Synthesis and characterization of a biotinylated organophosphorus ester for detection and affinity purification of a brain serine esterase: neuropathy target esterase

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We have synthesized a novel stable precursor, saligenin phosphorotrichloridate, which, on reaction with *N*-monobiotinyldiamines, generates a series of biotinylated covalent inhibitors of serine esterases. A homologue designated S9B [1-(saligenin cyclic phospho)-9-biotinyldiaminononane] was selected to allow detection and rapid isolation of neuropathy target esterase (NTE). This enzyme is the primary target site for those organophosphorus esters (OPs) which cause delayed neuropathy. NTE comprises about 0.03% of the total protein in brain microsomal fractions and has resisted purification attempts over many years. S9B is a potent progressive inhibitor of NTE esteratic activity (second-order rate constant 1.4×10^7 $M^{-1} \cdot min^{-1}$). Incubation of S9B with brain microsomes led to specific covalent labelling of NTE as determined by detection of a biotinylated 155 kDa polypeptide on Western blots. Specificity of S9B labelling was further demonstrated by inhibition with the neuropathic OP mipafox. Biotinyl-NTE in SDS-solubilized S9B-labelled microsomes was adsorbed on to avidin–Sepharose and subsequently eluted, yielding a fraction enriched approx. 1000-fold in NTE by a single step with recoveries of 30%. Essentially pure NTE was obtained after separation from two endogenous biotinylated polypeptides (120 and 70 kDa) in avidin–Sepharose eluates by preparative SDS/PAGE. Other biotinylated saligenin phosphoramidates derived from the same precursor may be useful for detection and isolation of other serine esterases and proteinases.

INTRODUCTION

Organophosphorus esters (OPs) such as di-isopropyl fluorophosphate (DFP) have been widely used as inhibitors in biochemical studies of serine esterases and proteases (Aldridge and Reiner, 1972; Powers and Harper, 1986). On a much larger scale, OPs are used as pesticides and industrial intermediates. Certain OPs induce a delayed neuropathy with degeneration of spinal and peripheral nerve axons and resulting paralysis (Johnson, 1975a; Lotti, 1992). The primary target protein modified by neuropathic OPs is neuropathy target esterase (NTE). NTE was originally defined as a population of [^{32}P]DFP labelling sites and as an esterase activity in brain homogenates; the labelling sites and the esterase can be blocked by a neuropathic OP such as mipafox, but not by a non-neuropathic OP such as paraoxon (Johnson, 1969a,b). Later, SDS/PAGE of [^{3}H]DFP-labelled brain microsomes indicated that NTE had a subunit mass of 155 kDa (Williams and Johnson, 1981; Thomas et al., 1989); this is twice the subunit mass of better-characterized esterases such as acetylcholine esterase and endoplasmic-reticulum carboxyesterases.

Elucidation of the structure, subcellular localization and normal functions of NTE are of interest, not only for toxicology, but also for fundamental neurobiology, since the protein may play a role in normal axonal maintenance (Lotti, 1992). However, progress in this area has been very limited for many years, as NTE has resisted purification attempts by several laboratories. This is primarily due to low abundance ([^{3}H]DFP labelling indicates that NTE comprises about 0.03% of brain microsomal protein; Williams and Johnson, 1981), and poor recovery of detergent-solubilized NTE esterase activity on chromatographic fractionation, possibly due to loss of an activating factor (Pope and Padilla, 1989). Similarly, ion-exchange, hydrophobic-interaction, metal-chelate, hydroxyapatite and lectin-affinity chromatography were ineffective for the isolation of [^{3}H]DFP-labelled NTE, while a combination of procedures ending with preparative SDS/PAGE yielded a fraction in which NTE comprised 2% of the total protein (Ruffer-Turner et al., 1992).

It has become clear that isolation of NTE requires development of active-site-directed reagents which will allow use of a highly selective affinity-chromatography step. Recently, Kay et al. (1992) described the synthesis of biotinylated aminoacylchloromethanes (aminoalkyl chloromethyl ketones) for the detection of serine proteinases. A biotinylated covalent inhibitor of a serine proteinase or esterase allows the possibility not only for detection of the enzyme by Western blotting, but also for selective isolation via capture of the specifically biotinylated protein on solid-phase avidin matrices. We describe here the synthesis of a novel stable precursor from which a series of biotinylated OPs can be easily generated, and we demonstrate the use of one homologue, S9B, for detection and affinity isolation of NTE. A preliminary report of this work has appeared in abstract form (Johnson and Glynn, 1993).

Abbreviations used: OP, organophosphorus ester; NTE, neuropathy target esterase; DFP, di-isopropyl fluorophosphate; TFMK, trifluoromethyl ketone; SCl_3, saligenin phosphorotrichloridate; 9B, 1-biotinyl-1,9-diaminononane; S9B, 1-(saligenin cyclic phospho)-9-biotinyldiaminononane; DMF, dimethylformamide; DTT, dithiothreitol; TE, 50 mM Tris/HCl/1 mM EDTA, pH 8.0; ECL, enhanced chemiluminescence.

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EXPERIMENTAL

Materials

Saligenin (2-hydroxybenzyl alcohol), 2,6-lutidene, 1,9-diaminononane and other diaminoalkanes were purchased from Aldrich. Enzygraphic Web was obtained from IBI Ltd., enhanced chemiluminescence (ECL) reagents and streptavidin–biotin–peroxidase complex from Amersham International and cellulose nitrate paper (0.45 μm pore size) from Millipore. Avidin–Sepharose was prepared by allowing avidin (Sigma) to react with CNBr-activated Sepharose 4B (Pharmacia), at a ratio of 0.5 mg/ml of swollen gel, under conditions recommended by the manufacturers. Phenyl valerate and mipafox, used as substrate and inhibitor respectively in the NTE esterase assay (see below) were purified as described previously (Johnson, 1977). All other reagents were purchased from Sigma or BDH and used without further purification.

Synthesis of the biotinylated OP reagent S9B

Synthesis of the stable precursor saligenin phosphorotrichloridate [4-*H*-1,2,3-benzodioxaphosphorin 3,3,3-trichloride (SCl_3)]

Saligenin (42 g = 0.35 mol) was added slowly (over 2 h) to a stirred suspension of powdered phosphorus pentachloride (84 g = 0.4 mol) in dry toluene under an argon atmosphere at 0 °C. When no powdered material remained, the solution was decanted away from a small amount of sticky orange solid and warmed to 27 °C under nitrogen over 2 h. Solvent was then stripped twice by rotary evaporation at about 1.99–2.66 MPa (15–20 mmHg), first at 40–55 °C and subsequently, after addition of fresh dry toluene, at 60 °C. The residual straw-coloured liquid was vacuum-distilled by raising the bath temperature to 140 °C over 30 min, to produce an initial fraction [108–112 °C; 0.15 mmHg (19.9 kPa)] which was discarded, and then further increasing the bath temperature over the range 147–164 °C, during which time three fractions distilling over the range 108–110 °C at 15.9–19.9 kPa (0.12–0.15 mmHg) were collected. The combined yield of the very pale yellow to colourless liquid in these fractions was 34.5 g. The identity of the product as saligenin phosphorotrichloridate was confirmed by m.s.; it can be stored stably at 15 °C by excluding moist air.

Synthesis of 1-biotinyl-1,9-diaminononane (9B)

Equal volumes of dry dimethylformamide (DMF) containing 40 mM diaminononane and 8 mM *N*-hydroxysuccinimidylbiotin were mixed and incubated for 2 h at 37 °C. Aliquots (0.7 ml) of the reaction mix were dried under vacuum (Savant Speed-Vac) and were stored at 4 °C. Dried aliquots were redissolved in 0.4 ml of aq. 90% methanol immediately prior to injection on to a Waters 650E Advanced Protein Purification System with a 250 mm × 4.6 mm Synchropak RP-PC18 column (Hichrom Ltd.). A gradient of acetonitrile (solvent A) in 10 mM triethylammonium acetate, pH 4.0 (solvent B), was programmed as follows: 0–5 min, 0%A–15%A; 5–50 min, 15%A–25%A; 50–65 min, 95%A (isocratic). The desired product, 9B, was eluted at about 40 min and was immediately dried under vacuum. The yield of 9B was 54 ± 5% (mean ± S.D. for four preparations).

Synthesis of 1-(saligenin cyclic phospho)-9-biotinyldiaminononane (S9B)

Lutidine (40 mM, 1 vol.) in dry acetonitrile was added to 3 vol. of 15 mM 9B in dry DMF, followed by 1 vol. of 40 mM SCl_3 in dry acetonitrile. The reaction was allowed to proceed at 37 °C for 40 min and was terminated by mixing with 1 vol. of an aq. 10 mM triethylammonium acetate, pH 5.0. Aliquots of the reaction mixture were loaded on to the same RP-PC18 column with the same solvent system described above, but using the following program: 0–5 min, 0%A–20%A; 5–55 min, 20%A–55%A; 55–60 min, 55%A–95%A; 60–70 min, 95%A (isocratic). The desired product, S9B, was eluted at about 50 min, was dried under vacuum, redissolved in dry DMF and stored at 4 °C. The yield of S9B was 19.3 ± 3.4% (mean ± S.D. for three preparations) and its identity was confirmed by m.s.: $(M\text{H})^+$ = 553; mass spectrum (m/z) (relative intensity, %): 553 (100), 535 (33), 493 (6.8), 473 (21), 447 (30), 429 (4.3), 367 (78), 327 (88), 310 (18), 226 (25), 198 (2.5).

Determination of biotin concentrations

Concentrations of both the intermediate, 9B, and the final reagent, S9B, were determined as biotin using a colorimetric assay (Green, 1970).

Phenyl-valerate hydrolase (NTE esterase) assays

NTE activity has been operationally defined as that portion of the phenyl-valerate hydrolase activity in tissue homogenates or fractions that is sensitive to the neuropathic OP mipafox and resistant to the non-neuropathic OP paraoxon (Johnson, 1977). Paraoxon-treated hen brain microsomes were prepared as described below and preincubated (37 °C; 20 min) at 0.1 mg of protein/ml in 1.0 ml of TE buffer (50 mM Tris/HCl/1 mm EDTA, pH 8.0) in the presence or absence of mipafox (50 μM). S9B was then added in 10 μl of DMF to give the final concentrations noted in the Figures, and the preincubation continued for a further 20 min at 37 °C. Substrate (phenyl valerate, 10 mM) was then added and the reaction terminated 20 min later and the phenol liberated was determined as described by Johnson (1977). NTE activity was calculated as the difference between phenyl valerate hydrolysis in the absence and presence of mipafox.

Preparation of paraoxon-treated microsomes from hen brain

Brains from white hens (3 weeks old–adult) were processed as described previously (Ruffer-Turner et al., 1992) with minor modifications. Briefly, 10% (w/v) homogenates in TE buffer containing 1 mM dithiothreitol (DTT) were centrifuged (11000 g; 20 min; 4 °C) and then microsomes were recovered from the resulting supernatant by high-speed centrifugation (100000 g; 40 min; 4 °C). The microsomal pellet was resuspended to 10% of the original homogenate volume of TE and was incubated (37 °C; 20 min) in the presence of 160 μM paraoxon. The incubation was terminated by dilution with 5 vol. of cold TE buffer containing 0.9% NaCl and centrifugation (100000 g; 40 min; 4 °C). The paraoxon-treated pellets were resuspended in TE buffer at 5–8 mg protein/ml and stored as small aliquots at −80 °C.

NTE labelling with S9B and isolation of S9B-labelled NTE

Paraoxon-treated microsomes (2 mg protein/ml) were incubated in TE buffer with S9B (160 nM) for 20 min at 37 °C, washed by 4-fold dilution in TE buffer and centrifugation (100000 g; 1 h; 4 °C) and then resuspended in their original volume of TE containing 10 mM DTT. SDS (10%, w/v, stock solution) was added to give a final concentration of 0.15%, then the mixture was boiled for 7 min and, after cooling, was centrifuged

(550 *g*, 10 min). Aliquots (15 ml) of the clear supernatant were mixed overnight at room temperature with 100 μl of avidin–Sepharose. Each 100 μl of avidin–Sepharose was washed once in 25 ml, then four times in 1 ml of 0.15% SDS/TE and finally boiled (7 min) in 0.3 ml of 1% SDS/10 mM DTT/0.2 mM EDTA/10 mM Tris/HCl, pH 8.0. Eluates were pooled, dried under vacuum, then reconstituted in 20% of their original volume and applied to preparative SDS/PAGE (Bio-Rad Labs) with a 3% stacking gel and 4.4% running gel. Polypeptides in aliquots of each fraction were precipitated by incubating (18 h; −20 °C) with an equal volume of acetone and were analysed by analytical SDS/PAGE.

Analytical SDS/PAGE and Western blotting

Samples (30 μl) were subjected to SDS/PAGE (Laemmli, 1970) and were either stained with Coomassie Blue or transferred to nitrocellulose blots (Towbin et al., 1979). The blots were incubated (30–60 min) with streptavidin–biotin–peroxidase complex (diluted 1:300). Biotinylated polypeptides on the blot were revealed by using either Enzygraphic Web or ECL reagents as the peroxidase substrate. SDS/PAGE-fractionated polypeptides were quantified by densitometry (Molecular Dynamics Computing Densitometer) of Coomassie Blue-stained gels on which standard amounts of BSA had also been run.

RESULTS

Inhibition of NTE (phenyl valerate hydrolase) activity by S9B

The general structure of *N*-biotinylsaligenin phosphoramidates (SnB) is shown in Figure 1. In preliminary experiments, a homologous series of monobiotinyl alkyldiamines (n = 5–12) were allowed to react with saligenin phosphorotrichloridate by steps analogous to those described for 9B in the Experimental section. Aliquots of the crude unfractionated reaction mixtures were assayed for inhibition of NTE (phenyl valerate hydrolase) activity to establish a rank potency order: $n = 9, 10 > 12, 8 > 7 \gg 5$. Thereafter, attention was focused on the $n = 9$ homologue which we have designated 'S9B'.

NTE (phenyl valerate hydrolase) activity in dilute suspensions of paraoxon-treated microsomes (0.1 mg of protein/ml) was progressively inhibited by S9B with a second-order rate constant (k_a) of $1.4 \times 10^7\ M^{-1} \cdot min^{-1}$ (Figure 2). The I_{50} value over a standard 20 min preincubation was 1.6±0.84 nM (mean±S.D. for five preparations). By contrast, in concentrated suspensions of paraoxon-treated microsomes (2 mg of protein/ml), S9B was substantially less potent (I_{50} = 15–20 nM). We recently noted an analogous effect using other (non-saligenin) organophosphoroamidates with apparent increases of up to 10-fold in the I_{50} for concentrated versus dilute membrane preparations and ascribed this, in part, to enzymic degradation of the OP (Jokanovic and Johnson, 1993).

Labelling of NTE by S9B

Incubating paraoxon-treated microsomes (2 mg of protein/ml) with S9B led to covalent labelling of NTE, detected as a biotinylated 155 kDa polypeptide band on Western blots (Figure 3). The labelling of this band increased with S9B concentration over the range 10–100 nM, but did not increase further when 300 nM S9B was used. Labelling was markedly inhibited (> 80%) by prior incubation with the neuropathic OP mipafox. Two endogenous biotinylated polypeptides (120 and 70 kDa) were also observed on these blots (Figure 3).

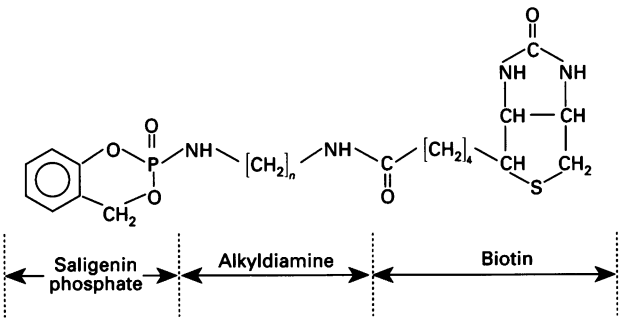

Figure 1 General chemical structure of *N*-biotinylated saligenin phosphoramidates

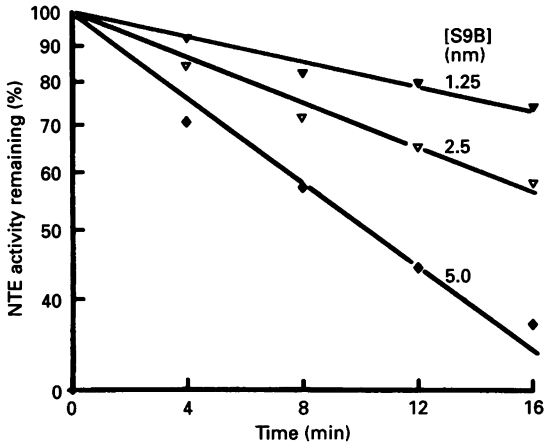

Figure 2 Progressive inhibition of NTE (phenyl valerate hydrolase) activity by S9B

Paraoxon-treated microsomes were incubated (37 °C; 20 min) in the absence or presence of 50 μM mipafox (see the Experimental section), then washed by centrifugation and resuspended at 0.1 mg of protein/ml. S9B was added to give the concentrations indicated in the Figure and incubation was continued. At the indicated times, phenyl valerate (final concn. 10 mM) was added, and after a further 20 min the reaction was terminated and phenol liberated determined as described in the Experimental section. Results are shown as the fraction of uninhibited NTE activity remaining after the indicated times.

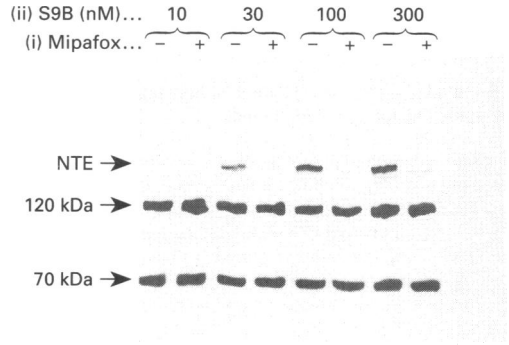

Figure 3 Detection of S9B labelling of NTE by Western blotting

Paraoxon-treated microsomes (2 mg of protein/ml) were preincubated (37 °C; 20 min) in the presence or absence of mipafox (100 μM). S9B was then added to give the indicated concentrations and, after a further 20 min at 37 °C, reactions were terminated by addition of SDS/PAGE sample buffer and boiling (7 min). Samples were subjected to analytical SDS/5%-PAGE, blotted, and biotinylated polypeptides were revealed using streptavidin–biotin–peroxidase complex and Enzygraphic Web (see the Experimental section).

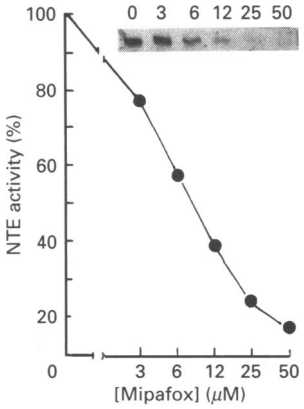

Figure 4 Inhibition of S9B labelling of NTE and of phenyl valerate hydrolase activity by mipafox

Paraoxon-treated microsomes (2 mg of protein/ml) were incubated (37 °C) with mipafox at the concentrations (μM) indicated in the Figure. After 20 min, an aliquot of each incubation mixture was incubated (20 min) with 250 nM S9B, then analysed by SDS/PAGE and blotting as described in Figure 3, except that ECL reagent was used as peroxidase substrate; these data (inset) show increasing inhibition of S9B labelling by mipafox concentrations from 0 to 50 μM. Another aliquot of each mipafox incubation mixture was diluted to 0.1 mg of protein/ml and assayed for phenyl valerate hydrolase activity as described in Figure 2.

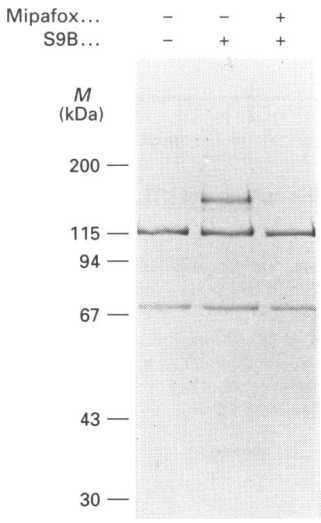

Figure 5 Avidin–Sepharose capture of polypeptides solubilized from S9B-labelled paraoxon-treated microsomes

Paraoxon-treated microsomes (2 mg of protein/ml) were incubated (37 °C): (i) for 20 min in the absence or presence of 100 μM mipafox, followed by: (ii) 20 min in the absence or presence of 160 nM S9B. The microsomes were then washed by centrifugation and solubilized by boiling in 0.15% SDS/10 mM DTT. Biotinylated polypeptides were isolated from the SDS extract by using avidin–Sepharose and detected by SDS/7.5%-PAGE and Coomassie Blue staining as described in the Experimental section. The migration of standard proteins of indicated molecular mass (*M*) is shown on the left-hand side of the gel.

The specificity of S9B labelling was also examined by comparing its inhibition after preincubation in the presence of increasing concentrations of mipafox with the inhibition of NTE esterase activity under the same conditions. Esterase activity was increasingly inhibited by mipafox over the range 3–50 μM, and although precise quantification by the Western-blot procedure was not possible, S9B labelling of NTE was clearly inhibited by mipafox over the same concentration range (Figure 4).

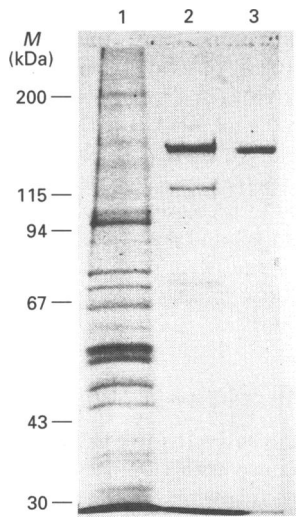

Figure 6 Isolation of S9B-labelled NTE from paraoxon-treated microsomes

Paraoxon-treated microsomes were labelled with S9B, washed, solubilized and biotinylated polypeptides were captured on avidin–Sepharose as described in Figure 4. Subsequently, avidin–Sepharose eluates were fractionated by preparative SDS/PAGE, and NTE-containing fractions were acetone precipitated and pooled (see the Experimental section). The Figure shows a Coomassie Blue-stained 7.5% gel with the positions of molecular-mass (*M*) markers on the left-hand side. Lane 1, solubilized paraoxon-treated microsomes, 48 μg of protein; lane 2, avidin–Sepharose eluate, 0.44 μg of protein; lane 3, pooled preparative-PAGE 155 kDa fraction, 0.165 μg of protein.

Isolation of S9B-labelled NTE

After S9B labelling and solubilization of paraoxon-treated microsomes, both biotinyl-NTE (155 kDa) and the two endogenous biotinylated polypeptides (120 and 70 kDa) could be adsorbed to, and subsequently eluted from, avidin–Sepharose (Figure 5). Preincubating paraoxon-treated microsomes with mipafox before addition of S9B inhibited biotinylation of NTE and thus no 155 kDa was present in avidin–Sepharose eluates from these samples (Figure 5). NTE comprised at least 30% of the total Coomassie Blue-staining protein on SDS/7.5%-PAGE of the avidin–Sepharose eluates. Thus, from microsomes in which NTE comprises about 0.03% of the total protein (Williams and Johnson, 1981), a purification of approx. 1000-fold was achieved in a single-step. Yields of NTE in the avidin–Sepharose eluates were 96 ± 18 ng/mg of microsomal protein (mean $\pm$ S.D. for five experiments) representing a recovery of about 30%.

The two endogenous biotinylated polypeptides were substantially less abundant in the microsomal fraction used for S9B labelling than in the initial 11000 *g* pellet of brain homogenates (see the Experimental section). The 155 kDa polypeptide was separated from the endogenous biotinylated 120 and 70 kDa polypeptides by fractionating the avidin–Sepharose eluates by preparative SDS/PAGE. By this means a preparation was obtained in which the 155 kDa polypeptide was the only band visible on Coomassie Blue-stained analytical SDS/PAGE gels (Figure 6, lane 3).

In order to partially characterize the isolated biotinyl-NTE a peptide mapping experiment was performed using limited digestion with endoproteinase Glu-C (staphylococcal V8 pro-

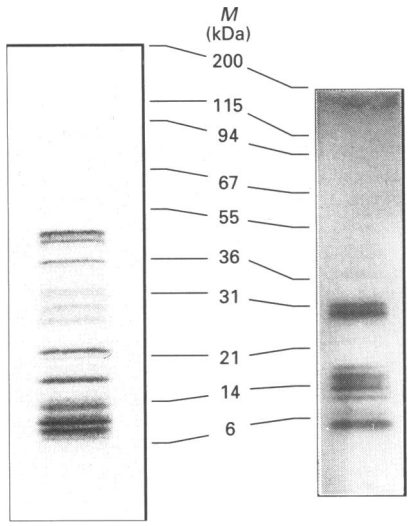

Figure 7 Digestion of biotinyl-NTE with endoproteinase Glu-C

Purified biotinyl NTE was incubated (37 °C; 2 h) with endoproteinase Glu-C in 0.1% SDS/1% DTT/50 mM ammonium bicarbonate at a ratio of 6:1 (NTE/proteinase; w/w). Aliquots of the digest were run on a 4–20% gradient gel, which was then either stained with Coomassie Blue (**a**) or blotted and probed for biotin as in Figure 4 (**b**). The migration of standard proteins of molecular masses (*M*) 6–200 kDa is indicated.

teinase). This generated Coomassie Blue-staining polypeptides of about 45 kDa (doublet), 30 kDa (three bands), 21 kDa, 17 kDa and a cluster of smaller fragments between 8 and 12 kDa (Fig. 7a). Western blotting of this V8-proteinase digest of biotinyl-NTE revealed three major clusters of biotin-containing polypeptides: very small fragments (< 6 kDa) and two groups of larger fragments centred around 16 kDa and 30 kDa (Figure 7b). The significance of this profile of active-site labelled NTE fragments is discussed below.

DISCUSSION

The present paper describes the synthesis of saligenin phosphorotrichloridate, a stable precursor from which a variety of substituted organophosphoramidates may be generated (provisional patent applications have been filed covering synthesis and use of saligenin phosphorotrichloridate), and the characterization of one biotinylated homologue, S9B, as a reagent to allow active-site labelling and affinity purification of NTE. Two molecular features of S9B determine its potency as an inhibitor of NTE esterase activity. The first is the saligenin phosphate moiety involving a slightly strained dioxaphosphoran ring. The second feature is the 9-carbon alkyl chain which links the phosphoramidate to biotin; short ($n < 7$) alkyl chain homologues of S9B were markedly less potent inhibitors of NTE esterase activity. The efficacy of OPs with hydrophobic substituents as NTE inhibitors has been well documented (Johnson, 1975b; 1988). Thus phenyl saligenin phosphate is potently neuropathic (Johnson, 1975a; Jortner and Ehrich, 1987) and, *in vitro*, inhibits NTE esterase activity with an I_{50} (20 min) of 5 nM (Johnson, 1975b). By contrast, methyl saligenin phosphate is an anticholinesterase agent with no selectivity for NTE (Ohkawa et al., 1980).

S9B labelling of paraoxon-treated brain microsomes was highly selective. By Western blotting, only one polypeptide band was biotinylated by S9B. The sensitivity to mipafox inhibition of this reaction (Figures 3 and 4), and the migration on SDS/PAGE of the biotinylated polypeptide (155 kDa) strongly suggests a specific labelling of NTE. In addition to selectivity, the other essential feature of S9B labelling of NTE is its covalent nature. Thus, following S9B treatment, microsomes could be boiled in 0.15% SDS, resulting in almost quantitative solubilization of microsomal protein with biotin still attached to NTE and available to bind avidin. Since the solubilization procedure inactivates unwanted proteinase activities, the affinity adsorption on to avidin–Sepharose could be allowed to proceed for 18 h at 20 °C to permit interaction between the solid phase and a dilute solution of biotinyl-NTE.

The final preparative SDS/PAGE step served to separate S9B-labelled NTE from the two endogenous biotinylated polypeptides. The identities of the latter are unknown, but their abundance in the low-speed pellet of brain homogenates suggests they may be mitochondrial. [Biotin-containing carboxylases have been isolated from rat liver mitochondria using avidin–Sepharose chromatography (Oei and Robinson, 1985).] While we have no unequivocal proof that the final product is homogeneous, it seems reasonable to assert that, since (a) only a 155 kDa polypeptide was labelled by S9B, (b) only biotinylated polypeptides appear to be eluted from avidin–Sepharose and (c) biotinyl-NTE was clearly separated from the 120 and 70 kDa endogenous biotinylated polypeptides by SDS/PAGE, then any remaining microheterogeneity of the final product that there may be could represent naturally occurring isoforms of NTE itself. For example, several isoforms of insect juvenile-hormone esterase are present in affinity-purified preparations which migrate as a single band on SDS/PAGE (Hanzlik and Hammock, 1987).

Our laboratory previously reported isolation of a preparative SDS/PAGE fraction in which [^{3}H]DFP-labelled NTE comprised about 2% of the total protein (Ruffer-Turner et al., 1992). SDS/PAGE fractionation of an endoproteinase Glu-C digest of the partially purified [^{3}H]DFP-labelled material revealed two major peaks of ^{3}H-labelled fragments centred around 30 and 16 kDa (Glynn et al., 1993). In the present study an endoproteinase Glu-C digest of the homogenous biotinyl-NTE preparation yielded two groups of biotin-labelled polypeptides centred around 30 and 16 kDa (Figure 7). Thus we have generated consistent peptide fingerprints of the putative active site of NTE using two different active-site reagents ([^{3}H]DFP and S9B) and NTE preparations of very different initial purity. Furthermore, the prominent Coomassie Blue-staining polypeptide bands in proteinase-V8 digests of purified biotinyl-NTE are sufficiently well resolved by SDS/PAGE to yield unambiguous N-terminal-sequence data, and this information is to be used to initiate molecular cloning of NTE.

The saligenin phosphotrichloridate (SCl_3) precursor is easily made, and is stable, but sufficiently reactive to yield potential affinity reagents simply by mixing it with an appropriately labelled amine. The relatively large amounts of naturally biotinylated polypeptides would hinder detection of NTE in brain tissue sections using S9B as a histochemical reagent. We have therefore used conditions essentially identical with those described in the Experimental section to allow SCl_3 to react with a conjugate of diaminononane and a commercially available *N*-hydroxysuccinimide activated derivative of digoxigenin. This generates a potent NTE inhibitor which can covalently attach digoxigenin to a 155 kDa brain microsomal polypeptide as detected by Western blotting with a peroxidase-labelled antibody to digoxigenin (results not shown). We are now evaluating this digoxigenin-labelled inhibitor as a reagent for histochemical localization of NTE. The applicability of analogous reagents to detection and isolation of serine esterases and proteinases other

than NTE remains to be determined. Clearly, however, features of optimal substrates for a particular enzyme could be incorporated in the labelled amine which is allowed to react with SCl_3. In this regard it is noteworthy that OPs are considered specific inhibitors of serine esterases/proteinases (Powers and Harper, 1986), while chloromethanes, for example, are moderate alkylating agents and also attack some cysteine proteinases (Kay *et al.*, 1992).

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