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FUNCTIONAL IDENTITY OF CATALYTIC SUBUNITS OF ACETYLCHOLINESTERASE

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Summary

11 S acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) from the electric eel *Electrophorus electricus* essentially consists of four catalytic subunits which appear to be identical structurally but to be assembled with slight asymmetry. During isolation and storage of the enzyme, proteolysis cleaves a portion of the subunits into major fragments containing the active site and minor fragments containing no active sites without change in the enzyme molecular weight. A previous report (Gentinetta, R. and Brodbeck, U. (1976) Biochim. Biophys. Acta 438, 437—448) indicated that the intact and the fragmented subunits reacted with diisopropylfluorophosphate at different rates and that the reaction rate in the presence of excess phosphorylating agent was not strictly first order. Those findings could not be reproduced in this report. Intact and fragmented subunits were observed to react at the same rate with diisopropylfluorophosphate. In addition, the overall reaction kinetics both of 11 S and 18 S plus 14 S acetylcholinesterase were found to be strictly first order in the presence of an excess of diisopropylfluorophosphate throughout the course of reaction. These results are consistent with several previous reports that only one type of active site can be detected in acetylcholinesterase. The proteolysis which fragments a portion of the catalytic subunit has no apparent effect on the catalytic properties of the enzyme.

Introduction

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) from the electric eel *Electrophorus electricus* can be isolated in several molecular forms. 18, 14

Abbreviation: iPr_2P-F, diisopropylphosphorofluoridate.

and 8 S forms apparently consisting of 12, 8 and 4 catalytic subunits attached to an elongated collagen-like tail structure can be obtained from extracts of fresh electric tissue [1—3; see [4]). In contrast, apparent autolysis on storage of crude enzyme solutions or treatment of extracts with proteases produce 11 S forms in which the tail structure is degraded [1,2,5—7]. Detailed structural studies of the 11 S forms show that they consist of four 75 000 dalton catalytic subunits of similar if not idential composition [8,9].

During purification and storage of either 11 S or 18 S plus 14 S acetylcholinesterase, the 75 000 dalton catalytic subunit undergoes proteolytic cleavage at a specific site to generate both a 50 000 dalton fragment containing the reactive serine residue at the active site [8] and a fragment of either 27 000 or 23 500 daltons. This proteolysis of catalytic subunits does not release fragments from the native 11 S enzyme but is detectable only after the enzyme is denatured and subjected to disulfide bond reduction [8,9]. While the extent of this cleavage is small and fairly constant in 18 S plus 14 S enzyme preparations [3,10], it varies from about 30% to nearly 100% in 11 S enzyme preparations, depending on the purification and storage conditions [7,9,11].

These structural variations have no apparent effects on the catalytic properties of acetylcholinesterase. 18 S, 14 S, and 11 S enzyme and catalytically active dimers and monomers produced from these forms show no significant differences in kinetic behavior towards substrates and inhibitors [1,4,12]. Furthermore, the catalytic subunits of acetylcholinesterase appear to be kinetically indistinguishable. The reaction of the enzyme with active site titrants has generally been found to be strictly first order in active enzyme concentration during the entire course of the titrations [13—15]. Recently, Gentinetta and Brodbeck [16] reported an apparent disagreement with these conclusions. By analysis of ^{3}H-labelled diisopropylfluorophosphate ([^{3}H]-iPr_2P-F) inhibited enzyme on sodium dodecyl sulfate-polyacrylamide gels after disulfide reduction they found two peaks that incorporated [^{3}H]iPr_2P-F at different rates. When they reacted iPr_2P-F with either 18 S plus 14 S or 11 S enzyme, they observed curvature in semilogarithmic plots of enzyme activity versus time. This curvature was most pronounced during the first few minutes of the reaction. These authors concluded that there are two different catalytic subunits in acetylcholinesterase that react at different rates which iPr_2P-F. Since these were the first reported differences in catalytic activity among subunits of acetylcholinesterase, we attempted to reproduce their novel findings. We find that the 75 000 dalton subunit and the 50 000 dalton fragment of 11 S enzyme react with [^{3}H]iPr_2P-F at precisely the same rate. In addition the kinetics of reaction of iPr_2P-F with either 11 S or 18 S plus 14 S enzyme is strictly first order in active enzyme concentration over the entire course of the reaction. Our results indicate that there is only one type of active site in acetylcholinesterase.

Methods

Both 11 S and 18 S plus 14 S acetylcholinesterase, free of detectable protein contaminants, were purified by affinity chromatography as described previously [3,11].

Phosphorylation of 11 S acetylcholinesterase with [^{3}H]iPr_2*P*-F (0.9 Ci/mmol, New England Nuclear) was carried out at 23°C in 0.1 M sodium phosphate (pH 7.4) by the addition of stock 1.11 mM [^{3}H]iPr_2*P*-F in propylene glycol to an enzyme solution. The use of acetonitrile [16] was avoided because this solvent acts as a weak competitive inhibitor of acetylcholinesterase activity (Rosenberry, T.L., unpublished data). The loss of enzyme activity was monitored by a modification [3] of the procedure of Ellman et al. [17]. At various times aliquots of the partially inhibited enzyme were removed and made 200 mM in butyrylcholine iodide (Sigma) to block further phosphorylation. These aliquots were dialyzed in 0.1 M sodium phosphate (pH 7.4) containing 5 μM tensilon (Hoffmann-LaRoche) and appropriate portions were taken for scintillation counting in Scintisol (Isolab) and protein determination. The remainder of each aliquot was dialyzed extensively against water and lyophilized. Protein determination, liquid scintillation counting, and electrophoresis in sodium dodecyl sulfate-polyacrylamide gels were carried out as described previously [8]. Gel electrophoresis of a 20-μg protein sample was followed by staining in Coomassie brilliant blue R [18], while gels run with 10-μg protein samples were soaked briefly in 10% glycerol/10% trichloroacetic acid, frozen and sliced [8]. Individual gel slices (1—5 mm) were prepared for liquid scintillation counting by digestion in NCS (Amersham) and water at 50°C, followed by addition of scintillation cocktail and ascorbic acid [19]. In this digestion procedure, recovery of tritium was quantitative.

The kinetics of reaction of iPr_2*P*-F with acetylcholinesterase is most conveniently measured by monitoring the simultaneous hydrolysis of an acetic acid ester substrate [14,20—22]. To 2.51 ml 0.10 mM *p*-nitrophenyl acetate, enzyme, 0.1 M sodium phosphate, (pH 7.0), 1% CH_3OH at 23°C was added 4 μl 10 mM iPr_2*P*-F (Sigma) in propylene glycol. The reaction was monitored by the appearance of *p*-nitrophenol (ϵ_{400nm} = 8400 $M^{-1} \cdot cm^{-1}$). This concentration of *p*-nitrophenyl acetate is far below its apparent K_m of 4 mM (unpublished data using the enzyme from eel; also see ref. 14). Furthermore, the enzyme concentration was adjusted such that the phosphorylation reaction was complete before 10% of the substrate was hydrolysed. Under these conditions the ratio of the velocity of hydrolysis of *p*-nitrophenyl acetate to the free enzyme normality is essentially constant during the course of the phosphorylation reaction, and the phosphorylation rate constant can be determined directly according to Eqn. 1 [21,22].

$$\ln (A_\infty - A_t) = -kt + \ln (A_\infty - A_0) \quad (1)$$

where k is the pseudo first order rate constant for phosphorylation and A is the observed absorbance at the time denoted by the subscript.

Results

When [^{3}H]iPr_2*P*-F was reacted with 11 S enzyme, the incorporation of tritium was directly proportional to the loss of enzyme activity (Fig. 1). This result suggests the presence of only one type of iPr_2*P*-F reacting site. Gel electrophoresis of labelled enzyme in sodium dodecyl sulfate after complete disulfide reduction (Fig. 2) gave a pattern similar to that previously reported

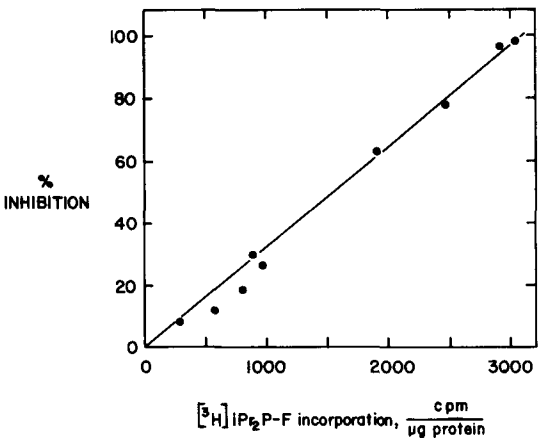

Fig. 1. Loss of enzymatic activity of 11 S acetylcholinesterase as a function of [^{3}H]iPr$_2$*P*-F incorporation. To 1 ml enzyme solution (0.83 mg, 11 nequiv.) was added 5 nmol stock [^{3}H]iPr$_2$*P*-F at time zero, an additional 10 nmol, after 80 min, and an additional 20 nmol, after 135 min.

for pure 11 S enzyme [8]. The most prominent band is the intact catalytic subunit with an apparent molecular weight of 75 000. Catalytic subunit fragments are also present: the 50 000 dalton fragment containing the active site and two smaller fragments of 27 000 and 23 500 daltons [8]. The faint bands above the monomer are trace amounts of catalytic subunit dimers and tetramers that are not dissociated by the disulfide reducing agent. The visual appearance of the stained bands was not affected by the degree of enzyme phosphorylation.

The relative incorporation of [^{3}H]iPr$_2$*P*-F into the 75 000 dalton subunit and the 50 000 dalton fragment was independent of the extent of phosphorylation. The phosphorylation patterns in two gels corresponding to two extreme phosphorylation levels are shown in Figs. 2B and 2C. In both gels, the ratio of tritium in the 75 000 dalton peak to that in the 50 000 dalton peak was essentially the same 1.42 for the slightly phosphorylated enzyme and 1.38 for the nearly totally phosphorylated sample. Enzyme phosphorylated to intermediate levels corresponding to 29% and 62% inhibition gave similar ratios, 1.39 and 1.38, respectively. Clearly, both the 75 000 dalton catalytic subunit and the 50 000 dalton subunit fragment react with [^{3}H]iPr$_2$*P*-F at the same rate.

In contrast to the report of Gentinetta and Brodbeck [16], we observed that the reaction of iPr$_2$*P*-F with both 11 S and 18 S plus 14 S acetylcholinesterase is strictly first order in active enzyme concentration when [iPr$_2$*P*-F] >> [enzyme] (Fig. 3). The initial curvature observed by those authors in their plots of log active enzyme versus time is not apparent here. They attributed the curvature to the presence of two different subunits, and they analyzed this curvature to assign fast and slow phosphorylation rates which differed by about a factor of 4. The respective second order reaction rate constants that we observed for 11 S and 18 S plus 14 S enzymes, $3.0 \cdot 10^2$ and $2.8 \cdot 10^2$ $M^{-1} \cdot s^{-1}$, are between the slow and fast rate constants reported by Gentinetta and Brodbeck [16].

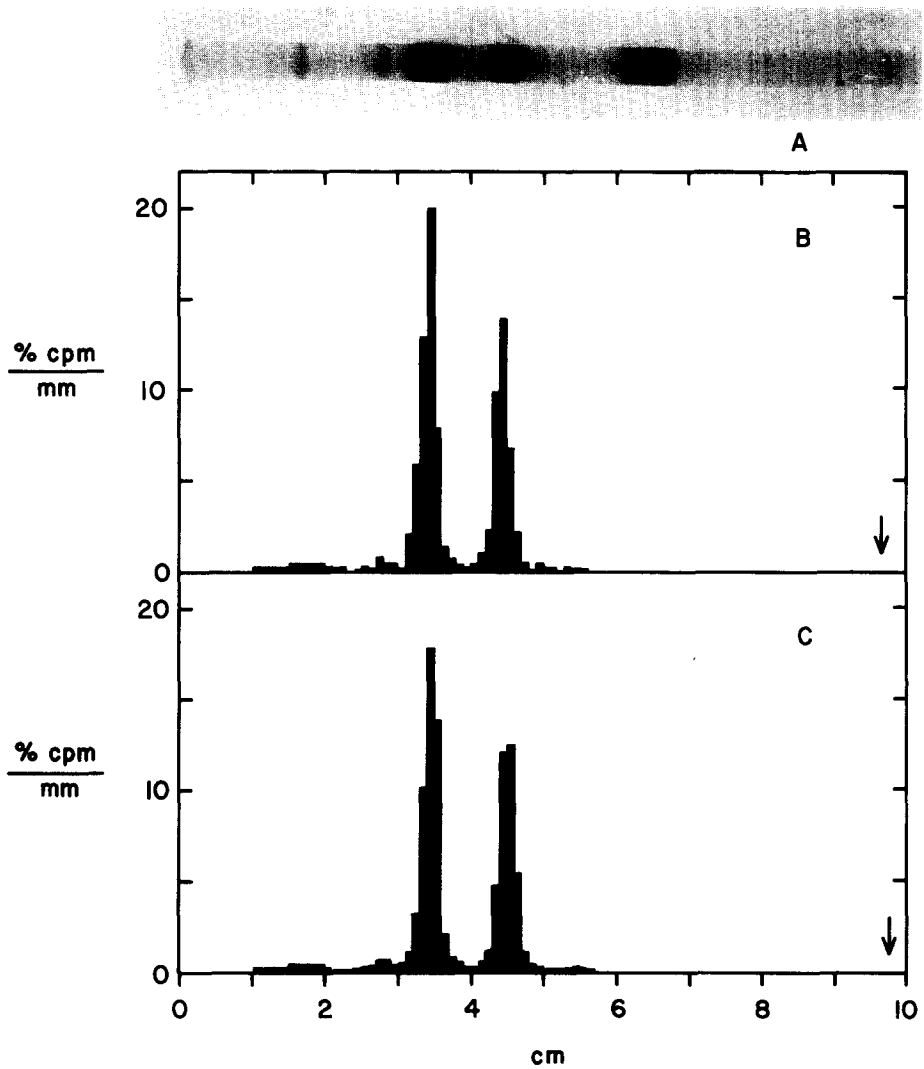

Fig. 2. Distribution of ^3H among [^3H]iPr_2*P*-F-labelled 11 S acetylcholinesterase subunit components. Samples were treated with 40 mM dithiothreitol at 50°C for 30 min in buffered 1% sodium dodecyl sulfate [8] and electrophoresed on 5.8% polyacrylamide gels in 1% sodium dodecyl sulfate. (A) Photograph of a Coomassie blue stained gel containing 20 μg enzyme. (B, C) Distribution of ^3H in slices of unstained gels containing 10 μg enzyme that has been labelled with [^3H]iPr_2*P*-F to a level of either 2% (B) or 98% (C) inhibition. The arrows indicate the dye fronts.

Discussion

Analyses of the reactions of either 11 S or 18 S plus 14 S eel acetylcholinesterase with iPr_2*P*-F are consistent with a single kind of active site in these enzyme forms. Proteolytic cleavage of part of the intact 75 000-dalton catalytic subunits, while generating fragmented subunits in which the reactive serine is found on a 50 000 dalton polypeptide, has no measureable effect on the reactivity of the enzyme towards iPr_2*P*-F (Fig. 2). The apparent non-

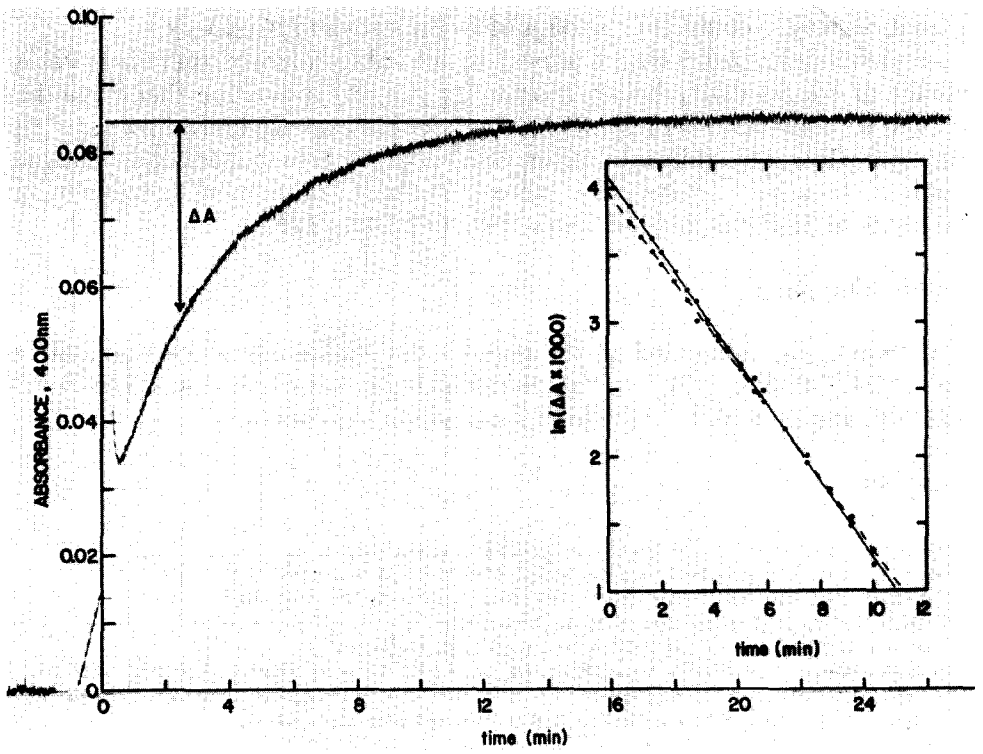

Fig. 3. Kinetics of reaction of iPr_2P-F with acetylcholinesterase. Shown is the actual absorbance trace for the hydrolysis of *p*-nitrophenyl acetate by 11 S acetylcholinesterase ($\approx 10^{-9}$ N in active sites) in the presence of 15.9 μM iPr_2P-F. The insert shows the agreement of the experimental traces to the first order plots corresponding to Eqn. 1 for 11 S enzyme, ●———●, and for 18 S plus 14 S enzyme, ○- - - - -○, under identical conditions. The extrapolated velocity at time zero in the presence of enzyme, *p*-nitrophenyl acetate, and iPr_2P-F ($\Delta A_{t=0} \cdot k_{obs} = 0.0199\ \Delta A_{400}$/min) corresponded precisely to the observed velocity prior to the addition of iPr_2P-F at time zero (0.0199 ΔA_{400}/min), indicating that 0.16% propylene glycol did not affect the enzyme activity. The inactivation was strictly first order over the entire observable trace, i.e. from 70% to 0% of the initial enzyme activity.

homogeneous reaction of [^{3}H]iPr_2P-F with acetylcholinesterase claimed by Gentinetta and Brodbeck [16] on the basis of their gel electrophoresis in sodium dodecyl sulfate may have arisen from protein impurities in their enzyme preparation. They neither correlate phosphorylation with enzyme inhibition nor identify phosphorylated gel bands with known enzyme subunits.

Our study of the kinetics of iPr_2P-F phosphorylation utilized an inherently precise continuous spectrophotometric determination of enzyme inactivation [21,22]. The rapid sampling procedure used by Gentinetta and Brodbeck [16], in addition to introducing more scatter to the kinetic plots, also requires a relatively high enzyme concentration during phosphorylation. We find a strict pseudo-first order inactivation of enzyme activity, in agreement with other active site titrations of acetylcholinesterase when the titrant is present in excess [13—15]. The deviation from strict pseudo-first order inactivation reported by Gentinetta and Brodbeck is closely analogous to similar phenomena observed by Hart and O'Brien [23], which were shown to arise from impurities in the phosphorylating agents applied to acetylcholinesterase. A similar

explanation may account for the data of Gentinetta and Brodbeck.

Acetylcholinesterase subunits appear to act independently; there is as yet no evidence of cooperativity between subunits, nor any indication that more than one type of active site is present [4]. Acetylcholinesterase does show substrate inhibition. However, this inhibition does not involve cooperativity between subunits, because catalytically active subunit monomers prepared by proteolysis of 18 S enzyme also show this inhibition [12].

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