Molecular Forms of Purified Cytoplasmatic and Membrane Bound Bovine-Brain-Acetylcholinesterase Solubilized by Different Methods

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Membrane bound, Triton X-100 solubilized bovine nucleus caudatus acetylcholinesterase is sedimenting in presence of Triton X-100 concentrations higher than the CMC as a 10.5 S-detergent-enzyme complex. There is evidence that this complex does neither represent the molecular enzyme arrangement present in the membrane, nor the molecular form originally released from the membrane.

The purified, cytoplasmatic acetylcholinesterase is sedimenting as a 10.5 S-form too. This form is clearly to be distinguished from the detergent enzyme complex, for it is obviously not capable of aggregating, whereas the 10.5 S-detergent-enzyme complex aggregates on detergent removal to defined water soluble oligomers with sedimentation coefficients of 16 S (70000 ± 10000), 20.6 S (960000 ± 60000) and 23.3 S (~ 1200000). In contrast to acetylcholinesterase from erythrocytes this aggregation is not easily reversibly by incubation with Triton X-100, reflecting differences in the hydrophobic part of the enzymes.

Purified acetylcholinesterase solubilized without detergent under autolytic or tryptic conditions is mainly sedimenting as a 4.5 S-form. Such slow sedimenting forms detected in crude solubilisates of neuronal tissues, may originate at least partially form autolytic solubilization.

Introduction

It is known that membrane proteins solubilized in the presence of a detergent aggregate to higher oligomers on removal of the detergent using their hydrophobic domains for interaction. It depends on the nature of the protein, but on the kind of the detergent too, as well as on the method of detergent removal, wether the oligomers formed are water soluble or insoluble [1].

Triton X-100 solubilized, highly purified AcChE from nucleus caudatus, depleted of the detergent by an affinity chromatographic procedure was shown to exist in several characteristic water soluble, high molecular forms, which have been separated by preparative gel electrophoresis [2, 3]. We were interested in the question, wether these oligomers are related to the socalled assymetric-, globular-, ES- (easy to solubilize), HS- (hard to solubilize) forms observed by others [4–6], or represent another set of AcChE-oligomers created on detergent depletion, and originating from a single detergent-enzyme complex.

The solubilization of membrane bound brain AcChE has been hitherto achieved not only by use of detergents [2, 7] but also by prolonged, repeated incubations with low salt buffers [8, 9], and several molecular forms ranging from 60000–420000 dalton have been described in such preparations. We have repeated this presumable autolytic solubilization procedure and extended it by using defined tryptic solubilization conditions.

It is known that about 10% of the total AcChE present in nucleus caudatus remains in the cytoplasmatic supernatant on subcellular fractionation. It would be of interest to look for differences in the sedimentation behaviour of these AcChE preparations solubilized in a different way and purified in order to elimate possibly interfering lipid material. The results could help to bring further light into the socalled somewhat confused discussion about molecular forms of mammalian CNS-AcChE [7].

Methods

TMA (Merck) was used recrystallized from ethanol; Triton X-100 was obtained from Serva (Hei-
delberg), trypsin (type III), trypsin inhibitor (type I-S), β-galactosidase (grade VI), catalase (C40) were obtained from Sigma (Munich).

Enzym activity: AcChE-activity was determined according to Ellman as described in [2], catalase activity according to [10] and β-galactosidase at 405 nm in 50 mM Tris pH 7.0, 1 mM MgCl₂, 0.1 M NaCl, 0.6% Mercaptoethanol with 2.6 mM O-Nitrophenyl-β-D-galacto-pyranoside (Sigma).

Solubilisation and purification of AcChE

I. Purification of Triton X-100 solubilized AcChE [2]

1. Homogenization, solubilization (10 mM Tris/HCl pH 7.0, 15 mM NaCl, 1.15% Triton X-100, 17.3 g nucleus caudatus/g Triton X-100, 30 min at 4 °C) and centrifugation (53000 x g, 3 h) to a clear supernatant containing 4 U/ml with a yield of nearly 90%.

2. Affinity chromatography, buffer washes and elution with 0.4 M TMA as described in [2]; concentration and dialytic removal of TMA against homogenization buffer (all procedures mentioned in the presence of 1% Triton X-100) to an AcChE-concentration of 800 U/ml with a yield of 80%.

3. Adsorption on affinity gel and subsequent detergent removal by repeated washings with detergent free buffers [2]; elution with 0.4 M TMA and concentration to 1300 U/ml with a yield of nearly 100%.

4. Dialytic removal of TMA against 10 mM Tris/HCl pH 7.0, 0.2 M NaCl and concentration to 2000 U/ml with a yield of 75%.

II. Purification of AcChE solubilized under autolytic conditions

1. Homogenization (15% w/v, 0.32 M Sucrose, 1 mM EDTA pH 7) and centrifugation (600 x g, 10 min) with twofold rehomogenization; centrifugation of the combined supernatants at 80000 x g, 90 min, (removal of water soluble AcChE), resulting in a pellet containing membrane bound AcChE in an over all yield of only 33% (58% was lost in the 600 x g-pellet, 9% was lost as water soluble AcChE).

2. Solubilization of membrane bound AcChE by two fold incubation of the membranes in homogenization buffer (6% w/v, 15 h and 30 h at 4 °C) with centrifugations at 80000 x g, 90 min, resulting in combined supernatants containing 0.3 U AcChE/ml; yield 40%.

3. Affinity chromatography, buffer washes, elution, concentration and dialysis as described under I, but without detergent, resulting in an enzyme concentration of 20 U/ml; yield 20% (much activity was lost during the buffer washes and the concentration-dialysis steps).

III. Purification of AcChE solubilized under tryptic conditions

1. Homogenization (7% w/v in 0.32 M Sucrose pH 7.0) and solubilization by adding trypsin (1 mg/g nucleus caudatus) and incubating for 12 h at room temperature; than addition of 1 mg trypsininhibitor/mg trypsin and centrifugation at 54000 x g, 4 h giving a clear supernatant containing 0.5 U AcChE/ml with a yield of 21% (loss of activity due to inactivation by trypsin was about 30% after 6 h and 55% after 12 h).

2. Affinity chromatography and subsequent steps as described under II, resulting in an enzyme concentration of 120 U/ml; yield 40%.

IV. Purification of cytoplasmatic AcChE

1. Homogenization (15% w/v, 10 mM Tris pH 7, 15 mM NaCl) and centrifugation (53000 x g, 3 h) to a clear supernatant containing 0.8 U AcChE/ml; yield 10%.

2. Affinity chromatography and subsequent procedures as described under II resulting in an enzyme concentration of 200 U/ml; yield 40%.

Gelelectrophoresis

All AcChE-preparations were checked for purity after the last purification step by analytical discontinuous polyacrylamid gel electrophoresis, staining for protein and AcChE-activity performed as described in [2].

Sedimentation analysis

Triton X-100 solubilized and cytoplasmatic AcChE were analysed after each purification step, AcChE solubilized under autolytic and tryptic conditions were analysed after the last purification step according to Martin and Ames [11]. Linear sucrose gradients were made up in 10 mM Tris pH 7.0 with salt concentration corresponding to the respective purification step and detergent concentration if considered mentioned in the legend.
100 μl of the enzyme solution mixed with 20 μl of marker protein solution (5 mg/ml β-galactosidase and catalase) was applied on top of a SW 56 gradient, 0.8-1 ml on top of a SW 27 gradient. Each enzyme solution was run in triplicate. Centrifugation was performed at 4°C either at 16000 × g_{max} for 16 h in a SW 56 rotor with 25-29 fractions of ~ 140 μl collected from the bottom, or at 120000 × g_{max} for 22 h in a SW 27 rotor with 40 fractions of 900 μl. Recovery of AcChE-activity from the gradients was up to 95%.

Results and Discussion

There is no information on the structure of the various possible protein-lipid-detergent-complexes which are released from neuronal membranes on exposure to detergents. Depending on the protein-lipid-detergent-ratio there can exist “not solubilized” as well as “solubilized” protein-lipid-detergent-, lipid-detergent-, or protein-detergent-complexes [1].

The great variety of these constituents and their numerous possibilities of interactions, may lead to questionable results in studying multiple molecular forms of neuronal membrane enzymes in such crude detergent treated membrane suspensions, without having considered membrane-detergent-ratio and suitable conditions of centrifugation in the solubilization step as well as in sedimentation analysis. We decided to follow the rate of molecular forms of brain AcChE solubilized by different methods after or in the course of purification. Solubilization of membrane bound AcChE was accomplished with Triton X-100, a detergent of low CMC, at a lipid-detergent-ratio accepted to give complete solubilization of membranes, and at such a high membrane concentration that the free, not membrane bound detergent constitutes only a small portion of the total detergent present in the sample [1]. Studying protein-complexes originally released from the membrane under such conditions should be done in the presence of the same free, not membrane bound detergent concentration which of course is not easily determined. It is however plausible and in some cases proofed, that this concentration remains below the CMC of the detergent, if the concentration of the membranes is relatively high (~ 2 mg/ml) [1]. It is for this reason that we have studied the sedimentation of crude detergent solubilized AcChE at detergent concentrations lower and higher than the CMC (~ 0.015% for Triton X-100). If the detergent concentration is chosen higher than the CMC – for example 0.5-1% for Triton X-100 as in many studies published [4-7] – there exists the possibility that the protein-lipid-detergent-complexes or the protein-detergent-complexes originally released from the membrane, are successively delipidated or cleaved forming new protein-detergent-complexes of potentially changing compositions and S-values depending on the conditions accidentally met.

These considerations are supported by comparing the sedimentation profiles shown in Fig. 1A/B. The active AcChE-species present in crude solubilizates is sedimenting in a gradient containing Triton X-100 below the CMC as a broad peak centered at 14.2 S demonstrating some heterogeneity of different complexes, whereas at detergent concentrations higher and much higher than the CMC there exists a homogenous AcChE-detergent-complex sedimenting in a sharp peak at 10.5 ± 0.3 S. Therefore it has to be stressed that this 10.5 S-form, also observed by others under similar conditions [4-7], does neither represent the AcChE-complex originally released from the membrane, nor a potential AcChE molecular form existing in the membrane.

In Fig. 1C it is demonstrated that during the subsequent purification procedure performed at a detergent concentration higher than the CMC, the 10.5 S-AcChE-detergent-complex is preserved and that the small amount of 5 S-form present in the crude extract is lost. As it is shown by the distinct change of the sedimentation profile shown in Fig. 1D the different high molecular forms of AcChE [3] are created from this 10.5 S-AcChE-detergent-complex during detergent removal by the affinity procedure described under methods. The previously dominating 10.5 S-AcChE-detergent-complex strongly decreases in favour of mainly 16 S-, little 20 S- and sometimes 23 S-species, formed even at a high concentration of the eluting agent TMA. The removal of TMA did not alter the sedimentation profile shown in Fig. 1D.

Separation of the different molecular forms present in detergent free preparations by sedimentation on sucrose gradients could be scaled up to obtain enough material for further studies of the single molecular forms. They were shown by gel electrophoresis to be identical with the oligomers separated by preparative gel electrophoresis [3], and with an improved technique of gradient gel electro-
Fig. 1. Sedimentation profiles of cytoplasmatic and solubilized membrane bound bovine nucleus caudatus AcChE treated by different methods as described in Methods. The resulting enzyme preparations were subjected to 6–20% sucrose density gradient centrifugation together with catalase (11.4 S) and β-galactosidase (16 S) under conditions described in Methods. A) Triton X-100 solubilized crude AcChE solution; sedimentation in presence of Triton X-100 below CMC; 0.4 U AcChE applied; gradient containing 0.01% Triton X-100, 10 mM Tris/HCl pH 7, 15 mM NaCl. B) Triton X-100 solubilized crude AcChE solution; sedimentation in presence of Triton X-100 above CMC; 0.4 U AcChE applied; gradient containing 1% Triton X-100, buffer as in A. C) Triton X-100 solubilized AcChE after purification step 2 (affinity chromatography, dialysis and concentration in presence of 1% Triton X-100); 80 U AcChE applied; gradient containing Triton X-100 and buffer as in B. D) Triton X-100 solubilized AcChE after purification step 4 (removal of Triton X-100 and TMA, dialysis and concentration); 200 U AcChE applied; gradient containing 0.2 M NaCl in buffer as in A without Triton X-100. E) Cytoplasmatic AcChE after purification step 2 (affinity chromatography, dialysis and concentration); 20 U AcChE applied; gradient containing salt and buffer as in D. F) Autolytically solubilized AcChE after purification step 3; 20 U AcChE applied; gradient containing salt and buffer as in D.

This is in contrast to water soluble, detergent depleted molecular forms of AcChE from erythrocytes, which seem to be of variable, accidental composition — although created by the same way of detergent removal [12] — and which show a fast disaggregation on reincubation with detergents, reflecting differences in the hydrophobic regions of these enzymes [13]. It is in contrast too to the water insoluble, undefined aggregates observed with the rat brain enzyme after detergent removal by dialysis or chromatography [7]. In our opinion it is not justified to classify defined oligomers created by

phoresis molecular weights of 400000 (10.5 S), 700000 (16 S), 960000 (20 S) and > 1100000 (23 S) could be assigned.

Disaggregation of the isolated 16 S-molecular form by incubating with Triton X-100 at a concentration above the CMC (0.1%) was rather slow (10% 10.5 S-species was formed during a four hour incubation) but noticeable. This and the mode of formation of the high molecular weight forms described here make it probable that these forms are water soluble, detergent depleted oligomers with a defined characteristic number of protomers.
self-micellization as artefacts [7]. One should be aware that protein-detergent complexes in this sense are artefacts as well and that on the other side dissociation-association processes of membrane bound proteins could be of importance in membrane function.

In all detergent depleted preparations analysed by sedimentation analysis a small 10.5 S peak was still present and it is not clear whether this peak represents residues of the 10.5 S-AcChE-detergent-complex, not converted into higher oligomers because of insufficient detergent removal, or wether it originates from an enzyme species not capable of aggregating on detergent removal. Testing the first hypothesis, further extensive washing of the enzyme preparation bound to the affinity gel was performed, but no changes of the sedimentation profile could be detected. Testing the last hypothesis, the 10% of cytoplasmatic AcChE was separated from the original brain homogenate, purified and subjected to sedimentation analysis. The results shown in Fig. 1E demonstrate that this water soluble AcChE is sedimenting in a somewhat broad 10.5-S peak with little amounts of 16 S-form present. The sedimentation behaviour of this AcChE-species was not influenced by the presence of 1% Triton X-100, confirming that besides the 10.5 S-AcChE-detergent-complex described before there exists a detergent free 10.5 S-AcChE species not capable of aggregating at low salt, detergent free conditions.

The tailing of the cytoplasmatic AcChE 10.5 S-peak led to the supposition, that there were little amounts of molecular forms with lower S-values present in the AcChE-preparations obtained without having applied detergent. That such forms might be created by autolytic processes, probable to occur under solubilization conditions as described in [8, 9], was confirmed by studying the sedimentation behaviour of AcChE solubilized under autolytic (Fig. 1F) and under tryptic (not shown; identically with Fig. 1F) conditions and subsequently purified by affinity chromatography. The results of these investigations are shown in Fig. 1F demonstrating that large amounts of a slow sedimenting 4.8 ± 0.2 S-form are created under such solubilization conditions.

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