4 S).

treatment with a reducing agent and trypsin. Since our present result showed that partial monomerization could be achieved solely by reduction and alkylation, we investigated whether tryptic digestion alone could also produce catalytically active monomers. Preliminary experiments showed that native bovine caudate nucleus AChE in the presence of edrophonium (a reversible inhibitor of the enzyme) is very stable to trypsin treatment, whereas AChE partially denatured by 2 M-urea was digested to give small peptides within minutes. Optimal conditions for trypsin treatment were established at 5% (w/w) trypsin in presence of 20 mM-edrophonium. When bovine brain AChE was incubated at 37 °C under these conditions, enzyme activity was fully preserved up to 4 h of incubation, and 87% of its activity was recovered after 16 h of incubation. SDS/PAGE in non-reducing conditions revealed that the G_4 enzyme gradually became monomerized by trypsin. Monomeric enzyme appeared on the SDS gel after only 15 min, and after 4 h of trypsin treatment virtually no more dimers and tetramers would be seen (Fig. 3a). From the Coomassie Blue-stained gel it is further seen that the light dimer, which is devoid of the anchor, disappeared faster than the heavy dimer containing the anchor. Similarly, the band at 300 kDa disappeared faster than the band at 240 kDa. In

the Coomassie Blue-stained gel only the light monomer was seen, whereas the corresponding autoradiogram revealed both heavy and light monomers. Trypsin treatment also freed the anchor from the catalytic subunits (Fig. 3b). However, the anchor appeared much later than the monomers, indicating differences in the susceptibility towards trypsin of the inter-subunit disulphide bonds, compared with those linking the anchor to the catalytic subunits. On comparing lanes 7 and 8 of Fig. 1 with lanes 4 and 5 of Fig. 3(b), it is seen that trypsin cleaved the anchor into at least two [125 I]TID-labelled peptides. It is also shown in Fig. 3(b) that incubation with trypsin for 16 h removed most of the [125 I]TID label attached to the catalytic subunits.

The apparent molecular mass of the monomer produced by trypsin was diminished by only a few kDa, indicating that the cleavage had occurred near one of the ends of the polypeptide chain (Fig. 3a). In order to ascertain that the cleavage had occurred before cysteine-580 near the C-terminus and not N-terminally, the enzyme was blotted on to PVDF membranes and subjected to automated Edman degradation. Both control AChE and that treated for 16 h with trypsin (5%, w/w) at 37 °C displayed identical amino acid sequences (results not shown). Sucrose density gradient centrifugation of trypsin-treated brain AChE in the presence of Triton X-100 showed that increasing amounts of catalytically active monomers were obtained with increasing duration of trypsin treatment (Figs. 4a, 4b and 4c). In the absence of Triton X-100 in the gradient, the enzyme no longer aggregated, and it lost most of its activity.

DISCUSSION

G_2 and G_4 AChE not only differ in their subunit assembly, but also in the way they are anchored to membranes and to extracellular structures. In animals from insects to mammals there is only one copy of the AChE gene within the genome of an individual, and the polymorphism of AChE observed within an individual is solely a result of C-terminal splicing (Chatonnet & Lockridge, 1989). As a result of this splicing, the G_2 form of AChE from *Torpedo* is 38 amino acids shorter at the C-terminus than the asymmetric A_{12} form (Sikorav *et al.*, 1988; Gibney & Taylor, 1990). Since the C-terminal sequence of the asymmetric form of *Torpedo* AChE is highly similar to mammalian G_4 forms (Doctor *et al.*, 1990; Rachinsky *et al.*, 1990; Soreq *et al.*, 1990), it is reasonable to assume that a similar splicing event leads to the G_2 and G_4 forms in mammals (Roberts *et al.*, 1991). From the difference at the C-terminus of human AChE (Haas *et al.*, 1986; Soreq *et al.*, 1990), we concluded that the C-terminal region in G_4 AChE must be responsible for the strong inter-subunit contact observed in this form of the enzyme.

In order to obtain information on the forces holding the subunits together, several groups have, in the past, investigated the conditions under which oligomeric forms of AChE could be monomerized. The conversion of the glycosyl-phosphatidylinositol (G-PI)-anchored G_2 form of AChE into enzymically active monomers was achieved with AChE from *Torpedo californica* (Lee *et al.*, 1982), with AChE from human erythrocytes (Ott *et al.*, 1983) and with AChE from human lymphocytes (Bartha *et al.*, 1987). In the case of the G-PI-anchored G_2 form of AChE, the mere reduction of the inter-subunit disulphide bond was sufficient to render the enzyme monomeric, indicating that there are no additional forces holding the subunits together (Ott *et al.*, 1983). On the other hand, monomerization of the G_4 forms of AChE could only be achieved by reduction and proteolytic degradation (Vigny *et al.*, 1979) or by denaturation (Gennari & Brodbeck, 1985; Fuentes *et al.*, 1988).

From our results, it is seen that a 5-fold molar excess of DTT over catalytic subunits was sufficient to reduce the inter-subunit

disulphide bonds as well as the bonds linking the enzyme to the hydrophobic anchor. Since in non-denaturing conditions the enzyme remained tetrameric, and even at a 10-fold higher excess of DTT only small amounts of monomer could be detected, it is obvious that disulphide bonding is not the only force involved in the assembly of the amphiphilic tetramers. This characteristic is clearly distinct from that of the G_2 form of AChE from erythrocytes, in which non-denaturing conditions result in complete monomerization (Ott *et al.*, 1983). Since density gradient centrifugation carried out in high ionic strength buffer did not yield increased amounts of monomers of brain AChE (results not shown), we conclude that the inter-subunit contact area is probably hydrophobic.

As shown previously, treatment of G_4 brain AChE with proteinase K yielded an enzyme that, under denaturing conditions, was monomerized without disulphide bond reduction (Gennari & Brodbeck, 1985; Fuentes *et al.*, 1988). Under these conditions the hydrophobic anchor was also liberated, suggesting that the peptide cleaved by proteinase K contained the inter-subunit disulphide bond as well as the disulphide bonds between the subunits and the anchor. We have now shown that treatment of G_4 AChE with trypsin yields catalytically active monomers in which the anchor was released. As shown in Fig. 3(a), trypsin decreased the subunit molecular mass only slightly, indicating that the cleavage must have taken place near one end of the polypeptide chain. The identical *N*-terminal amino acid sequences determined before and after trypsin treatment clearly showed that the cleavage by trypsin took place near the *C*-terminus. Our finding corroborates that of Roberts *et al.* (1991), who showed that cysteine at position 580 is involved in the inter-subunit disulphide bonding. As shown in Figs. 3(b) and 4, trypsin not only led to monomerization of the enzyme but also released the anchor from the catalytic subunits. This result supports the model of Roberts *et al.* (1991), which postulates that the *C*-terminal cysteine residue is involved in both inter-subunit disulphide bonding and anchor attachment. As further shown in Fig. 3(b), treating the enzyme for 16 h with trypsin released the anchor as well as most of the [125 I]TID label found in the light monomer. The [125 I]TID label thus reflects the intact light monomers, while the Coomassie Blue stain depicts the small decrease in molecular mass caused by trypsin.

It was previously shown by us (Heider *et al.*, 1991b) that the [125 I]TID label attached to the catalytic subunits is located in two cyanogen bromide fragments originating *C*-terminally from the active site serine. One of the peptides consists of residues 478–564, and our present results suggest that trypsin removed the [125 I]TID label attached to this part of the primary sequence. Therefore trypsin removed not only the inter-subunit disulphide bond near the *C*-terminus, but also a hydrophobic area providing the force for the assembly of pairs of dimers to give the tetramer as well as for hydrophobic bonding of the membrane anchor to the subunits. This tryptic cleavage site does not seem to be accessible to proteinase K, since treatment with this enzyme did not result in monomerization upon density gradients (see above).

Membrane-anchored brain AChE and the collagen-tailed form are both oligomers which are asymmetrically assembled. Presently, little is known about the sequence of events leading to the formation of the different disulphide bonds within these oligomers. One possible sequence of assembly might involve the formation of the disulphide bonds between pairs of subunits, followed by disulphide interchange between one pair of subunits and the anchor. Alternatively, two individual subunits may first be attached to the anchor, followed by hydrophobic bonding of a disulphide-linked dimer.

Since in brain AChE we did not observe the formation of catalytically active dimers upon reduction and alkylation or

Fig. 5. Model of the hydrophobic assembly of bovine caudate nucleus AChE

The light grey structures represent the two catalytic subunits of bovine caudate nucleus AChE, which at their *C*-terminus are disulphide-bonded to the hydrophobic membrane anchor. The dark grey structures are the other two catalytic subunits, which are *C*-terminally disulphide-bonded to each other. The central part of the enzyme comprises the four *C*-terminal ends of the catalytic subunits and the catalytic-subunit-binding domain of the hydrophobic anchor. The arrows point at the *C*-terminal tryptic cleavage sites within catalytic subunits as well as within the membrane anchor (note that identical trypsin-sensitive sites exist on all four catalytic subunits).

upon limited tryptic treatment of the enzyme, we conclude that the hydrophobic forces between pairs of dimers are stronger than the interaction between two disulphide-bonded monomers. Under the conditions of our tryptic treatment, a small piece of the sequence was removed at the *C*-terminal end, suggesting that the tetrameric enzyme, together with the anchor, is tightly connected to a hydrophobic core consisting of the *C*-termini of the four subunits (Fig. 5) and one part of the hydrophobic anchor (represented by the black bar in Fig. 5).

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