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Removal of covalently bound inositol from *Torpedo* acetylcholinesterase and mammalian alkaline phosphatases by deamination with nitrous acid

Evidence for a common membrane-anchoring structure

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Our earlier evidence suggested that both acetylcholinesterase and alkaline phosphatase are anchored to the cell surface via covalently-attached phosphatidylinositol [Low, Futerman, Ferguson & Silman (1986) Trends Biochem. Sci. **11**, 212–215]. We now present chemical data, based upon a nitrous acid deamination reaction, showing that in both proteins the phosphatidylinositol moiety is attached through a glycosidic linkage to a sugar residue bearing a free amino group.

INTRODUCTION

The anchoring of proteins to membranes by covalent attachment to phosphatidylinositol or a related molecule now appears to be a well-established phenomenon (reviewed in Low *et al.*, 1986; Low, 1987). Proteins currently believed to be anchored by this mechanism include APase (Low & Zilversmit, 1980), AChE (Low & Finean, 1977; Futerman *et al.*, 1985*a*,*b*,*c*), 5′-nucleotidase, alkaline phosphodiesterase I, trehalase, variant surface glycoprotein (VSG) of *Trypanosoma brucei*, decay accelerating factor, T-cell activating protein, *Leishmania* p63 protease and the Thy-1, RT-6, Qa and ThB antigens (for references see Low *et al.*, 1986; Low, 1987). A particularly powerful tool in these studies has been phosphatidylinositol-specific phospholipase C (PI-PLC) which removes the 1,2-diacylglycerol from the phosphatidylinositol moiety that is covalently attached to these proteins and thus releases them from the membrane. Since many of the proteins listed above are not available in amounts sufficient for detailed chemical analyses, their susceptibility to PI-PLC is currently the only evidence that they are anchored via covalently attached phosphatidylinositol.

In addition to their sensitivity to PI-PLC, several of these proteins share additional unusual structural features. On the basis of these similarities, we have proposed that all the phosphatidylinositol-anchored proteins located at the cell surface are attached to the phosphatidylinositol molecule in the membrane through a similar intervening structure. This model (Low *et al.*, 1986) proposed that the phosphatidylinositol is glycosidically linked to glucosamine which is, in turn, linked through an oligoglycan to the *C*-terminal amino acid. In the case of the VSG of *T. brucei* this glucosamine is unusual inasmuch as it is not *N*-acetylated (Strang *et al.*, 1986) and therefore, upon deamination with nitrous acid, undergoes a specific rearrangement resulting in cleavage of the glycosidic bond with inositol (Ferguson *et al.*, 1985). Recent indirect evidence demonstrating immunological cross-reactivity in the anchoring domains of several of these proteins has tended to support the proposed model (Stieger *et al.*, 1986; Bordier *et al.*, 1986; Davitz *et al.*, 1986).

In the following, we provide additional support for the generality of this model by demonstrating that the *myo*-inositol in both APase and AChE is attached to the protein by a nitrous acid-sensitive linkage.

MATERIALS AND METHODS

Materials

AChE solubilized from *Torpedo* membranes by PI-PLC was purified by affinity chromatography as described previously (Futerman *et al.*, 1985*b*). Purified bovine intestinal alkaline phosphatase (specific activity $\geqslant$ 1000 units/mg) was obtained from Sigma Chemical Co. (type VIIL) and from Miles Scientific (labelling grade). On the basis of Triton X-114 partition and gel filtration studies the intestinal APase preparations used here consisted almost completely (i.e. > 98%) of the non-aggregated hydrophilic form and are thus equivalent in their physical properties to the APase solubilized from human placenta by PI-PLC (Malik & Low, 1986). PI-PLC was purified from *Staphylococcus aureus* culture supernatants as previously described (Malik & Low, 1986). *myo*-Inositol 2-phosphate was obtained from Sigma.

Purification of human placental alkaline phosphatase

A crude particulate fraction of human plancenta was prepared essentially as described by Malik & Low (1986) but with 0.25 M-sucrose/10 mM-Tris/HCl, pH 7.4, for homogenization. The particulate fraction from one placenta was frozen overnight, thawed, resuspended in homogenization buffer (approx. 0.1 ml/g of original tissue), centrifuged at 150000 *g* for 40 min, resuspended in the same volume of homogenization buffer and then incubated with PI-PLC (2 μg/ml) and 10 mM-Hepes/

Abbreviations used: AChE, acetylcholinesterase; APase, alkaline phosphatase; PI-PLC, phosphatidylinositol-specific phospholipase C.

Table 1. Effect of nitrous acid on the *myo*-inositol content of acetylcholinesterase and alkaline phosphatase

Control samples were hydrolysed with 6 M-HCl directly and analysed for inositol by g.l.c.–m.s. The experiments with acetate buffer alone or with $NaNO_2$ in the acetate buffer were, following incubation, precipitated with 5% (w/v) trichloroacetic acid (10 min, 0 °C) and then centrifuged at 1500 *g* for 10 min, the supernatant was discarded and the pellet analysed for inositol after hydrolysis with 6 M-HCl. The amount of AChE was estimated by amino acid analyses and A_{280} values as described previously (Futerman *et al.*, 1985*b*). The amount of placental APase was estimated from amino acid analyses; the number of residues/mol was estimated by comparison with the cDNA sequence (Kam *et al.*, 1985; Millan, 1986). The amount of intestinal APase was estimated from the protein value supplied by the manufacturer and assigning a subunit M_r of 70000. Each line of the Table refers to an independent experiment; the experiments with *Torpedo* AChE and human placental APase were done with two different preparations of protein. Values given are mean ± S.D. and values in parentheses indicate the number of replicate treatments.

Protein	Preincubation conditions ...	*myo*-Inositol (mol/mol of protein) Control	Acetate buffer	Acetate buffer/$NaNO_2$
Torpedo AChE		0.98 ± 0.03 (6)*	0.83 ± 0.08 (3)	0.31 ± 0.01 (4)
		1.12 ± 0.02 (3)†	1.08 ± 0.03 (3)	0.41 ± 0.02 (3)
Human placental APase		1.28 ± 0.05 (5)†	1.23 ± 0.01 (3)	0.33 ± 0.01 (3)
		1.20 ± 0.01 (3)†	1.17 ± 0.03 (3)	0.25 ± 0.01 (3)
Bovine intestinal APase		0.36 ± 0.06 (3)*	0.30 ± 0.01 (3)	0.10 ± 0.01 (3)
			0.27 ± 0.03 (3)	0.09 ± 0.01 (3)

* Used *scyllo*-inositol as internal standard.
† Used *myo*-[$^2H_6$]inositol as internal standard.

NaOH, pH 7.0, for 30 min at 37 °C. After centrifugation at 150000 *g* for 40 min the supernatant was concentrated to approx. 20 ml by ultrafiltration in an Amicon cell with a YM-30 membrane. This was applied to two Sephacryl S-300 columns linked in series (each 2.5 cm × 120 cm; V_0 approx. 410 ml). The columns were equilibrated and eluted with 50 mM-NaCl/10 mM-Hepes/0.1 mM-$MgCl_2$/0.01 mM-zinc acetate/NaOH, pH 7.4, at a flow rate of 12 ml/h; 18 ml fractions were collected. The alkaline phosphatase peak fractions (elution volume approx. 630 ml) were pooled, the pH adjusted to 8.0 with 1 M-Tris base and loaded at a flow rate of 1 ml/min on a Mono-Q HR5/5 column (0.5 cm × 5 cm; Pharmacia) equilibrated in 10 mM-Hepes/0.1 mM-$MgCl_2$/0.01 mM-zinc acetate/NaOH, pH 7.4. The column was eluted with a 25 ml linear gradient of 0.05–0.5 M-NaCl in the equilibrating buffer; 1 ml fractions were collected. Alkaline phosphatase peak fractions (at approx. 9 ml elution volume) were pooled and dialysed exhaustively against distilled water. The experiments reported here utilized two preparations each consisting of the alkaline phosphatase purified individually from two placentas and then combined.

Inositol analysis

Protein samples (approx. 100–200 μg), 200 ng of a *scyllo*-inositol (Calbiochem) or *myo*-[$^2H_6$]inositol (M.S.D. Isotopes, Montreal, Canada) internal standard were hydrolysed in 0.25 ml of 6 M-HCl at 110 °C for 24 h in sealed ampoules. The internal standard was shown to be stable to the conditions of hydrolysis. A 0.2 ml sample was placed in a silanized 0.3 ml conical vial and taken to dryness in a stream of air or N_2 at 45 °C, and then in a rotary pump vacuum to ensure complete drying and removal of HCl. Unhydrolysed sampled were prepared by adding the protein and internal standard directly to the vials and then dried. Samples were then converted to the pertrimethylsilyl derivative and analysed by g.l.c.–m.s. as described previously (Futerman *et al.*, 1985*b*). Poor recovery of inositol standards was experienced when 1 μg/ml solutions of these standards, in distilled water, were dried in glass vials; this was due presumably to their adsorption to the glass which was not prevented by use of silanized glass vials. However, it was found that recovery was markedly improved by addition to the standards of 50–100 μl of 100 mM-Tris/HCl (pH 8.0) prior to drying. The Tris buffer was, therefore, routinely added, prior to hydrolysis or drying, to all samples analysed for inositol and inositol phosphate.

Inositol phosphate analysis

Samples, including *myo*-[$^2H_6$]inositol 1-phosphate as an internal standard, were treated with nitrous acid (see below); after this treatment the pH was adjusted to 7.0 with NH_4OH and the samples dried as above. The samples were then derivatized and analysed as described previously (Sherman *et al.*, 1986).

Nitrous acid treatment

Protein samples (approx. 0.5 mg/ml) were incubated at 25 °C for 5 h in the presence of 0.25 M-sodium acetate or ammonium acetate buffer (pH 3.5) with or without freshly prepared 0.2 M-$NaNO_2$. The reaction was terminated either by precipitating the protein with trichloroacetic acid (see Table 1) or by neutralizing with NH_4OH; samples were then analysed for either inositol or inositol phosphate, respectively (see above). Initial analyses of inositol phosphates were complicated by low recovery of the internal standard, perhaps due to the large amounts of salt in the dried samples taken for derivatization; this problem was not encountered in the inositol analysis since most of the salt was removed during the trichloroacetic acid precipitation of the protein after the

Table 2. Analysis of inositol and inositol phosphates released by nitrous acid treatment of acetylcholinesterase and alkaline phosphatase

Values are expressed as a percentage of total *myo*-inositol content determined by 6 M-HCl hydrolysis of untreated protein samples.

Protein		Content (% of total *myo*-inositol) of: Inositol	Inositol 1-phosphate	Inositol 2-phosphate
Torpedo AChE*				
Expt. I.	Acetate buffer	0.4±0.3	0	0
	Acetate buffer/$NaNO_2$	1.2±0.7	67.6±6.6	10.0±5.4
Expt. II.	Acetate buffer	0.5±0.1	0	0
	Acetate buffer/$NaNO_2$	2.9±0.2	57.3±5.2	4.8±0.6
Human placental APase†				
Expt. I.	Acetate buffer	0.1±0.2	—	—
	Acetate buffer/$NaNO_2$	56.8±1.3	—	—
Expt. II.	Acetate buffer	0.1±0.1	—	—
	Acetate buffer/$NaNO_2$	65.3±2.2	—	—

* Two independent experiments with different preparations of AChE and *myo*-[$^2H_6$]inositol 1-phosphate as internal standard; values are means±S.D. of quadruplicate treatments.

† Two independent experiments with the same preparation of APase but using different procedures; in expt. II 10 mM-ammonium phosphate (pH 4.0) and 1 mM-EDTA was added to the preincubation mixture in an attempt to inhibit the action of the APase. Since extensive degradation of the *myo*-[$^2H_6$]inositol phosphate internal standard occurred in all samples, quantification of inositol phosphates was not accurate and these data are not presented. The inositol values were corrected for recovery of the *myo*-[$^2H_6$]inositol generated by hydrolysis of the internal standard; values are means±S.D. of triplicate treatments.

nitrous acid treatment. Recovery of the inositol phosphates was greatly improved, in subsequent experiments, by using ammonium acetate buffer instead of sodium acetate.

RESULTS AND DISCUSSION

In a previous report, we demonstrated for the first time that *myo*-inositol was present in stoichiometric amounts in purified AChE released from *Torpedo* membranes by PI-PLC (Futerman *et al.*, 1985*b*). We now report similar observations for APase. Thus, human placental APase was found to contain approx. 1.2–1.3 mol of *myo*-inositol/mol (i.e. controls in Table 1); analysis of unhydrolysed control samples indicated a free *myo*-inositol content of less than 5% of these values. Purified bovine intestinal APase from two different commercial sources was similar analysed. Significant amounts of inositol were detected which were, however, substantially lower than the expected value of 1 mol/mol and varied considerably from batch to batch (i.e. 0.13–0.36 mol/mol). As with the placental APase, less than 5% of the total *myo*-inositol could be detected in unhydrolysed controls. The reason for the relatively low and variable content of *myo*-inositol in the commercial intestinal APase preparations is not clear at present; it could be due to proteolysis during extraction from the membranes with butanol, to contamination by other proteins or to the presence of a subpopulation of APase molecules not anchored by phosphatidylinositol which nevertheless co-purified with the phosphatidylinositol-anchored molecules. It should be emphasized, with reference to the latter point, that both the placental APase and the *Torpedo* AChE used here were solubilized by PI-PLC prior to purification, which would necessarily select only those molecules anchored via phosphatidylinositol or related structures.

Hydrolysed samples were also analysed for *chiro*-inositol (results not shown). This was done since in our earlier study (Futerman *et al.*, 1985*b*) with *Torpedo* AChE some preparations were shown to contain significant amounts of this relatively rare inositol isomer. In the present study it was detected in the two human placental APase preparations at levels of approx. 3% and 20% of the amount of *myo*-inositol; for the single preparations of AChE and bovine intestinal APase that were analysed this value was approx. 10%. *chiro*-Inositol was barely detectable in either the unhydrolysed samples of placental APase or in those which had been precipitated by 5% trichloroacetic acid (results not shown), suggesting that it is covalently attached either to the protein by a acid-labile linkage or to a substance which co-purified with the protein. The presence of *chiro*-inositol in this human enzyme as well as the *Torpedo* AChE (Futerman *et al.*, 1985*b*) suggests that it is not an artifact, but rather associated with a biologically functional molecule the structure of which is unknown.

The effect of nitrous acid on the attachment of inositol to the protein was investigated. Each enzyme was incubated at pH 3.5 in the presence or absence of $NaNO_2$; trichloroacetic acid was added to precipitate the proteins, which were then acid-hydrolysed and analysed for *myo*-inositol. The values obtained are compared with those for control samples which were acid-hydrolysed directly (Table 1). These data confirm that the *myo*-inositol is tightly associated with all three proteins since 80–95% was precipitated by trichloroacetic acid after preincubation in the pH 3.5 buffer. The amount of bound inositol was substantially reduced if $NaNO_2$ was

included in the preincubation mixture. However, in all cases, substantial amounts of *myo*-inositol (approx. 20–40%) remained bound to the protein (Table 1). The failure to remove all of the inositol by nitrous acid deamination was not unexpected since cleavage of the glycosidic linkage in glucosamine-containing glycosides is not quantitative. Thus, deamination of methyl 2-amino-2-deoxy-α-D-glucopyranosides yields approx. 25% methyl 2-deoxy-2-*C*-formyl-α-D-ribofuranoside (in which the glycosidic linkage is retained) in addition to the major product, 2,5-anhydro-D-mannose (Williams, 1975; Lindberg *et al.*, 1975).

Since the AChE and human placental APase used in these studies were released from membranes by PI-PLC the inositol remaining with the protein should be present as a phosphate ester. If the original lipid was phosphatidyinositol then this ester should be inositol 1-phosphate, inositol 1,2-cyclic phosphate or a mixture of both (see Ferguson *et al.*, 1985). The relatively mild deamination technique was therefore used to demonstrate that the inositol attached to the protein was in fact a phosphate ester. In these experiments the products of the deamination reaction were analysed directly without trichloroacetic acid precipitation or acid hydrolysis (Table 2). In the case of AChE substantial amounts of inositol 1-phosphate were detected in the samples treated with pH 3.5 buffer and $NaNO_2$ but not in the pH 3.5 buffer controls. Similarly, inositol 2-phosphate could only be detected after the deamination reaction; however, the amounts were about 10–20% of those detected for inositol 1-phosphate (Table 2). The inositol 2-phosphate may be the result of hydrolysis of inositol 1,2-cyclic phosphate at pH 3.5 since the cyclic inositol phosphate is one of the likely products of a phospholipase C mediated hydrolysis of the phosphatidylinositol anchor (Ferguson *et al.*, 1985). Much smaller amounts of *myo*-inositol were also observed, and these increased slightly upon nitrous acid treatment.

The experiments with placental APase were more difficult to interpret since less than 2% of the *myo*-[$^2H_6$]inositol 1-phosphate internal standard was recovered. Increases in inositol phosphates were observed after nitrous acid treatment but loss of the internal standard meant they could not be quantified accurately and are not therefore presented in Table 2. This problem was not encountered with the AChE samples prepared by a similar procedure (internal standard recovery generally 50–100%), and we therefore attribute the loss of the internal standard to enzymic autohydrolysis by the APase during preparation of the samples for analysis. In this regard, it is relevant to note that the APase concentration during these manipulations was of the order of 500 μg/ml and consequently degradation of inositol phosphate by this relatively non-specific phosphomonoesterase was not unexpected. Furthermore, separate experiments showed that substantial APase activity remains after nitrous acid treatment. In one experiment (Table 2) an unsuccessful attempt was made to minimize degradation of the internal standard by addition of the APase inhibitors EDTA and inorganic phosphate. However, since nitrous acid did release large amounts of *myo*-inositol from the placental APase, these data (quantified by the *myo*-[$^2H_6$]inositol released by degradation of the internal standard) are shown in Table 2.

The total recovery of inositol and inositol monophosphates in these experiments (Table 2) amounted to approx. 60–80% of that predicted from total *myo*-inositol content, as determined by 6 M-HCl hydrolysis, and is thus quite similar to the amount of inositol released from these proteins when determined by the trichloroacetic acid precipitation procedure (Table 1). This suggests that extensive, additional substitution on the inositol ring by nitrous acid-insensitive linkage (e.g. by phosphate in AChE or glycosidic linkages to other sugars in AChE and APase) is unlikely in the proteins studied here.

The results presented in this report support our proposal (Low *et al.*, 1986) that phosphatidylinositol-anchored proteins share common structural features in addition to the phosphatidylinositol moiety. Thus, inositol, inositol phosphate or phosphatidylinositol have now been shown to be released by nitrous acid treatment of variant surface glycoprotein (Ferguson *et al.*, 1985), *Torpedo* AChE and two genetically distinct types of mammalian APase; and a preliminary report suggests that the same may be true for human erythrocyte AChE (Roberts & Rosenberry, 1986) in spite of its insensitivity to PI-PLC (Low & Finean, 1977; Futerman *et al.*, 1985*c*). Although the detailed chemical composition of the membrane-anchoring domains of the proteins studied here are not yet known, the data presented suggest that, as in variant surface glycoprotein, the phosphatidylinositol is glycosidically linked to the protein via a non-*N*-acetylated amino sugar, perhaps glucosamine. This conclusion is supported by preliminary studies which indicate that some non-*N*-acetylated glucosamine is present in *Torpedo* AChE (S. Rees, A. Futerman & I. Silman, unpublished work). The recent findings that PI-PLC releases soluble mediators of insulin action (Saltiel *et al.*, 1986; Saltiel & Cuatrecasas, 1986) from a nitrous acid-sensitive glycolipid increases the likelihood that eukaryotic cells use this mechanism for the hydrophobic binding and release of biologically important molecules in more instances than have thus far been detected. The structures of the elements that link the phosphatidylinositol to the proteins and to the insulin mediators remain unknown and of great interest.

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