AN ACTIVE MONOMERIC FORM OF *ELECTROPHORUS ELECTRICUS* ACETYLCHOLINESTERASE

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1. Introduction

Acetylcholinesterase from *Electrophorus electricus* exists in several different molecular forms [1–4]; all appear to be derived from the largest, the 'D' form (18.5 S, 1 100 000 daltons), which consists of twelve catalytic subunits closely organized in three tetrameric groups, attached to a rod-like tail [2–4]. Degradation of this form can yield active globular derivatives, among which the tetrameric 'G' form (11.8 S) is the most abundant and has been the most extensively studied. In addition to the tetramer, sonication of the D form produces a low yield of the dimeric form, G' (7.7 S). Grafius et al. have obtained a similar form (7.4 S) which they called 'half-monomer' [5], by sonicating acetylcholinesterase in Triton X-100.

The catalytic properties (K_M, substrate specificity) of the different forms of acetylcholinesterase are identical [6]. Although some conflicting results have appeared in the literature on the number of subunits and active sites on the enzyme, it is now firmly established that the G form contains four active sites [7]. The analogous 'lytic' form of *Torpedo californica* acetylcholinesterase also contains four active sites [8].

In this paper, we report a yet smaller, monomeric, form of active acetylcholinesterase, G''. We describe its catalytic properties with particular reference to peripheral binding sites and site–site interactions.

Abbreviations: TDF, *p*-(trimethylammonium) benzenediazonium fluoroborate; SDS, sodium dodecyl sulfate; IPA, indophenylacetate; DTNB, 2,2'-dinitro-5,5'-dithiodibenzoic acid; DFP, diisopropylphosphofluoridate; DTT, dithiothreitol; AchE, acetylcholinesterase.

2. Materials and methods

Acetylcholinesterase, solubilised by homogenizing electric organs in saline buffer (1 M NaCl, 0.05 M $MgCl_2$, 0.01 M Tris, pH 7), was purified as previously described [3]. After several months' storage at −20°C, a sample of this preparation was centrifuged in a 5–20% sucrose gradient (SW 27, 25 000 rev/min for 45 h, at 4°C), and the 3–7 S region of the gradient, containing the G'' form, was collected and lyophilised. The contaminating G and G' forms were separated by molecular sieve chromatography on a Biogel A 1.5 m column (diameter 2 cm, length 1 m, applied pressure about 75 cm). The G'' form of acetylcholinesterase was kept in saline buffer containing 0.5 mg/ml bovine serum albumin to prevent inactivation.

Purified G'' labelled with [^{3}H]DFP was prepared by molecular sieve chromatography of a sonicated preparation of the labelled asymmetric forms (2 min sonication at 15 s. intervals, with an MSE generator at maximum power, 20 000 Hz; same Biogel A 1.5 m column as above).

Gel electrophoresis in 1% SDS-polyacrylamide was carried out according to Fairbanks et al. [9], using *N,N'*-diallyltartardiamide instead of *N,N'*-methylenebisacrylamide. Gels were cut into 2 mm thick slices, dissolved in 1% periodic acid, and counted in 10 ml of Bray's scintillation fluid in an Intertechnique scintillation counter.

Acetylcholinesterase activity was measured by the Ellman method, at 20°C. The absorbancy variations at 412 nm, measured in a Zeiss PMQ II spectrophotometer, were recorded with a Sefram

servotrace recorder, and corrected for spontaneous hydrolysis of acetylthiocholine. Indophenylacetate hydrolysis was recorded at 525 nm. Kinetic studies of G, G′ and G″ were always done under identical experimental conditions. In each case, the enzyme content was adjusted so that the hydrolysis rate was equal.

3. Results and discussion

Storage of the asymmetric forms of acetylcholinesterase leads to a spontaneous degradation of the enzyme into globular forms which retain their catalytic activities. An analysis of these degradation products on Biogel A 1.5 m column (see Materials and methods) showed the presence of three active enzyme fractions, which had Stokes radii of 8.7, 5.9 and 3.66 nm. Their sedimentation constants were determined in a sucrose gradient (5–20% sucrose) and found to be respectively 11.8 S, 7.7 S and 5.3 S (fig.1). From these data, the first two fractions were identified as the tetramer G and the dimer G′. A mol. wt. of 70 000 was determined for the third one, named G″, by comparison with the heavier forms of the enzyme [10].

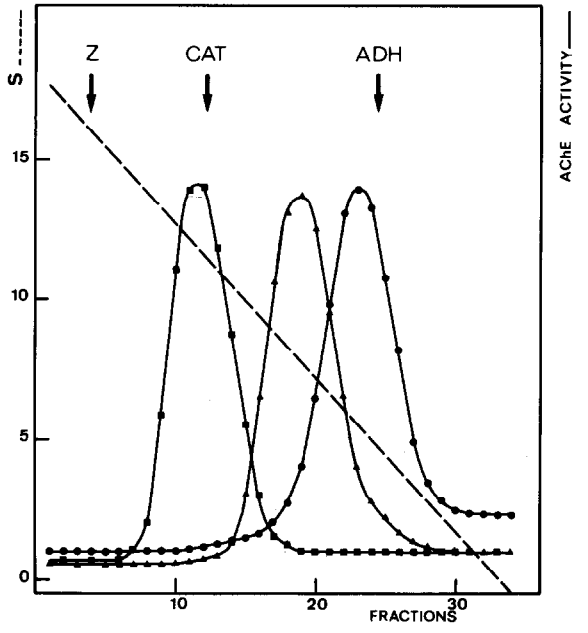

Fig.1. Sucrose gradient centrifugation of the globular forms of *Electrophorus* acetylcholinesterase. (■) G. (▲) G′. (●) G″. The top of each active fraction on Biogel A 1.5 m (see Materials and methods) was centrifuged in a 5–20% weight/volume sucrose gradient in 1 M NaCl, 0.05 M $MgCl_2$, 0.01 M Tris-HCl, pH 7. Centrifugation 40 000 rev/min, at 2°C, for 16 h in an SW 60 Beckman rotor. Marker enzymes: *E. coli* β-galactosidase (16 S); catalase (11.4 S); yeast alcohol dehydrogenase (7.4 S).

This mass is very close to that of the subunits of acetylcholinesterase, as determined by polyacrylamide gel electrophoresis in a dissociating medium: the values given by different authors vary from 70 000 to 100 000 daltons [11,14]. It thus appears that G″ consists of a single subunit.

Additional evidence for this has been obtained from SDS polyacrylamide gel electrophoresis of [^{3}H]DFP labelled G″. [^{3}H]DFP labelled G″ was obtained as a degradation product after sonication of the asymetric forms of acetylcholinesterase labelled with [^{3}H]DFP. Its idendity with catalytically active G″ was assessed by its physical parameters (Stokes radius, sedimentation constant).

Fig.2 shows that, in the DTT reduced state, the

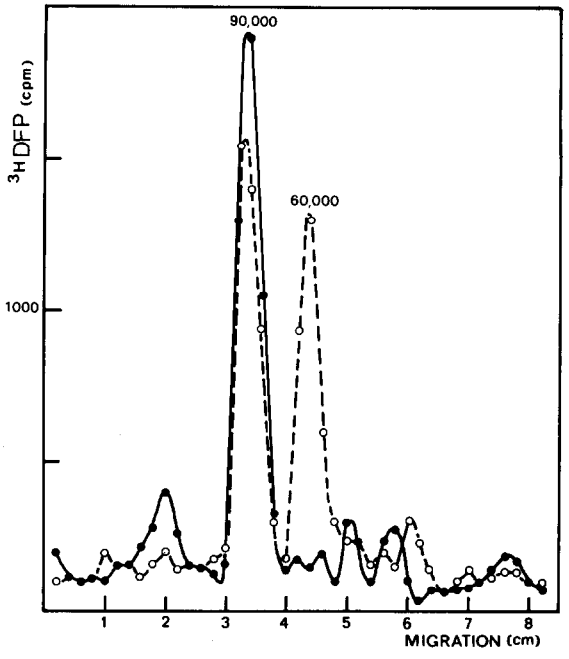

Fig.2. SDS-polyacrylamide gel electrophoresis of [^{3}H]DFP labelled G″ acetylcholinesterase. (●————●) Non-reduced. (○– – – –○) DTT reduced. Calibration of the gels was done using as mol. wt. standards: alkaline phosphatase, bovine serum albumin, catalase, pepsin and Bromophenol blue.

G'' form exhibited both the 90 000 and 60 000 dalton bands, corresponding respectively to an intact subunit, and a radioactively labelled degradation fragment of this subunit. This pattern is identical to that obtained for other molecular forms isolated from the same acetylcholinesterase preparation [11]. In the non-reduced state, only the 90 000 dalton band was found (fragments do no show up separately, as they are linked to their complementary non labelled peptides by disulfide bonds) [11].

From these observations, we conclude that G'' consists of one single catalytic subunit, identical to those which constitute the more complex forms of the enzyme.

3.1. *Catalytic properties of monomeric acetylcholinesterase*

Fig.3 shows Dixon plots for the three globular enzymes G, G' and G''. Their K_Ms for acetylthiocholine were very similar, that of G'' being, however, slightly higher than that of G' or G (60 μM compared to 40 μM). Inhibition by excess substrate was observed with G'', as with the other forms of acetylcholinesterase, but became apparent only at a higher substrate concentration (fig.4); the optimal substrate concentration was 0.4 mM for both G and G', and 1.7 mM for G''.

The effect of the alkylating inhibitor TDF on the activity of G, G' and G'' is summarized in table 1. The hydrolysis of acetylthiocholine by all three forms of acetylcholinesterase was completely blocked after a 15 minute treatment with this agent. During the onset of this inhibition the catalytic hydrolysis of indophenylacetate (IPA) was also strongly inhibited, but it recovered during a second slower phase. Although each form (G, G'and G'') displayed this recovery of IPA hydrolyzing activity, it was found to be more rapid in the case of the tetrameric G form.

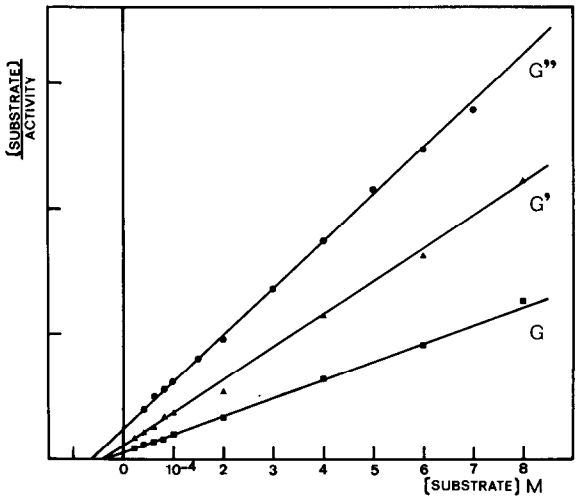

Fig.3. Dixon plot for the hydrolysis of acetylthiocholine by the globular forms of acetylcholinesterase. (■) G. (▲) G'. (●) G''. Ellman's reagent medium: 1 mM DTNB, 0.05 M phosphate pH 7, 0.5 mg/ml bovine serum albumin. Reaction was done at 20°C, monitored at 412 nm, and corrected for spontaneous hydrolysis of acetylthiocholine. Substrate concentration over rate ratios, given in ordinates, are in arbitrary units.

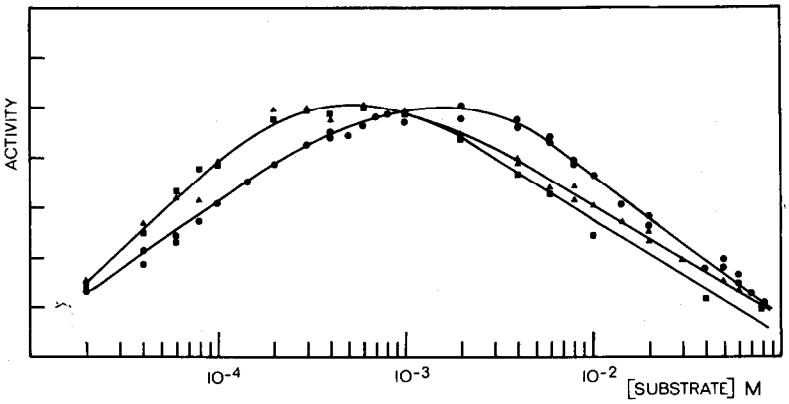

Fig.4. Variation of acetylthiocholine hydrolysis rate with substrate concentration, for the globular forms of acetylcholinesterase: excess substrate inhibition (■ – ■) G. (▲ – ▲) G'. (● – ●) G''. Conditions as in fig.3. Rates are given in arbitrary units, the curves being normalized assuming that V_{max}, (as obtained from the linear Dixon plots, cf. fig.3) is identical for the three enzymes.

Table 1
Effects of alkylation of acetylcholinesterase by TDF on the hydrolysis rates of acetylthiocholine and indophenylacetate

Molecular form	G		G′		G″	
Substrate	Acetyl-thiocholine	Indophenyl acetate	Acetyl-thiocholine	Indophenyl acetate	Acetyl-thiocholine	Indophenyl acetate
Control	100	100	100	100	100	100
2 min	50	35	50	60	60	60
8 min	10	5	10	5	10	5
1 h	0	5	0	–	0	5
3 h	0	50	0	–	0	27
20 h	0	80	0	38	0	35

Hydrolysis rates were determined at 20°C, at variable times after addition of 0.3 mM TDF to the enzyme. The reaction media contained 0.75 mM acetylthiocholine iodide and 0.5 mM DTNB in one case, 0.75 mM indophenylacetate in the other. The rate of absorbancy variation at 525 nm for indophenylacetate hydrolysis was 0.2% that in the Ellman assays (412 nm), for G and G′, and only 0.1% for G″. All activities were normalized to 100 for the control. The activity of the G″ preparation was such that 1 μl of enzyme produced a variation of 0.010 OD per min, in 1 ml Ellman assay medium for 1 cm path length.

4. Conclusion

Low sedimentation constant forms of *Electrophorus* acetylcholinesterase have been reported by previous authors: a combination of acid precipitation and chromatographic procedures, used by Hargreaves et al. [15] yielded a 4 S preparation; Grafius et al. also reported a 4 S catalytically active minor component in their sonicated material [5].

We have shown that the G″ form of acetylcholinesterase (5.3 S) consists of a single monomer. This enzyme is catalytically active; its K_M for acetylthiocholine is approximately equal to that of all other forms of acetylcholinesterase, globular or asymmetric [6].

It was of particular interest to investigate the features of acetylcholinesterase kinetics which might reveal interactions between catalytic and peripheral sites [7,8]. Inhibition by excess substrate probably results from binding of substrate molecules at a peripheral site [16]. We found that monomeric acetylcholinesterase showed substrate inhibition, demonstrating that both interacting sites exist on the same monomer. Affinity for acetylcholine on both sites seems decreased, so that the optimal substrate concentration is markedly shifted towards higher values. The 'two-phase' reaction kinetics following TDF treatment was originally taken as indicating the existence of regulatory and catalytic sites, possibly interacting in an allosteric manner [17,18]. It was not clear whether they were located on the same or different subunits. The fact that both the initial inhibitory phase and the slower recovery phase of indophenylacetate hydrolysis were observed with the G″ monomers indicates that the two types of alkylated sites occur on the same unit.

Each subunit is thus found to possess the whole complement of catalytic and peripheral (perhaps regulatory) sites, and exhibits the characteristic features of acetylcholinesterase catalysis. This is in agreement with the established fact that there are four active sites and four peripheral sites per tetramer [7], and with the observation that following SDS polyacrylamide gel electrophoresis only one kind of subunit can be detected [11,14].

Acknowledgements

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