Monomers of the catalytic domain of human neuropathy target esterase are active in the presence of phospholipid

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NEST is a hydrophobic recombinant polypeptide comprising the catalytic domain (residues 727–1216) of neuropathy target esterase. NEST in bacterial lysates has potent esterase activity, which is lost after its solubilization and purification in detergent-containing solutions. Activity in purified NEST preparations was restored by the addition of phospholipids before the removal of detergent by dialysis. The pattern of digestion by proteinase K of NEST–phospholipid complexes suggested that NEST might incorporate in a topologically random fashion into nascent liposomes and that the bulk of each NEST molecule might be exposed either to the liposome lumen or the external medium. Significant quantities of NEST were liberated from NEST–phospholipid complexes by treatment with dilute acid or alkali, suggesting that charge interactions might contribute to the

association; however, NEST was irreversibly denatured at these pH values. Treatment of NEST–phospholipid complexes with glutaraldehyde afforded some protection against the inactivation of esterase activity by detergent but the pattern of cross-linked forms of NEST generated did not indicate pre-existing oligomers. Similarly, the inactivation of esterase activity in NEST–phospholipid complexes by radiation indicated that NEST monomers are catalytically active. The foregoing observations are not compatible with structural algorithms predicting that the catalytic serine residue lies at the centre of one of three transmembrane helices in NEST.

Key words: interfacial activation, membrane, radiation inactivation, secondary structure prediction.

INTRODUCTION

Neuropathy target esterase (NTE) is an integral membrane protein in vertebrate neurons [1] and was originally identified as the primary site of action of those organophosphates that, in humans and other adult animals, cause axonal degeneration [2,3]. Elucidation of the role of NTE in this syndrome might shed light on processes involved in normal neuronal function and in some human neurodegenerative diseases. *In vitro*, NTE catalyses the hydrolysis of phenyl valerate but its physiological substrate is unknown [1]. Examination of the primary sequence of human NTE suggests that the protein comprises at least two domains: a C-terminal serine esterase domain and an N-terminal putative regulatory domain with some similarity to cyclic AMP-binding proteins [4]. We expressed in *Escherichia coli* a recombinant polypeptide corresponding to residues 727–1216 of human NTE. This polypeptide, called NEST (NTE esterase domain), reacted with phenyl valerate and organophosphate inhibitors in a manner very similar to that of native NTE; Ser⁹⁶⁶ was identified as the catalytic serine residue [5].

Hydropathy analysis of the sequence of 1327 amino acid residues that comprise human NTE predicts a single transmembrane helix (TM) between residues 10 and 32. Surprisingly, although hydropathy analysis does not predict any TMs within NEST's sequence, the protein associates with particulate fractions of *E. coli*, and detergent is required during its solubilization and purification from bacterial lysates. After solubilization in Triton X-114 and phase-partitioning [6], purified NEST was concentrated in the detergent phase, a characteristic of an integral membrane protein [5]. Furthermore, purified NEST

showed very little esterase activity unless dioleoylphosphatidylcholine (DOPC) was added and detergent was removed by dialysis. The aim of the present study was to investigate further the phospholipid/membrane association of NEST.

Figure 1 Phospholipids enhance NEST's esterase activity

Purified NEST (0.1 mg/ml in 0.3% CHAPS) was mixed with solutions of phospholipids (◆, phosphatidylcholine; ■, phosphatidylinositol; ▲, phosphatidylserine) in CHAPS to produce the final concentrations shown. The mixtures were dialysed for 18 h at room temperature (20–25 °C) against detergent-free PEN buffer, pH 7.8. Aliquots of dialysed samples were diluted 1:8000 in TE buffer, pH 8.0, for assay of esterase activity. Separate aliquots of each sample were run, in duplicate, on SDS/PAGE and stained with Coomassie Blue; densitometry was then used to determine protein concentrations relative to standards (of BSA) run on the same gel.

Abbreviations used: DOPC, dioleoylphosphatidylcholine; G6PDH, glucose-6-phosphate dehydrogenase; NTE, neuropathy target esterase; NEST, NTE esterase domain; TM, transmembrane helix.

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Figure 2 Phospholipid protects NEST against digestion with proteinase K

(a, b) Purified NEST (0.1 mg/ml in 0.3% CHAPS/PEN buffer) (a) or BSA (0.1 mg/ml in 0.3% CHAPS/PEN buffer) (b) was mixed with DOPC (0.5 mg/ml final concentration) and dialysed against detergent-free PEN buffer. Separate aliquots of NEST and albumin were dialysed in the absence of DOPC. Aliquots of the dialysed mixtures were then incubated at 4 °C with proteinase K (4 µg/ml). After the indicated times (min), proteinase activity was terminated by the addition of PMSF (final concentration 4 mM) and incubation for a further 20 min at 4 °C. SDS/PAGE sample buffer was then added [final SDS concentration 2% (w/v)] and the mixture was boiled for 5 min. Samples were run on SDS/PAGE and stained with Coomassie Blue. (c) Relative NEST concentrations were determined by densitometry.

EXPERIMENTAL

Materials

CHAPS, phospholipids and glutaraldehyde were from Sigma. The sources of all other materials were as described previously [5].

Generation, isolation and reconstitution of NEST

These methods have been detailed previously [5]. In brief, a section of NTE cDNA corresponding to amino acid residues 727–1216 was amplified by PCR and cloned into a pET21 vector. The encoded polypeptide with N-terminal T7 and C-terminal His₆ tags was expressed in *E. coli* BL21 pLysS and is termed NEST. Bacterial lysates were treated with PEN buffer [50 mM sodium phosphate (pH 7.8)/0.5 mM EDTA/300 mM NaCl] containing 2% (w/v) CHAPS and NEST was isolated from the solubilized extract by sequential nickel-chelation and gel-filtration chromatography in PEN buffer containing 0.3% CHAPS.

Figure 3 NEST is liberated from complexes with DOPC by aqueous medium only under denaturing conditions

(a) NEST–DOPC complexes (approx. 0.05 mg/ml protein) were centrifuged (100 000 *g*, 45 min, 4 °C). The resulting pellets were resuspended in their original volumes of buffers at the pH values shown in the Figure, then re-centrifuged. Aliquots of the resulting supernatant were neutralized by the addition of 1 M Tris/HCl, pH 7.5, then run on SDS/PAGE and stained with Coomassie Blue. Dilutions of a NEST–DOPC pellet quantitatively solubilized in 0.2% SDS are shown at the right of the Figure. (b) Pelleted NEST–DOPC complexes were resuspended in buffers at the pH values shown. After 1 h at 4 °C the samples were neutralized by the addition of 1 M Tris/HCl, pH 7.5, then diluted (1:100) and assayed for esterase activity at pH 8.0 (◆). Alternatively, NEST–DOPC complexes were diluted (1:100) into buffers at the indicated pH values and then assayed for esterase activity at that pH (■). Activities are expressed relative to the maximum value for each experimental set. The following buffers (all at 50 mM and all containing 1 mM EDTA) were used: glycine/HCl (pH 3.0), sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0 and 7.0); Tris/HCl (pH 8.0), glycine/NaOH (pH 9.0 and 10.0) and triethanolamine/HCl (pH 11.0). We did not control for any possible effects of changing ionic strength with these buffers.

Purified NEST (0.1 mg/ml in 0.3% CHAPS/PEN buffer) was mixed with a solution (10 mg/ml in 10% CHAPS) of DOPC to give a protein-to-DOPC ratio of 1:3–4 (w/w); CHAPS was then removed by dialysing the mixture for 18 h at room temperature (20–25 °C) against PEN buffer containing 1 mM dithiothreitol.

Phenyl-valerate hydrolase assays

These assays were done as described previously [5]. NEST-containing preparations were diluted in TE buffer [50 mM Tris/HCl (pH 8.0)/1 mM EDTA] and incubated with phenyl valerate for 20 min at 37 °C; the phenol liberated was determined as described [5]. The data shown (in Figures 1, 3b and 4) are means of duplicate determinations (the range was less than 10% of the mean value) and are representative of at least two separate experiments.

SDS/PAGE and densitometry

Samples were run on SDS/PAGE, stained with Coomassie Blue and NEST polypeptide bands were quantified by laser densitometry (Molecular Dynamics Personal Densitometer SI). Data shown (in Figures 2, 3a and 5) are representative of at least two separate experiments.

Radiation inactivation experiments

NEST–DOPC preparations were diluted in TE buffer containing 10 % (v/v) glycerol and 10 mM dithiothreitol to a concentration of approx. 1 $\mu\text{g}/\text{ml}$ NEST. Glucose-6-phosphate dehydrogenase (G6PDH, from *Leuconostoc mesenteroides*; Sigma) was included in the mixture at 25 units/ml as an internal standard. Aliquots (0.4 ml) of the mixture were frozen in glass ampoules which were heat-sealed and subsequently subjected to various doses of radiation at -135°C as described previously [7]. On opening, the air in the ampoules was purged with nitrogen, after which the samples were thawed and assayed for phenyl-valerate hydrolase activity as above and G6PDH activity with a Sigma kit. G6PDH activity was inactivated with a target size of 132 ± 24 kDa (mean $\pm$ S.D. for four experiments), which is consistent with reported values [8].

RESULTS

After purification from bacterial lysates in the presence of the detergent CHAPS (see the Experimental section), NEST was mixed with phospholipid dissolved in CHAPS and dialysed against detergent-free buffer; the resulting preparations were assayed for esterase activity. Dialysis in the presence of phosphatidylcholine, phosphatidylserine or phosphatidylinositol resulted in preparations with esterase activities at least an order of magnitude greater than those dialysed without phospholipid (Figure 1). Reconstitution of the esterase activity of NEST was not achieved by mixing (even for up to 24 h) separately dialysed preparations of NEST and phospholipid: this suggests that NEST adopted a catalytically active conformation simultaneously with the formation of phospholipid liposomes on the removal of detergent.

We investigated the sensitivity to proteinase K of preparations of NEST dialysed with or without DOPC. Dialysis (at pH 7.8) of purified NEST in the absence of phospholipid generated a protein suspension that could be pelleted by centrifugation (100 000 g , 45 min). In the absence of DOPC, approx. 75 % of the NEST suspension was degraded within 20 min at 4°C . The presence of DOPC afforded substantial protection against proteolysis so that about half of the NEST polypeptides in the mixture were intact after 1 h and, even after 4 h, more than 35 % of NEST remained (Figures 2a and 2c). In either the absence or the presence of DOPC, NEST was degraded by proteinase K without the formation of any large (more than 20 kDa) fragments (Figure 2a). The protection afforded by DOPC against proteolysis was not simply due to the occlusion of NEST within the lumen of newly formed liposomes, because a soluble protein, BSA, was degraded identically after dialysis in either the absence or the presence of DOPC (Figure 2b).

We next examined whether NEST associates with liposomes partly by charge interactions with the phospholipid head group: such interactions are characteristic of peripheral membrane proteins and can be disrupted by dilute acids and alkalis [9]. NEST–DOPC preparations in aqueous buffer at pH 7.8 were sedimented almost quantitatively by centrifugation at 100 000 g for 45 min. When the pellet from this centrifugation was resuspended in aqueous buffer at various pH values and re-centrifuged, significant amounts of NEST protein were found in the supernatant at pH 4.0 and 11.0 (approx. 25 % of the total) and at pH 3.0 (approx. 50 % of the total) (Figure 3a). If NEST–DOPC complexes were exposed to these various pH values for a comparable period (1 h at 4°C), then neutralized and diluted (1:100) into an esterase assay (at pH 8.0), substantial enzymic activity was detected in all samples except those exposed to pH

Figure 4 Inhibition of NEST esterase activity by detergent and the effect of glutaraldehyde

(a) Two series of incubations of NEST–DOPC with detergent were set up: for the direct series, NEST–DOPC (approx. 10 ng/ml NEST) and detergents at the indicated concentrations were incubated in a phenyl-valerate esterase assay; for the preincubated series (preinc.), NEST–DOPC (20 $\mu\text{g}/\text{ml}$ NEST) was mixed with detergents at the indicated concentrations for 1 h at room temperature, then aliquots of the mixture were diluted 1:2000 into an esterase assay. (b) NEST–DOPC (0.1 mg/ml NEST) was incubated for 2 h at 4°C with glutaraldehyde at the concentrations indicated in the Figure. Aliquots were then diluted 1:10 000 into a phenyl-valerate esterase assay in the absence or presence of 0.5 % CHAPS.

values of less than 5.0 and more than 9.0 (Figure 3b). Interestingly, when NEST–DOPC complexes were assayed directly for esterase activity at various pH values, activity dropped sharply between pH 7 and 5 (Figure 3b) but this loss of activity was reversible. By contrast, the pH values (less than 5.0 and more than 10.0) at which significant amounts of NEST were liberated from NEST–DOPC complexes were those that caused irreversible denaturation.

Addition of detergents to NEST–DOPC preparations caused a concentration-dependent inhibition of esterase activity (Figure 4a). CHAPS at 0.1 % caused very little inhibition, whereas 0.5 % decreased activity to less than 20 % of control. Triton X-100 was more potent as an inhibitor in the esterase assay, causing approx. 80 % inhibition at only 0.05 %. The concentrations of CHAPS and Triton X-100 inhibitory in the NEST esterase assay coincided with their critical micelle concentrations, which are in the range 0.25–0.5 % and 0.01–0.02 % respectively. When NEST–DOPC preparations were preincubated with detergents and then diluted 1:2000 into the esterase assay, similar inhibition was observed, suggesting that the effect was not rapidly reversible (during 20 min at 37°C ; Figure 4a). Similarly, it was found that esterase activity was not restored by diluting NEST preparations in 0.3 % CHAPS or in 0.03 % Triton X-100 into a large excess of buffer containing a detergent-free suspension of DOPC (results not shown). Pretreatment of NEST–DOPC preparations with the cross-linking agent glutaraldehyde before incubation with

Figure 5 Treatment of NEST–DOPC complexes with glutaraldehyde generates cross-linked oligomers

NEST–DOPC preparations (0.1 mg/ml NEST) were incubated for 2 h at 4 °C in PEN buffer, pH 7.8, with the concentrations of glutaraldehyde indicated (in mM) at the top of (a), then excess glutaraldehyde was quenched by addition of Tris/HCl, pH 8.0 to a final concentration of 30 mM. Samples were run on SDS/PAGE, stained with Coomassie Blue (a) and relative quantities of monomers (approx. 55 kDa) and the sum of discrete oligomers (i.e. the pairs of bands centred around 100 and 200 kDa) were quantified by densitometry (b). The positions of molecular mass markers are indicated (in kDa) at the left of (a).

CHAPS partly protected the esterase activity from detergent-mediated inactivation. When assayed in 0.5 % CHAPS, esterase activity in the presence of 10 mM glutaraldehyde was approx. 2.5-fold greater than in its absence (Figure 4b).

Analysis of glutaraldehyde-treated NEST–DOPC preparations by SDS/PAGE revealed the formation of cross-linked oligomers with discrete pairs of polypeptide bands centred on approx. 100 and 200 kDa, which might have been dimers and tetramers respectively (Figure 5a). Densitometry of these gels showed that treatment with 20 mM glutaraldehyde resulted in the cross-linking of more than 80 % of the NEST molecules in the preparation, much of these to large aggregates that failed to enter the gel. Over the range 1–20 mM glutaraldehyde, the combined amount of putative cross-linked dimers and tetramers was 20–40 % of the original NEST (Figure 5b). Taken together with the data in Figure 4(b), these findings indicate that covalently cross-linked NEST oligomers not only retained esterase activity but were also more resistant than NEST monomers to detergent-mediated inactivation.

The foregoing results suggest that phospholipid liposomes provided an environment in which, to a limited extent, NEST oligomer formation was possible and that oligomers were catalytically active. However, to determine unequivocally the size of the smallest species in NEST–DOPC preparations with esterase activity we used radiation inactivation analysis. Doses of radiation from 10 to 1000 kGy caused a decrease in esterase activity consistent with a single species with a target size of 53 ± 4 kDa (from four experiments; results not shown). This result clearly indicates that phospholipid-requiring monomers of NEST had esterase activity.

DISCUSSION

Some serine hydrolases that catalyse the hydrolysis of ester bonds exhibit interfacial activation: that is, they show maximal catalytic activity only when the enzyme is adsorbed at an oil/water interface, such as at the cytoplasmic face of cell

membranes for group IVA phospholipase A_2 [10] or the surface of lipid micelles for triacylglyceride lipase [11]. Similarly, NTE and NEST both seem to require interfacial activation. Thus, when detergent extracts of brain membranes are fractionated by ion-exchange chromatography, much of the associated NTE-like esterase activity is lost but this can be partly restored by the addition of phospholipid [12]. Our present observation of the promotion of esterase activity in purified NEST by phospholipids indicates that this effect is independent of the N-terminal transmembrane domain of NTE. However, whereas group IVA phospholipase A_2 and triacylglyceride lipase are soluble proteins, NEST seems to be much more hydrophobic and the catalytically active form is not soluble in aqueous solution.

NEST seems to adopt a catalytically active conformation during the period in which protein–phospholipid complexes form as detergent is removed by dialysis. These complexes can be sedimented almost quantitatively at 100000 *g* for 45 min and show greater resistance to proteinase K digestion than NEST alone: this resistance is not absolute because about half the NEST molecules in NEST–DOPC complexes are degraded relatively rapidly. This might indicate that NEST is incorporated randomly into nascent DOPC liposomes so that the bulk of each NEST molecule is exposed either to the liposome lumen or to the external medium.

Different conformations are adopted by NEST in the presence of detergent micelles and in the presence of phospholipid. The CHAPS-associated conformation lacks catalytic activity and, as we have shown previously, exhibits a pattern of degradation by proteinase K that proceeds via formation of several large fragments, whereas the susceptible NEST molecules in complexes with DOPC are rapidly degraded to relatively small (less than 20 kDa) polypeptides [5].

The ability of dilute aqueous acid or alkali to liberate NEST from complexes with DOPC suggests that its association with phospholipid might be mediated in part by charge interactions. However, we have shown here that the protein is irreversibly denatured at the relatively extreme pH required to dissociate

Figure 6 Predicted TMs in NTE and NEST

NTE is represented with an N-terminal regulatory domain (grey) and a C-terminal catalytic domain (white). NEST (human NTE residues 727–1216) comprises the catalytic domain and also has N-terminal T7 and C-terminal His₆ tags. A hydropathy analysis of the NTE protein sequence predicts a single TM between residues 10–32. By contrast, analysis of NTE by the TM-pred (www.ch.embnet.org/cgi-bin/tmpred) and TopPred2 (www.biokemi.su.se/~server/toppred2) programs predicts four TM segments (shown as thick vertical lines) at the following residues: 11/12–32 (TM1; score 2375), 733/734–753 (TM2; score 958), 922/925–942/943 (TM3; score 1087), 956–976 (TM4; score 1276). The catalytic serine residue (Ser⁹⁶⁶) is at the centre of putative TM4. Abbreviation: aa, amino acid residues.

NEST–DOPC complexes. In addition, the fact that we previously demonstrated [5] that NEST, solubilized in Triton X-114, partitions into a detergent-rich phase emphasizes that simple biochemical tests extracting proteins into detergents or media of different pH can provide only operational definitions of integral or peripheral membrane proteins.

The outer-membrane phospholipase A of *E. coli* is an example of a serine esterase that is an integral membrane protein. This enzyme is continually in contact with its substrate and is activated by dimerization, triggered by deformation of the membrane in which it resides [13]. Treatment of the phospholipase with glutaraldehyde achieves an almost quantitative conversion into cross-linked dimers; these are stabilized against inactivation by detergent [14]. By contrast, although treatment of NEST with glutaraldehyde forms cross-linked oligomers with enhanced resistance to detergent inactivation, neither dimers nor any other single discrete oligomeric form predominate. Consistent with this is the demonstration by radiation inactivation that phospholipid-requiring NEST monomers have esterase activity.

Finally, the question remains as to the structural basis for NEST's affinity for phospholipid. Although standard hydropathy analysis detects no TMs within the primary sequence of NEST, other algorithms predict three TMs (Figure 6). One postulate of the three-TM model for NEST is that the catalytic serine residue (Ser⁹⁶⁶) lies at the centre of one of the putative TMs. It is difficult to envisage how sufficient water could be present at this location to allow the efficient hydrolysis of substrates unless

Ser⁹⁶⁶ faced into the aqueous lumen of a transmembrane pore. It seems probable that oligomers of a polypeptide with only three TMs would be required to form a functional pore. Thus our present demonstration that monomers of NEST have esterase activity makes a three-TM model very unlikely. A definitive description of the nature of NEST's association with phospholipid membranes will require the determination of its tertiary structure. Because the conformation of NEST in detergent is clearly different from its membrane-associated form, this might require crystallization within a lipid bilayer [15].

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REFERENCES

- Glynn, P. (1999) Neuropathy target esterase. *Biochem. J.* **344**, 625–631
- Johnson, M. K. (1969) The delayed neurotoxic effect of some organophosphorus compounds: identification of the phosphorylation site as an esterase. *Biochem. J.* **114**, 711–717
- Johnson, M. K. (1974) The primary biochemical lesion leading to the delayed neurotoxic effects of some organophosphorus esters. *J. Neurochem.* **23**, 785–789
- Lush, M. J., Li, Y., Read, D. J., Willis, A. C. and Glynn, P. (1998) Neuropathy target esterase and a homologous *Drosophila* neurodegeneration-associated mutant protein contain a domain conserved from bacteria to man. *Biochem. J.* **332**, 1–4
- Atkins, J. and Glynn, P. (2000) Membrane association of and critical residues in the catalytic domain of human neuropathy target esterase. *J. Biol. Chem.* **275**, 24477–24483
- Bordier, C. (1981) Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* **256**, 1604–1607
- Schoonderwoerd, K., Hom, M. L., Luthjens, L. H., Vieira van Bruggen, D. and Jansen, H. (1996) Functional molecular mass of rat hepatic lipase in liver, adrenal gland and ovary is different. *Biochem. J.* **318**, 463–467
- Hill, J. S., Davis, R. C., Yang, D., Wen, J., Philo, J. S., Poon, P. H., Phillips, M. L., Kempner, E. S. and Wong, H. (1996) Human hepatic lipase subunit structure determination. *J. Biol. Chem.* **271**, 22931–22936
- Findlay, J. B. C. (1987) The isolation and labelling of membrane proteins and peptides. In *Biological Membranes: A Practical Approach* (Findlay, J. B. C. and Evans, W. H., eds.), pp. 179–217. IRL Press, Oxford
- Dessen, A. (2000) Structure and mechanism of human cytosolic phospholipase A₂. *Biochim. Biophys. Acta* **1488**, 40–47
- Derewenda, Z. S. and Sharp, A. M. (1993) News from the interface: the molecular structures of triacylglyceride lipases. *Trends Biochem. Sci.* **18**, 20–25
- Pope, C. N. and Padilla, S. S. (1989) Modulation of neurotoxic esterase activity in vitro by phospholipids. *Toxicol. Appl. Pharmacol.* **97**, 272–278
- Snijder, H. J. and Dijkstra, B. W. (2000) Bacterial phospholipase A: structure and function of an integral membrane phospholipase. *Biochim. Biophys. Acta* **1488**, 91–101
- Dekker, N., Tommassen, J., Lustig, A., Rosenbuch, J. P. and Verheij, H. M. (1997) Dimerisation regulates the enzymatic activity of *Escherichia coli* outer membrane phospholipase A. *J. Biol. Chem.* **272**, 3179–3184
- Landau, E. M. and Rosenbuch, J. P. (1996) Lipidic cubic phases: a novel concept for the crystallization of membrane proteins. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 14532–14535