Amphiphilic Forms of Butyrylcholinesterase in Mucosal Cells of Rat Intestine

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ABSTRACT: The properties of a cholinesterase from mucosal cells of rat intestine have been characterized. The enzyme was identified as butyrylcholinesterase because it was more sensitive to iso-OMPA (IC_{50} = 1.0×10^{-6} M) than to BW284C51 (IC_{50} = 5.5×10^{-5} M) and was not inhibited by substrate excess. It displayed a higher affinity for acetylthiocholine than for butyrylthiocholine. A major molecular form was observed sedimenting at 5.9 S. Two other minor molecular forms were identified as a hydrophilic tetramer (G_4, sedimenting at 10.5 S) and a monomer (G_1, sedimenting at 4.3 S). The 5.9S component was referred to as "G" form (G for globular) and not "G_2" as usual dimers for the following reasons: (i) the G form was unaffected by the reducing agents, β-mercaptoethanol and dithiothreitol, which converted disulfide-linked dimers of acetylcholinesterase into monomers, (ii) the G form was shifted from 5.9 to 3.4 S when the sucrose gradient contained Triton X-100. This value of 3.4 S (in Triton X-100) appeared too low for a typical G_2 form. The shift in the S value was partly reversible: the 3.4 S form resedimented at 5.2 S in the absence of detergent. The behavior of the G form in sucrose gradients indicated that it was amphiphilic. This was confirmed in nondenaturing electrophoreses and also by quantitative binding of the G form to octyl-Sepharose. The hydrophobic domain of the G form was not a glycolipid, as shown by its insensitivity to *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C and its nonaggregating properties in the absence of nondenaturing detergent. Proteinase K failed to remove the hydrophobic domain(s). In contrast, bromelain rapidly generated active peptides significantly less amphiphilic than the native form. Indirect estimation of G and G_1 molecular weights from Stokes radii obtained from HPLC and sedimentation coefficients gave respectively 126 000 and 72 000, which were compatible with those of dimers and monomers. The G_1 form was also amphiphilic but was more readily eluted from octyl-Sepharose column than the G form. Taken together these data suggested that the G form was a dimer of amphiphilic catalytic subunits not linked by a disulfide bond and with a special type of hydrophobicity. Depending on their type of hydrophobic domain, amphiphilic cholinesterase dimers (G_2 forms) have been referred to as class I (glycolipid anchor) or class II (hydrophobic sequence of the catalytic peptide) by Bon et al. [Bon et al. (1988) *J. Neurochem. 51*, 776–785, 786–794]. The major molecular form of rat mucosal cells is tentatively characterized here as the first example in mammals of very rare amphiphilic butyrylcholinesterase dimers of class II.

Vertebrate acetylcholinesterase (AChE, EC 3.1.1.7)[1] and butyrylcholinesterase (BChE, EC 3.1.1.8) present parallel molecular polymorphisms including asymmetric "collagen-tailed" and globular forms (Massoulié & Bon 1982; Massoulié & Toutant, 1988). Globular forms (monomeric, G_1, dimeric, G_2, and tetrameric, G_4) are either hydrophilic ("soluble") or amphiphilic (interacting directly with nondenaturing detergents). The structure of the hydrophobic domain in amphiphilic globular forms has been characterized in some instances. The amphiphilic G_4 form of bovine caudate nucleus AChE associates four hydrophilic subunits with a noncatalytic element that mediates the membrane attachment (Inestrosa et al., 1987). For the amphiphilic G_2 forms, two classes have been defined (Bon et al., 1988a,b). Class I is constituted of dimeric cholinesterases only soluble in the presence of Triton X-100. The amphiphilic properties of these enzymes are due to the presence of a glycosylphosphatidylinositol domain [for a review, see Silman and Futerman (1987)]. Specific phospholipases C (PI-PLC) or phospholipases D (PI-PLD) may convert the amphiphilic molecules into nonamphiphilic derivatives (Futerman et al., 1983; Davitz et al., 1987; Rosenberry et al., 1989). The dimeric AChE of mammalian erythrocyte plasma membrane belongs to class I. Class II is formed by dimeric cholinesterases, insensitive to PI-PLC or PI-PLD, which are soluble in media devoid of detergent but whose apparent sedimentation coefficients are decreased in the presence of nondenaturing detergent. The amphiphilic properties of the enzymes belonging to this second class suggest a different structure of the hydrophobic domain.

BChE globular forms have been generally described as soluble species except in human central nervous system (Atack et al., 1986) and in *Torpedo* heart and nervous sytem (Bon et al., 1988b), where amphiphilic forms have been reported [for a review, see Chatonnet and Lockridge (1989)]. *Torpedo* heart BChE dimers belong to class II [the hydrophobic domain is not a glycolipid; Bon et al. (1988b)]. Further studies of amphiphilic dimers of BChE have been generally hampered by their low abundance. At the gene level also, "hydrophilic" transcripts have been characterized (McTierman et al., 1987; Prody et al., 1987; Arpagaus et al., 1990) but BChE transcripts coding for amphiphilic products have not been reported so far

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[1] Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; ChE, cholinesterases; AcSCh, acetylthiocholine; BuSCh, butyrylthiocholine; LS, low-salt; LSS, low-salt-soluble; DS, detergent-soluble; DOC, sodium deoxycholate; DTT, dithiothreitol; PI-PLC, phosphatidylinositol-specific phospholipase C; SR, Stokes radius.

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[for a review, see Chatonnet and Lockridge (1989)]. This situation contrasts with that of the AChE gene in *Torpedo* (Sikorav et al., 1988; Schumacher et al., 1988) and mammals (Rachinsky et al., 1990; Li et al., 1991), where the production of different transcripts by alternative splicing leads to both hydrophilic and amphiphilic subunits.

We are interested in amphiphilic BChE. The intestine mucosal cells, which are rapidly renewed and mainly involved in nutrient absorption, display AChE and/or BChE activity. In vertebrates, various levels of activity have been detected (Sine & Colas, 1985; Sine et al., 1988, 1989, 1991a). Rodents generally possess a high cholinesterase (ChE) activity compared with avian, amphibian, and fish (Sine et al., 1991b). There is no apparent correlation between the activity levels of AChE or BChE and the dietary habits of the vertebrates (Sine et al., 1988). However, the implication of mucosal cell ChE in a digestive process may not be ruled out, as suggested by starvation and refeeding experiments (Leparoux et al., 1992).

The distribution of ChE globular forms differs in the intestine mucosal cells according to the animal species. These molecular forms occur as soluble or amphiphilic species (Sine & Colas, 1985, 1987; Sine et al., 1988, 1989). In the latter case, preliminary results suggest that the mucosal cells of mouse and rat small intestine contain BChE molecular species having a hydrodynamic behavior which may be explained by amphiphilic properties (Sine et al., 1988, 1991b). Thus, significant changes of the S value have been observed when Triton X-100 was used during homogenization. In this paper, we characterized the rat small intestine molecular forms. Our findings indicate the presence of amphiphilic forms of BChE.

MATERIALS AND METHODS

Chemicals. Acetylthiocholine iodide (AcSCh), aprotinin, bacitracin, benzamidine hydrochloride, butyrylthiocholine iodide (BuSCh), 1,5-bis(allyldimethylammoniophenyl)pentan-3-one dibromide (BW284C51), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), eserine, tetraisopropylpyrophosphoramide (iso-OMPA), proteinase K, and sodium deoxycholate were obtained from Sigma Chemical Co. Bromelain and phosphatidylcholine-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* were purchased from Boehringer. Triton X-100 was from Aldrich. CH Sepharose 4B and octyl-Sepharose CL-4B were purchased from Pharmacia Fine Chemicals. All other chemicals were of analytical grade. Procainamide hydrochloride and 1-(*N*,*N*,*N*-trimethylammonio)-6-hexylamine were gifts of Dr. P. Masson (CRSSA, La Tronche, France).

Extraction of Cholinesterase Activity. Male Wistar rats, 2 months old, weighing 250–300 g, were killed by cervical disruption. The intestinal segment corresponding to duodenum, jejunum, and ileum was removed, longitudinally opened, and washed with an ice-cooled 10 mM potassium phosphate buffer (pH 7.4) containing a mixture of protease inhibitors: 1 mg/mL bacitracin, 1 mM benzamidine, and 25 units/mL aprotinin. Mucosal cells were gently scraped off using a glass slide. Scrapings were weighed and 12% (w/v) homogenates were prepared, using a Potter Teflon–glass homogenizer (10 passes at 1500 rpm). Extracts prepared in 10 mM potassium phosphate (pH 7.4) buffer containing 0.15 M NaCl were referred to as low-salt-soluble (LSS) fractions. Extracts prepared in 10 mM potassium phosphate (pH 7.4) buffer containing 0.15 M NaCl and 1% Triton X-100 were referred to as detergent-soluble (DS) fractions. Homogenates were filtered through gauze and LSS and DS extracts were obtained after centrifugation for 1 h at 105000*g*.

Enzyme Assays. Cholinesterase activity was measured according to the colorimetric method of Ellman et al. (1961) at 25 °C in 10 mM potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl. When specific inhibitors were used, preincubation with the inhibitors was performed for 10 min. One enzymatic unit was defined as the amount of enzyme which catalyzed the hydrolysis of 1 μmol of substrate/h at 25 °C. Protein content was determined according to Lowry et al. (1951). BChE specific activity was expressed as units per milligram of protein.

Sucrose Gradient Centrifugation. Sedimentation analyses were performed in 2–20% (w/v) linear sucrose gradient prepared with 10 mM potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl. Extracts (LSS or DS) were analyzed without detergent or in the presence of 1% Triton X-100. Samples were mixed with sedimentation markers (alkaline phosphatase, 6.1 S; catalase, 11.3 S; β-galactosidase, 16 S), carefully layered onto gradients, and centrifuged for 15 h at 119000*g* and 4 °C in a Beckman SW40 Ti rotor. Gradients were fractionated from top to bottom, and fractions of 0.250 mL were collected.

Nondenaturing electrophoresis. Homogenates of rat intestinal mucosal cells in LS medium (10 mM potassium phosphate buffer, pH 7.4, containing 0.15 M NaCl) were run on preparative sucrose gradients containing 1% Triton X-100. Peak fractions were pooled, dialyzed against 10 mM Tris pH 7.4 for 1 h at 4 °C, and concentrated (3:1, v/v) in a speed vac. Nondenaturing PAGEs were performed in 7.5% polyacrylamide gels in the presence of Triton X-100 (0.5% in gel and running buffer) or sodium deoxycholate (0.2%) + Triton X-100 (0.2%) as described previously (Toutant et al., 1988). BChE or AChE activity was stained according to the method of Karnovsky and Roots (1964) with BuSCh or AcSCh as substrate. BChE and AChE were digested for 1 h at 37 °C with stock PI-PLC diluted to 5 units/mL.

HPLC. LSS extracts (100–200 μL) were analyzed by HPLC (Waters 600E, Waters 991, Waters Ultra Wisp 715) using a gel filtration column (TSK-Gel G 3000 SW, 7.5 × 300 mm). The elution medium contained 50 mM potassium phosphate (pH 6.8). For DS extracts, the elution medium contained 50 mM potassium phosphate (pH 6.8) and 0.5% Triton X-100. The flow rate was 0.5 mL/min. Calibration was carried out with β-galactosidase [Stokes radius (SR), 8.2 nm; molecular weight (MW), 540 000), urease (SR, 6.1 nm; MW, 483 000), catalase (SR, 5.22 nm; MW, 232 000), alcohol dehydrogenase (SR, 4.6 nm; MW, 150 000), bovine serum albumin (SR, 3.55 nm; MW, 68 000), ovalbumin (SR, 3.05 nm; MW, 43 000), and cytochrome *c* (SR, 1.74 nm; MW, 12 400)] as standards. Stokes radius and molecular weight determinations were carried out as described by Siegel and Monty (1966). For determining BChE activity, fractions were collected each 0.4 min and assayed according to the method of Ellman et al. (1961).

Octyl-Sepharose Chromatography. Eight milliliters of octyl-Sepharose CL-4B gel was packed in a column (6 cm × 1.5 cm) and equilibrated with 10 mM potassium phosphate buffer (pH 7.4). The G-form fraction obtained from preparative sucrose gradients was chromatographed. The elution of nonretained components was carried out at a flow rate of 0.2 mL/min. The column was washed overnight with the above buffer. A Triton X-100 gradient (0–2%) was used to remove the bound components. Fractions of 0.5 mL were collected and assayed for BChE activity according to the method of Ellman et al. (1961). The Triton X-100 concen-

Table I: Mutual Competition Analysis between Acetyl- and Butyrylthiocholine by LSS Extracts of Mucosal Cells

rate of substrate hydrolysis[a]						
AcSCh		BuSCh		calculated		
0.05 mM	0.1 mM	0.05 mM	0.1 mM	distinct sites	common site	observed
1.764		0.970		2.734[b]	2.034[b]	2.105[b]
	2.734		1.588	4.322[c]	2.870[c]	2.911[c]

[a] Rates of hydrolysis were expressed as micromoles per hour of liberated thiocholine. [b] In the presence of 0.05 mM AcSCh and 0.05 mM BuSCh. [c] In the presence of 0.10 mM AcSCh and 0.10 mM BuSCh.

tration of each fraction was determined by the optical density measured at 280 nm.

Affinity Chromatography. Procainamide hydrochloride or 1-(*N*,*N*,*N*-trimethylammonio)-6-hexylamine was coupled to CH Sepharose 4B using the carbodiimide coupling procedure (Pharmacia). The affinity gel was equilibrated with 20 mM Tris–HCl buffer (pH 7.3). The G form, isolated from LSS extracts centrifuged on preparative sucrose gradients, was applied to the column. Elution of nonretained components was carried out in the equilibration medium at a flow rate of 0.250 mL/min for 15 h. The BChE activity, quantitatively retained, was eluted with a NaCl gradient (0–2.5 M).

Proteolytic Digestions. The G form, obtained from preparative sucrose gradients, was incubated with proteinase K (0.1 mg/mL) at 25 °C for 20 h in 10 mM potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl. Bromelain was added to the G-form fraction at a final concentration of 0.5 unit/mL. Incubation was performed for 50 min at 37 °C in 10 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM DTT and 0.15 M NaCl. Digestion was stopped with 4 mM iodoacetamide and by cooling the incubation medium in ice.

RESULTS

Kinetic Properties and Inhibitor Effects. Kinetic experiments involving acetylthiocholine and butyrylthiocholine as substrates were carried out on LSS extracts obtained from homogenates of rat intestine mucosal cells (Table I). The results were treated according to Dixon and Webb (1979). The total rate of choline ester hydrolysis, measured in mixed-substrate incubations, agreed quite well with the theorical data calculated from the common site reaction and disagreed with those calculated from a reaction scheme involving two distinct catalytic sites—or two enzymes (Table I). These results showed that only one type of cholinesterase was present in the extracts.

The extracts were assayed for ChE activity with various concentrations of AcSCh or BuSCh (10^{-5}–10^{-1} M). As shown in Table II, the activity was higher when measured in the presence of AcSCh than in the presence of BuSCh. The ratio of maximal velocities V_{AcSCh}/V_{BuSCh} was 1.3 ± 0.1. The values of the V/K_m ratio, known to be a more significant parameter of the catalytic efficiency, showed clearly that AcSCh was a better substrate than BuSCh (Table II). However, no inhibition was found with a large excess of either AsSCh or BuSCh. In the presence of increasing concentrations of BuSCh, the activity of the enzyme did not reach a plateau but increased in a continuous manner, even when the substrate concentration was 1000-fold the value of K_m.

Selective inhibitors were used to characterize the type of ChE. In all experiments the concentration of AcSCh was 5 × 10^{-4} M. Eserine inhibited the hydrolysis of BuSCh with

Table II: Kinetic Parameters of Cholinesterase in Mucosal Cells of Rat Intestine[a]

substrate	max activity V (unit/mg of protein)	Michaelis const 10^4K_m (M)	$10^{-3}V/K_m$
AcSCh	4.8	0.80 ± 0.07	60
BuSCh	3.6	1.00 ± 0.05	36

[a] One enzymatic unit was defined as the amount of protein which catalyzed the hydrolysis of 1 μmol of substrate/h at 25 °C.

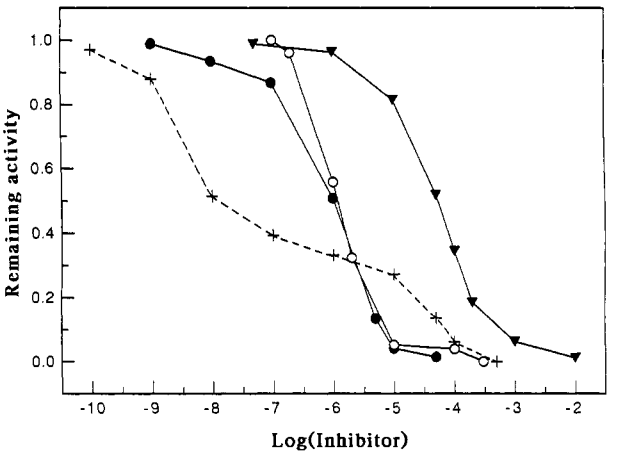

FIGURE 1: Effect of specific inhibitors on the mucosal cell cholinesterase activity of rat small intestine. Activities, measured with 5 × 10^{-4} M AcSCh in the presence of eserine (●), iso-OMPA (○), and BW284C51 (▼) were expressed as percentages of control without inhibitor. The BW284C51 effect on cholinesterase activities of rat serum (+– –) was tested for comparison.

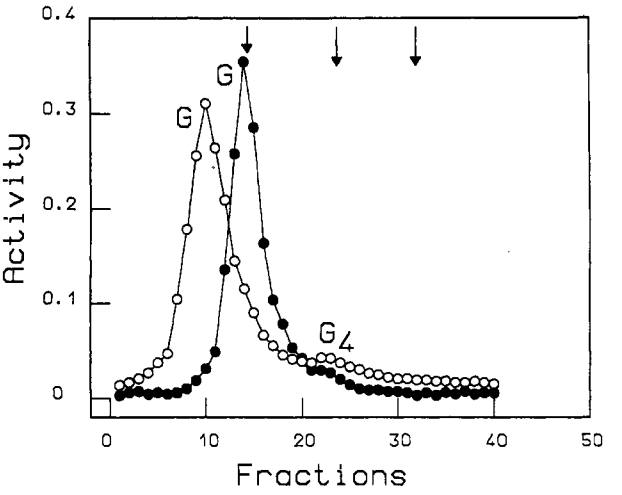

FIGURE 2: Distribution of BChE molecular forms from mucosal cells. LSS extract (●) was analyzed in sucrose gradient without detergent, and DS extract (○) was analyzed in the presence of 1% Triton X-100. Vertical arrows indicate the position of sedimentation markers, from left to right, alkaline phosphatase (6.1 S), catalase (11.3 S), and β-galactosidase (16 S).

an IC_{50} of 1.0 × 10^{-6} M (Figure 1). In addition, iso-OMPA (IC_{50} = 1.0 × 10^{-6} M) was found to be a more potent inhibitor than BW284C51 (IC_{50} = 5.5 × 10^{-5} M). All inhibition curves were monophasic. Under the same experimental conditions, samples of rat serum, which is known to contain both AChE and BChE (Silver, 1974; Arpagaus et al., 1991), assayed in the presence of BW284C51 revealed a biphasic inhibition curve demonstrating the contributions of AChE (IC_{50} = 3.2 × 10^{-9} M) and BChE (IC_{50} = 5.5 × 10^{-5} M). Such biphasic inhibition curves of ChE activities have also been reported for other rat tissues using diisopropyl fluorophosphonate as inhibitor (Vigny et al., 1978). Taken together, our results

Table III: Effect of Various Agents on the Sedimentation Coefficient of the G Form[a]

agent	sed coeff (S)	agent	sed coeff (S)
none	5.9	cholate (0.5%)	4.5
Brij 96 (0.5%)	5.9	deoxycholate (0.5%)	4.4
Lubrol WX (0.5%)	5.9	Triton X-100 (1%)	3.4
NaCl (1M)	5.8	Emulphogen (1%)	3.3
Tween 20 (1%)	5.4	Nonidet P40 (1%)	3.3
octyl D-glucoside (0.5%)	4.8	sulfobetaine SB14	3.2

[a] LSS extracts, prepared as described under Materials and Methods were analyzed on sucrose gradients in the presence of various agents.

indicated that the enzyme of mucosal cells involved in choline ester hydrolysis was BChE, not AChE.

Molecular Forms. Sucrose gradient centrifugation of LSS extracts revealed the presence of two globular forms (Figure 2): a preponderant form, called G in this study, which sedimented as a dimeric form (5.9 S) and a minor tetrameric form (G_4, 10.5 S) which appeared to be variable in amount and, in a few cases, not detected. Homogenization of the scrapings in a high-salt medium [10 mM potassium phosphate buffer (pH 7.3) + 1 M NaCl] failed to display heavier molecular forms such as asymmetric forms.

When scrapings were homogenized in LS medium containing 1% Triton X-100 (DS fraction) and centrifuged in the presence of detergent, the sedimentation pattern showed a preponderant form sedimenting at only 3.4 S (Figure 2). It should be noted that the peak asymmetry of the 3.4S form *was* more apparent than that of the 5.9S form, considering the fact that the activities of each sample (LSS and DS extracts) layered on sucrose gradients were similar and that Triton X-100 did not produce any significant change of the BChE activity. A minor G_4 form (10.5 S) was also observed in the DS extracts.

As shown in Table III, various nonionic detergents other than Triton X-100 (Emulphogen, Nonidet P40) induced a decrease of the sedimentation coefficient of the G form, but this was not observed with Brij 96 or Lubrol WX. Ionic detergents such as cholate, deoxycholate, and sulfobetaine SB14 were also effective in producing such a change.

Solubility Properties. LS extraction of the enzyme was achieved from homogenate by centrifugation (105000*g*, 1 h). The resulting pellet was again homogenized in the same medium and submitted to identical centrifugation (105000*g*, 1 h). Figure 3 (upper panel) shows the proportion of BChE activity found in each supernatant and pellet for six repetitive extractions (steps 1–6). The activity progressively decreased in the successive supernatants. At step 6, a very low BChE activity was found in the supernatant (0.6%) and the enzyme was assumed to be completely extracted.

Partition between G and G_4 Forms. The different supernatants, analyzed by sucrose gradient centrifugation in the absence of detergent, showed an increase in the relative amount of the G_4 form which represented 9, 16, 18, 25, and 33% of the supernatant BChE activity from steps 1 to 5, respectively (Figure 3, lower panel). The step 6 pellet (about 4% of the total activity), analyzed in the presence of 1% Triton X-100, contained a G_4 form corresponding to 16% of the BChE activity layered onto the sucrose gradient.

Study of the G Form. (a) Sedimentation. Two preparative centrifugations were performed, one (Figure 4A) in the absence and the other (Figure 4C) in the presence of 1% Triton X-100 in the homogenate and the sucrose gradient medium. The presence of detergent induced a shift of the S value of the G form from 5.9 (Figure 4A) to 3.4 (Figure 4C).

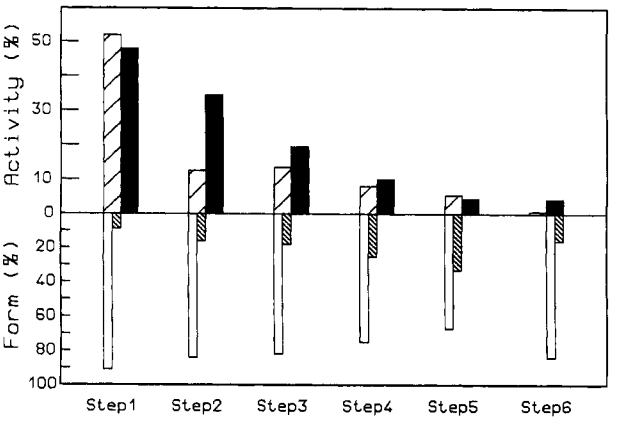

FIGURE 3: Repetitive extractions of mucosal cell BChE. Activity in each supernatant (▨) and pellet (■) was expressed as percentage of total initial activity of BChE (upper panel). The molecular forms were analyzed in the supernatants (steps 1–5) in the absence of detergent and in the pellet (step 6) in the presence of detergent. The relative amount of the G form (▩) and the G_4 form (□) was estimated from sedimentation profiles (lower panel).

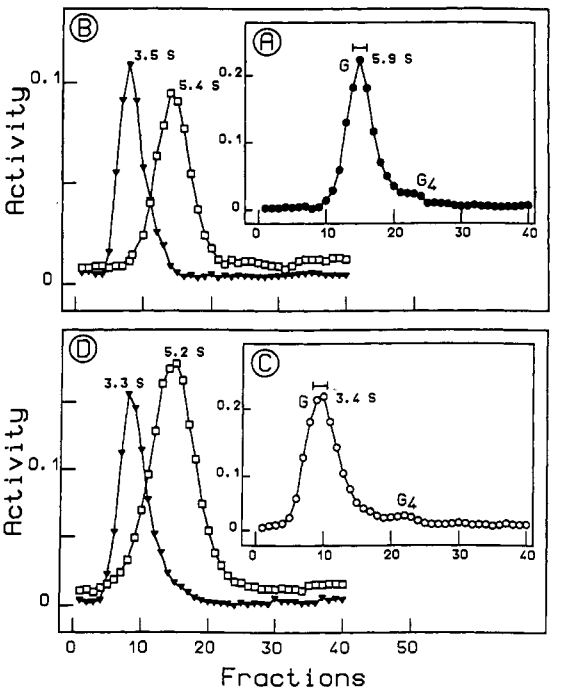

FIGURE 4: Hydrodynamic properties of the G form. The G form of LSS extract (A) or DS extracts (C), collected as shown by the horizontal bar, was reanalyzed by sucrose gradient centrifugation (B, D) without detergent (□) and in the presence of 1% Triton X-100 (▼). Activities were expressed in arbitrary units.

The 5.9S form preparation, reanalyzed in a new sucrose gradient without detergent (Figure 4B), was characterized by a lower S value (5.4 S), suggesting that this species binds small molecules. The same 5.9S form preparation, centrifuged in a sucrose gradient containing 1% Triton X-100 (Figure 4B), sedimented at 3.5 S. This value appeared very close to that determined for the G form (3.4 S) obtained from DS extracts centrifuged in sucrose gradients containing Triton X-100 (Figures 2 and 4C). When the 5.9S form was treated with 0.1% DTT and analyzed in sucrose gradient centrifugation, no change of the S value was observed.

A very close S value (3.3 S) was obtained when the 3.4S form preparation (Figure 4C) was reanalyzed in a new sucrose gradient containing Triton X-100 (Figure 4D). The same 3.4S form preparation, resedimented in a sucrose gradient

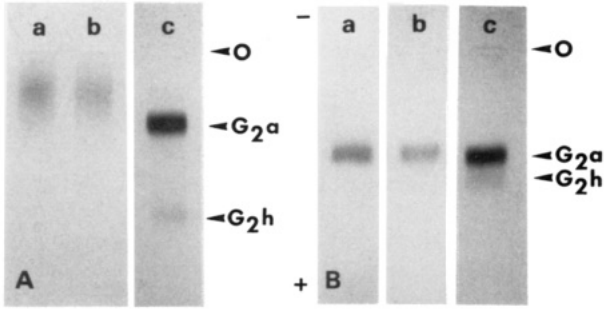

FIGURE 5: Nondenaturing electrophoresis of LSS extracts in the presence of 0.5% Triton X-100 (A) or 0.2% Triton X-100 + 0.2% deoxycholate (B). Peak fractions of BChE (5.9 S) from an LSS extract run on a preparative sucrose gradient were used (see Figure 4A). Control (lanes a) and PI-PLC-treated samples (lanes b) were run as well as a detergent-soluble AChE sample from *Drosophila* (lanes c). Gels were stained with either BuSCh (lanes a and b) or AcSCh (lane c) as substrates. O, origin of migration; G_2^a and G_2^h, amphiphilic and hydrophilic dimers of *Drosophila* AChE. Note that the electrophoresis in (B) was run for a shorter time than in (A) as exemplified by the shorter migration of the *Drosophila* G_2^h form.

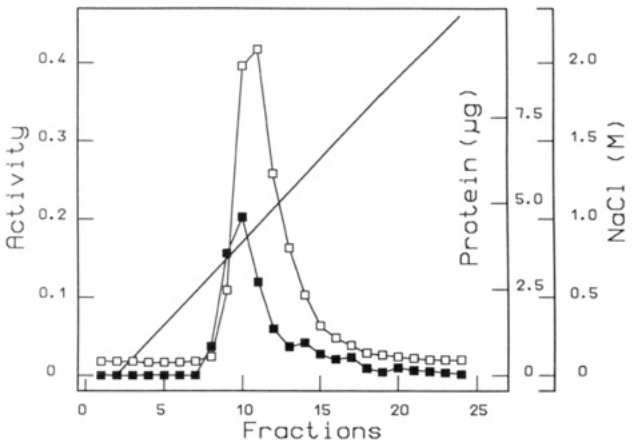

FIGURE 6: Affinity chromatography of the G form on procainamide coupled to CH Sepharose 4B. Conditions are indicated under Materials and Methods. The elution of the enzyme (□) was achieved with a NaCl gradient (0–2.5 M). Protein (■) was determined in parallel.

without detergent, was characterized by an increase of its S value (5.2 S).

The hydrodynamic behavior of the G form might be explained by a complex between the enzyme and a noncatalytic component (hypothesis 1) or by the binding of nondenaturing detergent molecules to a hydrophobic domain of the enzyme (hypothesis 2). A noncatalytic subunit (hypothesis 1) might dissociate from the enzyme, in the presence of nondenaturing detergent, explaining the shift to lower S values in sucrose gradient centrifugation. Considering this hypothesis, centrifugations of longer duration (24 h), in the presence of Triton X-100, were attempted in order to isolate the light form from its hypothetical binding components. The resulting preparation, recentrifuged in the absence of detergent, again sedimented at 5.2 S.

(*b*) *Nondenaturing Electrophoresis*. In the system used, the presence of DOC, an anionic detergent, was shown to increase the migration of amphiphilic components, but not that of hydrophilic components, as compared to their migration in the presence of Triton X-100 only (Massoulié et al., 1988). BChE samples prepared as indicated under Materials and Methods were run on a gel either containing 0.5% Triton X-100 (Figure 5A) or containing 0.2% Triton X-100 and 0.2% DOC (Figure 5B). In Figure 5A, BChE activity appeared as a smear of low mobility (lane a) unaffected by a treatment

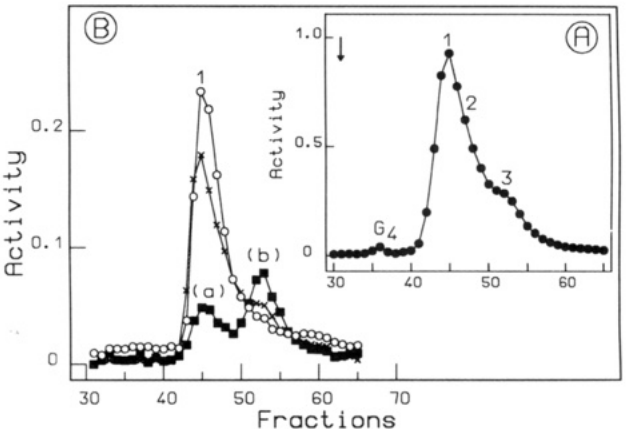

FIGURE 7: HPLC chromatography of the G form. LSS extract was analyzed by gel filtration chromatography (A). Fractions 1–3 in (A) were rechromatographed under the same experimental conditions (B). Fractions 1 (○) and 2 (×) gave a major peak (peak 1). Fraction 3 (■) showed two active peaks (a and b). The arrow in (A) corresponds to the exclusion volume.

with PI-PLC (lane b). In Figure 5B, BChE appeared as a narrow band of increased migration (lane a). PI-PLC had no effect (lane b). A sample of *Drosophila* AChE containing a major amphiphilic dimer (G_2^a) and a minor hydrophilic counterpart (G_2^h) was run in parallel to show the typical migration of amphiphilic AChE dimers [lanes c in Figure 5A and B and Toutant et al. (1988)]. The presence of β-mercaptoethanol in both sample and gel did not result in any variation in BChE mobility.

(*c*) *Affinity Chromatography*. Affinity chromatography, performed with procainamide or 1-(*N*,*N*,*N*-trimethylammonio)-6-hexylamine as ligands, retained the G form, which was eluted at 0.9 M NaCl (Figure 6). The fraction containing the maximal activity was analyzed by sucrose gradient centrifugation without detergent or in the presence of 1% Triton X-100. S values of 5.5 or 3.5 were found, respectively. Attempts to remove a possible noncovalently bound amphiphilic part of the enzyme failed because the same S values (5.5 and 3.5 S) were obtained when Triton X-100 was used during either step of enzyme binding to ligand or step of washing. Inhibitor and substrate specificities were tested on the purified enzyme; results similar to those shown in Figure 1 for crude extracts were obtained.

(*d*) *HPLC Study*. LSS extracts were analyzed by HPLC using a gel filtration column without Triton X-100. The elution profile (Figure 7A) displayed two peaks corresponding to the G_4 and G forms. The heterogenous G peak was subdivided into three fractions (Figure 7A). Each of them was rechromatographed under the same experimental conditions. Fractions 1 and 2 showed similar elution profiles with one major peak (peak 1: SR, 5.2 nm; MW, 126 000). In contrast, fraction 3 displayed two active species [(a) and (b) in Figure 7B]. Fraction a corresponded clearly to the main active form (peak 1). Fraction b, corresponding to about 8.6% of the total activity in (A), was eluted as a monomeric form (G_1: SR 3.9 nm, MW 72 000). Analyzed in sucrose gradient centrifugation, the G_1 fraction sedimented at 4.3 S in the absence of Triton X-100 and at 3.7 S in the presence of detergent.

Similar HPLC experiments, performed in the presence of Triton X-100 in the elution buffer, led to similar results (i.e. a major peak, SR 5.2 nm, and a very minor component, SR 3.9 nm).

(*e*) *Octyl-Sepharose Chromatography*. The G form was isolated from LSS extracts centrifuged on preparative sucrose

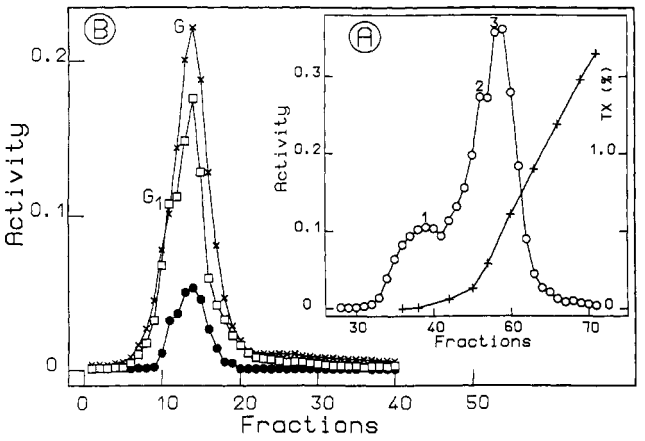

FIGURE 8: Hydrophobic chromatography of the G form on octyl-Sepharose gel. In (A), the enzyme elution (○) was performed with a 0–2% Triton X-100 (TX) gradient (+). Fractions 1–3 were collected and analyzed by sucrose gradient centrifugation (B) in the absence of detergent: fraction 1 (●), fraction 2 (□), and fraction 3 (×). Activities were expressed in arbitrary units.

Table IV: Proteolytic Digestion of the G Form

G form	Stokes radius (nm)	MW	S value without Triton X-100	S value with 1% Triton X-100	shift
control	5.2	126 000	5.9	3.4	2.5
proteinase K					
digest product A	4.5	88 000	4.7	3.3	1.4
bromelain					
digest product B	4.5	90 000	4.8	4.3	0.5
digest product C	3.0	54 000	4.3	3.9	0.4
digest product D	2.4	37 000	3.7	3.5	0.2

gradients in the absence of detergent (Figure 4A) and chromatographed on octyl-Sepharose gel. The enzyme was strongly and quantitatively bound to the gel, even when salt solutions, such as $(NH_4)_2SO_4$, were omitted. A 0–2% Triton X-100 gradient was able to elute the enzyme (Figure 8A). The chromatographic profile presented three active regions (Figure 8A). Analysis by sucrose gradient centrifugation in the absence of detergent (Figure 8B) showed that fraction 3 was essentially composed of the G form (5.2 S) whereas discrete shoulders of the G_1 form (4.3 S) were apparent in both fractions 1 and 2. Fractions 1–3 were eluted respectively with 0.02, 0.21, and 0.50% Triton X-100 (Figure 8A), indicating that the G_1 form, mainly found in fractions 1 and 2, needed a lower detergent concentration than the G form to be eluted.

(f) Protease Digestion. The G form appeared highly insensitive to trypsin, chymotrypsin, pepsin, thermolysin, collagenase, endoprotease Glu-C, and papain. Proteinase K had a slow action on the G form. In this case, HPLC chromatography revealed that the G form was partially converted into a lower mass protein (digest product A, Table IV). The active digest product A was centrifuged in a sucrose gradient with or without Triton X-100. In the absence of detergent, it sedimented at 4.7 S; however in the presence of Triton X-100, a lower S value (3.3 S) close to control (3.4 S) was obtained (Table IV). This result showed that proteinase K failed to remove the bulk of the hydrophobic domain of the molecule responsible for the sedimentation shifts. Under the same experimental conditions, the action of bromelain was more effective than that of proteinase K. Chromatographic analysis showed a complete conversion of the G form into three active digest products (B–D). Analyzed in sucrose gradient, the digest product B appeared less hydrophobic than the digest product A obtained with proteinase K. Digest products C and D were characterized by low Stokes radii and reduced sedimentation shifts.

DISCUSSION

The enzyme responsible for cholinesterase activity in the mucosal cells from rat small intestine hydrolyzed AcSCh as well as BuSCh but presented a higher affinity for the former substrate. No inhibition was observed with substrate excess. In addition, the IC_{50} value of iso-OMPA (inhibitor specific to BChE) was 55 times lower than that of BW284C51 (inhibitor specific to AChE). The enzyme was thus referred to as butyrylcholinesterase. It should be noted that peculiar substrate affinities in the order AcSCh > BuSCh have already been reported for *Torpedo* BChE (Toutant et al., 1985).

The BChE activity was soluble under LS conditions. Repetitive extractions allowed a quantitative recovery of the activity without detergent. For each step, LSS extracts contained two globular forms (G_4 and G) of BChE in variable amounts. The G_4 form did not present any shift in its S value in centrifugations performed either in the presence of Triton X-100 or in the absence of detergent; this was typical of a hydrophilic form.

Structure of the G form. The G form sedimented like a dimeric form in the absence of detergent (5.9 S) and had a Stokes radii (5.2 nm) higher than that of the classic G_1 form (3.7 nm) and compatible with that of a dimeric form (Bon et al., 1988a). Sedimentation coefficient and Stokes radius values gave an estimated molecular weight of 126 000 for the G form (and 72 000 for G_1). Thus the G form could be a dimer of BChE catalytic subunits. However, the usual thiol reagents (β-mercaptoethanol, DTT) which are effective in converting disulfide-linked dimers of ChEs into monomers had no effect on the G form. In addition, the S-value shift when the G form was centrifuged in the presence of Triton X-100 (5.9 to 3.5 S) was much more important than that observed for *Torpedo* BChE dimers, for example [0.7 S, Bon et al. (1988a)]. This might be an indication that the G form was an association of one catalytic subunit with one noncatalytic element as noted recently for the C_2 component of human plasma BChE, which was shown to be an albumin conjugate of the catalytic subunit (Masson, 1989). In our case, the noncatalytic element should be highly hydrophobic to explain the large shift in S value. We do not rule out such an hypothesis although it appears unlikely that such an amphiphilic complex should be naturally soluble in LS buffer (Figure 3).

An alternative possibility is that the G form is a dimer of BChE catalytic subunits linked either by strong hydrophobic interactions not dissociated by nondenaturing detergents or by an interchain covalent bond not sensitive to DTT (Roberts et al., 1991). In this view, the linkage of the subunits in the dimeric G form should resemble that involved between the two disulfide-linked dimers in the tetrameric G_4 form of AChE (Vigny et al., 1979) or BChE (Lockridge et al., 1979; Lockridge & La Du, 1982). In addition, such a lack of intermolecular disulfide bridges has been reported for certain dimers of *Torpedo* AChE (Witzemann & Boustead, 1983). If the G form is dimeric, its amphiphilic properties are due to a hydrophobic domain of each catalytic subunit and this domain is not a glycolipid anchor, as shown by the resistance to PI-PLC treatment (Figure 5). Thus the G form (now G_2) should be related to the class II dimers as defined by Bon et al. (1988b). This is further supported by the absence of aggregation of the G form in the absence of detergent. The nature of the hydrophobic domain is unknown, and the reason for the large shift in S values in the presence of Triton X-100 is unclear.

We observed comparable shifts by using other nondenaturing detergents (see Table III). It is known that Brij 96 induces a higher sedimentation shift of amphiphilic forms than Triton X-100 (Toutant et al., 1988; Bon et al., 1990, 1991). Surprisingly, in our case, no shift of the G-form S value was observed with this detergent. In addition, HPLC chromatography did not reveal a significant increase of the G-form Stokes radius in the presence of Triton X-100.

The G form could only be digested by a few proteolytic enzymes. The resistance to various proteases might be related to the intestinal origin of this BChE form. In particular, proteinase K did not remove the amphiphilic domain and did not abolish BChE activity even after long digestion periods. Papain allowed the conversion of erythrocyte AChE into a nonamphiphilic molecule (Weitz et al., 1984; Dutta-Choudhury & Rosenberry, 1984). Our results show that only bromelain (a nonspecific protease related to papain) and not papain generated, by a limited proteolysis, several digest products whose amphiphilic properties were significantly decreased as compared to the proteinase K product and control (Table IV). This suggests that the hydrophobic cleavage sites of the G form might be different from those of AChE dimers of class I and that the hydrophobic domain might be represented by several dicrete amphiphilic regions.

G_1 Form. The G-form peak observed in HPLC was heterogenous and contained a small amount of the G_1 form, which was characterized as an amphiphilic species. As indicated by octyl-Sepharose chromatography and by sucrose gradient centrifugation, the G_1 form possessed a less marked hydrophobicity than the G form. Such a G_1 form was also observed in the mucosal cells of colon and caecum of rat intestine. However, in these two intestinal segments, no G form could be detected. This observation raises the question of the significance of the amphiphilic forms. The marked difference in amounts of G_1 and G forms might be the result of a possible metabolic pathway in which the G form accumulates. In this biosynthetic scheme, the G_1 form might represent a short-lived precursor. It should be noted that the nonamphiphilic G_4 form belongs to another and distinct metabolic pathway whose precursor remains unknown. Multiple biosynthetic pools of AChE (nonamphiphilic forms, amphiphilic forms belonging to classes I and II) have been reported in different tissues (Grassi et al., 1982; Lazar et al., 1984; Bon et al., 1991). A similar situation might occur for the BChE of mucosal cells of rat small intestine.

CONCLUSION

The present report shows that G_1 and G_2 amphiphilic forms of BChE exist in the mucosal cells of rat intestine. Although we do not exclude that some amphiphilic properties may arise from posttranslational modifications, they may come from the peptidic sequence itself. Together with the previous report of an amphiphilic dimer of *Torpedo* heart BChE (Bon et al., 1988a,b), this work suggests that the vertebrate BChE gene produces not only major "hydrophilic" transcripts but also other discrete transcripts (differently spliced for the same gene) that result in amphiphilic proteins.

ADDED IN PROOF

While this article was in proof, a paper by Duval et al. (1992) demonstrated that COS cells transfected with the cDNA coding for the hydrophilic catalytic subunit of *Torpedo* AChE synthesized several molecular forms, including an amphiphilic dimer of class II. Such a possibility might be considered for the origin of amphiphilic BChE forms described here.

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