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CHROMATOGRAPHIC CHARACTERIZATION OF NEUROTOXIC ESTERASE

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Abstract—Neurotoxic esterase (neuropathy target enzyme, NTE) is an enzyme whose irreversible inhibition is the apparent first step in the induction of organophosphorus-induced delayed neuropathy. NTE is an integral membrane protein and thus must be solubilized before isolation can be attempted. This study describes solubilization of active chicken brain NTE with the nondenaturing detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and characterization of the detergent-solubilized enzyme by gel exclusion chromatography. When detergent-solubilized membranes were chromatographed on Sepharose gel exclusion media, NTE activity eluted with an apparent molecular weight of 880–970 kD. When [^{3}H]diisopropylphosphorofluoridate-radiolabeled membranes and unlabeled microsomal membranes were CHAPS-solubilized, combined and chromatographed on Sepharose 4B, NTE activity coeluted with two radiolabeled proteins (M_r = 148 kD and M_r = 112 kD using sodium dodecyl sulfate-polyacrylamide gel electrophoresis with reducing conditions). Another radiolabeled protein (M_r = 92 kD) coeluted exclusively with inhibitor-resistant esterase activity. This study provides strong evidence that the 148 and 112 kD proteins are subunits of a multicomponent NTE complex.

Organophosphorus (OP) compounds, widely used in agriculture and industry, pose significant health hazards throughout the world [1, 2]. Acute neurotoxicity from exposure to OP compounds is characterized by autonomic and central nervous system hyperexcitability and is due to inhibition of acetylcholinesterase (AChE), the enzyme responsible for hydrolysis of the neurotransmitter acetylcholine [3]. Delayed neurotoxicity from exposure to some OP compounds is expressed as progressive degeneration of certain long nerve tracts in the central and peripheral nervous systems [4–7], a syndrome referred to as organophosphorus-induced delayed neuropathy (OPIDN). While OP compounds which cause OPIDN may be potent AChE inhibitors, AChE inhibition is not essential for its development [8]. Conversely, extensive (greater than 65%) inhibition of neurotoxic esterase (NTE), another serine esterase, is highly correlated with the development of OPIDN [9–12].

NTE activity is measured *in vitro* by a differential assay based on its relative "sensitivity" to inhibition by mipafox (an OP which causes OPIDN) and "insensitivity" to inhibition by paraoxon (an OP which does not cause OPIDN) [13]. The ability of an OP compound to inhibit NTE at least 65% and then undergo "aging" (i.e. loss of alkyl group from the covalently-bound residue) has proven to be an accurate indicator of the delayed neuropathic potential of numerous OP compounds [7, 14]. Little is known, however, regarding the physiological role of NTE or how the irreversible binding of a neuropathic OP compound to NTE and subsequent aging may alter neuronal function and stability. Isolation and characterization of NTE could clarify its role in pathological as well as normal conditions.

Because NTE is an integral membrane protein, any isolation procedure must begin with solubilization. Solubilization of active NTE has been achieved with the detergents sodium cholate [15], Triton X-100 [16, 17] and *n*-octyl-glucoside [18, 19] and with the organic solvent dimethyl sulfoxide [20]. A systematic study of detergent solubilization of the enzyme has been published recently [21]. In some of the above studies, isolation of the solubilized NTE was attempted. Ishikawa and coworkers [17] partially separated paraoxon-sensitive and mipafox-sensitive phenyl valerate hydrolases from Triton X-100 solubilized chicken brain membranes by sucrose density gradient centrifugation. Partial separation by gel exclusion chromatography of detergent solubilized NTE from other hen brain phenyl valerate hydrolases was reported by Chemnitius *et al.* [19]. Neither of these studies, however, reported enrichment of NTE specific activity by these procedures. This study describes the solubilization of chicken brain NTE with the nondenaturing detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) [22] followed by gel exclusion chromatography of the detergent-solubilized enzyme.

METHODS

Chemicals. Paraoxon (diethyl-*p*-nitrophenyl phosphate) was purchased from the Aldrich Chemical

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Co. (Milwaukee, WI). Mipafox (*N,N'*-diisopropylphosphorodiamidic fluoride) was purchased from Chemsyn Science Laboratories (Lenexa, KS). Phenyl valerate was synthesized by Dr. S. Wyrick of the University of North Carolina at Chapel Hill, NC. Protosol and [1,3-^{3}H]-diisopropylphosphorofluoridate (4.1 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Sodium dodecyl sulfate (99% purity) was purchased from the Fluka Chemical Corp. (Hauppauge, NY). Sepharose 4B, Sepharose 6B and CHAPS were purchased from the Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade.

Assay of esterase activity. NTE activity was assayed essentially by the method of Johnson [13] as modified by Soliman *et al.* [23], but the reaction volume was reduced 4-fold to 0.75 ml. Preincubation with paraoxon and/or mipafox was for 20 min at 37° and incubation with substrate was for 30 min; with dilute samples (e.g. Sepharose fractions) incubation was usually extended to 60 min. Nonenzymatic hydrolysis was always monitored and used as a blank for enzymatic hydrolysis. Paraoxon-sensitive esterase (PSE) activity was measured as phenyl valerate hydrolysis which was inhibited by 40 μM paraoxon. NTE activity was defined as phenyl valerate hydrolysis resistant to 40 μM paraoxon but sensitive to 50 μM mipafox. Inhibitor-resistant esterase (IRE) activity was defined as phenyl valerate hydrolysis which was resistant to both OP inhibitors at the given concentrations. One unit of esterase activity was defined as that amount hydrolyzing 1 nmole of phenyl valerate/min at 37°. Mipafox IC_{50} values were determined at a paraoxon concentration of 100 μM, whereas the mipafox concentration was varied between 1 and 70 μM.

Solubilization of native neurotoxic esterase. Adult (1–2 years, 1.5–2 kg body weight), female White Leghorn chickens (Feather Down Farms, Apex, NC) were used throughout. Hens were decapitated, and freshly dissected brain was weighed and homogenized on ice in 4 vol. (w:v) of ice-cold phosphate buffer (0.1 M potassium phosphate, pH 7.2, containing 0.2 M potassium chloride and 1 mM disodium EDTA). This homogenate was centrifuged at 12,000 *g* for 5 min at 4°. The resulting supernatant (S1) was used for solubilization trials with the detergent CHAPS. The protein concentration of S1 was diluted to 5 mg/ml [24] with phosphate buffer and detergent (10% in phosphate buffer) was added to give final detergent concentrations of 0.01, 0.03, 0.10, 0.30, 1.00, and 3.00%. These protein-detergent suspensions were mixed gently with magnetic stir bars at 0° for 30 min, followed by centrifugation at 100,000 *g* for 60 min. The supernatants were collected and the pellets were resuspended in phosphate buffer containing the appropriate detergent concentration. Aliquots of the original suspension, solubilized protein (100,000 *g* supernatant) and nonsolubilized protein (100,000 *g* pellet) were assayed for protein content and for esterase activity. When NTE activity was solubilized from crude microsomal membranes, the S1 fraction was centrifuged at 100,000 *g* for 60 min, the pellet (microsomal membranes) was resuspended in the original S1 volume with phosphate buffer containing 0.3% CHAPS and solubilized as before. Percent solubilization was calculated as the [enzyme activity in the 100,000 *g* supernatant/the activity in the original suspension] × 100. Percent recovery was calculated as [(the activity in the 100,000 *g* supernatant + the activity in 100,000 *g* pellet)/activity in the original suspension] × 100.

Gel exclusion chromatography of detergent-solubilized neurotoxic esterase. Sepharose 4B or Sepharose 6B was suspended in CHAPS solubilization buffer (phosphate buffer plus 0.3% CHAPS) and degassed before preparing a column (3 cm × 77 cm). The column was calibrated with proteins of known molecular weight (thyroglobulin, 660 kD; ferritin, 440 kD; catalase, 232 kD; aldolase, 158 kD) using the same elution buffer. The 100,000 *g* supernatant fraction from CHAPS-treated microsomal membranes (S3) was applied to the column and eluted with the above buffer. Absorbance at 280 nm was monitored, and fractions were assayed for PSE, NTE and IRE activities.

Labeling of CHAPS-solubilized proteins with [^{3}H]DFP and subsequent electrophoretic separation. NTE activity was solubilized with 0.3% CHAPS as described before, and 0.25 ml of the soluble preparation (S3) was preincubated with 0.75 ml of 50 mM Tris–hydroxyaminomethane (Tris–HCl) buffer (pH 8.0, 25°) containing 0.2 mM EDTA with either (a) no inhibitors, (b) 100 μM paraoxon, or (c) 100 μM paraoxon plus 50 μM mipafox for 30 min at 37°. Following incubation, [^{3}H]DFP was added (2.5 μM final concentration, 10 μCi/ml), and the incubation was continued for an additional 60 min. The incubation was stopped by transferring the tubes to an ice bath. Samples (0.1 ml) were mixed with 0.025 ml of gel solubilization buffer (0.1 M Tris–HCl, pH 7.4, containing 5 mM 2-mercaptoethanol, 5% sodium dodecyl sulfate (SDS), 5 mM EDTA, 30% glycerol and 0.025% bromophenol blue) and separated by discontinuous polyacrylamide slab gel electrophoresis (5.0, 6.0 or 7.5% acrylamide gels) according to the method of Laemmli [25]. To locate the radiolabeled proteins on the gel, slices (1.1 mm) were made of the individual lanes with a Bio-Rad Electric Gel Slicer (model 195) and solubilized overnight at 37° in scintillation vials with 0.5 ml Protosol. Xylene-based scintillation fluid (5 ml) was added, and samples were counted by liquid scintillation at 29% efficiency. Molecular weights of unknown proteins were estimated from their migration relative to standards (Bio-Rad high molecular weight standards).

[^{3}H]DFP labeling of intact membranes prior to chromatography. Microsomal membranes were preincubated in Tris–HCl buffer, pH 8.0, containing 0.2 mM EDTA with 100 μM paraoxon for 4 hr at 0°. The suspension was then divided into two samples: [^{3}H]DFP (10 μCi/ml, 2.5 μM final concentration) was added to one sample (radiolabeled membranes), while an equivalent amount of buffer was added to the other (unlabeled membranes). Following incubation at 0° for 16 hr, both samples were centrifuged at 100,000 *g* for 60 min. The pellets were surface washed, resuspended in the original S1 volume with phosphate buffer, and solubilized with 0.3% CHAPS as before. Solubilized proteins from both labeled and unlabeled membranes were combined and con-

centrated by filtration in a stirred cell at 4° (Amicon YM-100 membrane, 100 kD cut-off) prior to chromatography on Sepharose 4B. Alternate fractions from the column were concentrated by filtration and either assayed for esterase activity or separated on 5% polyacrylamide gels and analyzed for radiolabeling.

RESULTS

Solubilization. Enzymatically active, soluble (i.e. not pelleted after centrifugation at 100,000 *g* for 60 min) NTE was obtained from hen brain fractions following treatment with 0.1–3.0% CHAPS in a phosphate buffer (Fig. 1). Optimal solubilization was achieved with 0.3–1.0% CHAPS. Treatment of crude microsomal membranes with CHAPS (0.3%, 30 min at 0°) resulted in 41 ± 4% (mean ± SD) solubilization with 89 ± 4% recovery of the original enzyme activity. The mipafox IC_{50} values of the membrane bound and CHAPS-solubilized NTE were identical (5 μM; data not shown).

Although CHAPS-solubilized NTE activity was relatively stable at 0–4° with 60–70% activity remaining after 7 days, simple manipulations caused substantial loss of activity. The 100,000 *g* superntant fraction from CHAPS-treated membranes (S3) dialyzed overnight (6 kD cut-off dialysis membrane) against solubilization buffer lost 43–85% of its NTE activity. Extensive losses (70–90%) in NTE activity of the S3 fraction were seen with hyperbaric ultrafiltration (nitrogen, 55 mm Hg) in a stirred cell. Adding back the ultrafiltrate to the concentrate failed to regain activity. Ultrafiltration (nitrogen, 55 mm Hg) of the [^{3}H]DFP-labeled S3 fraction resulted in accumulation of radiolabel on the membrane. Concentration of NTE activity was possible without substantial loss of activity, however, with filtration in the same apparatus under gravity rather than under hyperbaric conditions.

Chromatography. When standard proteins were chromatographed on Sepharose 4B, a plot of V_e/V_0 (i.e. elution volume of a standard protein divided by column void volume) versus log molecular weight was linear and was used to estimate the molecular weights of unknown proteins (Fig. 2A). NTE activity eluted in two peaks; one peak was associated with the void volume while the other peak eluted with an estimated molecular weight of 880 kD (Fig. 2B). [Sepharose 6B chromatography of the S3 fraction yielded an estimated molecular weight of 970 kD for NTE (data not shown)]. IRE and PSE activities eluted with estimated molecular weights of 270 and 158 kD, respectively, resulting in partial separation of NTE, IRE and PSE activities. PSE activity represented the majority of the detergent-solubilized phenyl valerate hydrolase activity (note difference in scales for esterase activities in figure legend). Whereas NTE and PSE activities eluted in a reproducible pattern from one separation to another, IRE activity showed a more variable elution profile. NTE specific activity was increased about 2-fold from 65 units NTE activity/mg protein in the S3 fraction to 138 units NTE activity/mg protein in the concentrated Sepharose pool (fractions 34–40, Fig. 2B). Specific activity before solubilization (i.e. crude microsomal membranes) was estimated at 82 units/mg protein.

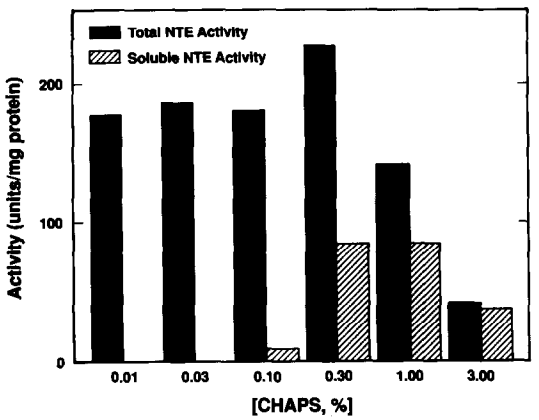

Fig. 1. Solubilization of chicken brain neurotoxic esterase activity with CHAPS. Fresh brain was homogenized in phosphate buffer, centrifuged, and the protein concentration of the supernatant (S1) adjusted to 5 mg/ml with buffer and CHAPS (10% in phosphate buffer) as described in Methods. One unit of esterase activity is defined as that amount hydrolyzing 1 nmole of phenyl valerate/min at 37°. The specific activity of the starting material (i.e. S1) was 212 units/mg protein.

[^{3}H]*DFP labeling of membrane proteins.* Labeling of the CHAPS-treated microsomal membrane soluble fraction (S3) with [^{3}H]DFP after preincubation with either (a) no OP inhibitors (b) 100 μM paraoxon or (c) 100 μM paraoxon plus 50 μM mipafox revealed one major [^{3}H]DFP-binding site that was resistant to paraoxon but sensitive to mipafox (Fig. 3). This protein had an apparent molecular weight of 148–160 kD on either 5.0, 6.0 or 7.5% SDS-polyacrylamide gels. Another minor [^{3}H]DFP binding protein (M_r = 112 kD) was detected occasionally. By combining the S3 fraction from membranes that were preincubated with paraoxon and then labeled with [^{3}H]DFP with a similarly prepared, unlabeled S3 fraction, both esterase activity and protein-bound radiolabel could be followed concurrently by gel exclusion chromatography. When assayed for esterase activity, NTE eluted as expected with an estimated molecular weight of about 900 kD (Fig. 4A). When individual fractions possessing NTE activity were analyzed for covalently bound radiolabel by polyacrylamide gel electrophoresis (PAGE), the [^{3}H] radiolabel was confined to two proteins with molecular weights of 148 kD and 112 kD under denaturing and reducing conditions (i.e. SDS-PAGE). In contrast, another radiolabeled protein (M_r = 92 kD, SDS-PAGE) coeluted exclusively with IRE activity (Fig. 4B).

DISCUSSION

Active NTE, solubilized from hen brain microsomal membranes with the zwitterionic detergent CHAPS, exhibited mipafox sensitivity similar to the unsolubilized NTE activity. Davis and Richardson [21] have reported solubilization of ≤35% of the hen brain microsomal NTE activity also using CHAPS detergent but in a non-buffered system. CHAPS-solubilized NTE activity was relatively stable when

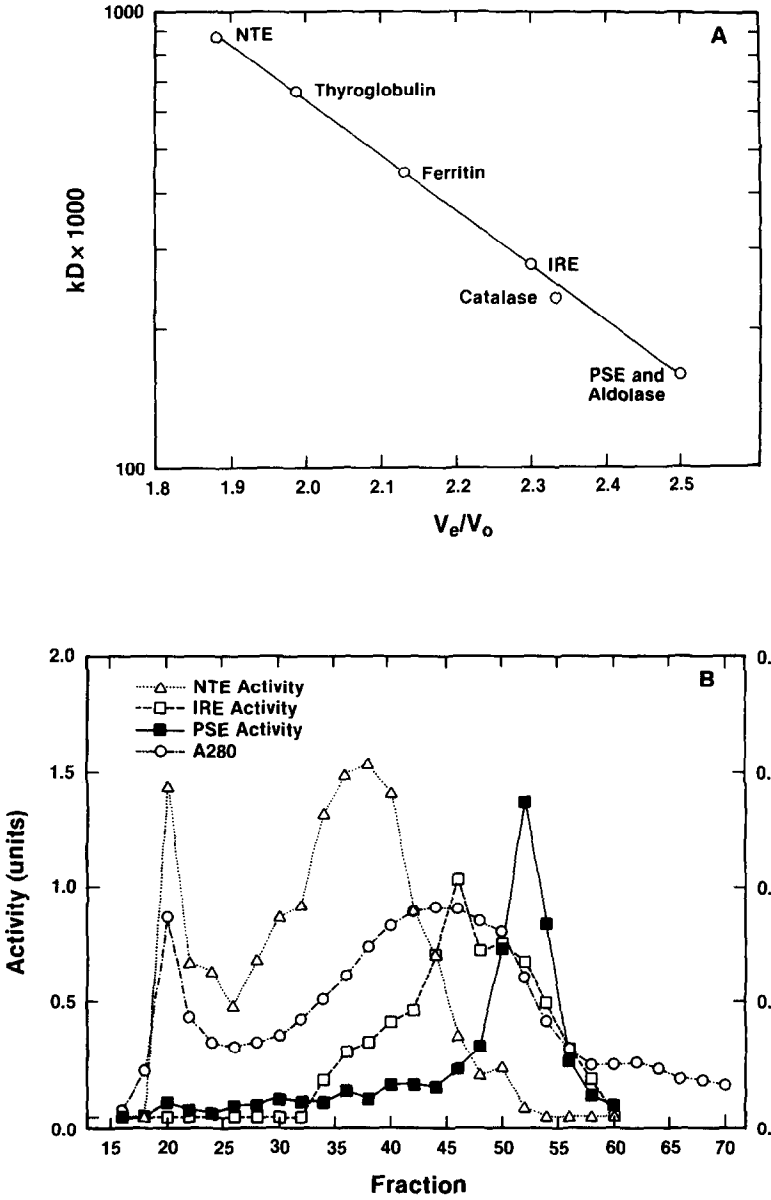

Fig. 2. Sepharose 4B chromatography. (A) Calibration curve for the Sepharose 4B column. Purified standard proteins of known molecular weight (thyroglobulin, 660 kD; ferritin, 440 kD; catalase, 232 kD; and aldolase, 158 kD) were used to calibrate the column (3 cm × 77 cm), and a plot of V_e/V_0 (i.e. elution volume/void volume) versus log molecular weight was constructed. Void volume (V_0) of the column was estimated at 147 ml. (B) Sepharose 4B chromatography of CHAPS-solubilized esterases. Seven milliliters of the 100,000 *g* supernatant fraction from CHAPS-solubilized membranes (S3) was applied to the column and eluted in phosphate buffer containing 0.3% CHAPS. NTE, IRE and PSE activities were assayed, and absorbance at 280 nm was monitored in individual fractions. NTE and IRE activities are expressed as units esterase activity/ml, whereas PSE activity is expressed as units esterase activity/0.1 ml.

stored at 0–4°, yet a remarkable loss of NTE activity was noted following simple manipulations such as dialysis against the same buffer or ultrafiltration. NTE activity could be concentrated without extensive loss of activity by filtration under gravity using the same membrane that resulted in 70–90% loss of activity when done under pressure. Coincidentally, when the [^{3}H]DFP-labelled S3 fraction was subjected to ultrafiltration under pressure, radiolabel accumulated on the membrane. These observations suggest that CHAPS-solubilized NTE has an affinity for dialysis tubing, filters, etc., and that it aggregates under pressure, in which case either condition could impair recovery of enzyme activity. Nonspecific binding and aggregation appear to be common problems in the study of membrane-bound esterases [27].

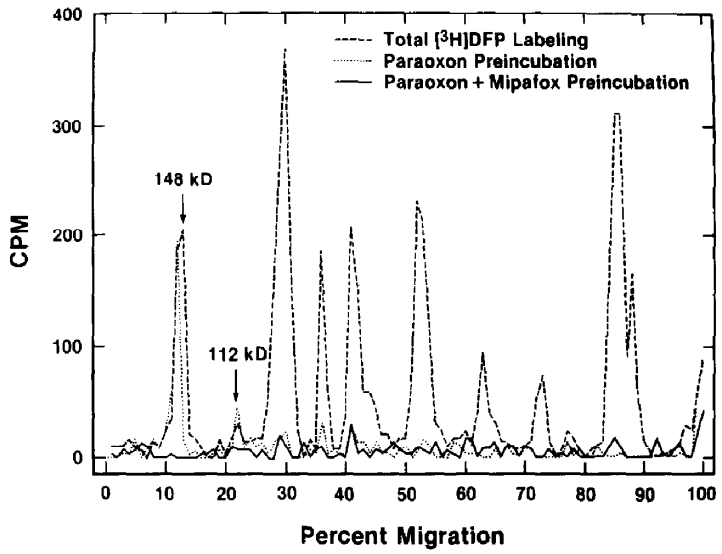

Fig. 3. Labeling of CHAPS-solubilized proteins with [^{3}H]diisopropylphosphorofluoridate (DFP) and subsequent electrophoretic separation. The soluble fraction (S3) was labeled with [^{3}H]DFP after preincubation with either (a) no OP inhibitors (b) 100 μM paraoxon or (c) 100 μM paraoxon plus 50 μM mipafox. Proteins were separated by discontinuous SDS-PAGE, and radiolabeling was determined in gel slices as described in Methods.

The presence of NTE activity in the void volume (Figs. 2B and 4A) suggests that a proportion of the solubilized NTE activity exists as a large aggregate. Void volume peaks of IRE activity were also occasionally observed. Indeed, some of the molecular weight marker proteins showed void volume elution peaks. Due to this aggregation, the esterases eluting in the void volume may exhibit different [^{3}H]DFP binding characteristics relative to those eluting in the fractionation range. For example, the void volume peak of IRE activity showed negligible [^{3}H]DFP labeling of the 92 kD protein relative to the fractionated IRE activity (Fig. 4B). Alternatively, the lack of 92 kD radiolabeling in the void volume peak of IRE activity could indicate the presence of another inhibitor-resistant esterase which does not bind [^{3}H]DFP under the present conditions.

Our estimate of the molecular weight of NTE is lower than that of Chemnitius *et al.* [19], who, using gel exclusion chromatography, calculated the molecular weight of *n*-octyl-glucoside solubilized hen brain NTE to be 1.8 million daltons. Although these differences in size of the solubilized enzyme could be due simply to the relative size of the micellar structure formed by the different solubilization protocols, another factor to consider is the gel exclusion medium used in the two studies. In the investigation by Chemnitius *et al.* [19], Sephacryl S-300 medium was used, which has a useful working range between 10,000 and 800,000 daltons [28], whereas we used Sepharose 4B for our gel exclusion chromatography because its effective fractionation range (60,000 daltons to 20,000,000 daltons [28]) was more appropriate for separation of proteins larger than 800,000 daltons. In addition, chromatography of CHAPS-solubilized NTE activity on Sepharose 6B (with an effective fractionation range between 10,000 and 4,000,000 daltons) yielded an apparent molecular weight for NTE of 970 kD. Regardless of the true size of the micellar form, detergent-solubilized NTE appears to exist as a large macromolecular complex consisting of multiple components. This large size may indicate that a certain degree of membrane integrity is necessary for solubilized NTE activity, and may underlie some of the difficulties encountered in isolation of active NTE.

Ishikawa *et al.* [17] reported separation of mipafox-sensitive and paraoxon-sensitive esterases by sucrose density centrifugation. In the present study, CHAPS-solubilized NTE was partially separated from paraoxon-sensitive and inhibitor-resistant esterases by gel exclusion chromatography. It is apparent that these operationally-defined esterase activities are chromatographically separable.

Multiple [^{3}H]DFP-binding sites were present in the CHAPS-solubilized proteins but only one (M_r = 148–160 kD) was detected consistently by SDS-PAGE as paraoxon-resistant, mipafox-sensitive (i.e. inhibitor characteristics of NTE). This 148–160 kD protein is most likely the 155–178 kD protein previously proposed to be the active site subunit of NTE [29–33].

When gel exclusion chromatography was performed on proteins that were labeled with [^{3}H]DFP so that esterase activity and radiolabel could be monitored simultaneously, two radiolabeled proteins (M_r = 148 kD and 112 kD, SDS PAGE) coeluted exclusively with NTE activity. The 148 kD protein eluted with approximately four times the radiolabel of the 112 kD protein, suggesting that the 112 kD protein exists in a lower concentration or has a lower affinity for DFP; thus, its presence was more difficult

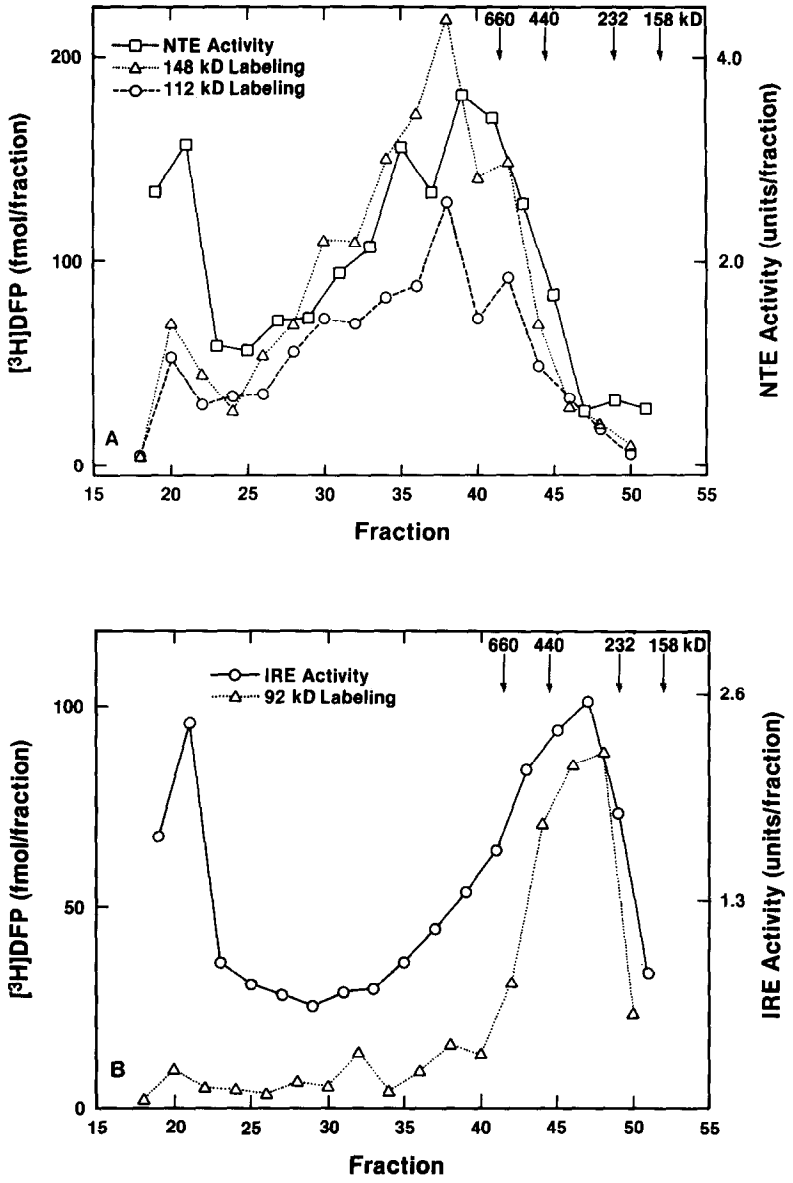

Fig. 4. Sepharose 4B chromatography of CHAPS-solubilized [^{3}H]DFP-labeled esterases. Crude microsomal membranes were preincubated with 100 μM paraoxon and then divided into two samples: one sample was labeled with [^{3}H]DFP, the other with buffer only. Following solubilization, the 100,000 g supernatants (S3) were combined and chromatographed on Sepharose 4B. Column fractions were concentrated and assayed either for esterase activity or for covalently bound radiolabel as described in Methods. The amount of radiolabel in each protein was estimated by the trapezoidal rule for area under the curve [26]. (A) Distribution of NTE activity and radiolabeling in the 148 kD and 112 kD proteins. NTE activity is expressed as units/7-ml fraction. Radiolabeling of the 148 kD protein is expressed as fmol [^{3}H]DFP bound/fraction, whereas radiolabeling of the 112 kD protein is expressed as 2 × (fmol [^{3}H]DFP bound/fraction). (B) Distribution of IRE activity and radiolabeling in the 92 kD protein. IRE activity is expressed as units/7-ml fraction, whereas radiolabeling of the 92 kD protein is expressed as fmol [^{3}H]DFP bound/fraction.

to detect. While the 148 kD protein is probably the same paraoxon-resistant, mipafox-sensitive DFP-binding protein previously noted by other investigators, the 112 kD protein may be identical to a minor DFP-binding protein (M_r = 115 kD) reported [32] to be sensitive to mipafox but unaffected by paraoxon. Using radiation inactivation, Carrington *et al.* [34] estimated the target size of NTE activity to be 105 kD, providing evidence that this smaller protein may possess the esterase activity.

Another radiolabeled protein (M_r = 92 kD, SDS-PAGE) coeluted with IRE activity. Williams and Johnson [29] reported a 92 kD paraoxon-resistant, DFP-binding protein that may be identical to the

92 kD IRE-associated protein that we detected. All three of these DFP-labeled proteins have, therefore, been reported previously, but their relationships to the various esterase activities have not been well defined.

Coelution of two [^{3}H]DFP-radiolabeled proteins with the NTE activity in gel exclusion chromatography suggests that two phosphorylation sites are associated with NTE and thus are potential sites for the initiation of OPIDN. One or both of the [^{3}H]DFP-labeled proteins associated with NTE activity may be the active site subunit(s) responsible for hydrolysis of carboxylesters: both of these proteins are radiolabeled with DFP in a paraoxon-resistant, mipafox-sensitive manner. The role of these two proteins in catalytic function and the development of OPIDN remain to be elucidated.

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