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AFFINITY CHROMATOGRAPHY OF NEUROPATHY TARGET ESTERASE

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SUMMARY

Neuropathy target esterase (NTE) is a membrane-bound protein which has been proposed as the target site in nerve tissue for initiation of organophosphate induced delayed neuropathy (OPIDN). Efforts to characterize NTE and to determine the mechanism of its involvement in OPIDN have been hampered by the lack of a suitable method for its purification. We describe here the development of a trifluoromethyl ketone liganded affinity gel which selectively binds NTE. Triton X-100/NaCl extracts of NTE from chick embryo brain microsomal membranes were adsorbed to an affinity gel prepared by attachment of 3(9′-mercaptononylthio)-1,1,1-trifluoropropan-2-one to epoxy-activated Sepharose CL4B (MNTFP-Sepharose). Typically 70 - 80% of NTE activity is bound under conditions in which undetectable quantities of total protein bound (< 4%). It proved difficult to elute active NTE under non-denaturing conditions, but SDS-PAGE analysis of MNTFP-Sepharose bound proteins eluted with 2% SDS identified a 155 kDa NTE-like protein that bound in a trifluoromethylketone- or mipafox-sensitive but paraoxon-insensitive manner. The levels of inhibition of binding correlated with the inhibition of activity and suggested that the 155-kDa band was composed of a single protein. MNTFP-Sepharose affinity chromatography in combination with preparative SDS-PAGE therefore holds promise as a method for obtaining microgram quantities of NTE for chemical analysis and sequencing.

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Abbreviations: CAPS, 3(cyclohexylamino)propanesulfonic acid; DFP, di-isopropylfluorophosphate; DTFP, 3-decylthio-1,1,1-trifluoropropan-2-one; mipafox, *N,N′*diisopropylphosphorodiamidic fluoride; MNTFP-Sepharose, 3-(9′-mercaptononylthio)-1,1,1-trifluoropropan-2-one attached to epoxy-activated Sepharose CL4B; NTE, Neuropathy Target Esterase; OPIDN, Organophosphate Induced Delayed Neuropathy; OTFP, 3-octylthio-1,1,1-trifluoropropan-2-one; paraoxon, phosphoric acid diethyl 4-nitrophenyl ester.

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Key words: Organophosphate – neuropathy – affinity – chromatography – trifluoromethylketone

INTRODUCTION

Neuropathy target esterase (NTE) is a membrane-bound protein with esterase activity that is proposed as the target site in nerve tissue for initiation of Organophosphate induced delayed neuropathy (OPIDN). This activity is identified as phenyl valerate hydrolysis that is resistant to treatment with paraoxon and sensitive to co-incubation with paraoxon and mipafox. Experiments with radiolabeled diisopropylfluorophosphate, a potent inhibitor of NTE activity, have identified NTE as a protein with an apparent molecular weight on SDS-PAGE of 155 kDa [1–3]. In addition Triton X-100-solubilized NTE has been observed to have a sedimentation coefficient of 9.6 S on 5–20% sucrose density gradients [3,4]. Beyond the determination of these features very little progress has been made toward physically characterizing NTE or determining the mechanism by which chemical modification of NTE leads to OPIDN. The slow progress in these two areas is directly attributable to the lack of a method for isolating a purified preparation of NTE.

Attempts to purify NTE by gel filtration, sucrose gradient centrifugation, ion exchange chromatography and preparative isoelectric focusing have so far failed to yield significantly purified preparations of NTE [3–8]. Gel filtration was the only method by which even a relatively modest increase in specific activity was observed [6,8]. The failure of other methods to yield fractions with significantly increased specific activity was primarily due to their failure to recover enzymatic activity rather than their inability to resolve NTE from other proteins.

The inability to obtain high yields of active protein is a commonly observed problem in the purification of integral membrane proteins. A recent review of the literature indicated that the method which has been employed most successfully toward the purification of integral membrane proteins is affinity chromatography [9]. We report here on the development of 3-(9′-mercaptononylthio)-1,1,1-trifluoropropan-2-one as an affinity ligand for the purification of NTE. A similar ligand, 3-(4′-mercaptobutylthio)-1,1,1-trifluoropropan-2-one, was previously employed in the affinity purification of juvenile hormone esterase [10,11].

MATERIALS AND METHODS

Synthesis of 3-(9′-mercaptononylthio)-1,1,1-trifluoropropan-2-one (MNTFP)

MNTFP was prepared by a modification of the method previously employed by Ashour and Hammock [12]. Two mmol of 1,9-nonanedithiol (Aldrich Chemical Co., Milwaukee, WI) and 2 mmol of 3-bromo-1,1-1-trifluoropropan-2-one (PCR Research Chemicals Inc., Gainesville, FL) were dissolved in 3 ml of dichloromethane under nitrogen. The solution was cooled in an ice bath and then stirred

continually while 2.4 mmol of triethylamine (Aldrich) were added in a dropwise manner over a period of 5 min. The flask was then flushed with nitrogen and the solution stirred overnight at room temperature. Precipitated triethylamine hydrobromide was removed by filtering the solution through a sintered glass funnel. Dichloromethane was removed from the filtered product using a rotary evaporator and the product was redissolved in 2 ml of methanol. Previous studies with trifluoromethylketone columns for the purification of other esterases indicated that the free thiol compounds tended to become oxidized during chromatographic purification. Therefore no further purification was performed on this final product. The expected side reactions also resulted in products like the bis-trifluoromethylketone of dimercaptononane, which will not bind to the epoxy-activated gel, and the unreacted dithiol which will simply serve as a hydrophobic ligand with no specific affinity for esterases. TLC and GLC analysis of the crude reaction mixture indicated the expected 1:2:1 ratio of the resulting products (unreacted dithiol:monosubstituted product:disubstituted product) with traces of polysulfides from oxidative polymerization. Crude yield based on weight was approximately 130%, largely due to the presence of triethylamine as determined by NMR. For subsequent calculations the recovery of products was assumed to be 100% with half of the recovered product the desired monosubstituted trifluoromethylketone based on NMR integration.

Preparation of epoxy-activated gel and attachment of ligand

The 3-(9′-mercaptononylthio)-1,1,1-trifluoropropan-2-one liganded Sepharose affinity gel (MNTFP-Sepharose) was prepared essentially as described by Abdel-Aal and Hammock [11] with care being taken to ensure that washing steps were carried out under neutral conditions (Fig.1). Epoxy-activated Sepharose CL4B was first prepared with 5.9 ± 0.7 μequivalents of epoxide per g of moist suction filtered gel (n = 3). Epoxy-activated gel was then resuspended in 0.6 mM $NaHCO_3$ and allowed to settle for 1 h. Fifty ml of settled resin were combined with 50 ml of 0.1 M $NaHCO_3$:methanol (1:1) and 50 ml of ligand (approx. 40 mM). This suspension was placed in a flask flushed with nitrogen and swirled vigorously on an orbital shaker for 21 h. Subsequently, unreacted epoxides were cleaved by incubation for several hours with excess β-mercaptoethanol. The suspension was then packed into a column, washed with 20 volumes of 0.1 M $NaHCO_3$:methanol (1:1) followed by 20 volumes of methanol, and stored at 4°C. Fluorine analysis of two batches found that between 7 and 13% of the available epoxides reacted with ligand. Prior to use, MNTFP-Sepharose was packed into a column and washed successively with 20 volumes of 0.6 mM $NaHCO_3$:methanol (1:1), 20 volumes of 0.6 mM $NaHCO_3$, and 5 volumes of 50 mM Tris–HCl buffer, 0.2 mM EDTA, pH 8.0. For the majority of experiments the affinity gel was resuspended at a concentration of 167 mg moist resin/ml of buffer (16.7%).

Preparation of detergent-solubilized NTE

Microsomal membranes from chicken embryo brains were prepared as described by Thomas et al. [8]. Two basic methods were used to prepare soluble NTE for affinity binding experiments. The first method involved extracting brain mi-

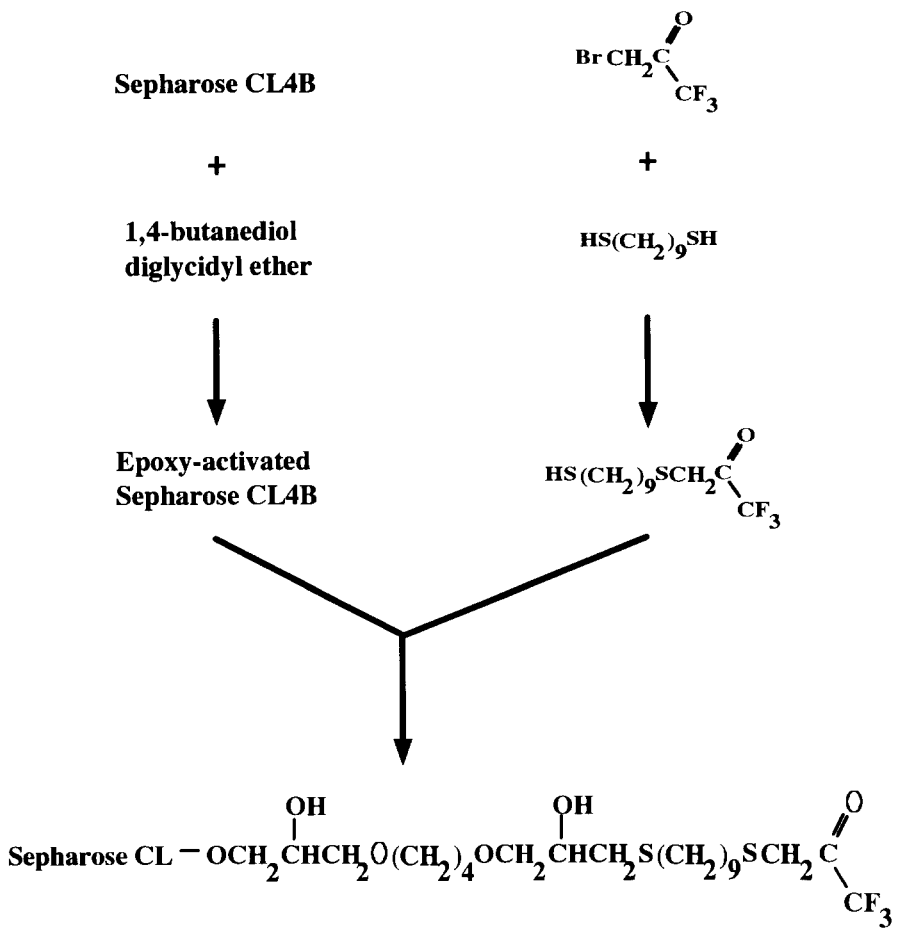

Fig. 1. Synthesis of MNTFP-Sepharose Affinity Gel. Reaction conditions were as described in the Materials and Methods section.

crosomal membranes for 1 h at 4°C with Triton X-100 in a buffer containing 0.5 M NaCl, 1 mM EGTA, 1 mM EDTA and 1 mM Tris–HCl, pH 7.2. The Triton X-100 concentration was 3 mg/ml in samples containing 5 mg protein per ml and 6 mg/ml in samples containing 10 mg protein per ml, thereby maintaining the detergent to protein ratio at 0.6 (wt/wt). Soluble NTE was recovered in the supernatant after centrifugation at 100 000 × *g* in either a type 60 Ti or type 75 Ti rotor (Beckman) for 1 h at 4°C. The second method involved further purifying soluble NTE preparations by gel filtration. Soluble NTE samples (200 μl) were applied to a Superose 12 column (HR 10/30, Pharmacia) equilibrated in 0.02% Triton X-100, 0.5 M NaCl, 0.1 mM DTT, 1 mM EDTA, 1 mM EGTA, 20 mM Tris–HCl, pH 7.2, at a flow rate of 0.25 ml/min. Fractions of 0.5 ml each were collected and those containing NTE activity, but not other types of phenyl

valerate hydrolyzing activities, were combined (typically fractions 17 to 21 or 22).

Batch binding and elution experiments

Acid-washed glass vials with Teflon lined caps were used for binding and elution experiments. In a typical experiment, 80 μl of a 16.7% suspension of MNTFP-Sepharose and 400 μl of sample were added to each vial. Vials were placed on their sides and shaken at 4°C for 16 – 25 h (most often 20 h). Supernatant fractions containing non-bound proteins were recovered after centrifuging the suspensions in 1.5 ml tubes for 2 min. The pelleted affinity gel was then washed with 1 ml of 0.02% Triton X-100, 0.5 M NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM Tris – HCl, pH 7.2, and recovered by centrifugation as above. Methods for eluting proteins bound to the MNTFP-Sepharose gel varied and these will be discussed in the results section.

Phenylvalerate hydrolysis and protein determination

Phenylvalerate hydrolysis was assayed colorimetrically by the method of Johnson [13] with modifications described by Thomas et al. [3,8]. The preparation of paraoxon, mipafox and phenylvalerate was described previously [4]. NTE activity was identified as the difference between paraoxon-resistant and paraoxon plus mipafox-resistant activity. Units of activity are reported as μmoles of phenol produced per min (I.U.). Protein was quantitated using the method of Lowry et al. [14] with 1.8% SDS present to prevent precipitation of Triton X-100 [15]. Bovine albumin (Fraction V, Sigma) was used as a standard.

Gel electrophoresis

Non-bound, bound and eluted proteins were analyzed on 1.5 mm thick, 7.5% or 5% polyacrylamide gels according to the Laemmli method [16]. Soluble samples were prepared for electrophoresis by adding 1 volume of 0.125 M Tris – HCl, pH 6.8, 4% SDS (w/v), 20% glycerol (w/v), 40 mM DTT, and 0.002% bromophenol blue (2× sample buffer) to 1 volume of sample and incubating at 60°C for 1 h with occasional mixing. Proteins bound to the affinity gel were eluted for analysis by adding 1 volume of 2X sample buffer to an equal volume of pelleted gel and incubating as above. Suspensions were then centrifuged for 2 min in a microfuge and the supernatant was isolated for SDS-PAGE. Molecular weight markers contained a mixture of 'High Molecular Weight Markers' from Sigma and RNA Polymerase from Pierce. Gels were stained either with Coomassie Blue R250 or silver [17].

RESULTS

Binding of NTE to MNTFP-Sepharose

An affinity gel for the purification of NTE was prepared in two steps (Fig. 1). First, 1,9-nonanedithiol was reacted with 3-bromo-1,1,1-trifluoropropan-2-one to form crude 3-(9′mercaptononylthio)-1,1,1-trifluoropropan-2-one (MNTFP). This was then attached to epoxy-activated Sepharose CL4B to form MNTFP-

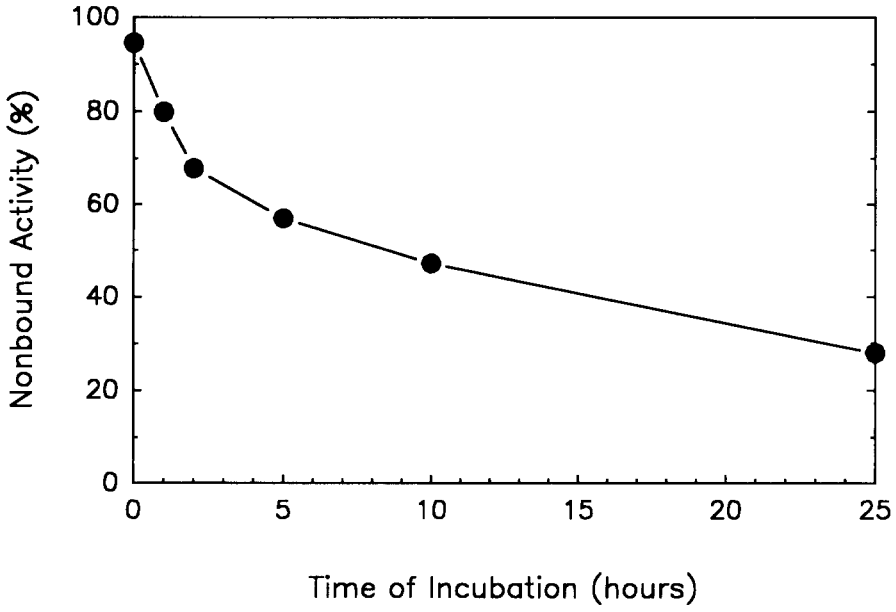

Fig. 2. Time course of NTE binding to MNTFP-Sepharose affinity gel. Crude membranes from chicken embryo brain were extracted at 1 mg/ml in 0.2% Triton X-100, 0.5 M NaCl, 1 mM EDTA, 1 mM EGTA and 1 mM Tris–HCl, pH 7.2 while on ice for 1 h. Soluble proteins were isolated by centrifugation at 100 000 × *g* for 1 h, and then incubated with MNTFP-Sepharose affinity resin at 4°C for the indicated times. Non-bound fractions were then assayed for remaining NTE activity. Activities are reported as percentages of the activity found in control samples incubated for equal periods of time with buffer rather than affinity resin. At 25 h the control sample had lost 29.3% of its original NTE activity.

Sepharose. Initially the level of Triton X-100/NaCl-solubilized NTE that bound to the MNTFP-Sepharose affinity gel was determined by monitoring the decrease in the concentration of NTE activity in the non-bound fraction. The level of non-bound NTE activity was found to decrease in a time-dependent manner (Fig. 2). After 25 h of incubation at 4°C the non-bound fraction contained 72.1% less NTE activity than a control sample incubated simultaneously without MNTFP-Sepharose. The loss of NTE activity in this control sample was only 29.3% compared to its activity prior to incubation. In order to rule out the possibility that NTE activity was inhibited by ligand that had leached off the affinity gel, some of the MNTFP-Sepharose was incubated with buffer for 20 h at 4°C. An aliquot of NTE was then diluted twofold with this buffer but no inhibition of NTE activity was observed. The effect of salt concentration on binding to the affinity gel was also examined. In one experiment, when a buffer containing 100 mM NaCl was compare to the standard buffer containing 500 mM NaCl the amounts of NTE which bound were 45% and 89%, respectively.

Specificity of binding to MNTFP-Sepharose

The specificity of NTE binding to MNTFP-Sepharose was investigated in several ways. First, the protein concentrations of a control and non-bound frac-

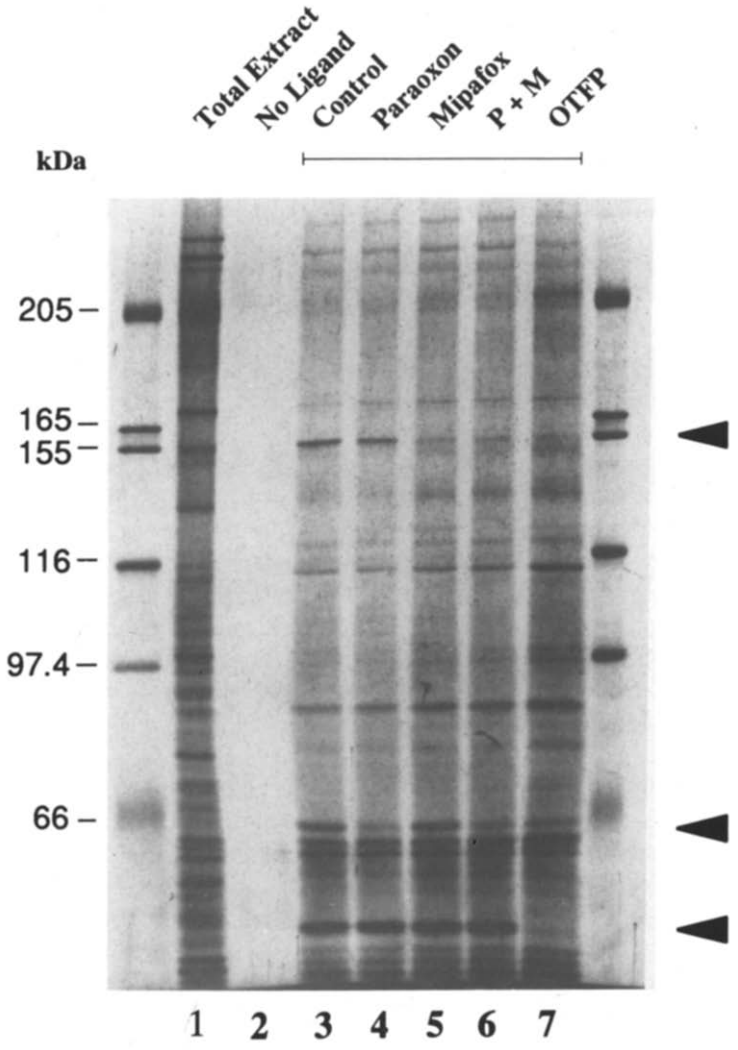

Fig. 3. Differential inhibition of binding to MNTFP-Sepharose. Membranes were incubated with 50 mM Tris–HCl, 0.2 mM EDTA, pH 8.0 containing either paraoxon (100 μM), mipafox (50 μM), or paraoxon + mipafox for 20 min at 37°C. Non-bound inhibitors were removed by twice diluting the samples 25-fold with ice cold buffer followed by centrifugation at 100 000 × g for 40 min at 4°C. Pelleted membranes were resuspended at 1 mg protein per ml in 0.2% (w/v) Triton X-100, 0.5 M NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM Tris-HCl, pH 7.2., and incubated at 4°C for 1 h. The detergent-solubilized proteins were isolated by centrifugation in a Beckman Airfuge for 10 min at 30 psi and then incubated with MNTFP-Sepharose for 20 h at 4°C. The bound proteins were eluted from buffer washed gels by addition of an equal volume of SDS-PAGE sample buffer (containing 2% SDS) followed by incubation at 60°C for 1 h. These were then analyzed by SDS-PAGE (see bracketed lanes) on a 5% acrylamide gel and visualized by silver staining. Lane 1, total Triton X-100/NaCl extract from membranes (Total Extract); lane 2, proteins bound to β-mercaptoethanol-treated epoxy-activated Sepharose (No Ligand); lanes 3–6, proteins bound to MNTFP-Sepharose from buffer (Control), paraoxon, mipafox, and paraoxon plus mipafox treated membranes; lane 7, proteins from buffer-treated membranes bound to MNTFP-Sepharose in the presence of 1 mM OTFP. The positions of the 155-kDa, 65-kDa and 40-kDa OTFP sensitive bands are indicated with arrows.

tion were compared. No reduction in the protein concentration of the non-bound fraction was observed indicating that less than 3.8% of the total protein had bound to the affinity gel. In the same experiment there was an 81% reduction in non-bound NTE activity compared to a similarly incubated control sample that did not contain MNTFP-Sepharose. Since NTE activity was not being inhibited by leached ligand this indicated that the soluble NTE activity had selectively bound to the MNTFP-Sepharose. When the affinity gel with bound NTE was assayed directly no NTE activity was observed. This suggested that NTE was bound to the affinity gel through a direct interaction between the affinity ligand and the catalytic site of NTE thereby inhibiting the activity.

Identification of a specific NTE-like protein bound to the MNTFP affinity resin

The finding that NTE binds to MNTFP-Sepharose through a direct interaction between the affinity ligand and the catalytic site of NTE suggested that trifluoromethyl ketones and other inhibitors of NTE should be able to inhibit binding. We therefore employed a strategy of differential inhibition to identify specific proteins that could be prevented from binding to MNTFP-Sepharose by either 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP), a soluble trifluoromethylketone inhibitor of NTE, or mipafox, an organophosphate inhibitor of NTE, but not by paraoxon, a non-inhibitory organophosphate (Fig.3). In the case of the irreversible organophosphate inhibitors, membranes were first treated with either paraoxon, mipafox, or both prior to detergent solubilization of proteins. In the case of OTFP, which has one less methylene group than the affinity ligand and is a reversible inhibitor of NTE activity ($I_{50} = 5.9 \times 10^{-8}$ M [8]), the inhibitor was included during the incubation with MNTFP-Sepharose. Proteins bound to the affinity gel were then eluted with SDS and analyzed by SDS-PAGE. See the legend of Fig. 3 for details of the inhibition, binding and elution conditions.

Analysis of the MNTFP-Sepharose bound proteins that were extracted from buffer-treated membranes (Control, Fig.3) indicates that a large number of proteins bound to the affinity gel despite the fact that no detectable decrease in the total protein concentration of the non-bound fraction was observed. The relative proportions of many of these proteins in the bound fraction appeared to have been enhanced compared to the Triton X-100/NaCl-solubilized fraction from membranes (Total Extract, Fig.3). Inclusion of 1 mM OTFP during incubation of samples with MNTFP-Sepharose inhibited the binding of only three proteins which had relative mobilities on SDS-PAGE of 155 kDa, 65 kDa and 40 kDa. The inhibition by OTFP of the binding of these three proteins suggests that they contain catalytic sites which bind directly to the affinity ligand.

The abilities of paraoxon and mipafox to inhibit binding were also examined in order to identify a specific protein that binds to MNTFP-Sepharose in an NTE-like manner. The binding of only two proteins was inhibited by either paraoxon or mipafox. These were two of the three affected by OTFP and their inhibition by these organophosphates suggests that they have serine containing catalytic sites. Binding of the 65-kDa protein was sensitive to paraoxon but insensitive to mipafox. This protein therefore does not bind to the affinity gel in an NTE-like manner. On the other hand, the 155-kDa protein binds in an NTE-

like, mipafox-sensitive but paraoxon-insensitive manner. The protein band at 155 kDa appears as a single sharp band, the majority of which was eliminated by mipafox treatment of the membranes prior to detergent solubilization. The small amount of residual staining observed at 155 kDa in the bound fractions from both the mipafox- and paraoxon plus mipafox-inhibited samples is consistent with the fact that these samples retained 11% and 14% of their NTE activity, respectively compared to that observed in the buffer treated sample.

Analysis of the soluble NTE fraction by SDS-PAGE revealed the presence of a significant band of protein at 155 kDa (Fig. 3, Total Extract). The intensity of staining of this band was not diminished in non-bound fractions after incubation with MNTFP-Sepharose (result not shown). This indicated that either these samples contained a considerable amount of inactive NTE or NTE is a relatively minor component of the protein migrating in this region.

As mentioned above, a large number of proteins were bound by MNTFP-Sepharose in a manner that was unaffected by the presence of 1 mM OTFP. As one step toward determining whether these OTFP-insensitive proteins were being bound in a non-specific manner, β-mercaptoethanol was attached to epoxy activated Sepharose CL4B rather than the affinity ligand. A protein sample was then incubated with this βME-Sepharose and bound proteins were analyzed by

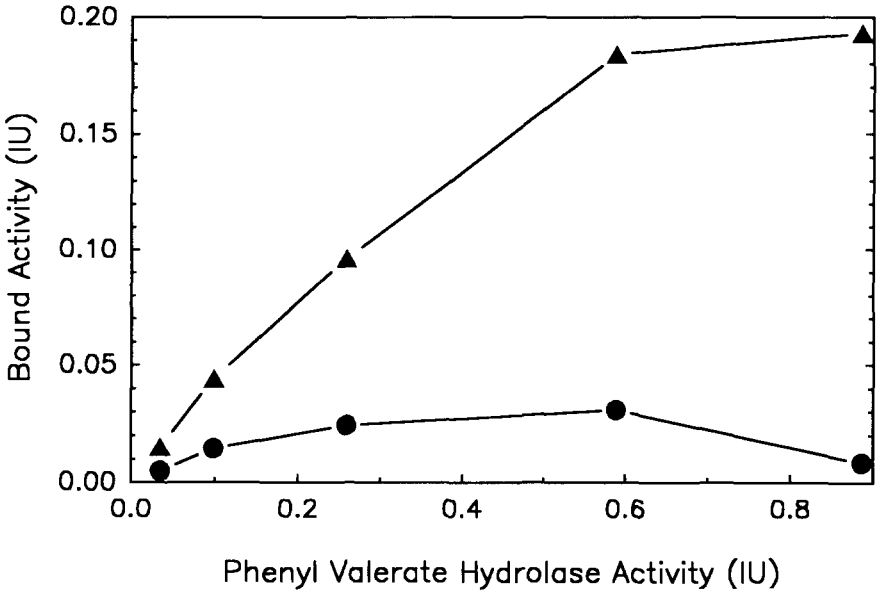

Fig. 4. Saturation of MNTFP-Sepharose affinity gel. Crude membranes from chicken embryo brain were extracted at 5 mg/ml in 0.3% Triton X-100, 0.5 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Tris–HCl, pH 7.2 on ice for 1 h. Soluble proteins were isolated by centrifugation at 100 000 × *g* for 1 h. The designated amounts of phenylvalerate hydrolase activity were incubated with a fixed amount of MNTFP-Sepharose for 20 h at 4°C. Non-bound fractions were assayed to determine the amount of total phenylvalerate hydrolase activity (▲-▲) and NTE activity (●-●) that had bound to the affinity gel.

SDS-PAGE (No Ligand, Fig.3). This analysis indicated that none of the proteins which bound to MNTFP-Sepharose were bound to βME-Sepharose.

Therefore, neither the Sepharose CL4B nor the butane diglycidyl ether spacer arm attached to it are primarily responsible for the non-specific binding to MNTFP-Sepharose.

Saturation of binding

If NTE does bind specifically to the MNTFP-affinity ligand then binding should be a saturable phenomenon. This was tested by incubating constant quantities of affinity resin with increasing volumes of sample. In the experiment presented in Fig. 4 both the quantity of NTE activity and of total phenylvalerate hydrolyzing activity binding to the MNTFP-affinity resin were determined. It can be seen that before the level of total phenylvalerate hydrolase binding reached a plateau the level of NTE binding dropped. The sample used in these experiments was a Triton X-100/0.5 M NaCl soluble protein fraction extracted from crude brain membranes, which contained at least four different types of phenylvalerate hydrolases including NTE [8,13]. It appears that some of these phenylvalerate hydrolases have a stronger affinity for the ligand than NTE. As these other hydrolases saturate the resin the amount of NTE which is able to bind decreases. We have previously shown that gel filtration chromatography can be used to separate NTE from these other types of hydrolases. It should therefore be possible to increase the amount of NTE bound/g of affinity gel by using NTE samples which have been purified first by gel filtration.

Elution of NTE activity and the 155-kDa protein MNTFP-Sepharose

The majority of elution experiments described here involved incubation of a Superose 12 purified NTE fraction (see Methods) with either MNTFP-Sepharose or MDTFP-Sepharose (3-(10′-mercaptodecylthio)-1,1,1-trifluoropropan-2-one attached to epoxy-activated Sepharose). Since the trifluoromethylketones are rapidly reversible inhibitors of NTE, we expected that NTE could be eluted from these affinity gels either by incubation with fresh buffer or by competition with a soluble trifluoromethylketone inhibitor, such as OTFP or DTFP (3-decylthio-1,1,1-trifluoropropan-2-one). However, incubation for 1–24 h at 4°C with or without OTFP or DTFP failed to elute any of the 155-kDa band. The NTE activity in eluates was determined after removing the soluble inhibitor by gel filtration, but no NTE activity was recovered. Since the trifluoromethylketones were identified as rapidly reversible inhibitors of NTE activity at 37°C (conditions of NTE assay) we also attempted to elute NTE by incubation at 37°C, but no elution of either NTE activity or the NTE-like 155-kDa protein was observed. The proteins which remained bound after treatment with the above buffers were then analyzed by SDS-PAGE after elution with SDS. The results indicated that the 155-kDa protein and most other proteins had remained bound to the affinity gel during incubation with the soluble inhibitors.

A wide variety of elution buffers were then tested that contained reagents capable of reducing the strength of either electrostatic, hydrophobic or hydrogen bonds. Table I gives an overview of the various reagents or buffer conditions that

TABLE I

ATTEMPTS TO ELUTE NTE ACTIVITY AND THE 155-kDa PROTEIN FROM MNTFP-SEPHAROSE AFFINITY GEL

Binding conditions:

0.2% Triton X-100, 0.5 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Tris-HCl, pH 7.2. Inc. at 4°C for 20 h.

Elution conditions:

(1) Binding buffer (1-4 days, 4°C).
(2) Octylthiotrifluoropropanone (OTFP, 1×10^{-3} M). This is a soluble analog of the affinity ligand which was used to competitively elute NTE.
(3) DFP, an irreversible inhibitor of NTE.
(4) SDS or Triton X-100. These were used to disrupt hydrophobic bonds and maintain NTE in solution.
(5) NaCl (0-2 M), used to disrupt electrostatic interactions.
(6) pH (4.5-10), used to disrupt electrostatic interactions.
(7) Dimethylformamide or dimethylsulfoxide (20 or 50%, respectively), used to disrupt hydrogen bonds.
(8) Urea (1-4 M), reduces strength of hydrophobic interactions by disordering water.
(9) Combinations of OTFP and above conditions.

were tested for their ability to elute the 155 kDa protein*. None of the solutions tested however were successful at eluting the 155 kDa protein under conditions that do not inactivate NTE activity. The replacement of OTFP, a reversible inhibitor of NTE, with DFP, an irreversible inhibitor, failed to improve the elution of the 155 kDa protein. SDS and combinations of Triton X-100 and urea were capable of eluting the 155 kDa protein but only at concentrations that inactivated NTE activity in control samples. High salt concentrations (1-2 M) had no effect on the elution of the 155 kDa protein. Lowering the NaCl concentration in the elution buffer from 500 mM to 100 mM or less also had no effect on the ability to elute the 155 kDa protein. Buffers containing 20 mM acetate at pH 4.5 and 20 mM CAPS at pH 10.0 were also employed but these had very little effect on elution of the 155 kDa protein from the affinity resin. The pH 10 buffer did elute a significant amount of the OTFP-insensitive proteins. Barth and Richardson [18] had previously reported that hydrogen ion concentration outside this range irreversibly inactivated NTE activity. Organic solvents such as dimethylsulfoxide and dimethylformamide were also ineffective. Incubation of soluble NTE with 50% dimethylsulfoxide on ice for 1 h reduced the NTE activity by 8.1%. Dimethylformamide in concentrations of 20% and 50% reduced NTE activity by 8.5% and 52%, respectively.

*The specific details of the reagent concentrations, incubation times, and incubation temperatures of all the elution buffers investigated can be found in Dr. Thomas' doctoral dissertation (University of California, Davis, 1991).

DISCUSSION

Work from this laboratory has previously shown that trifluoromethylketone derivatives can be potent reversible inhibitors of NTE activity [8]. Those derivatives with an alkyl chain ranging in length from 8 to 12 carbons were found to be more inhibitory than derivatives with shorter alkyl chain lengths. We therefore prepared MNTFP-Sepharose by attaching 3-(9′-mercaptononylthio)-1,1,1-trifluoropropan-2-one to epoxy-activated Sepharose CL4B (Fig. 1) and tested its suitability for affinity purification of NTE. NTE was found to bind slowly (Fig. 2) in a manner that was both specific (Fig. 3) and saturable (Fig. 4). However, many additional proteins were found to bind non-specifically to MNTFP-Sepharose in a manner that was insensitive to competition with the soluble trifluoromethylketone OTFP. NTE binding to MNTFP-Sepharose is sensitive to organophosphate inhibitors of NTE which is consistent with a model in which the ligand forms a covalent bond with the active site serine of NTE. However, the ability to elute NTE from the affinity gel under denaturing conditions suggests that the bond must be very unstable and rapidly reversible in the absence of a catalytic site.

A strategy of differential inhibition prior to binding identified only one protein that bound to the affinity resin in an NTE-like paraoxon-insensitive, paraoxon + mipafox-sensitive manner (Fig.3). This NTE-like protein migrated on SDS-PAGE with the same apparent molecular weight as the a 155-kDa protein that is labeled with [^{3}H]DFP in an NTE-like manner [1–3]. Binding of the 155-kDa protein was also inhibited by 8-octylthio-1,1,1-trifluoropropan-2-one (OTFP), which inhibits NTE activity and has the same basic structure as the affinity ligand. This inhibition of binding by inhibitors of NTE activity suggests that NTE specifically binds the affinity ligand at its active site.

The primary strategy used to elute NTE from MNTFP-Sepharose was to competitively elute it using a high concentration of a soluble trifluoromethyl ketone inhibitor of NTE. However, neither OTFP (soluble ligand) nor DFP, an irreversible organophosphate inhibitor of NTE, were sufficient to displace NTE. Therefore, a variety of other conditions were tested, in combination with OTFP, for their ability to enhance dissociation of NTE from the affinity resin. These included lowering the NaCl concentration, raising the glycerol concentration, adding detergents such as SDS and Triton X-100, changing the pH, adding organic solvents such as dimethylformamide and dimethylsulfoxide, and adding urea. The only conditions which successfully eluted the 155-kDa protein were those which irreversibly inactivated NTE. The failure to elute active NTE from MNTFP-Sepharose prompted investigation of less inhibitory trifluoromethyl ketones as affinity ligands. Affinity gels with the less inhibitory ligands, 3-(4′-mercaptobutylthio)-1,1,1-trifluoropropan-2-one and 3-(6′-mercaptohexylthio)-1,1,1-trifluoropropan-2-one (MBTFP- and MHTFP-Sepharose, respectively), bound significantly less NTE activity while still binding high levels of the non-specifically bound proteins. This lowered the fraction of NTE in the bound fraction and reduced or eliminated our ability to detect a specific 155-kDa band following SDS-PAGE. Given the lower yields of bound NTE and lower specificity

for NTE binding observed with both MBTFP-Sepharose and MHTFP-Sepharose further attempts to specifically elute NTE from these gels were not pursued.

The slow binding of NTE to MNTFP-Sepharose and the failure to elute NTE under non-denaturing conditions is inconsistent with the fact that the trifluoromethylketones have been shown to be rapidly reversible inhibitors of NTE [8]. Nevertheless, differential inhibition experiments indicated that NTE does bind specifically to MNTFP-Sepharose through an interaction between the ligand and the active site of NTE. We looked for additional non-specific interactions between NTE and the spacer arm attached to Sepharose CL4B but no binding of NTE or any other protein was observed. One likely explanation for the specific and tight association of NTE with the MNTFP-Sepharose affinity gel is that NTE is bound both specifically at its active site and non-specifically through hydrophobic interactions with other ligands. If this explanation is correct then preparing MNTFP-Sepharose with a much lower density of ligand might improve our ability to elute active NTE under non-denaturing conditions.

The advantage of affinity chromatography is that proteins can generally be eluted under physiological conditions using a soluble ligand to compete with the immobilized ligand. This makes affinity chromatography both a very specific and a gentle method for purifying proteins. So far, the affinity method described here has failed to provide a purified preparation of active NTE. The only conditions which have succeeded in eluting NTE have been denaturing. Nevertheless, the application of this affinity purification method has led to some important progress. Inactive NTE can now be obtained in one simple step from crude detergent extracts at a sufficient level of purity that it can be identified in polyacrylamide gels after staining with either silver or Coomassie Blue. Differential inhibition experiments indicate that the MNTFP-Sepharose bound, NTE-like 155-kDa band is most likely a single homogeneous protein. If this is confirmed then it should be possible to purify NTE by preparative SDS-PAGE of the MNTFP-Sepharose bound fraction. In a preliminary experiment, recently presented by Mackay et al. [19] from this laboratory, this method was tested for its ability to isolate sufficient quantities of NTE for amino terminal sequencing. Amino acid analysis confirmed that 15 μg of the 155-kDa protein were recovered after electroelution from a 3-mm-thick polyacrylamide gel onto a ProBlot membrane. This experiment therefore demonstrates the feasibility of using MNTFP-Sepharose affinity chromatography and preparative SDS-PAGE to isolate NTE for amino acid sequencing. Once this information is obtained a search of the protein sequence databases may reveal that NTE is a unique and as yet uncharacterized protein or that NTE is a previously characterized protein or even part of a known family of proteins. Either finding will open up new avenues of investigation into the characteristics of NTE and the mechanisms causing OPIDN.

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