Tritiation of α -bungarotoxin with *N*-succinimidyl [2,3- ^3H]propionate

A useful reagent for labelling proteins

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Mono[^3H]propionyl- α -bungarotoxin, prepared with *N*-succinimidyl [2,3- ^3H]propionate (sp. radioactivity 50 Ci/mmol) and purified to homogeneity by electrofocusing, retains its biological activity and stability. Rate constants for its binding to acetylcholine receptor were 4.4-fold lower than for unlabelled toxin; no dissociation was detectable. Analysis of enzymic digests of toxin showed ^3H is located mainly or entirely in ϵ -propionyl-lysine.

Radiolabelling of proteins is commonly achieved by iodination with ^{125}I , due to the very high specific radioactivities obtainable. However, alternative methods using ^3H are needed, since: (a) iodination frequently alters biological activity of proteins and commonly produces variable mixtures of labelled products exhibiting somewhat different properties; (b) the half-life of ^{125}I is 60 days compared with 12.3 years for ^3H ; (c) the much higher radiation energy of ^{125}I entails safety problems and also irradiation damage on storage over short periods, necessitating frequent preparation or re-purification. In the case of the post-synaptic neurotoxins (e.g. α -bungarotoxin) that specifically react with nicotinic acetylcholine receptors, a number of tritiation methods have been employed, as reviewed by Fulpus (1976). The first such derivative, mono[^3H]acetyl- α -bungarotoxin, described by Barnard *et al.* (1971), had the same affinity as native toxin for muscle receptors (Barnard *et al.*, 1975), but had a specific radioactivity too low for sensitive analyses of most cellular receptors. Therefore, we have investigated the use of *N*-succinimidyl [2,3- ^3H]propionate, an acylating reagent that is now available commercially at a specific radioactivity of 40–60 Ci/mmol, for tritiation of α -bungarotoxin. Under easily controlled conditions it can introduce one propionyl group per toxin molecule, with high retention of biological activity. Further, as covalent radiolabelling of β -bungarotoxin and acetylcholine receptor can be achieved also with this reagent (J. O. Dolly, J. Spokes & E. A. Barnard, unpublished work), it could prove more useful than radioiodination for labelling proteins in general.

Methods

Purification and tritiation of α -bungarotoxin

From the crude venom of *Bungarus multicinctus*

(Miami Serpentarium, Miami, FL, USA) α -bungarotoxin was purified by chromatography on CM-Sephadex (Spokes & Dolly, 1980) and CM-cellulose (Lee *et al.*, 1972). Purity of the labelling reagent used, *N*-succinimidyl [2,3- ^3H]propionate (sp. radioactivity 50 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.), was greater than 95% on t.l.c. on silica gel G in toluene/ethyl acetate (1:1, v/v) (R_f 0.66, as for unlabelled reagent). The solvent was evaporated from this reagent with a stream of N_2 and α -bungarotoxin was added in 0.1–1.0 ml of 0.02 M-sodium phosphate buffer, pH 7. After thorough mixing, the solution was stirred continuously for 2 h at 23°C; where stated, additional toxin was then added. The reaction mixture was gel-filtered on a Sephadex G-25 column (1.5 cm $\times$ 28.0 cm) equilibrated with 0.025 M-ammonium acetate, pH 5, and the labelled toxin in the void volume was collected.

Separation of [^3H]propionylated and native toxins

The mixture of labelled and unlabelled α -bungarotoxins was applied to a small column of CM-cellulose and eluted with a linear gradient of ammonium acetate, pH 5. Mono[^3H]propionylated species were purified from selected fractions by preparative isoelectric focusing in a flat-bed of Ultrodex (Pharmacia, Uppsala, Sweden) using the Multiphor apparatus and Ampholines (LKB, Bromma, Sweden) as described by the supplier. The single gel segment containing the peak of radioactivity was layered on to a Sephadex G-25 column, and gel-filtered as above.

Characterization of [^3H]propionylated α -bungarotoxin

Chemical specific activities of the labelled toxins were determined from their radioactive content and the amount of protein present, as measured colori-

metrically (Lowry *et al.*, 1951), was confirmed by the alanine and valine contents determined by amino acid analysis (Threlfall & Thomas, 1979), using pure α -bungarotoxin as standard in both cases. Biological activities of all toxins were assessed from their ability to bind irreversibly to acetylcholine receptor of cat denervated leg muscle (Dolly & Barnard, 1977) and electric organ of *Torpedo marmorata* (Lo *et al.*, 1981); the receptor was solubilized with Triton X-100 and its binding to the tritiated toxins (15–30 nM) was measured at 25°C or 37°C by the DEAE-cellulose disc assay and appropriate blanks plus controls (Dolly & Barnard, 1977). To identify the labelled residues in the mono-[³H]propionylated toxin, the latter (50 pmol, with 10 μ g of serum albumin) in Tris/HCl (pH 8.2)/8 M-urea was treated with 5 mM-2-mercaptoethanol for 45 min at 25°C, and then with 20 mM-iodoacetamide (17 h at 25°C). After separation of the protein by gel filtration, it was digested overnight at pH 7.8, 37°C, with 30 μ g of Pronase AS (Sigma)/ml and for 3 h with 30 μ g of prolidase (Sigma)/ml, and finally for 16 h at 37°C with 20 μ g of trypsin (Worthington Corp., New York, NY, U.S.A.)/ml. The digest was freeze-dried and resuspended in water (0.1 ml) and subjected to electrophoresis at 3 kV for 40 min at pH 2.1. The two most acidic peaks obtained were eluted and re-digested as above at 37°C with 100 μ g of both Pronase and prolidase/ml for 72 h, whereas the more basic peak was incubated at pH 5.5 with 50 μ g of carboxypeptidase Y (Boehringer)/ml for 18 h at 25°C before electrophoresis. Marker ϵ -propionyl-lysine was made by reacting lysine, CuSO₄ and succinimidyl propionate (at a molar ratio of 1:1.5:5) for 2 h at room temperature.

Results and discussion

A homogeneous preparation of α -bungarotoxin, which showed a single N-terminal residue (Ile), and which in gel electrophoresis and isoelectric focusing (Spokes & Dolly, 1980) gave one protein band, was made to react with *N*-succinimidyl [2,3-³H]propionate for 2 h at 23°C. With 25 nmol or 1 μ mol of toxin together with 50 nmol or 0.33 μ mol of reagent the amounts of radioactivity associated with the toxin represented 69% and 96% respectively of the total recovered after gel filtration.

Chromatography on CM-cellulose was found to be effective in separating the reaction products, as judged by the separation, for comparison, of purified samples (Dolly & Barnard, 1977) of mono-, di- and tri-[³H]acetyl- α -bungarotoxin (the more basic of two peaks of the latter) (Fig. 1a). Using these conditions, chromatography of a sample labelled with a 3-fold excess of toxin over reagent separated a broad peak of tritiated toxin (Ia) and a very much smaller

Fig. 1. Chromatographic separation of tritiated α -bungarotoxin derivatives on CM-cellulose

Samples were applied to a column (1 cm × 4 cm) of CM-52 CM-cellulose equilibrated with 25 mM-ammonium acetate (pH 5) and eluted with a linear gradient (as indicated) of the latter; the fractions were counted for radioactivity. (a) shows the positions of mono-(□), di-(●) and tri-[³H]acetylated toxins (○). (b) shows [³H]propionylated species separated after labelling 1 μ mol of toxin with 0.3 μ mol of *N*-succinimidyl [2,3-³H]propionate (●) and those obtained (○) by reacting 10 nmol of toxin and 60 nmol of tritiating reagent for 2 h at 23°C followed by the addition (in the latter case only) of a large excess of α -bungarotoxin; the elution profile of unlabelled toxin was determined by *A*₂₈₀ (□).

one (Ib) from unmodified toxin (Fig. 1b). Likewise, a sample labelled with excess of reagent, with the later addition of excess toxin, showed again that a large proportion of the ³H was eluted at the position of mono-substituted toxin (Ia); in addition, a smaller second peak (Ib) was partially resolved (Fig. 1b). Measurement of the amino acid and radioactivity contents of the most basic derivative (in peak Ia) established that it had a chemical specific radioactivity of 48 Ci/mmol; this is very close to the nominal value of 50 Ci/mmol stated for the labelling reagent, thereby confirming that this sample contained equimolar amounts of toxin and label.

Although using an excess of toxin over *N*-succinimidyl [2,3-³H]propionate resulted in preferential formation of mono-labelled toxin, preparative isoelectric focusing (Fig. 2) was needed to separate completely the most basic tritiated species (pI = 9.9) from the variable amounts (Fig. 1) of peak Ib material (pI = 9.5) present in the major peak from the ion-exchange chromatography; it was also effective in removing any contaminating unlabelled toxin (pI = 10.6). Determination of the chemical specific activity of the most basic species purified in

Fig. 2. Complete purification of mono[³H]propionylated α -bungarotoxin by preparative isoelectric focusing. Eluted material from the peak fraction of 1a (○) and 1b (●) of the CM-cellulose column (Fig. 1b; ○) was subjected to isoelectric focusing for 5 h at 2°C using 3W constant power, on a flat-bed (7 cm $\times$ 13 cm) of Ultradex containing 2% Ampholine (1% each of pH 9–11 and pH 7–9 ranges). The pH (□) in the gel slices (3 mm) was measured at 20°C with a microelectrode and samples were counted for radioactivity. (b) shows the electrofocused profile of the pooled eluted fractions of peak 1a (Fig. 1b; ●); the arrow represents the focused position of native toxin.

this manner, by measuring its protein and radioactive contents, confirmed that it contained 1 mol of label/mol of toxin. Similarly, purification of the more acidic mono-labelled species from the less abundant 1b peak (Fig. 1) was accomplished (Fig. 2). Analysis by electrophoresis of the products of the initial enzymic digest of the most basic mono-[³H]propionyl- α -bungarotoxin showed two peaks migrating slower and one moving faster than ϵ -propionyl-lysine; on further exhaustive digestion

these were largely converted into material with the same mobility as that marker. This was evidence that a propionyl group was introduced at the lysine side-chain. It has not been proven that this mono-propionylated α -bungarotoxin is labelled at a unique lysine residue; however, in the case of the most abundant mono-acetylated toxin derivative formed using [³H]acetic anhydride in a parallel manner (Barnard *et al.*, 1971), complete sequence analysis showed that the substitution is on Lys-51 (E. A. Barnard & T. H. Chiu, unpublished work). This residue is hence the most reactive to acylation, consistent with the highly accessible position of a homologous residue apparent in the three-dimensional structure of a related toxin (Walkinshaw *et al.*, 1980). Thus it seems probable that Lys-51 is also the labelled site in the equivalent mono-propionylated derivative described here, although this requires confirmation.

Reaction of the most basic toxin derivative with an excess of *Torpedo* acetylcholine receptor, followed by gel filtration to separate inactive free toxin if present, has demonstrated that it is 100% biologically active (Lo *et al.*, 1981), a finding consistent with its homogeneity and with only a single type of residue being identified as modified. With two batches of the reagent at different specific radioactivities, the biological specific activities of the monopropionylated species prepared as above were calculated from the amount of receptor-³H-labelled-toxin complex measured after reaction (1 h at 37°C, i.e. to saturation) of a 5-fold excess of ³H-labelled toxin with a precisely known amount of receptor. The latter was determined by using purified mono-[³H]acetylated α -bungarotoxin, prepared with [³H]acetic anhydride of independently known specific radioactivity, and gave values of 50 and 15 Ci/mmol for the [³H]propionylated toxins. Also, the chemical specific radioactivity (as shown above) for the first of these two preparations agreed very well. The biological specific activities obtained for peaks II and III (Fig. 1) were 1.9 and 2.8 times higher respectively than for the two mono-labelled derivatives, indicating that they represent di- and tri-substituted derivatives, as expected from their chromatographic behaviour.

The reaction (Lo *et al.*, 1981) of acetylcholine receptor from cat denervated muscle with the most basic [³H]propionylated toxin was analysed (Fig. 3); the association rate constants calculated were $(1.8 \pm 0.6) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $(2.7 \pm 0.9) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (Fig. 3b) at 25°C for the oligomeric forms of the receptor showing $s_{20,w}$ values of 9S and 4S respectively. No dissociation of either receptor-³H-labelled-toxin complex was detectable after 50 h at 25°C, under conditions where allowance was made for a slight inactivation of receptor in this period; this is consistent with electrophysiological

evidence that tri[³H]acetyl toxin binds irreversibly to muscle receptor (Dolly *et al.*, 1977). The association rate of mono[³H]propionyl toxin with an excess of receptor, which contained only the 4S form, was monophasic for at least 90% of the reaction (Fig. 3b), so that it behaves as a homogeneous species; the 4S form was used as this oligomeric species can be more readily separated (Fig. 3a). With that toxin (up to 30 nM) the extent of non-specific binding was less than 2% and the amount of radioactivity obtained in receptor-free blank assays was never more than 0.5% of the total ³H-labelled toxin added; pooled fractions from peaks Ia and Ib gave similar results. The monopropionylated toxins were stable on storage at -20 or -80°C in buffered solutions, pH 7-8, for up to 2 years.

To compare the affinities of the mono[³H]propionyl and native toxins, we measured the amounts of receptor-³H-labelled toxin complex formed after incubation for 1 h of *Torpedo* receptor (4 nM) with 20 nM-³H-labelled toxin alone or with the latter diluted to the same concentration with various amounts of native α-bungarotoxin. As the toxin binds irreversibly to the receptor, the relative activity (R) of native (T) to ³H-labelled toxin (T*) is given by

$$\frac{1}{R} = 1 + \frac{k_T[T]}{k_{T^*}[T^*]}$$

where k_T and k_{T^*} are their association rate constants. For the most basic mono[³H]propionylated toxin, a plot of $1/R$ against $[T]/[T^*]$ gave a straight line; the slope of k_T/k_{T^*} was 4.4 or 4.7 for determinations at 25 or 37°C respectively. This indicates that mono-propionylation, unlike acetylation, of a lysine residue decreases somewhat the reaction rate with receptor, but the extent of this is minimal and less than that reported for toxin labelled with [³H]pyridoxamine phosphate (Jones & Thompson, 1980).

In conclusion, it should be emphasized that the conditions needed for tritiation to high relative specific activities are very mild, unlike most iodination procedures, and the introduction of a single label left 100% of the molecules with high biological activity and stability. Moreover, a high degree of labelling was readily achieved without using the large relative concentrations of radioactive reagent used in other methods (Fulpius, 1976) and the resultant change in charge facilitates complete purification of the mono[³H]propionylated toxin. Thus, this method should be suitable for tritiation of other polypeptides and proteins, provided the reactive amino groups are non-essential for biological activity; this is exemplified by active [³H]propionylated protein A, which is now commercially available.

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Fig. 3. Time course of the reaction of mono-³H-propionyl-α-bungarotoxin with an excess of muscle acetylcholine receptor

To isolate a single oligomeric form of the receptor a Triton X-100 extract of cat denervated leg muscle was stored at 2°C for 24 h before preparative sucrose-density-gradient centrifugation (Lo *et al.*, 1981); fractions containing 4S peaks were used. A portion of this extract was labelled with a 20-fold molar excess of ³H-labelled toxin for 1 h at 25°C, and gel filtered to remove unbound toxin; its sedimentation profile showed that it contained predominantly the 4S form of receptor (a). The inset shows the rate of formation of receptor-³H-labelled-toxin complex (●) at 25°C using 4.5 nM-toxin and 5.6 nM-receptor; ■, control value obtained in the presence of excess unlabelled toxin. (b) shows a second-order plot for the results shown in the insert (●), where $[T]_0$, $[R]_0$, $[T]_t$ and $[R]_t$ are the concentrations of toxin and receptor present initially and after time t ; k is the association rate constant, which is calculated from the slope of the line. The kinetic data are averages for quadruplicate determinations.

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