Activation of the glycosyl-phosphatidylinositol-anchored membrane dipeptidase upon release from pig kidney membranes by phospholipase C

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Incubation of pig kidney microvillar membranes with *Bacillus thuringiensis* or *Staphylococcus aureus* phosphatidylinositol-specific phospholipase C (PI-PLC) resulted in the release of a number of glycosyl-phosphatidylinositol (GPI)-anchored hydrolases, including alkaline phosphatase (EC 3.1.3.1), aminopeptidase P (EC 3.4.11.9), membrane dipeptidase (EC 3.4.13.19), 5'-nucleotidase (EC 3.1.3.5) and trehalase (EC 3.2.1.28). Of these five ectoenzymes only for membrane dipeptidase was there a significant (approx. 100%) increase in enzymic activity upon release from the membrane. Maximal activation occurred at a PI-PLC concentration 10-fold less than that required for maximal release. In contrast solubilization of the membranes with n-octyl β -D-glucopyranoside had no effect on the enzymic activity of membrane dipeptidase. A competitive e.l.i.s.a. with a polyclonal antiserum to membrane dipeptidase indicated that the increase in enzymic activity was not due to an increase in the amount of

membrane dipeptidase protein. Although PI-PLC cleaved the GPI anchor of the affinity-purified amphipathic form of pig membrane dipeptidase there was no concurrent increase in enzymic activity. In the absence of PI-PLC, membrane dipeptidase in the microvillar membranes hydrolysed Gly-D-Phe with a K_m of 0.77 mM and a V_{max} of 602 nmol/min per mg of protein. However, in the presence of a concentration of PI-PLC which caused maximal release from the membrane and maximal activation of membrane dipeptidase the K_m was decreased to 0.07 mM while the V_{max} remained essentially unchanged at 624 nmol/min per mg of protein. Overall these results suggest that cleavage by PI-PLC of the GPI anchor on membrane dipeptidase may relax conformational constraints on the active site of the enzyme which exist when it is anchored in the lipid bilayer, thus resulting in an increase in the affinity of the active site for substrate.

INTRODUCTION

Over 100 eukaryotic cell-surface proteins, including a number of hydrolases, are now known to be anchored in the plasma membrane by a covalently attached glycosyl-phosphatidylinositol (GPI) moiety [reviewed in (Englund, 1993; McConville and Ferguson, 1993)]. In addition to attachment of the protein to the lipid bilayer a number of other functions have been proposed for GPI anchors. Thus GPI anchors have been shown to be involved in intracellular sorting, transmembrane signalling, act as mediators of hormone action and in the novel endocytic process of potocytosis (for reviews see Hooper, 1992a; Rodriguez-Boulant and Powell, 1992; Anderson, 1993; Brown, 1993; Kilgour, 1993; Romero and Larner, 1993). The presence of a GPI anchor may also allow the selective release of certain cell-surface proteins by the action of endogenous phospholipases. In protozoa a GPI-specific phospholipase C has been purified, cloned and sequenced (Hereld et al., 1986, 1988). Phospholipase C-mediated release has been reported for the SSp-4 antigen and sialidases in *Trypanosoma cruzi*, and for the p76 serine protease in *Plasmodium* sp. (reviewed in McConville and Ferguson, 1993). In the case of p76, cleavage of the protein by phospholipase was required for activation of the protease (Braun-Breton et al., 1988). In mammalian systems there is some evidence for a GPI-specific phospholipase C activity in rat liver plasma membranes (Fox et al., 1987; Stieger et al., 1991), although a recent report failed to find such an activity but instead suggested that the observed action was due to the combined activities of a phospholipase D and a phosphatase (Heller et al., 1992). However, indirect evidence for endogenous phospholipase C release has been demonstrated for

the rat pancreatic zymogen granule protein GP-2 (Paul et al., 1991) and bovine brain 5'-nucleotidase (Vogel et al., 1992).

Membrane dipeptidase (MDP; dehydropeptidase-I; renal dipeptidase; microsomal dipeptidase; EC 3.4.13.19) is a zinc-metalloenzyme found predominantly in the brush-border membrane of the kidney and in the lung. The enzyme is capable of hydrolysing a wide variety of dipeptides, including those with a D-amino acid in the C-terminal position (Campbell, 1970; Armstrong et al., 1974), and may have a role in the metabolism of glutathione and leukotriene D₄ (Kozak and Tate, 1982; Campbell et al., 1990). MDP is the only known mammalian enzyme to exhibit β -lactamase activity (Kropp et al., 1982). The enzyme has been cloned and sequenced from several species including human (Adachi et al., 1990), pig (Rached et al., 1990), rat (Adachi et al., 1992) and mouse (Satoh et al., 1993).

Pig MDP was the first mammalian peptidase found to be anchored via GPI (Hooper et al., 1987) and subsequently human MDP was also shown to be GPI-anchored (Hooper and Turner, 1988a). The GPI anchor on both pig and human MDP has been characterized in terms of its hydrolysis by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) and a serum phospholipase D (Hooper and Turner, 1989; Littlewood et al., 1989; Hooper et al., 1990), and polyclonal antisera have been generated against the cross-reacting determinant of both proteins (Hooper et al., 1991; Broomfield and Hooper, 1993). More recently we have determined the glycan core structures of the GPI anchors on pig and human MDP, representing the first inter-species comparison of the GPI-anchor structure on the same protein (I. A. Brewis, M. A. J. Ferguson, A. J. Turner and N. M. Hooper, unpublished work).

Abbreviations used: GPI, glycosyl-phosphatidylinositol; MDP, membrane dipeptidase; octyl glucoside, n-octyl β -D-glucopyranoside; PI-PLC, phosphatidylinositol-specific phospholipase C.

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In the course of these studies we observed that during its purification from pig or human kidney cortex release of MDP from the membrane by *Bacillus cereus* phospholipase C, which contains a PI-PLC contaminant (Ferguson et al., 1985; Hooper, 1992b), caused an increase in the activity of the enzyme. A similar increase in activity was noted on PI-PLC release of MDP from sheep lung membranes (Campbell et al., 1990). The aim of the current study was to characterize in more detail this activation of MDP on release from the membrane by PI-PLC and to examine whether it was a general phenomenon with GPI-anchored hydrolases. Thus we used pig kidney microvillar membranes which are rich in a number of other GPI-anchored hydrolases, including alkaline phosphatase (EC 3.1.3.1), aminopeptidase P (EC 3.4.11.9), 5'-nucleotidase (EC 3.1.3.5) and trehalase (EC 3.2.1.28) (Turner and Hooper, 1990).

EXPERIMENTAL

Materials

Pig kidneys were kindly provided by ASDA Farmstores, Lofthousegate, W. Yorkshire, U.K. and were obtained within 10 min of death. Microvillar membranes were prepared from pig kidney cortex by the method of Booth and Kenny (1974). MDP was purified from pig kidney cortex by affinity chromatography on cilastatin-Sepharose after solubilization with either bacterial PI-PLC (hydrophilic form of MDP) or *n*-octyl β -D-glucopyranoside (octyl glucoside) (amphipathic form of MDP) (Hooper and Turner, 1989; Littlewood et al., 1989). Adult New Zealand White rabbits (2–3 kg), maintained by the University of Leeds Biomedical Services, were used to raise the polyclonal antibody against affinity-purified, hydrophilic pig kidney MDP (Littlewood et al., 1989). Cilastatin (MK 0791) was a gift from Dr. H. Kropp (Merck, Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A.). PI-PLC from *Bacillus thuringiensis* and from *Staphylococcus aureus* were gifts from Dr. M. G. Low (Department of Physiology and Cellular Biophysics, College of Physicians and Surgeons of Columbia University, New York, NY, U.S.A.). Units of PI-PLC activity are $\mu\text{mol/min}$. Triton X-114 was precondensed before use (Bordier, 1981). All other materials were from sources previously noted.

Enzyme assays

MDP enzymic activity was determined with Gly-D-Phe (1 mM) as substrate in 0.1 M Tris/HCl, pH 8.0. Reactions were terminated by heating at 100 °C for 4 min, and the substrate and reaction products resolved and quantified by reverse-phase h.p.l.c. (Hooper et al., 1987). Alkaline phosphatase was assayed with *p*-nitrophenyl phosphate (2.2 mM) as substrate in 25 mM glycine/NaOH, 0.25 mM MgCl_2 , pH 10.5 (Hooper, 1993). Aminopeptidase P was assayed with Gly-Pro-HyPro (1 mM) as substrate in 0.1 M Tris/HCl, 4 mM MnCl_2 , pH 8.0. Reactions were terminated by heating at 100 °C for 4 min, and the substrate and reaction products resolved and quantified by reverse-phase h.p.l.c. (Hooper and Turner, 1988a). Aminopeptidase N was assayed with Ala-*p*-nitroanilide (0.5 mM) as substrate in 50 mM Tris/HCl, pH 7.4 (Hooper, 1993). Trehalase and 5-nucleotidase were assayed as described in Dahlqvist (1964) and Heinonen and Lahti (1981) respectively. Protein content was determined using bicinchoninic acid with BSA as standard (Hooper, 1993).

Incubation of membranes with PI-PLC and detergent

Pig kidney cortex microvillar membranes (80 μg of protein) or affinity-purified MDP (2 μg of protein) were incubated in 10 mM

Hepes/NaOH, pH 7.4 (0.1 ml final volume) in the absence or presence of PI-PLC or octyl glucoside (60 mM final concentration). Following incubation for 1 h at 37 °C an aliquot (50 μl) was removed and adjusted to 0.5 ml with 10 mM Tris/HCl, 0.15 M NaCl, pH 7.4 for assay of total enzyme activities. In the case of the microvillar membranes the rest of the sample from the incubation was also adjusted to 0.5 ml with 10 mM Tris/HCl, 0.15 M NaCl, pH 7.4, and then centrifuged at 12000 *g* for 10 min. The supernatant was removed and the pellet resuspended in 10 mM Tris/HCl, 0.15 M NaCl, pH 7.4, to the same volume as the supernatant. Enzyme activities were then determined in the two fractions to calculate the percentage release. In the case of purified MDP the conversion of the amphipathic into the hydrophilic form of the protein was determined by temperature-induced phase separation in Triton X-114 as detailed in Hooper (1992b). The resultant aqueous and detergent-rich phases were then assayed for enzyme activity. Where indicated the PI-PLC was heat-inactivated by incubating at 100 °C for 15 min.

E.I.I.s.a.

A competitive e.i.i.s.a. was carried out essentially as described in Hooper et al. (1991). Affinity-purified, hydrophilic form of pig kidney MDP was bound to each well of a 96-well microtitre plate (1 μg per well). Dilutions of the microvillar membranes (untreated, PI-PLC-treated or detergent-treated) in 0.1 % (w/v) BSA in PBS (20 mM Na_2HPO_4 , 2 mM NaH_2PO_4 , 0.25 M NaCl, pH 7.4) were preincubated with anti-MDP antibody (1:8000 dilution in 0.1 % BSA in PBS) for 2 h at 23 °C. (The anti-MDP antibody was fractionated on a column of immobilized variant surface glycoprotein to remove those antibodies recognizing the cross-reacting determinant in the GPI anchor of other PI-PLC-released proteins.) Samples were centrifuged at 12000 *g* for 10 min, then triplicate 50 μl portions were transferred to the precoated microtitre plate wells and incubated for 16 h at 4 °C. The plates were washed, bound antibody detected with biotinylated rabbit immunoglobulin and streptavidin-biotinylated horseradish peroxidase complex and the assay developed with 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid).

RESULTS

During the purification of the hydrophilic forms of both pig and human MDP we consistently observed an increase in the enzyme activity of MDP in the soluble fraction after incubation of the kidney microsomal membranes with *Bacillus cereus* phospholipase C. This increase in activity was $43 \pm 6\%$ (mean $\pm$ S.E.M., $n = 21$) for pig MDP and $34 \pm 5\%$ ($n = 4$) for human MDP.

Pig kidney microvillar membranes were incubated in the presence of *B. thuringiensis* PI-PLC (Figure 1) and the activities of a number of GPI-anchored hydrolases assessed. Incubation of the microvillar membranes with *B. thuringiensis* PI-PLC resulted in the release of the GPI-anchored enzymes in a concentration-dependent manner (Figure 1a). Maximal release of the five GPI-anchored hydrolases occurred at a PI-PLC concentration of 0.1 unit/ml, and was 85 % for alkaline phosphatase, 80 % for aminopeptidase P, 72 % for trehalase, 63 % for MDP and 32 % for 5'-nucleotidase. The transmembrane polypeptide-anchored hydrolase, aminopeptidase N, was not released. In the presence of *B. thuringiensis* PI-PLC the activity of MDP was significantly increased (100–110 % greater than in the absence of PI-PLC) (Figure 1b). Maximal activation occurred at a PI-PLC concentration of 0.01 unit/ml. The activities of the other GPI-anchored hydrolases were not significantly altered in the presence

Figure 1 Effect of *Bacillus thuringiensis* PI-PLC on the release from microvillar membranes and enzyme activity of various cell-surface hydrolases

Pig kidney microvillar membranes were incubated in the presence of increasing concentrations of *B. thuringiensis* PI-PLC for 1 h at 37 °C as described in the Experimental section. (a) Release of enzymes from the membrane; (b) change in total enzyme activity. Each point is the mean of two determinations performed in duplicate. Key to symbols: ○, membrane dipeptidase; □, alkaline phosphatase; △, aminopeptidase P; ●, 5'-nucleotidase; ▲, trehalase; ◇, aminopeptidase N. Except in (b) where 5'-nucleotidase is represented by ■. Results are not presented for trehalase and aminopeptidase N in (b).

Figure 2 Determination of the amount of membrane dipeptidase present before and after treatment of the membranes with PI-PLC or detergent

Pig kidney microvillar membrane samples were incubated in the absence or presence of the indicated reagents at 37 °C for 1 h as described in the Experimental section. The ability of the samples to inhibit the binding of an anti-MDP serum to immobilized affinity-purified MDP was then determined by a competitive e.l.i.s.a. as described in the Experimental section. Each point is the mean of two determinations performed in triplicate. △, Untreated microvillar membranes; ○, microvillar membranes incubated with *B. thuringiensis* PI-PLC (1 unit/ml); □, microvillar membranes solubilized with octylglucoside (60 mM final concentration).

of PI-PLC (maximally 10% increase over the activity in the absence of PI-PLC). Incubation of the membranes with *S. aureus* PI-PLC also resulted in the release of the GPI-anchored proteins in a concentration-dependent manner (results not shown). Maximal release of the GPI-anchored hydrolases occurred at a PI-PLC concentration of 0.1 unit/ml, and was 82% for alkaline phosphatase, 81% for aminopeptidase P and 56% for MDP. Again only the activity of MDP was significantly increased (86% greater than in the absence of PI-PLC) with maximal increase occurring at a PI-PLC concentration of 0.01 unit/ml. Similar results were obtained upon incubation of the microvillar membranes with *B. cereus* PLC (results not shown).

The effect of detergent solubilization of the microvillar membranes on the activity of MDP was examined (Table 1). The activity of MDP did not change significantly after incubation of the membranes with the detergent octyl glucoside, although

Table 1 Effect of detergent solubilization and PI-PLC treatment on the activity of MDP in microvillar membranes

Pig kidney microvillar membranes were incubated in the absence or presence of the indicated reagents for 1 h at 37 °C as described in the Experimental section. The specific activity (mean $\pm$ S.E.M. of four determinations performed in duplicate) was determined on the total incubation sample before subjecting it to temperature-induced phase separation in Triton X-114 as described in the Experimental section. The phase separation results are the mean of two determinations performed in duplicate.

Treatment performed	Specific activity (nmol of o-Phe/min per mg of protein)	Phase separation (% of total activity in each phase)	
		Detergent-rich phase	Aqueous phase
None	130.5 $\pm$ 1.2	92	8
Octyl glucoside (60 mM)	132.4 $\pm$ 3.2	88	12
<i>B. thuringiensis</i> PI-PLC (1 unit/ml)	254.5 $\pm$ 2.1	14	86
Octyl glucoside (60 mM)	249.3 $\pm$ 1.3	12	88
Octyl glucoside (60 mM) followed by PI-PLC (1 unit/ml)			
Heat-inactivated PI-PLC (1 unit/ml)	129.7 $\pm$ 4.4	90	10

Table 2 Effect of PI-PLC on the activities of the affinity-purified hydrophilic and amphipathic forms of MDP

Affinity-purified hydrophilic or amphipathic MDP (2 μ g) were incubated either in the absence or presence of *B. thuringiensis* PI-PLC (10 units/ml) for 1 h at 37 °C. The specific activity (mean $\pm$ S.E.M. of 4 determinations performed in duplicate) was determined on the total incubation sample before subjecting it to temperature-induced phase separation in Triton X-114 as described in the Experimental section. The phase separation results are the means of two determinations performed in duplicate.

Treatment	Specific activity (μ mol of o-Phe/min per mg of protein)	Phase separation (% of activity in each phase)	
		Detergent-rich phase	Aqueous phase
Untreated hydrophilic MDP	81.8 $\pm$ 0.7	8	92
PI-PLC-treated hydrophilic MDP	82.7 $\pm$ 0.4	9	91
Untreated amphipathic MDP	60.3 $\pm$ 0.3	81	19
PI-PLC-treated amphipathic MDP	59.5 $\pm$ 0.4	12	88

Table 3 Kinetic parameters of the hydrolysis of Gly-o-Phe by MDP

Pig kidney microvillar membranes were incubated in the absence or presence of *B. thuringiensis* PI-PLC for 1 h at 37 °C as described in the Experimental section. Enzyme activity was determined on the total incubation sample. K_m and V_{max} values were determined from Lineweaver–Burke plots.

Enzyme source	K_m (mM)	V_{max} (nmol/min per mg of protein)
Microvillar membranes	0.77 $\pm$ 0.17	602 $\pm$ 7
Microvillar membranes + <i>B. thuringiensis</i> PI-PLC (1.0 unit/ml)	0.07 $\pm$ 0.03	624 $\pm$ 37

incubation of the membranes with *B. thuringiensis* PI-PLC resulted in an increase in the enzyme activity of MDP of 95 % compared with the untreated membranes. Solubilization of the membranes with octyl glucoside followed by incubation with PI-PLC resulted in an increase in MDP activity of 91 % compared with the untreated membranes. The activity of MDP in the membrane sample incubated with the detergent octyl glucoside partitioned predominantly into the detergent-rich phase on temperature-induced phase separation in Triton X-114 (Table 1), indicating that the hydrophobic fatty acids had not been cleaved from the GPI anchor. In contrast the activity of MDP in the membrane sample incubated with PI-PLC partitioned predominantly into the aqueous phase which is consistent with the removal of the hydrophobic fatty acids from the anchor. The activity of MDP did not alter when the microvillar membranes were incubated in the presence of heat-inactivated PI-PLC, and it was not released from the membrane as shown by phase-separation in Triton X-114 (Table 1).

A competitive e.i.s.a. was performed to assess the effect of either detergent solubilization or treatment with *B. thuringiensis* PI-PLC on the amount of MDP present in the microvillar membranes (Figure 2). Various concentrations of untreated membranes, octyl glucoside-solubilized membranes and PI-PLC-treated membranes all inhibited the binding of the anti-MDP serum to immobilized, affinity-purified MDP in an identical dose-dependent manner. All three membrane samples completely inhibited the binding of the antiserum at an amount of 1×10^{-5} g of total membrane protein and displayed an I_{50} (the amount of sample giving 50 % inhibition of antibody binding) of approx. 1×10^{-6} g of total membrane protein.

The effect of *B. thuringiensis* PI-PLC on the activity of the affinity-purified, hydrophilic and amphipathic forms of MDP was examined (Table 2). Treatment of amphipathic MDP with PI-PLC successfully cleaved off the fatty acids from the anchor, generating the hydrophilic form as determined by temperature-

induced phase separation in Triton X-114. However, this PI-PLC treatment did not significantly alter the activity of MDP. The activity of the hydrophilic form of MDP was unaffected by incubation with PI-PLC.

The effect of incubation of the pig kidney microvillar membranes with *B. thuringiensis* PI-PLC on the kinetic parameters of MDP for the substrate Gly-D-Phe was examined (Table 3). In the presence of a concentration of PI-PLC which caused maximal release and activation of MDP (Figure 1) there was a 10-fold decrease in the observed K_m compared with the untreated membranes. In contrast there was no significant difference in the V_{max} . Assuming Michaelis–Menten kinetics, these values for K_m predict an almost 2-fold change in specific activity as observed in Figure 1.

DISCUSSION

The present study arose from our observation that there was a significant increase in enzymic activity during the purification of MDP from both pig and human kidney following its release from the membrane by *B. cereus* phospholipase C in which there is a PI-PLC contaminant (Ferguson et al., 1985; Hooper, 1992b). Thus we attempted to characterize more precisely why cleavage of MDP by PI-PLC should result in an increase in enzymic activity and whether such an effect was common to the other GPI-anchored hydrolases present in the kidney microvillar membrane. Incubation of pig kidney microvillar membranes with *B. thuringiensis* or *S. aureus* PI-PLC resulted in the release of a number of GPI-anchored hydrolases, including alkaline phosphatase, aminopeptidase P, trehalase, 5-nucleotidase and MDP in agreement with our previous observations (Hooper and Turner, 1988a,b). Only in the case of MDP was a significant increase in its enzymic activity observed upon incubation of the microvillar membranes with either *B. thuringiensis* or *S. aureus* PI-PLC. As expected, the transmembrane-polypeptide-anchored

hydrolase, aminopeptidase N (EC 3.4.11.2) (Olsen et al., 1988), was not released from the membrane by PI-PLC treatment and was also not activated. Thus this increase in enzymic activity does not appear to be a GPI-anchored-protein-specific effect or even a general membrane protein effect but rather it appeared to be unique to MDP.

The effect of detergent solubilization compared with PI-PLC treatment on the enzyme activity of MDP was examined (Table 1). Incubation of the microvillar membranes with octyl glucoside at a concentration which is known to be effective at solubilizing MDP (Hooper and Turner, 1988b) had no effect on the enzyme activity, although incubation with PI-PLC caused both an increase in activity and release of the protein from the membrane. This activation of MDP was not a temperature-dependent effect as incubation of the membranes at 37 °C for 1 h had no effect on the enzyme activity of MDP (Figure 1, Table 1). Moreover the activating effect of the PI-PLC was dependent on its catalytic activity as incubation of the microvillar membranes with heat-inactivated PI-PLC had no effect on the enzymic activity of MDP.

The effect of PI-PLC treatment on affinity-purified samples of MDP was also investigated (Table 2). Incubation of the amphipathic form of MDP with PI-PLC resulted in the cleavage of the GPI anchor, as assessed by phase separation in Triton X-114. However, no increase in the enzymic activity of the MDP was observed, in contrast to the results obtained with the membrane preparations. It should be noted though that the purification of the amphipathic form of MDP is a relatively inefficient process (Hooper and Turner, 1989) which may be selecting a non-representative population of MDP which has already been activated.

One of the functions proposed for a GPI anchor is to allow selective release of the protein from the cell surface by phospholipases. Coupled with this release is the possibility of activation/deactivation of the GPI-anchored protein. Incubation of *Plasmodium falciparum* membranes with PI-PLC leads to the release of a number of GPI-anchored proteins including one of relative molecular mass 76000 (p76) (Braun-Breton et al., 1988). Only the PI-PLC-released soluble form of p76, and not the membrane-anchored form, displayed proteolytic activity, demonstrating that cleavage of a GPI anchor can lead to enzymic activation. In contrast it should be noted that MDP is enzymically active when anchored in the membrane (Table 1).

The activity of acetylcholinesterase in cultures of *Schistosoma mansoni* has been observed to increase significantly following incubation of the cells with PI-PLC (Espinoza et al., 1988). A subsequent study showed that this increase in activity was due to an increase in the rate of biosynthesis rather than due to a direct activation of the enzyme (Espinoza et al., 1991). An increase in the rate of biosynthesis of MDP cannot explain the observed increase in its activity upon incubation of the microvillar membranes with PI-PLC. However, it may be that PI-PLC treatment released a population of MDP sequestered in membrane vesicles. A competitive e.l.i.s.a was therefore performed to determine whether incubation of the microvillar membranes with PI-PLC caused an increase in the amount of MDP protein accessible to substrate (Figure 2). Treatment of the microvillar membranes with either PI-PLC or octyl glucoside had no effect on the amount of MDP available to bind to the anti-MDP antibody as compared with untreated membranes. Thus PI-PLC treatment of the microvillar membranes does not appear to cause an increase in the actual amount of MDP protein.

One possible explanation for the increase in the enzymic activity of MDP upon incubation of the membranes with PI-PLC is that the accessibility of the substrate to the active site was

somehow altered. This was initially suggested by Campbell et al. (1990) who proposed that the alteration of the relationship of MDP with the lipid bilayer might remove steric hindrances and facilitate an increased activity. The concept of removing steric hindrances has also been proposed as the reason for the activation of the hyaluronidase sperm PH-20 protein upon its release from the membrane by PI-PLC (Gmachl et al., 1993). The large substrate, a polysaccharide with a relative molecular mass of several million, might only have limited access to the active site when the membrane-bound PH-20 is densely clustered at the cell surface. Thus release of the enzyme by PI-PLC increases the accessibility of the active site and hence the apparent enzyme activity increases. However, the substrate for MDP used in the present study was the dipeptide Gly-D-Phe which is relatively small (M_r 222.2). The observation (Table 1) that solubilization of the membrane with octyl glucoside fails to cause activation of MDP would suggest that other proteins or lipids in the membrane are probably not responsible for any steric hindrance. Therefore the hypothesis that removal of external steric hindrances is responsible for the increased activity is somewhat less convincing in this case.

MDP exists in its native state as a disulphide-linked dimer (Hooper and Turner, 1989; Turner and Hooper, 1990). The disulphide bonds between the two subunits might cause conformational strain in the protein molecule when it is anchored in the membrane which lowers the enzymic activity of the catalytic site. Upon cleavage of even just one of the GPI anchors in the dimer this conformational strain is relaxed, leading to activation of the enzyme. Interestingly this hypothesis is supported in part by the observation that maximal activation of MDP occurs at a 10-fold lower concentration of PI-PLC than is required for maximal release (Figure 1). The observed decrease in the K_m of MDP for Gly-D-Phe when acted on by PI-PLC (Table 3) would also be consistent with the relaxing of some conformational strain on the active site of the enzyme increasing the affinity for the substrate, as opposed to an alteration in the catalytic efficiency of the enzyme. This phenomenon cannot be a general property of disulphide-linked GPI-anchored proteins as within the same membrane 5'-nucleotidase also exists as a disulphide-linked dimer (Zimmerman, 1992) and yet is clearly not activated upon release from the membrane by PI-PLC (Figure 1). Although incubation of Torpedo electric organ membranes with *B. thuringiensis* PI-PLC (1 unit/ml) caused a significant (approx. 100%) increase in the activity of the disulphide-linked, GPI-anchored acetylcholinesterase upon its release from the membrane (N. M. Hooper and A. J. Turner, unpublished work).

The observation that solubilization of the microvillar membranes with octyl glucoside had no effect on the activity of MDP (Table 1) could be explained if both GPI anchors of the disulphide-linked dimer were inserted into the same detergent micelle thus maintaining the conformational constraints on the dimer which exist in the membrane. Subsequent treatment of this detergent-solubilized form of MDP with PI-PLC leads to an increase in the enzyme activity as a result of removing the conformational constraints on the catalytic site as described above. A recent report has also suggested that a conformational rearrangement, which is induced by PI-PLC cleavage of the GPI anchor, is responsible for the increased affinity of ligand binding of the *Saccharomyces cerevisiae* cyclic AMP receptor protein (Muller and Bandlow, 1994). As with MDP, detergent solubilization of the receptor protein had no effect on ligand binding. In conclusion, it appears that cleavage by PI-PLC of the GPI-anchor on MDP may be relaxing conformational constraints on the active site of the enzyme which exist when it is anchored in the membrane.

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