Improved properties of bovine erythrocyte acetylcholinesterase, isolated by papain cleavage

C. Schmidt-Dannert *, H.M. Kalisz *, I. Šafařík, R.D. Schmid

* Department of Enzyme Technology, GBF – Gesellschaft für Biotechnologische Forschung, Mascheroder Weg 1, D-38124 Braunschweig, Germany

b Department of Analytical Chemistry and Biochemistry, Institute of Landscape Ecology, České Budějovice, Czech Republic

Received 30 March 1994; accepted 31 May 1994

Abstract

A simple and rapid procedure involving papain cleavage of the membrane anchor was used to isolate membrane-bound acetylcholinesterase from bovine erythrocytes. The solubilized enzyme was purified 930-fold by ion exchange chromatography and gel filtration. The properties of the papain-cleaved acetylcholinesterase were compared with those of a commercial acetylcholinesterase, solubilized from the erythrocyte membranes by detergents. Cleavage of the membrane anchor eliminated dimer aggregation, caused a pH shift in thermal stability and resulted in increased stability in organic solvents. Bovine serum albumin, used as stabilizer of the commercial enzyme preparation, increased the thermal stability but concomitantly decreased the activity of acetylcholinesterase at pH 6–8. The improved stability of the cleaved acetylcholinesterase, especially in organic solvents, may enhance the biosensor performance of the enzyme.

Key words: Acetylcholinesterase; Papain cleavage; Isolation; Bovine erythrocyte; Stability

1. Introduction

Several forms of acetylcholinesterase (ACHE; EC 3.1.1.7) are found in vertebrate tissues (Acheson and Quinn, 1990; Quinn, 1987; Rosenberry, 1975; Taylor, 1991). These forms are classified as either asymmetric if they contain a collagen-like structure or globular if this structure is devoid (Acheson and Quinn, 1990; Ott et al., 1975; Rosenberry and Scogglin, 1984; Rosenberry, 1982). The globular class includes ACHEs with a short glycolipid domain at the C-terminus (Rosenberry and Scogglin, 1984; Dutta-Choudhury and Rosenberry, 1984). This hydrophobic domain, which anchors the enzyme into the phospholipid bilayer of the membrane, is bound to the C-terminus of the enzyme via an inositol and a glycane or ethanolamine (Taguchi and Ikezawa, 1987).

Membrane-bound ACHE is normally isolated from the membranes by detergents (Großman and Liefländer, 1979; Berman, 1973) or high salt treatment (Mitchell and Hanahan, 1966; Heller and Hanahan, 1972). With both isolation methods...
the isolated enzyme is very hydrophobic due to the glycolipid anchor, readily forming high molecular weight aggregates and eluting as broad bands from liquid chromatography columns such as gel filtration (Großman and Liefländer, 1979; Sussman et al., 1991; Ott and Brodbeck, 1978). This makes the handling of the enzyme difficult and requires the addition of either detergents or stabilizing proteins, such as bovine serum albumin (BSA), to prevent micelle or aggregate formation.

Numerous membrane-bound enzymes have been solubilized by treatment with proteinases without loss of catalytic activity (Brunner et al., 1978; Bjerrum et al., 1979; Feracci and Maroux, 1980; Skovbjerg et al., 1981; Svensson et al., 1978; Danielsen et al., 1980). Moreover, limited proteolysis with papain of the pure human erythrocyte ACHE in vitro resulted in the conversion of the amphiphilic enzyme into a hydrophilic form (Dutta-Choudhury and Rosenberry, 1984; Weitz et al., 1984). Similar observations have been made with an ACHE from Torpedo marmorata with either pronase (Li and Bon, 1983) or proteinase K (Stieger and Brodbeck, 1985).

In this paper we report the isolation of membrane-bound ACHE from bovine erythrocytes using a simple and rapid procedure involving in vivo cleavage of the hydrophobic anchor with papain. The cleaved enzyme exhibited increased hydrophilicity and stability, especially in organic solvents, in comparison to the commercial ACHE.

2. Materials and methods

Papain isolation of ACHE

Fresh bovine blood was obtained from a local slaughterhouse and preserved in 0.38% (w/w) sodium citrate. The erythrocytes were separated by centrifugation at 20 000 ×g according to Taguchi et al. (1984) and Steck and Kant (1976), washed three to four times in isotonic buffer (5 mM sodium phosphate buffer containing 150 mM NaCl, pH 7.4) and suspended in 20 ml buffer. ACHE isolation was performed with 2 U ml⁻¹ papain (31.5 U mg⁻¹; Sigma) and 1 mM edrophonium chloride (Sigma) for 3–4 h at 23°C. The erythrocytes were then centrifuged at 15 000 ×g and the clear red supernatant was further purified.

Purification of isolated ACHE

Edrophonium chloride and papain were removed from the sample on an S-Sepharose column (50 × 79 mm) at a flow rate of 4 ml min⁻¹ using 50 mM sodium acetate buffer, pH 6. Unbound ACHE was then pooled, applied at a flow rate of 4 ml min⁻¹ on a Q-Sepharose column (25 × 285 mm) pre-equilibrated with 50 mM sodium phosphate buffer, pH 6 (buffer A), and eluted with buffer A containing 200 mM NaCl. Active fractions were pooled, concentrated by ultrafiltration (Omegacell, Filtron) and applied at a flow rate of 0.05 ml min⁻¹ on a Sephadex G-100 column (26 × 900 mm) pre-equilibrated with buffer A.

Partial purification and papain digestion of a commercial ACHE

Commercial ACHE from bovine erythrocyte membranes (3.9 U mg⁻¹ lyophilisate) was a gift from Boehringer Mannheim. The enzyme, containing 90% BSA as stabilizer, was partially purified to 98 U mg⁻¹ protein on a Sephadex G-100 Superfine column using buffer A under the conditions described above.

Papain digestion was performed for 3.5 h at 23°C using 4 mg ml⁻¹ papain, 1 mM edrophonium chloride, and 4 mg ml⁻¹ ACHE lyophilisate in 50 mM sodium phosphate buffer, pH 7.5. Partially digested ACHE was purified to 35 U mg⁻¹ on a Mono S column using 50 mM sodium acetate buffer, pH 6, and a TSK 4000 column with 50 mM sodium phosphate buffer, pH 7.5. Gel filtration was also used as a criterion for anchor cleavage. A shift in the elution profile was observed from a high molecular mass peak of the aggregates to a peak corresponding to the hydrophilic dimer upon cleavage of the anchor.

Analytical methods

ACHE activity was measured at 25°C according to Riddles et al. (1983) using 0.33 mM DTNB (2,2'-dinitro-5,5'-dithiobenzoic acid) in 50 mM sodium phosphate buffer, pH 7.3, with acetylthiocholine iodide as substrate. Protein was deter-
mined by the method of Bradford (1976) using Coomassie brilliant blue-G reagent (Bio-Rad) with BSA as standard. PAGE was performed on a Pharmacia Phast System (Pharmacia/LKB, Freiburg, Germany) according to the manufacturer’s recommendations. Gels were stained for protein with silver by the method of Butcher and Tomkins (1986).

**Characterization**

The properties of the following ACHE preparations were compared: Papain isolated ACHE (372 U mg\(^{-1}\)); commercial ACHE containing 90% BSA (3.5 U mg\(^{-1}\)); partially purified commercial ACHE (98 U mg\(^{-1}\)); papain digested and 10-fold purified commercial ACHE (35 U mg\(^{-1}\)).

### 3. Results and discussion

#### 3.1. Papain isolation of ACHE and purification of the isolated ACHE

Membrane-bound ACHE is normally isolated by treatment of the erythrocytes with detergents (Großman and Liefländer, 1979; Berman, 1973) or high salt concentrations (Mitchell and Hanahan, 1966; Heller and Hanahan, 1972). These preparations still contain the hydrophobic glycolipid anchor which causes aggregation and makes handling of the enzyme difficult. An alternative isolation method involves cleavage with a phosphatidyl-specific phospholipase C (Ott et al., 1975; Taguchi and Ikezawa, 1987; Taguchi et al., 1984). The isolated enzyme is considerably less hydrophobic and easier to handle, but the method is relatively expensive due to the high costs of the phospholipase C.

In order to facilitate the isolation of the membrane-bound ACHE and to improve its handling we used papain cleavage for the isolation of the enzyme from intact bovine erythrocytes. Preliminary experiments were performed as described by Dutta-Choudhury and Rosenberry (1984) and Weitz et al. (1984) for the in vitro cleavage of the hydrophobic anchor from purified human erythrocyte ACHE. However, these conditions were inadequate for the in vivo isolation of the bovine erythrocyte ACHE and had to be modified using higher concentrations of papain and edrophonium chloride and increased duration of incubation (see Materials and methods). Edrophonium chloride, a competitive inhibitor of ACHE, was essential for the retention of ACHE activity during papain cleavage. At inhibitor concentrations lower than 1 mM ACHE was virtually completely proteolysed.

The isolated ACHE was separated from papain, edrophonium chloride on an S-Sepharose column. Two further chromatography steps enabled a 930-fold purification of the enzyme to 97% purity and a specific activity of 372 U mg\(^{-1}\) (Table 1). This was about 8-fold lower than the specific activity of the anchor-containing bovine ACHE (Großman and Liefländer, 1979; Berman and Young, 1971; Beauregard and Roufogalis, 1979). A partial loss of activity on cleavage of the anchor has also been reported by Weitz et al. (1984) and may be attributed to conformational change of the enzyme upon removal of the hydrophobic anchor.

#### 3.2. Purification and cleavage of a commercial ACHE preparation

Commercial ACHE, containing 90% BSA as stabilizer, could only be partially purified to a

<table>
<thead>
<tr>
<th>Step</th>
<th>Total units</th>
<th>Total protein (mg)</th>
<th>Specific activity (U mg(^{-1}))</th>
<th>Yield (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-Sepharose</td>
<td>185</td>
<td>712.25</td>
<td>0.4</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>148</td>
<td>27.86</td>
<td>6.8</td>
<td>80</td>
<td>17</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>89</td>
<td>0.27</td>
<td>372.0</td>
<td>48</td>
<td>930</td>
</tr>
</tbody>
</table>
specific activity of 35 U mg\(^{-1}\). Different purification methods, such as ion exchange chromatography, affinity chromatography according to Berman and Young (1971) and gel filtration, failed to separate BSA from the enzyme. Although the best results were obtained using gel filtration on a Sephadex G-100 column, the partially purified enzyme still contained relatively high amounts of BSA. The strong hydrophobic interaction between the BSA and the glycolipid anchor of ACHE may have been responsible for the poor separation of the two proteins. However, despite cleavage of the membrane anchor, the cleaved ACHE was only partially purified to 98 U mg\(^{-1}\) by gel filtration. The incomplete separation of BSA from the cleaved commercial ACHE implies that not only the membrane anchor of the enzyme interacts with the BSA.

Papain cleavage of the membrane anchor from the commercial ACHE eliminated aggregate formation under electrophoretic and chromatography conditions. Consequently, the cleaved enzyme migrated in its dimeric form. This observation was used to assess the degree of anchor cleavage. The greater the degree of cleavage the higher the percentage of the dimer peak, with only the dimer being observed when anchor cleavage was complete.

### 3.3. Comparison of the properties of cleaved and native ACHE

The influence of the membrane anchor on the physicochemical properties of ACHE, such as hydrophobicity and temperature stability, has previously been investigated (Taguchi and Ikezawa, 1987; Taguchi et al., 1984). We have extended these studies in order to assess the effect of anchor cleavage and addition of BSA on the properties of ACHE. Four different ACHE preparations were used: papain isolated ACHE, papain digested commercial ACHE, partially purified commercial ACHE and commercial ACHE containing 90% BSA. Special attention was paid to the influence of the anchor and BSA on the kinetics and stability of ACHE for application in biosensors.

Cleavage of the membrane anchor considerably decreased the hydrophobicity of ACHE and eliminated the formation of high molecular mass aggregates. A molecular mass of 201 kDa was estimated for native ACHE and 180 kDa for the cleaved enzyme (Table 2).

The inhibition of ACHE by the specific cholinesterase inhibitors, carbofuran and malaoxon, and by the serine protease inhibitors, TPCK, TLCK and PMSF, was not affected by cleavage of the membrane anchor (results not shown). Cleavage also had no affect on either the kinetics or the pH optimum of ACHE activity (Table 2). However, the temperature optimum for ACHE activity increased from 45°C to 55°C. Moreover, optimum enzyme stability shifted from pH 7 to 9 (Table 2). Thermal stability was also affected by cleavage of the anchor. Papain digested ACHE was significantly more stable at 50°C than the native enzyme in the presence of BSA (Fig. 1). Similar observations were made with an ACHE isolated with a phosphatidylinositol-specific phospholipase C (Ott et al., 1975; Taguchi and Ikezawa, 1987; Taguchi et al., 1984). At elevated temperatures both the native and papain digested ACHE were stabilized by the added BSA at pH 6–8 (Fig. 2). However, above pH 8 BSA did not contribute significantly to the thermal stability of the enzyme. The papain isolated ACHE exhibited optimal stability at pH 9–10, being relatively unstable below pH 9 (Fig. 2). Addition of BSA to the cleaved enzyme increased its stability at neutral pH, making the enzyme more stable than the native ACHE under similar conditions. Saccharides, such as glucose and sucrose, exerted a similar stabilizing effect on ACHE as did BSA (results not shown). Thus, in applications where

<table>
<thead>
<tr>
<th>Properties</th>
<th>Papain isolate</th>
<th>Commercial ACHE</th>
<th>Purified commercial</th>
</tr>
</thead>
<tbody>
<tr>
<td>( M_r ) (dimer)</td>
<td>180 000</td>
<td>201 000</td>
<td>201 000</td>
</tr>
<tr>
<td>pH optimum</td>
<td>8.5</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Max. stability (pH)</td>
<td>9</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>( T ) optimum (°C)</td>
<td>55</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>( K_m ) (μmol 1(^{-1}))</td>
<td>708</td>
<td>728</td>
<td>610</td>
</tr>
</tbody>
</table>
Fig. 1. Effect of in vitro papain cleavage on the thermal stability of ACHE. Native (open symbols) and cleaved (closed symbols) commercial ACHE was incubated at 50°C at pH 6 (○, ●), pH 7 (□, ■) and pH 8 (○, ●). Aliquots were removed at the times indicated and activity measured as described in the text.

the purity of ACHE is essential, sugars could be used as stabilizers instead of BSA without any detrimental effect on enzyme activity.

BSA affected not only the thermal stability of ACHE but also its activity at neutral pH. The greater the amount of BSA in the sample the lower the activity at pH 6–8 (Fig. 3). The difference was most noticeable at pH 6, at which a 50% reduction in enzyme activity was observed in

Fig. 2. Effect of pH on the stability of commercial (●), purified commercial (■), papain cleaved (▲) and papain isolated (♦) ACHE at (a) 40°C and (b) 50°C. Samples were incubated for 24 h (40°C) or 2 h (50°C) in sodium acetate buffer, pH 4; sodium phosphate buffer, pH 6–8; glycine-NaOH buffer, pH 9–12 (all 50 mM). Activity was measured as described in the text and is expressed as percentage of the residual activity.

Fig. 3. Effect of papain cleavage and/or BSA concentration on the pH optimum of ACHE activity. Activity of commercial (●), purified (■), purified containing added BSA (□), papain cleaved (▲) and papain isolated (♦) ACHE was measured at the indicated pH as described in the text. Buffers used were: sodium acetate buffer, pH 4; sodium phosphate buffer, pH 6–8; glycine-NaOH buffer, pH 9–12 (all 100 mM). Activity is expressed as percentage of optimum.
Table 3
Stability of ACHE in the presence of organic solvents. ACHE was incubated 24 h at 23°C in 100 mM sodium phosphate buffer, pH 7.5, containing 15% solvent

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Papain isolate</th>
<th>Commer-</th>
<th>Purified commercial</th>
<th>Commercial, papain cleaved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>312</td>
<td>98</td>
<td>99</td>
<td>nd</td>
</tr>
<tr>
<td>Ethanol</td>
<td>400</td>
<td>51</td>
<td>46</td>
<td>106</td>
</tr>
<tr>
<td>Butanol</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hexanol</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Activity was measured as described in Materials and methods and is expressed as a percentage of the control in buffer only. nd = not determined

the presence of 90% BSA. In the absence of BSA activity at pH 6 was more than 90% of the optimum at pH 8.5. Readdition of BSA caused a decrease in the activity at pH 6–8. Cleavage of the membrane anchor did not affect the pH optimum of the enzyme (Fig. 3). Hence, although BSA stabilized ACHE at pH 6–8, it also affected a decrease in the enzyme activity in this pH range. It is thus possible that the strong hydrophobic interaction between BSA and the enzyme, especially with its membrane anchor, may cause a conformational change in ACHE structure contributing to the observed changes in activity and stability.

Cleaved ACHE exhibited greater stability in water soluble organic solvents, such as methanol and ethanol (Table 3), with the cleaved enzyme being 3–4-fold more stable in the presence of the solvents than in buffer. In water-insoluble solvents, such as butanol and hexanol, both the native and cleaved ACHE preparations were rapidly inactivated. The increased stability of the cleaved ACHE in soluble organic solvents may be advantageous in environmental control. The application of the cleaved ACHE in biosensor analysis is currently under investigation.

In conclusion, in vivo cleavage of the membrane anchor of ACHE was a rapid and convenient procedure for the isolation of the enzyme from the bovine erythrocytes. The disadvantages of a partial loss of activity caused by the papain cleavage of the anchor were offset by the improved handling and properties of the enzyme and by the speed and ease of isolation. Cleavage of the anchor did not influence the kinetic properties or the sensitivity of ACHE towards pesticides and insecticides. In addition, the cleaved enzyme exhibited higher thermal stability and higher stability towards water soluble solvents. The thermal stability of ACHE at neutral pH may be increased further by the addition of stabilizers such as BSA or preferably sugars. Moreover, the reduced tendency of the cleaved ACHE to aggregate makes the enzyme much easier to handle. This is important for the application of the cleaved enzyme in biosensors.

Acknowledgement

Ivo Šafarík gratefully acknowledges the awarding of an Alexander von Humboldt Foundation Scholarship.

References


