Membrane Association of and Critical Residues in the Catalytic Domain of Human Neuropathy Target Esterase*

Received for publication, April 6, 2000, and in revised form, May 12, 2000
Published, JBC Papers in Press, May 17, 2000, DOI 10.1074/jbc.M002921200

Jane Atkins‡ and Paul Glynn§
From the Medical Research Council Toxicology Unit, University of Leicester, Leicester LE1 9HN, United Kingdom

Neuropathy target esterase (NTE) is an integral membrane protein in vertebrate neurons and a member of a novel family of putative serine hydrolases. Here we show that NEST, a recombinant polypeptide expressed in Escherichia coli, reacts with an ester substrate and covalent inhibitors in a manner very similar to NTE. NEST comprises residues 727–1216 of human NTE, and site-directed mutagenesis revealed that serine 966 and two aspartate residues, Asp1086 and Asp960, are critical for catalysis. The results of mutating the 11 histidines in NEST suggest that NTE does not use a conventional catalytic triad. By reacting NEST with [3H]diisopropyl fluorophosphate, Ser966 was confirmed as the active-site serine, and evidence was obtained that an isopropyl group is transferred from the Ser966 adduct to an aspartate residue. Detergent was required both for solubilization of NEST from lysates of E. coli and during purification procedures. Catalytic activity was lost in detergent extracts, but was restored when purified NEST was incorporated into dioleoylphosphatidylcholine liposomes. Hydropathy analysis did not indicate the presence of membrane-spanning segments within the NEST sequence. However, biochemical evidence including detergent-phase separation experiments and the resistance of liposome-incorporated NEST to proteolysis indicated that, unlike most eukaryotic serine hydrolases, the catalytic domain of NTE has integral membrane protein properties.

Serine hydrolases comprise a very large class of enzymes that can hydrolyze ester, peptide, and amide bonds. The active-site nucleophilic serine residue of these enzymes attacks the carbonyl carbon atom of the substrate, forming a covalent acyl-enzyme intermediate, which is subsequently hydrolyzed (1). A consequence of this reaction mechanism is that serine hydrolases are susceptible to covalent inhibition by organophosphorus esters (OPs) with which they form an analogous phosphyl-enzyme intermediate. Diisopropyl fluorophosphate (DFP) has for many years been widely used in biochemical studies of serine hydrolases, and newer OPs have been proposed as agents for activity-based profiling of this class of enzymes (2). In man and some other vertebrates, DFP and certain other OPs cause degeneration of long axons in the spinal cord and peripheral nerves. The primary target for these OPs is neuropathy target esterase (NTE), an integral membrane protein in vertebrate neurons. The physiological substrate of NTE is unknown, but it is detected in vitro by catalyzing the hydrolysis of phenyl valerate (3). Elucidation of the molecular mechanisms involved in OP-induced neuropathy is of fundamental interest since it may yield information about normal axon maintenance.

DFP and other neuropathic OPs react covalently with the active-site serine residue of NTE, thereby inhibiting its esterase activity (see Scheme 1). Subsequently, a rapid intramolecular rearrangement, termed aging, leaves a negatively charged monoisopropyl phosphate species covalently attached to the serine. The aged enzyme, unlike the simple inhibited enzyme, cannot be reactivated by nucleophilic reagents such as oximes (see Scheme 1). The aging reaction in DFP-inhibited NTE differs from that in better characterized serine hydrolases such as acetylcholinesterase: it is considerably faster, and in an intramolecular transfer, one of the isopropyl groups forms a covalent adduct with another amino acid residue of NTE called site Z (see Scheme 1) (4, 5). Much evidence indicates that aging of NTE is the essential primary event in the initiation of OP neuropathy (5).

Molecular cloning and sequencing have revealed that NTE is unrelated to known serine hydrolases, but belongs to a novel protein family represented in species from bacteria to man (6). The Drosophila NTE homologue, the Swiss Cheese protein (SWS), is involved in neural development, and sus mutant flies show age-dependent neurodegeneration (7). Whether a genetic human neurological condition results from mutation of the NTE gene (on chromosome 19p13.3 (6)) remains to be determined. However, in contrast to the recessive genetic disorder in sus mutant flies, OP-induced neuropathy in adult vertebrates does not appear to result from a loss of the normal enzymatic activity of NTE, but instead may represent a toxic gain of function in the modified protein (3). In this study, to begin to elucidate the molecular basis for the unusual properties of NTE, we expressed portions of recombinant NTE in Escherichia coli to define both the minimal domain and some of the individual residues required for catalytic activity and reaction with OPs.

EXPERIMENTAL PROCEDURES

Chemicals—The E. coli BL21(DE3) pLysS strain and pET21b vector were purchased from Novagen. Pfu polymerase and the QuickChange™ site-directed mutagenesis kit were supplied by Stratagene. Nickel-nitrilotriacetic acid-agarose was purchased from Qiagen Inc. [1,3,5]-[3H]DFP ([3H]DFP, 310 Bq/mmole) was from NEN Life Science Products, and 2-isoni trosoacetophenone was from Aldrich. Phenyl saligenin phosphate and mipafox were supplied by Oryza Labs. All other reagents were purchased from Sigma or synthesized in this laboratory as de-
DNA Cloning and Mutagenesis—Polymerase chain reactions were carried out using Pfu polymerase and the NTE cDNA clone D16 as a template (6). Polymerase chain reaction products corresponding to amino acids 727–1216, 733–1216, 754–1216, and 753–1168 were cloned into pET21b vector, and DNA sequences were verified. Site-directed mutagenesis was performed using the QuikChange™ site-directed mutagenesis kit. Seventeen residues within construct 727–1216 were mutated, and all mutations were checked by DNA sequencing.

Protein Expression and Purification—All constructs in the pET21b vector were expressed in E. coli strain BL21(DE3) pLysS. An important polypeptide had an N-terminal 10-amino acid T7 tag to allow immunoochemical detection and a C-terminal His6 tag for affinity purification. Cultures were grown at 37 °C, and after 4–5 h of induction with 2 mM isopropyl-β-D-thiogalactopyranoside, cells were harvested by centrifugation. Cell pellets were freeze-thawed and resuspended in TE buffer (50 mM Tris-HCl and 1 mM EDTA, pH 8.0). Cell lysates were then analyzed either for solubility of the recombinant polypeptides (see Fig. 1b) or for NTE-like esterase activity (see below). Cells transformed with the construct containing amino acids 727–1216, called NEST (for NTE esterase domain), were cultured on a large scale for protein purification. Freeze-thawed cell pellets were extracted (90 min, 4 °C) in Buffer A (50 mM sodium phosphate, pH 7.8, 300 mM NaCl, and 0.5 mM EDTA) containing 2% CHAPS and then centrifuged at 100,000 × g for 60 min at 4 °C. The supernatant was layered onto nickel-nitritotriacetic acid-agarose and eluted with Buffer A containing 0.3% CHAPS and 300 mM imidazole. The eluate was concentrated 30-fold and run on a 300SW gel filtration column (Nihon Waters Ltd.) equilibrated in Buffer A containing 0.3% CHAPS. Fractions containing NEST were identified by Western blot analysis with an antibody to the T7 tag and were then pooled, mixed with diethylphosphatidylcholine (DOPC; 3:1 w/w lipid/protein ratio), and dialyzed at room temperature for 2 days against Buffer A containing 1 mM diithiothreitol. The resulting NEST-containing liposomes were centrifuged at 1000 × g for 10 min to remove any precipitate and assayed for NTE-like activity (see below). The catalytic activity of the NEST/DOPC preparations was somewhat labile; thus, dialysis at room temperature for 3 days resulted in preparations with specific activities 10-fold lower than those dialyzed for 2 days.

NTE-like Esterase Assay and Oxime Reactivation—NTE-like activity was assayed as the paraoxon-resistant and mipafox-sensitive hydrolysis of phenyl valerate (8). To compare the enzymatic character of NEST in E. coli lysates with that of native NTE, bacterial lysates and chicken brain microsomes (isolated as described previously (10)) were incubated in the presence of phenyl valerate (8). To compare the enzymatic character of NEST specific activities 10-fold lower than those dialyzed for 2 days.

RESULTS

Delimitation of the Recombinant NTE Esterase Domain (NEST) and Comparison with Native NTE—Translation of human NTE cDNA clone D16 indicates a 1327-amino acid polypeptide; the putative active-site serine residue lies in a C-terminal region, well conserved between proteins in a novel family (6). Although standard hydropathy plots of the NTE sequence predict only a single transmembrane segment (TM) near the N terminus, analysis by the TMpred program (13) predicted four potential membrane-spanning segments (6). The homology within the NTE protein family and the position of the predicted TMs informed our design of recombinant NTE constructs to obtain a polypeptide with esterase activity (Fig. 1a). The NTE cDNA clone D16 (6) was used as a template for polymerase chain reactions to generate constructs of varying length, which were cloned into the pET21b vector and expressed in E. coli BL21(DE3) pLysS.

E. coli lysates containing a recombinant polypeptide corresponding to residues 727–1216 of human NTE showed potent esterase activity. This construct was called NEST and was used for most of the subsequent experiments in this study. The approximate N-terminal limit of the NEST construct was suggested by our previous observation (6) that endoproteinase Glu-C cleavage of NTE generated a number of peptides initiated by Leu275, suggesting that this might lie in the area of a protein domain boundary. In addition, TM2, the second of the four putative transmembrane segments of NTE, begins at Leu733. A recombinant polypeptide corresponding to NTE residues 733–1216 was also catalytically active. By contrast, a recombinant polypeptide (NTE residues 754–1216) lacking putative TM2 had no detectable activity. The C-terminal boundary of NEST was not investigated in detail, but activity was not detected in a polypeptide corresponding to NTE residues 733–
Fig. 1. NTE and recombinant polypeptides used in this study. a, recombinant polypeptides comprising the NTE residues shown were expressed in E. coli, and phenyl-valerate hydrolyase activity in lysates was determined as under “Experimental Procedures.” Polypeptides are represented as boxes, and putative transmembrane segments predicted by the TMpred program (13) are shown as thick vertical bars. The region of NTE with significant sequence similarity to a homologue in yeast (YMP9) is shaded, and the 200-residue region conserved in bacterial homologues is boxed. b, E. coli lysates containing the recombinant polypeptides were extracted (4 °C, 30 min) either with 8 M urea and 1% CHAPS (C) or with 1% CHAPS only (U) or with 1% CHAPS only (C) and centrifuged (100,000 × g, 45 min); then extracts were run on SDS-polyacrylamide gel, and recombinant polypeptides were detected by Western blotting with an antibody to the N-terminal T7 tag epitope. For details, see “Experimental Procedures.” In this and all other figures of gels/blots, the migration of standard markers (molecular mass in kilodaltons) is shown on the left side.

1166. Both catalytically active constructs could be extracted from E. coli lysates by the detergent CHAPS, but strongly denaturing conditions were required for the inactive polypeptides (Fig. 1b).

Recombinant NEST in bacterial lysates and native NTE in brain microsomes showed the same rank order to covalent inhibition by a series of five compounds. I$_{50}$ values ranged over >4 orders of magnitude, with the neuropathic OP phenyl saligenin phosphate (I$_{50}$ values in the low nanomolar range) being the most potent and the non-neuropathic phenylmethylsulfonyl fluoride (I$_{50}$ ~ 100 μM) the least. Throughout, NEST was marginally less sensitive (1.5–2.5-fold) to these inhibitors than native NTE (Table I).

Recombinant NEST, both as a component of crude bacterial lysates and as a purified protein incorporated into DOPC liposomes, had catalytic center turnover numbers similar to those of native NTE in brain microsomes (Table II). Following inhibition by the non-neuropathic OP phenyl diphenylphosphinate (which cannot undergo the aging reaction) (Scheme 1), treatment of NEST in bacterial lysates and of NTE in brain microsomes with an oxime allowed reactivation of most of the esterase activity (Table II). By contrast, neither preparation could reactivates after inhibition by the neuropathic OP DFP, indicating that aging had occurred (cf. Scheme 1).

The OP aging reaction of DFP-inhibited NTE is rapid (t$_{1/2}$ of a few minutes) and does not result in quantitative liberation of an isopropyl group into free solution, as occurs with DFP-inhibited acetylcholinesterase; instead, this group is transferred to a second site within NTE called site Z (Scheme 1). The isopropyl groups attached to the active-site serine and site Z in aged, [3H]DFP-inhibited NTE can be distinguished because alkaline hydrolysis liberates non-volatilizable ([3H]monoisopropyl phosphate) and volatilizable ([3H]isopropyl alcohol) radioactive fractions, respectively (4, 11). In this study, following aging in [3H]DFP-labeled preparations, ~30% of the radioactivity associated with native NTE or NEST was found to be bound to site Z (Table II).

Critical Residues in the NTE Esterase Domain Identified by Site-directed Mutagenesis and [3H]DFP Labeling—Within the family of proteins related to NTE, we previously noted a core 200-residue region containing not only the putative active-site serine (Ser$_{966}$) but also histidine, aspartate, and glutamate residues conserved even in a homologous protein in E. coli (6). We suggested that Ser$_{966}$ in combination with a conserved histidine and acidic residue could compose a catalytic triad as found in most serine hydrolases (6). In the present study, we used site-directed mutagenesis of NEST to change these residues (Ser to Ala, His to Ala, Asp to Asn, and Glu to Gln) (Fig. 2).

Aliquots of bacterial lysates containing equal amounts of the recombinant mutant polypeptides (as determined by Western blotting) were assayed for phenylvalerate hydrolyase activity
and reactivity with [3H]DFP. The latter values were used to normalize more accurately esterase activities for mutants showing insignificant or minor changes (Table III). Phenyl valerate is used in standard NTE assays at a concentration of 1.4 mM, and its $K_m$ has been estimated to be $-10 \text{ mM}$ (14). This relatively high $K_m$ and the limited solubility of phenyl valerate preclude accurate kinetic analysis, and so esterase activities for the various mutant enzymes are expressed relative to the value for wild-type NEST (100%).

As expected, mutation of Ser$^{366}$ abolished the esterase activity of NEST. Mutation of either of two conserved aspartate residues, Asp$^{969}$ or Asp$^{1036}$, reduced activity 10,000-fold. By contrast, mutation of two other aspartates, Asp$^{1033}$ and Asp$^{1044}$, or of a conserved glutamate, Glu$^{1032}$, had no apparent effect. On the other hand, the D1004N mutant had significantly ($-6$-fold) lower esterase activity than wild-type NEST. Interestingly, the proportion ($14.8 \pm 4.5\%$, $n = 3$) of total tritium associated with site Z in the [3H]DFP-inhibited D1004N mutant was only about half that associated with site Z in wild-type NEST (cf. Table II).

Within the core 200-residue region, mutation of two conserved histidines, His$^{845}$ and His$^{1053}$, had no effect on activity (Table III). Subsequently, all the remaining nine histidines in NEST (Fig. 2) were individually mutated to alanine, but only the H860A and H885A mutants showed substantially lowered ($>300$-fold) catalytic activity (Table III). However, although the H860A mutant showed $<0.1\%$ of the activity of wild-type NEST, it is unlikely that this histidine is directly involved in a catalytic triad; this is because a tyrosine residue is found in the corresponding position in SWS, the Drosophila NTE homologue (cf. Fig. 2), and we have shown that a portion of SWS, colinear with NEST, expressed in E. coli has substantial NTE-like esterase activity.2

After [3H]DFP labeling, NEST-containing liposomes were denatured in SDS and treated with endoproteinase Glu-C. Fractionation of these digests on SDS-polyacrylamide gel resolved 12 polypeptide bands with molecular masses between 6 and 40 kDa (Fig. 3a). A combination of N-terminal sequencing of bands 6, 8, and 10 and predictions of the size of fragments generated by endoproteinase Glu-C cleavage of the NEST sequence allowed creation of a partial peptide map (Fig. 3b). The amounts of total and volatilizable radioactivities following NaOH treatment of excised polypeptide bands 6, 8, and 10 were determined. Very little tritium was associated with band 10, suggesting that both the active-site serine and site Z lie downstream of Ala$^{955}$. By contrast, abundant tritium was associated with band 6, and as expected, a peak of radioactivity was liberated with Ser$^{366}$ when band 6 was subjected to repeated cycles of Edman degradation (Fig. 4a).

The proportion of volatilizable tritium (81%) in polypeptide band 8 was much greater than that in band 6 (28%), suggesting that the former polypeptide contains site Z. Edman degradation of band 8 liberated a peak of radioactivity coincident with Asp$^{1044}$ (Fig. 4b). Surprisingly, however, the percentage of volatilizable counts in the D1044N mutant (27.0%, $n = 2$) was not significantly different from that in wild-type NEST, suggesting that in the absence of Asp$^{1044}$, the transferred [3H]isopropyl group is adducted to another residue.

Membrane Association of the Recombinant NTE Esterase Domain—NEST was firmly associated with particulate fractions of bacterial lysates and could be solubilized using CHAPS, but its catalytic activity was completely abolished. Purification of NEST was monitored by SDS-PAGE since, at all stages of the isolation in detergent, catalytic activity could not be detected. However, when purified NEST was incorporated into DOPC liposomes with removal of detergent by dialysis over a 2-day period, potently active preparations were obtained (see Table II). During isolation of NEST by nickel-nitrilotriacetic acid-agarose and gel filtration chromatography, detergent was required to maintain solubility and to prevent precipitation of the polypeptide. Even in the presence of detergent, NEST displayed highly anomalous migration on gel filtration chromatography, eluting with cytochrome c (molecular mass of 12.4 kDa) rather than as a 55-kDa polypeptide as indicated by its behavior on SDS-polyacrylamide gel (Fig. 5a). This suggests that NEST is markedly retarded on gel filtration due to hydrophobic interactions with the column matrix.

When NEST-containing liposomes were extracted with the nonionic detergent Triton X-114 and extracts were then

| Preparation | Catalytic center | Oxime reactivation | Site Z-associated tritium |
|-------------|----------------|-------------------|--------------------------|
| NTE in brain | $1.56 \pm 0.04$ | $75.1 \pm 9.7$ | $30.7 \pm 9.1$ |
| NEST in E. coli lysates | $1.87 \pm 0.56$ | $87.0 \pm 12.4$ | $29.9 \pm 4.8$ |
| NEST/DOPC liposomes | $1.24 \pm 0.28$ | ND | $31.8 \pm 7.2$ |

**Scheme 1. Reaction of NTE with DFP.** The NTE active-site serine is represented as -OH. During the aging reaction of DFP-inhibited NTE, an isopropyl group (iPr) is transferred to a second residue called site Z (see Ref. 5).

| Active NTE | DFP | Inhibited NTE | Aged NTE |
|------------|-----|--------------|---------|
| [Z] iPr | O | | O− |
| | | | iPr |
| O− | P = O | | O− |
| | | iPr | |
| O− | P = O | | iPr |
warmed to allow separation into aqueous and detergent-rich phases, the great majority of NEST was found in the detergent-rich phase, a characteristic of integral membrane proteins (Fig. 5b). Following reaction of NEST/DOPC liposomes with DFP, the proportion of the polypeptide solubilized by Triton X-114 was only \( \frac{3}{10} \) of that achieved with untreated preparations; this effect was not seen when the zwitterionic detergent CHAPS was used for solubilization (Fig. 5c). The effect of DFP on Triton X-114 solubilization of NEST from DOPC liposomes was compared with those of phenyl saligenin phosphate, phenyl dipentanylphosphate, and phenylmethylsulfonyl fluoride. Of these four NTE inhibitors, only DFP significantly reduced the amount of NEST solubilized by Triton X-114 (Fig. 5d).

To further investigate the association of NEST with the DOPC liposomes, these preparations were treated with proteinase K at 4 °C for up to 3.5 h. NEST was resistant to proteolysis under these conditions, but was rendered more sensitive by addition of CHAPS (Fig. 6). Even in the presence of CHAPS, proteolysis was limited, and small amounts of polypeptide fragments of \( \approx 24 \text{ kDa} \) appeared particularly resistant. However, proteinase K was clearly capable of degrading denatured NEST as, in the presence of SDS, digestion rapidly went to completion (Fig. 6).

**DISCUSSION**

This study reveals that residues 727–1216 of human NTE comprise a functional domain, called NEST, with the esterase activity of the protein. NEST reacts with the artificial substrate phenyl valerate and a series of covalent inhibitors and undergoes the OP aging reaction in very similar fashion to native NTE. Removal of either 100 residues from the C terminus of NEST (a region conserved in eukaryote homologues of NTE) or 27 residues from the N terminus resulted in polypeptides that could not be expressed in the catalytically active form in E. coli. To the N-terminal side of NEST, NTE (in the region of residues 486–700 and 120–260) shows modest sequence similarity to cyclic AMP-binding proteins (6). Thus, in NTE, an N-terminal regulatory domain may modulate the activity of a C-terminal catalytic domain. The NTE Drosophila homologue SWS has been suggested to play a role in a cell signaling pathway (7), and the presence of these two functional domains in NTE appears to support such a role.

Within the NTE esterase domain, Ser966 is confirmed as the active-site serine residue, and two aspartates, Asp960 and Asp1086, appear essential for enzymatic activity. Most serine

---

**Fig. 2**. Alignment of the NEST sequence with eukaryotic NTE homologues showing some residues critical for catalysis. Numbers on the right indicate the residue number in the individual proteins: NTE (human; GenBank™/EBI Data Bank accession number AJ004832), SWS (Drosophila; Z97187), YOL4 (Caenorhabditis elegans; M98552), and YMF9 (Saccharomyces cerevisiae; Q04958). Residues identical in all four homologues are shown white-on-black. The shaded area indicates the core 200-residue region conserved in related predicted proteins in bacteria. Site-directed mutations (see Table III) causing severe (X) or minor/insignificant (0) loss of esterase activity are shown.

**Table III**

**Effects of site-directed mutagenesis on phenyl-valerate hydrolase activity of NEST**

Phenyl-valerate hydrolase activities of NEST mutants in bacterial lysates were assayed, and values for mutants with insignificant/minor changes in activity compared with wild-type NEST were normalized using the values determined for \( ^{3} \text{HDFP} \) reactivity as described under “Experimental Procedures.” Values are the mean ± S.D. of three to four experiments.

| Mutation | Activity (% of wild-type) |
|----------|---------------------------|
| Insignificant/Minor |                           |
| D1038N   | 95.8 ± 18.5               |
| D1044N   | 102.8 ± 2.4               |
| E1032Q   | 96.7 ± 20.3               |
| D1004N   | 14.3 ± 3.1                |
| H945A    | 75.9 ± 12.0               |
| H1035A   | 96.2 ± 9.7                |
| Severe   |                           |
| S966A    | <0.001                    |
| D960N    | 0.0085 ± 0.0018           |
| D1086N   | 0.0082 ± 0.0033           |
| H860A    | 0.067 ± 0.048             |
| H885A    | 0.29 ± 0.11               |

This study reveals that residues 727–1216 of human NTE comprise a functional domain, called NEST, with the esterase activity of the protein. NEST reacts with the artificial substrate phenyl valerate and a series of covalent inhibitors and undergoes the OP aging reaction in very similar fashion to native NTE. Removal of either 100 residues from the C terminus of NEST (a region conserved in eukaryote homologues of NTE) or 27 residues from the N terminus resulted in polypeptides that could not be expressed in the catalytically active form in E. coli. To the N-terminal side of NEST, NTE (in the region of residues 486–700 and 120–260) shows modest sequence similarity to cyclic AMP-binding proteins (6). Thus, in NTE, an N-terminal regulatory domain may modulate the activity of a C-terminal catalytic domain. The NTE Drosophila homologue SWS has been suggested to play a role in a cell signaling pathway (7), and the presence of these two functional domains in NTE appears to support such a role.

Within the NTE esterase domain, Ser966 is confirmed as the active-site serine residue, and two aspartates, Asp960 and Asp1086, appear essential for enzymatic activity. Most serine
Hydrolases for which crystal structures have been solved use a catalytic triad in which a histidine is within hydrogen bond distance of both the nucleophilic serine and an acidic (Asp or Glu) residue \(^{(1)}\). His\(^{885}\) is conserved in all eukaryotic homologues of NTE, but the loss of catalytic activity resulting from mutation of this histidine is substantially less (30-fold) than in the D960N and D1086N mutants. This raises the possibility that NTE does not use a conventional catalytic triad. Interestingly, two mammalian serine hydrolases, fatty-acid-amide hydrolase and cytosolic phospholipase A2, have recently been shown to use a catalytic mechanism without a histidine residue \(^{(15, 16)}\).

Transfer of an isopropyl group from the active-site serine to another residue within the same DFP-inhibited enzyme is a feature of the aging reaction of NTE not shared by acetylcholinesterase \(^{(4)}\). In this study, we found that some of the transferred isopropyl group is adducted to Asp\(^{1044}\). However, in a mutant form of NEST in which Asp\(^{1044}\) is absent, intramolecular isopropyl transfer appears to occur to the same extent, implying a degree of non-selectivity in the process. The fact that one aspartate residue (Asp1044) has been demonstrated to act as an isopropyl group acceptor suggests that other aspartates within NEST sequence 955–1166 could do the same. The \[^3H\]DFP-inhibited D1004N mutant had only about half the proportion of volatilizable radioactivity of wild-type NEST; hence, Asp\(^{1004}\) may be another candidate as an acceptor for isopropyl transfer. Overall, although aging of one molecule of DFP-inhibited NTE results in intramolecular transfer of one isopropyl group to one aspartate residue, within a population of DFP-inhibited NTE molecules, isopropyl transfer may involve different aspartate residues.

In this study, the amount of tritium detected in volatilizable form (~30% of the total NEST-adducted tritium) fell short of...
CHAPS or SDS. Proteinase K action was terminated with phenylmethylsulfonyl fluoride; then samples were run on SDS-polyacrylamide gel, and polypeptides were detected by Coomassie staining.

the value (50%) expected for a quantitative transfer of one of the pair of $^3$H-isopropyl groups from the active-site serine. We suggest that this reflects an instability of the adduct at one or more of the site Z residues so that some of the $^3$H-isopropyl alcohol is shed into the medium during the incubation or boiling in SDS-PAGE sample buffer, rather than an incomplete aging reaction. This suggestion is supported by the observation that DFP-induced aging appeared to have gone to completion as judged both by complete resistance of the DFP-inhibited enzyme to oxime reactivation (Table II) and by the fact that when NaOH extracts of SDS-PAGE bands containing $^3$H-DFP-inhibited NEST were acidified and then partitioned against chloroform, >95% of the radioactivity was found in the aqueous phase (data not shown), a property of monoisopropyl phosphate rather than diisopropyl phosphate, which partitions into the organic phase (4).

All NPs that undergo the aging reaction with NTE form stable covalent adducts with site Z; aging of saligenin octyl phosphate-inhibited NEST liberates >85% of the $^3$H-labeled saligenin group into the incubation medium (17). The failure of saligenin groups to form stable adducts with site Z may underlie our observation that reaction of NEST/DOPC liposomes with DFP, but not with phenyl saligenin phosphate, inhibits subsequent solubilization of the polypeptide with a nonionic detergent. It seems possible that formation of an isopropyl-asparyl ester from an aspartate residue could augment the hydrophobicity of NEST.

NTE itself is an integral membrane protein. In phase-separatated detergent extracts of brain microsomes, NTE preferentially partitions into the detergent-rich phase; furthermore, its highly hydrophobic nature frustrated attempts at purification by conventional chromatography (18). This study demonstrates that at least part of the hydrophobic character of NTE is also a property of its catalytic domain. Of the four putative transmembrane segments predicted by the Tmperf program (13), only TM1, at NTE residues 10–22, is also predicted by a standard hydropathy plot. If this is correct, then NTE would be a type II membrane protein with a large cytoplasmic domain. However, NEST, which lacks putative TM1, has many properties expected of an integral membrane protein. NEST is insoluble in the absence of detergent and, even in its presence, appears to bind to a gel filtration matrix, probably by hydrophobic interactions. This anomalous behavior on gel filtration contrasts markedly with our previous observations of a type I membrane protein that, under similar running conditions (0.5% CHAPS, 0.5 mM NaCl, and 50 mM Tris-HCl, pH 7.4), eluted from a gel filtration column at a position consistent with its molecular size determined by SDS-PAGE (19). When Triton X-114-solubilized NEST was partitioned into aqueous and detergent phases, the great majority of the polypeptide was found in the latter phase. NEST incorporated in DOPC liposomes is resistant to degradation by proteinase K, suggesting that a large portion of the polypeptide is shielded by the lipid membrane.

The integral membrane properties of the NTE catalytic domain differ from those of the great majority of known eukaryotic serine hydrolases. An exception may be fatty-acyl-amide hydrolase, a serine hydrolase whose substrates include several neuromodulatory fatty acid amidases and that has an interesting structural similarity to NTE. Analysis of the 579-amino acid sequence of fatty-acyl-amide hydrolase predicts a type II membrane protein with a single transmembrane segment between residues 9 and 29 and a large cytoplasmic domain (20). However, a 30–579 mutant form of fatty-acyl-amide hydrolase expressed in COS cells showed the same membrane-associated properties as the wild-type protein with the N-terminal transmembrane segment (21). Therefore, residues 30–579 of fatty-acyl-amide hydrolase contain regions that favor membrane association. On the other hand, in marked contrast to our present observations with NEST, after expression in E. coli and purification, the truncated 30–579 fatty-acyl-amide hydrolase mutant protein migrated on gel filtration in 0.5% CHAPS as an oligomer and furthermore did not require the presence of phospholipid to display oleamide hydrolase activity (21).

Finally, it is interesting to consider analogies of NTE to prokaryotic enzymes. The 27-kDa outer membrane phospholipase A of E. coli forms a membrane-spanning β-barrel structure, and its active-site serine is located on the exterior of this barrel facing the outer leaflet side of the membrane (22). A highly conserved region of 200 residues in NTE containing Ser$^{166}$, Asp$^{166}$, and Asp$^{166}$ is presented in predicted proteins of other bacteria (6), and the presence of protease-resistant polypeptides of a similar size in NEST suggests that the NTE catalytic domain may have a tightly folded core.

Acknowledgments—We thank Kathryn Lilley for protein sequencing, Paul Richards for valuable criticism of the manuscript, and David Read for assistance in preparing the electronic manuscript and figures.

REFERENCES

1. Dodson, G., and Wlodawer, A. (1998) Trends Biochem. Sci. 23, 347–352
2. Liu, Y., Patricelli, M. P., and Cravatt, B. F. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14904–14909
3. Glynn, P. (1999) Biochem. J. 344, 625–631
4. Clothier, B., and Johnson, M. K. (1979) Biochem. J. 177, 549–558
5. Johnson, M. K. (1990) Toxicol. Appl. Pharmacol. 102, 385–395
6. Lush, M. J., Li, Y., Read, D. J., Willis, A. C., and Glynn, P. (1998) Biochem. J. 332, 1–4
7. Kretzschmar, D., Hasan, G., Sharma, S., Heisenberg, M., and Benzer, S. (1997) J. Neurosci. 17, 7425–7432
8. Johnson, M. K. (1977) Arch. Toxicol. 37 113–115
9. Meredith, C., and Johnson, M. K. (1988) J. Neurochem. 51, 1097–1101
10. Glynn, P., Read, D. J., Guo, R., Wylie, S., and Johnson, M. K. (1994) Biochem. J. 301, 551–556
11. Meredith, C., and Johnson, M. K. (1989) J. Neurochem. 52, 1248–1252
12. Bordier, C. (1981) J. Biol. Chem. 256, 1604–1607
13. Hofmann, K., and Stedel, W. (1993) Biol. Chem. Hoppe-Seyler 344, 44–57
14. Johnson, M. K. (1982) Rev. Biochem. Toxicol. 4, 141–212
15. Patricelli, M. P., Lovato, M. A., and Cravatt, B. F. (1999) Biochemistry 38, 9804–9812
16. Donnen, A., Tang, J., Schmidt, H., Stahl, M., Clark, J. D., Seehra, J., and Somers, W. S. (1999) Cell 97, 349–360
17. Yoshida, M., Tomizawa, M., Wu, S.-Y., Quistad, G. B., and Casida, J. E. (1995) J. Neurochem. 64, 1680–1687
18. Ruesch/Turner, M. E., Read, D. J., and Johnson, M. K. (1992) J. Neurochem. 58, 135–141
19. Chantry, A., Gregory, N. S., and Glynn, P. (1989) J. Biol. Chem. 264, 21303–21307
20. Giang, D. K., and Cravatt, B. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2238–2242
21. Patricelli, M. P., Lashuel, H. A., Giang, D. K., Kelly, J. W., and Cravatt, B. F. (1998) Biochemistry 37, 15177–15187
22. Snijder, H. J., Ubarretxena-Belandia, I., Blaauw, M., Kalk, K. H., Verheij, H. M., Egmond, M. R., Dekker, N., and Dijkstra, B. W. (1999) Nature 401, 717–721