STRUCTURAL STABILITY AND COMPOSITION OF ACETYLCHOLINESTERASE PURIFIED BY AFFINITY CHROMATOGRAPHY FROM FRESH ELECTROPLAX TISSUE OF ELECTROPHORUS ELECTRICUS.†

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SUMMARY

Acetylcholinesterase (AChE) has been purified from fresh electroplax tissue by affinity chromatography. SDS gel electrophoresis of purified AChE with reduction shows predominantly one component at 80,000 molecular weight. SDS gels run at various times after purification demonstrate that the 80,000 molecular weight polypeptide is susceptible to cleavage, generating peptides of 55,000, 28,000 and 25,000 molecular weight. Incubation of purified AChE with trypsin mimics this cleavage of the 'native' subunit. Sucrose gradient centrifugation of purified AChE shows it to be composed of two forms of 18S and 14S which, upon proteolysis, convert to a globular 11S form. Furthermore, conversion of the 18S, 14S molecular forms of AChE to the globular form occurs faster than proteolytic cleavage of the catalytic subunits.

INTRODUCTION

'Native' Acetylcholinesterase (AChE) obtained from fresh electric organ tissue of the electric eel and purified by affinity chromatography is composed primarily of two molecular forms characterised by their sedimentation coefficients of 18S and 14S. Another globular form of the enzyme, 11S, can be derived from the 'native' forms by proteolysis, but this must be thought of as a degradation product, as it has been shown to have lost a long assymetric structure called the "tail", which though it has no catalytic activity may be structurally important, particularly as AChE is a membrane bound protein (1,2).

Recently, two laboratories have reported investigations on the subunit heterogeneity of various preparations of the globular enzyme obtained

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from toluene-stored tissue (3,4). Briefly, different samples of AChE displayed different SDS gel electrophoretic patterns depending on how long the tissue had been stored under toluene and the method of enzyme purification. Proteolysis and/or autolysis has thus been shown to effect major differences in the composition of the disulfide reduced polypeptides of the catalytic subunits of this globular form of the enzyme.

In this study, we have dealt exclusively with fresh electroplax tissue, thus purifying the most native forms (18S,14S) of the enzyme. We have investigated the stability of this kind of preparation towards proteolysis by contaminating endogeneous protease which is present even after affinity chromatography of the enzyme (unpublished observations). In order to understand the constitution and assembly of the subunits of AChE, and gain information about the molecular events occuring upon proteolysis, we have treated the purified AChE with trypsin under various conditions. SDS gel electrophoresis and isokinetic sucrose gradients were used to monitor the resulting changes in enzyme structure.

MATERIALS AND METHODS

Acetylcholinesterase Preparations: Purified enzyme was obtained from fresh electric organ tissue by affinity chromatography utilising a resin described by Dudai, Silman, et al. (5). The inhibitors synthesised for covalent linkage were the meta and para isomers of N-(6aminocaproyl)-1-aminophenyltrimethylammonium bromide hydrobromide; the affinity chromatographic results for either of these inhibitors was the same. The inhibitors were coupled to cyanogen bromide activated Sepharose 2B, which had been previously cross-linked and reduced (6), to give a final concentration of 0.8 μmoles of ligand per ml of packed gel. After extensive washing to remove unabsorbed proteins, AChE was eluted from this resin by applying a linear gradient of 0-5 mM decamethonium. Preparations were routinely stored at 4°C in 20 mM phosphate buffer (pH = 6.90). Electrophorus electricus were obtained from Paramount Research Supply Co., Ardsley, N.Y. and kept alive in an aquarium prior to use.

Enzyme Assay: Assays were performed at pH 8.0 by the method of Ellman et al. (7). Protein concentrations were estimated spectrophotometrically using an $\varepsilon_{280}^{1\%} = 18.0$ (8,9)

Trypsin Treatment: Treatment of purified AChE with trypsin (Worthington) was done for varying lengths of time at 4°C and at different concentrations of trypsin. Proteolysis was quenched by the addition of soya-bean trypsin inhibitor (Sigma).

Polyacrylamide gel electrophoresis: Electrophoresis using 5.6% gels was performed in the presence of SDS by the method of Fairbanks et al. (10) with and without reduction with dithiothreitol (Sigma). Coomassie blue stained gels were scanned at 550 nm on a Gilford spectrophotometer. Gels were calibrated for molecular weight as in ref. (10).

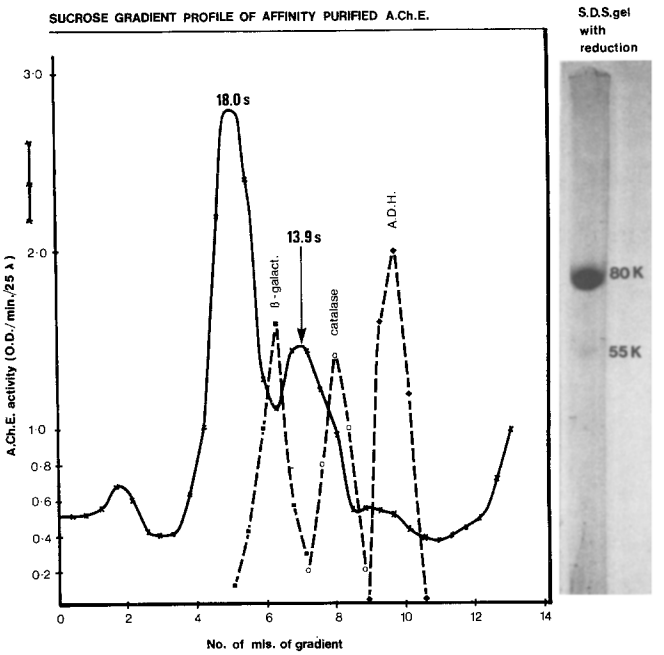

Figure 1 : The molecular forms and subunit composition of AChE purified by affinity chromatography from fresh electroplax tissue. Isokinetic sucrose gradients done in 0.5 M NaCl-0.02 M phosphate buffer (pH 6.9) were calibrated with standard proteins as described in text. Acrylamide gel electrophoresis was done in 10 cm, 5.6% gels in the presence of SDS and dithiothreitol.

Isokinetic sucrose gradients: Gradients were calculated and prepared for a Beckman SW 41 Ti rotor by the method of Noll (11). The experiments were run at 42,000 r.p.m. for 12 hours and the gradients were calibrated with standard proteins of known sedimentation coefficients centrifuged in the same tube: β-galactosidase (15.9S), catalase (11.4S) and alcohol dehydrogenase (7.6S). Enzyme activity assays for β-galactosidase and alcohol dehydrogenase were as described in ref. 12. Catalase was determined by its absorbance at 405 nm.

RESULTS AND DISCUSSION

Having purified the enzyme by affinity chromatography the preparation was characterised via isokinetic sucrose gradient centrifugation and polyacrylamide gel electrophoresis in the presence of SDS and dithiothreitol. The results for a typical preparation are shown in Fig. 1. This data indicates that we are dealing with the large assymetric forms (18S, 14S) composed of a long 'tail' structure and several groups of catalytic, tetrameric 'head' groups (1,2). Since the SDS gel consists primarily of one component, each subunit within the

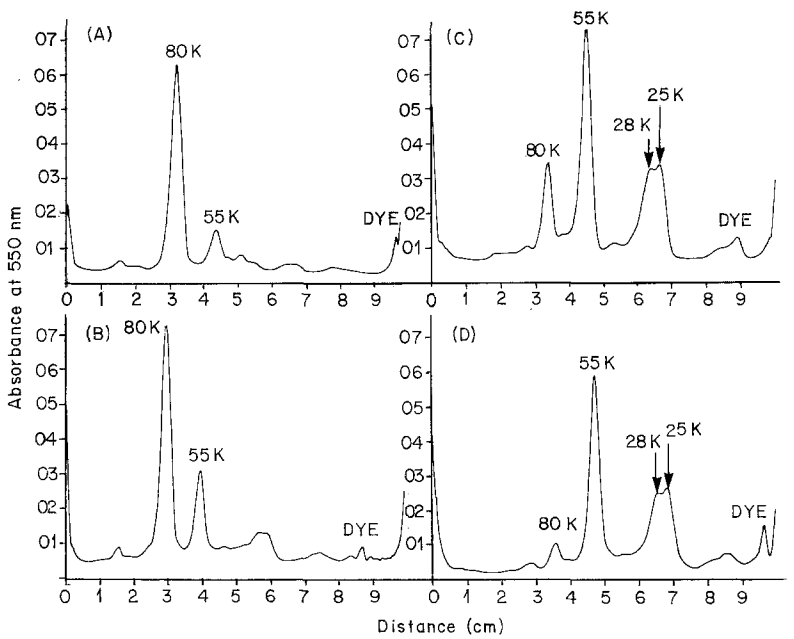

Figure 2 : SDS-acrylamide gel electrophoresis of the purified 18S, 14S forms of AChE under reducing conditions. Gels scanned from cathode (left) to anode (right) at 550 nm. A, AChE sample 1 week after affinity purification. B, 2 weeks. C, 3 weeks . D, 4 weeks.

catalytic tetramer is identical, and of molecular weight approximately 80,000. Samples prepared for SDS electrophoresis in the absence of reducing agents display no band at 80,000 molecular weight at all. In fact, a single band is observed with a molecular weight well in excess of 100,000. Although the 5.6% SDS acrylamide gels were not calibrated, under non-reducing conditions, for molecular weights in this region, this component may correspond to the dimeric structure of subunits (160,000) connected by intersubunit disulphide bonds, as reported by others for the 11S form of the enzyme (3,4).

SDS gels of AChE, with reduction, run as a function of time after purification indicate that the 80,000 subunit is susceptible to proteolytic cleavage by an endogenous protease which is present even after affinity chromatography. Fig. 2 summarises a typical experiment and shows scans of SDS gels of AChE from one to four weeks after purification. These results from a single sample show

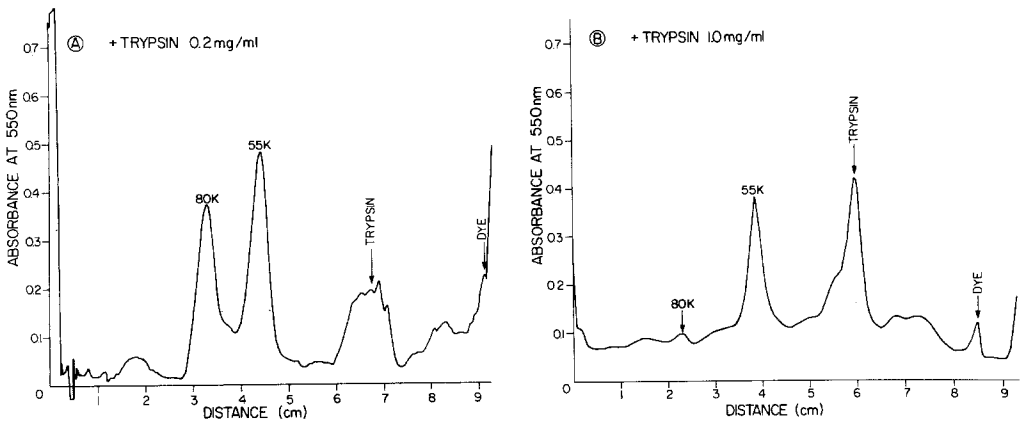

Figure 3 : SDS-Acrylamide gel electrophoresis in the presence of dithiothreitol of trypsin treated AChE samples. (A) and (B) represent incubations of the 18S, 14S forms of AChE with trypsin for 16 hours, 4°C, at the concentrations of trypsin noted in the figure. Gels scanned as in Fig. 2.

clearly that the 80,000 subunit is cleaved in a well defined way to generate a peptide of molecular weight 55,000 and two at 28,000 and 25,000 respectively. Parallel gels stained for carbohydrate shown the same 80,000 to 55,000 peptide conversion and indicate that these are glycopeptides. The 28,000 and 25,000 bands, however, do not stain for carbohydrate. These experiments which probe the subunit composition of the enzyme indicate that the tetramers of the 'native' forms are composed of catalytic subunits which undergo a well ordered proteolytic degradation.

Trypsin in high concentration, has been used to extract the globular 11S form of AChE from the electroplax tissue of both Electrophorus electricus and Torpedo californica (5,13). Using these same conditions on the already purified native forms of the enzyme we have found that it is possible to bring about marked changes in the subunit composition. Figure three shows gel scans of SDS gels where AChE has been incubated with two different concentrations of trypsin for 16 hours. A control sample with no trypsin added shows only the component at 80,000 but it can be seen that when trypsin is added and the concentration is increased this 80,000 peptide is cleaved to generate the

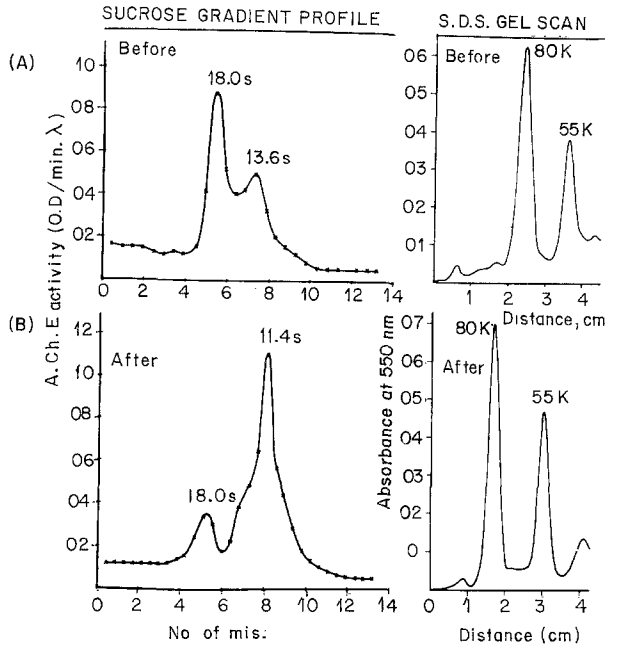

Figure 4 : The molecular forms and subunit composition of AChE before (A) and after (B) mild trypsin treatment. Samples were incubated with 0.01 mg/ml trypsin for 15 minutes prior to quenching with 0.2 mg/ml soyabean trypsin inhibitor. Isokinetic sucrose gradients in 0.5 M NaCl-0.02 M phosphate buffer (pH 6.9). Gels scanned as in Fig. 2.

55,000 peptide and bands around 25,000. Indeed it is possible using these high concentrations of trypsin to mimic the endogenous proteolytic cleavage of the native subunit observed to occur slowly after purification by affinity chromotography.

Variation of the proteolytic conditions reveals interesting differences in the rates of proteolysis of the different elements of the 18S,14S AChE structure. The effect of incubations of AChE with 0.01 mg/ml trypsin for 15 minutes as monitored by SDS gel electrophoresis and isokinetic sucrose gradients are shown in Fig. 4. It can be seen that before and after trypsin treatment there is no great change in the disposition of the 80,000 and 55,000 polypeptides. However, trypsin is known to convert the native forms of the enzyme to the globular (11S) form and indeed even this short incubation is sufficient to

convert the vast majority of the enyzme to 11S. Some 18S and 14S is, however, still present. Thus, the data seem to indicate that the purified 18S and 14S forms of the enzyme lose their long assymetric tail faster than cleavage of the catalytic subunits occurs.

It should be noted that our results on the "subunit heterogeneity" of the native (18S,14S) enzyme are in agreement with two recent papers on the globular, 11S, enzyme (3,4). Furthermore, our results support an inter-subunit disulphide model of the quaternary structure of the enzyme (model 2) proposed by Dudai and Silman (3). In this context, it should be noted that SDS gels run without reduction do not show any band at 80,000 when the enzyme is purified from fresh tissue. Rather, this band appears as a result of trypsin treatment or proteolysis/autolysis which occurs with storage (unpublished results from four experiments).

Finally, it is important and interesting to note that all the molecular changes observed in the experiments described in this paper occur without any detectable loss in activity of the enzyme.

ACKNOWLEDGEMENTS

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