Enzymes of the Cholinesterase Family

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IDENTIFICATION OF A 155 kDa FRACTION THAT POSSESSES NEUROPATHY TARGET ESTERASE ACTIVITY

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Neuropathy target esterase (NTE) is a protein that hydrolyses phenyl valerate in the presence of paraoxon, but is inhibited by mipafox. Many consider it the putative site of action for organophosphorus compounds that cause organophosphate induced delayed neuropathy (OPIDN). Williams and Johnson (1981) suggested that NTE was a 155 kDa protein. Thomas et al. (1993) used 3-(9'-mercaptononylthio)-1,1,1-trifluoropropan-2-one (MNTFP) bound to sepharose to obtain evidence that this protein was responsible for NTE activity. Recently, Glyn et al. (1994) isolated a 155 kDa peptide with a biotinylated saligenin phosphate analog. To date, no one has isolated the active enzyme.

The biological source for NTE was whole brains of 18-day old chick embryos. They were homogenized, centrifuged at 10,000xg for 20 minutes and the microsomes separated by centrifugation of the supernatant at 100,000xg for 60 min. NTE activity was solubilized from the resultant pellet by phospholipase A_2 treatment (Seifert and Wilson 1994).

Initial purification of NTE was based on gel filtration chromatography using a 5x100 cm S-400 HR column (Pharmacia). Solubilized chick brain extracts of 1 ml were combined 1:1 with a tris buffer containing 500 mM NaCl and 0.3% Triton X-100 or 0.1% W1 and applied to the gel filtration column. Samples were eluted with the same tris buffer used to dilute the extract. Comparing this to known commercial standards, it was possible to determine the mass of the activity to be about 200 ± 30 kDa. The Mipafox inhibition curves suggest it is the same enzyme responsible for the NTE activity in the initial extract. This is the first report of NTE partitioning in gel filtration with a mass less that about 1,000 kDa.

Subsequent purification involved preparative isoelectric focusing (IEF) and agarose native gel electrophoresis (AGE). IEF was performed on the 200 kDa eluates using a Rotofor (Biorad) containing 40 ml water and 5% 3/10 Biolyte. This permitted the isolation of the NTE activity within the precipitate at a pI of 4.5±0.6 and showed a 5-fold concentration of NTE over gel exclusion and a 4-fold increase over the initial extract.

Subsequent AGE separation on a 0.75% agarose gel containing 0.1% W1 and 250 mM sucrose demonstrated two distinct NTE bands: one that remained within 1 cm of the top of the gel with a PV hydrolase activity that was exclusively NTE, and another that ran with

the solvent front in which PV hydrolase activity accounted for only 12% of the total present. Analysis of the mipafox inhibition curves for NTE in these two fractions showed that the smaller one possessed a greater sensitivity to the inhibitor with an I50 of 85±10 μM compared to 280±24 μM for the larger.

The identity of the NTE activity was confirmed using immobilized MNTFP bound to sepharose. There was a time-dependant loss of NTE activity from the solution when the solubilized protein from the larger fraction was combined with the ligand that was not seen with the smaller NTE fraction. Elution of the ligand with Laemmli buffer and subsequent SDS-PAGE demonstrated a single protein band with an apparent mass of 138-157 kDa. This is in agreement with the observations of Williams and Johnson (1981), Thomas et al. (1993) and Glyn et al. (1994) suggesting that the activity in the larger band was exclusively NTE.

These results suggest that the earlier reports of solubilized NTE possessing a mass of greater than 1,000 kDa were the result of a hydrophobic aggregation and that the NTE activity observed was due to a 200 kDa subunit of that mass. Interestingly, although the fractions of NTE activity in our study possess the same approximate pIs, they exhibit different mipafox inhibition profiles and ligand affinities. The approach presented here will permit kinetic analysis on the natural enzyme to further characterize its activity and seek to identify its endogenous substrate.

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Front cover: A model of the complex between the snake venom toxin fasciculin (light color) and acetylcholinesterase (dark color), constructed by H.K.L. van den Born *et al.* [(1995) *Protein Science 4*, 703–715]. Photography by Z. Radić of the University of California at San Diego.

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