DIFFERENTIAL RADIOACTIVE LABELING OF AN 11S GLOBULAR FORM OF ACETYLCHOLINESTERASE

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1. Introduction

The structural stability and subunit composition of acetylcholinesterase (AChE) purified from the electric organ of *Electrophorus electricus* is of particular interest because of the proteins significant role in the neurochemistry of the cholinergic synapse. It has been demonstrated that a globular form of the enzyme with a sedimentation coefficient of approximately 11S is a degradation product obtained by proteolysis and/or autolysis of the assymetric 'native' forms (18, 14S) of the freshly, affinity purified protein [1].

The subunit composition of this 11S form of the protein also reflects this degradation. The 80 000 mol. wt catalytic subunit is cleaved to generate a component of 55 000 mol. wt and two lower mol. wt components at 28 000 and 25 000 as monitored by polyacrylamide gel electrophoresis in the presence of sodium dodecyl-sulphate (SDS) [2].

This communication describes two methods that have been employed to radioactively label the 11S form of the protein in an attempt to differentially probe the composition of the catalytic subunit.

2. Materials and methods

2.1. *Characterisation of the AChE preparations*

Electrophorus electricus were obtained from Paramount Aquarium, Tampa, Florida and kept alive in an aquarium in the laboratory prior to use. Purified enzyme was obtained from fresh electric organ tissue by affinity chromatography as described previously [2] and routinely stored at 4°C in 20 mM phosphate buffer (pH = 6.90). The molecular forms and subunit composition of the purified AChE were monitored via isokinetic sucrose gradients and polyacrylamide gel electrophoresis in the presence of SDS [2]. SDS gels which contained ^{125}I-radioactively labelled AChE were cut laterally in 2 mm slices and incubated for 16 h at room temperature with 1 ml of NCS tissue solubiliser (Nuclear Chicago) prior to the addition of 5 ml of dioxane based scintillation fluid. Samples were counted on a Nuclear Chicago Mark II scintillation counter and the results are reported as uncorrected counts per minute.

2.2. *Iodination techniques*

Several literature methods (3–5) on the enzymatic iodination of membranes and proteins were modified so as to incorporate labelled ^{125}I successfully into bovine serum albumin and myoglobin (Sigma) to a high activity (10^6 cpm/mg). In a typical enzymatic experiment with AChE, 10 μl of lactoperoxidase (2 mg/ml, Sigma) was added to 200 μl of the buffered AChE pH = 6.90 (A_{280} = 0.80) in the presence of 5 μl of NaI (10^{-5} M) and 10–100 μCi of carrier free Na^{125}I (New England Nuclear). The iodination was then initiated by adding 10 μl of 0.03% hydrogen peroxide and allowed to continue for 30 min at room temperature before being quenched by the addition of 25 μl of sodium azide (0.025 M). The samples were then dialysed overnight at 4°C against 20 mM phosphate (pH = 6.90) to remove the excess ^{125}I.

The preparation of the iodinated ester, ^{125}I-labelled 3-(4-hydroxyphenyl) propionic acid *N*-hydroxysuccinimide ester was as described by Bolton and Hunter [6] with the following modification: only 10–100

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μCi of $Na^{125}I$ was used in each preparation. Acetonitrile was used as the solvent for the starting material *N*-succinimidyl-3-(4-hydroxyphenyl)propionate and as a result no dimethylformamide was needed for quantitative extraction of the ester into benzene. The reaction vessel was silylated to ensure complete separation of the small benzene/water layers. Two 300 μl aliquots of benzene were used in the extraction and these fractions were evaporated off in a fume hood by blowing dry nitrogen gas over the solution. Complete removal of the benzene was achieved in about five min. The iodinated material was subsequently dried under vacuum for 2–3 h and used immediately for iodination. The AChE, 200 μl buffered now at pH = 8.2 was added to the solid ^{125}I-containing acylating agent was mixed by vortexing. The reaction was allowed to continue at room temperature for 10–15 min and the pH was then adjusted to 7.2. The samples were then dialysed against 20 μM phosphate (pH, 6.90) prior to running on SDS gels. Iodinated AChE was routinely concentrated on CF-25 Amicon centriflo membrane cones to a concentration of 1 mg/ml prior to loading onto SDS gels.

2.3. *Enzyme assays*

AChE assays were performed at pH 8.0 by the method of Ellman et al. [7]. Protein concentrations were estimated spectrophotometrically using an $\epsilon_{280}^{1\%} = 18.0$ [8].

Lactoperoxidase activity was measured according to the Worthington Biochemical catalogue (Worthington Biochemical corporation, Freehold, N. J.) assay for horseradish peroxidase at pH = 7.2 instead of 6.0 [5].

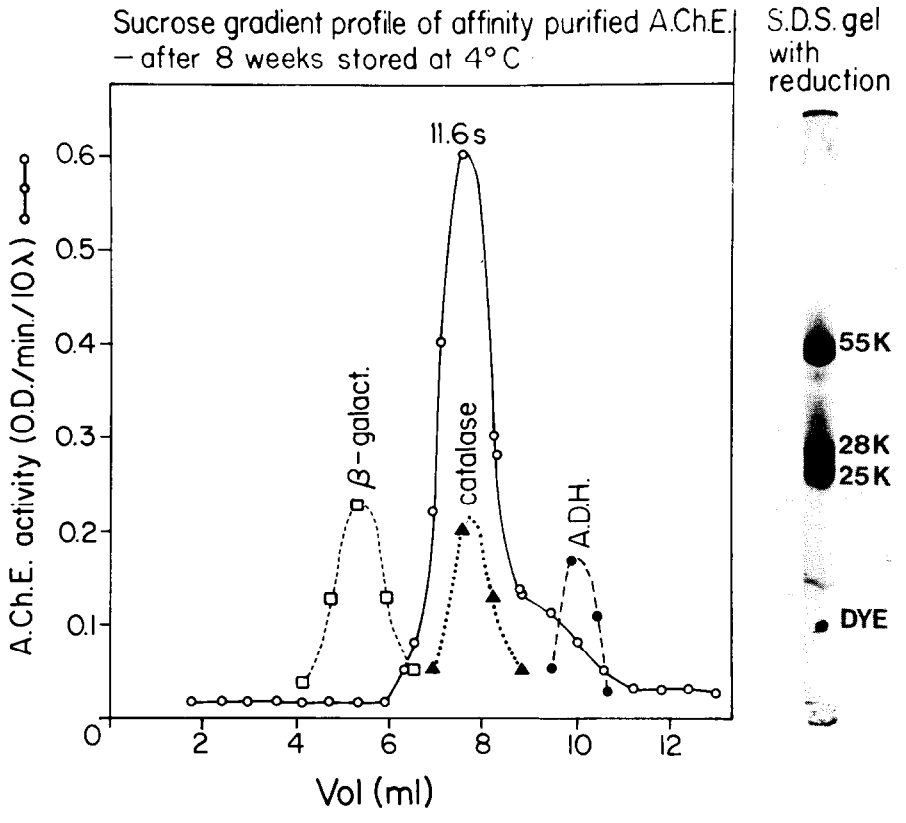

Fig.1. The molecular forms and subunit composition of a sample of AChE purified by affinity chromatography from fresh electroplax tissue and stored at 4°C for 8 weeks in 20 mM phosphate buffer (pH = 6.90). Isokinetic sucrose gradients were done in 0.5 M NaCl–0.02 M phosphate buffer (pH = 6.90) were calibrated with standard proteins as described in [2]. Acrylamide gel electrophoresis was done in 10 cm, 5.6% gels in the presence of SDS and dithiothreitol.

3. Results and discussion

The globular 11S form of AChE derived from the 18S and 14S molecular forms was used exclusively in the experiments described here. In fact, characterisation of the affinity purified material by isokinetic sucrose gradients and SDS gel electrophoresis, as shown in fig.1, demonstrated that the preparations were free of the 18S and 14S forms of the enzyme and that proteolytic and/or autolytic cleavage of the 80 000 mol. wt catalytic subunit had occurred yielding a major component at 55 000 mol. wt and two other fragments of 28 000 and 25 000 mol. wt. The complexity of this catalytic subunit composition has been discussed elsewhere [2,9,10] and the iodination experiments described here have attempted to probe the subunit structure further.

Two methods have been used to label the protein covalently with radioactive iodide (^{125}I); the first being an enzymatic iodination using lactoperoxidase whose specificity is for exposed tyrosine residues, and the second method being an esteratic method described by Bolton and Hunter [6] using ^{125}I-labelled 3-(4-hydroxy phenyl) propionic acid *N*-hydroxysuccinimide ester, whose site of action is the exposed amino groups of lysine and N-terminal amino acids. The two methods compliment one another by virtue of their different chemical specificity. The use of an ^{125}I-acylating agent is particularly attractive as the protein is never exposed directly to radioactive iodide.

The results of SDS gel electrophoresis, with reduction, of the iodinated AChE are summarised in figure two. The gel scans of the Coomassie blue stained gels (figs.2(c) and 2(d)) emphasise that for both of the iodination methods the catalytic subunit consists of the three 55 000, 28 000 and 25 000 mol. wt components. Figs.2(a) and 2(b) however demonstrate that these components behaved differently towards the two iodination techniques; specifically lactoperoxidase catalysed iodination resulted in greater than 90% of the label entering the low mol. wt 28 000 and 25 000 components while the esteratic iodination results in a uniform distribution of the covalent label between all three subunit components. Control experiments in which AChE is exposed only to ^{125}I in the absence of either lactoperoxidase or acylating agent show a completely non-specific low level of incorporation of ^{125}I throughout the SDS gel (less than 1000 cpm, total).

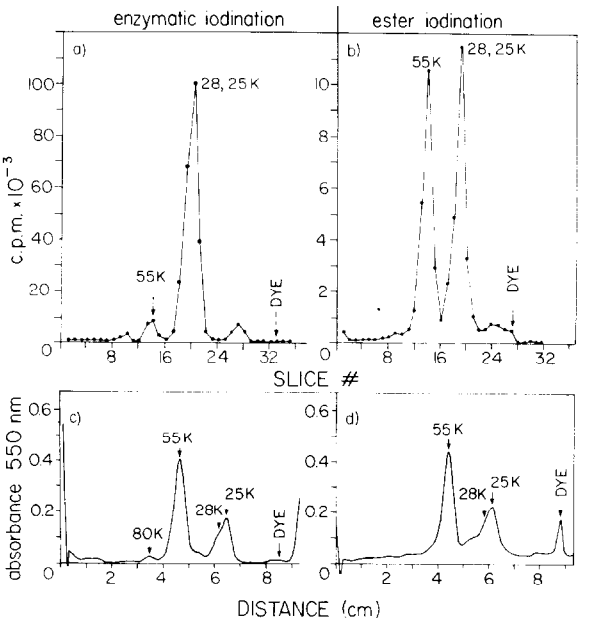

Fig.2. SDS-acrylamide gel electrophoresis of the 11S form of AChE *under reducing conditions.* (a) and (b): The distribution of ^{125}I after lactoperoxidase and esteratic iodination (see text), and (c), (d): corresponding gel scans at 550 nm from cathode (left) to anode (right).

Hence it appears that whereas all the components of the AChE subunit contain amino groups available for iodination by the esteratic method of Bolton and Hunter, only the low mol. wt components (28 000 and 25 000) possess the necessary number of exposed tyrosine residues for effective lactoperoxidase catalysed iodination.

Figure three summarises the results obtained when the iodinated AChE samples are run on SDS gels without prior reduction with dithiothreitol. The gel scans (3(c) and (d)) show major bands at 80 000 mol. wt and greater than 100 000 mol. wt. The acrylamide gels were not calibrated under non-reducing conditions for components greater than 100 000 mol. wt but this band has been observed by others and is thought to correspond to a dimeric structure of subunits connected via inter-subunit disulphide bonds [4]. Indeed, an SDS gel without reduction of the freshly affinity purified AChE displays only this one band at greater than 100 000 mol. wt. No band at 80 000 is observed but rather this appears, as shown here, upon storage

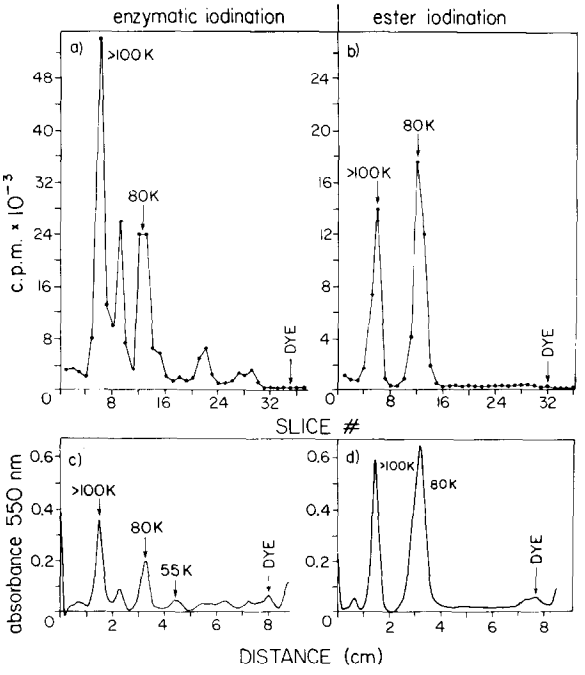

Fig.3. SDS-acrylamide gel electrophoresis of the 11S form of AChE *without prior reduction.* (a), (b), (c) and (d) as assigned in figure two.

of the protein (unpublished results from four preparations).

The iodination profiles, figs.3(a) and 3(b) mimic, for both iodination methods, the profile obtained by gel scanning the Coomassie blue stained gels; the extent of the iodination depending only on the amount of protein present. Hence from non-reduced samples, no great differences in the ^{125}I distribution in AChE is revealed.

In conclusion, it has been demonstrated that the globular 11S form of AChE can be chemically modified to incorporate ^{125}I covalently. Of particular interest, for future work, is the selectivity shown by lactoperoxidase catalysed labelling towards the low mol. wt components of the subunit. Work is now in progress using these iodination methods to study the 'native' molecular forms of the solubilised and membrane-bound enzyme.

Acknowledgements

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