MOLECULAR FORMS OF *ELECTROPHORUS* ACETYLCHOLINESTERASE THE CATALYTIC SUBUNITS: FRAGMENTATION, INTRA- AND INTER-SUBUNIT DISULFIDE BONDS

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1. Introduction

In previous publications, we have described the occurrence of five main molecular forms of *Electrophorus* acetylcholinesterase [1,2] and, more recently, of a sixth, monomeric form of this enzyme [3]. We have presented preliminary reports on the properties of these molecules which led us to distinguish between two classes among them [2]. The first class, that of the A (9 S), C (14.2 S) and D (18.4 S) forms possessed the hydrodynamic properties of highly 'asymmetric' particles, as judged from their Stokes radii and sedimentation constants [4]. The second class includes forms G (11.8 S), G′ (7.7 S) and G″ (5.3 S) which did not present such asymmetry. Electron micrographs [5,6] have shown that the asymmetric forms contain a rod-like structure, the 'tail'. We have deduced the number of catalytic subunits from molecular weight data for all forms [7], confirming a model previously proposed [5,6] according to which A, C, D are composed, in addition to a rod like 'tail', of one, two and three tetramers respectively. The forms G, G′ and G″ are tetramers, dimers and monomers. The catalytic properties of the monomer G″ have been recently described [3].

In this paper we analyze the polypeptide composition and subunit disulfide linkages for both asymmetric and globular forms by means of polyacrylamide gel electrophoresis after sodium dodecyl sulfate denaturation. A preliminary account of some of these results has been given [8].

Abbreviations: DFP: diisopropylphosphofluoridate, DTT: dithiothreitol, SDS: sodium dodecyl sulfate, Acetylcholinesterase (EC 3.1.1.7).

2. Materials and methods

The preparation and purification of the various forms of acetylcholinesterase have been described elsewhere [9,10]. Radioactive labelling of the active sites was carried out by reaction with 50 μM [^{3}H]DFP (CIS, 10 Ci/mM) without any carrier DFP, in 1 M saline buffer, at 4°C overnight. Enzymic activity was inhibited 95% under these conditions.

Electrophoresis in 5% polyacrylamide gels was performed according to the method of Fairbanks et al. [11], including 1% SDS in the gels. Denaturation and reduction of the protein was performed prior to electrophoresis, by incubating the sample at 100°C, for 10 min, in 10% SDS, 40 mM DTT, 0.01 M Tris pH 8.0. Iodoacetamide (100 mM) was added after 30 min, and allowed to react at 100°C, for 10 min.

In some experiments, reduction of disulfide bonds by 10 mM DTT, at 4°C overnight, and subsequent alkylation of –SH groups (20 mM iodo-acetamide for 30 min at 20°C), were performed before denaturation in otherwise identical conditions. When [^{3}H]DFP labelled enzyme was analyzed in gels, $N \rightarrow N'$-diallyltartardiamide was used instead of $N \rightarrow N'$-methylene bisacrylamide [12]. After electrophoresis, 2 mm thick slices of gels were dissolved in 2% periodic acid, and radioactivity determined in Bray's scintillation fluid in an Intertechnique Scintillation counter.

About 200 μg of protein were loaded onto the gel and stained with Coomassie Brilliant Blue or periodic acid-Schiff reagent. Scanning of the gels was performed in a Varian densitometer. Calibration of molecular weight estimation was carried out using standards of alkaline phosphatase, bovine serum albumin, catalase,

ovalbumin, pepsin, trypsin, phosphorylase *a*, kinase, chymotrypsin, cytochrome *c* and β-galactosidase.

3. Results and discussion

3.1. *A common catalytic subunit and its fragments*

We first examined the distribution of the serine carrying polypeptides, after phosphorylation of acetylcholinesterase with [^{3}H]DFP. After thorough dissociation of acetylcholinesterase by SDS denaturation and DTT reduction, several labelled polypeptide chains were found (fig.1). With an enzyme which had been freshly prepared by affinity chromatography [9] we detected one main polypeptide chain α, (90 000 dalton) and a minor component, α′ (60 000 dalton). In trypsin-treated acetylcholinesterase, or in aged preparations, we found decreased proportions of the α component, accompanied by increasing amounts of α′ and smaller chains. Our conclusion was that the α band corresponded to the original state of the active catalytic subunit.

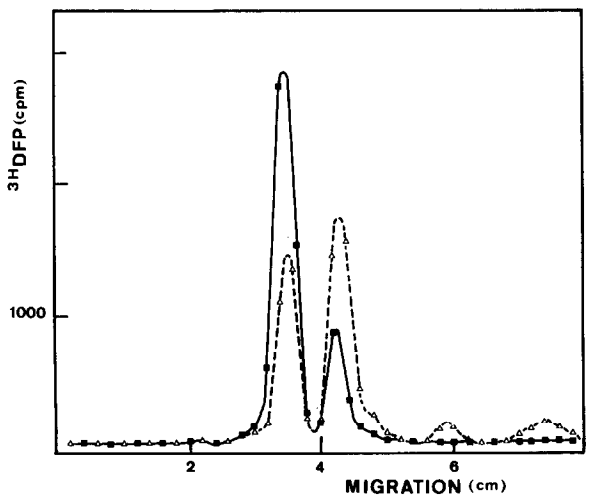

Fig.1. SDS polyacryalmide gel electrophoresis of reduced, [^{3}H]DFP labelled acetylcholinesterase. (——) Freshly prepared enzyme: An homogenate was prepared from fresh electric organs, precipitated with ammonium sulfate, and chromatographed on an affinity column [9] within 10 h. The purified enzyme was then dialysed overnight, lyophilised, and reacted with [^{3}H]DFP. It then contained D, C and A forms in roughly equal quantities. The main band was α (90 000 dalton). (– – –) Enzyme which had been prepared by conventional methods [10] and stored for 6 months before DFP reaction (form D). α′ (60 000 dalton), α″ (30 000 dalton) and α‴ (15 000 dalton) fragments were apparent.

We have previously established that trypsin brings about a stepwise conversion from the most complex asymmetric forms to the tetrameric G molecule [13]. Trypsin has also been found by Morrod et al. [14] to cleave the catalytic subunits into fragments, such as α′ in fig.1.

Therefore, quaternary structure dissociation and cleavage of intra-subunit peptide bonds may both be obtained through the action of the same enzyme. However, it appeared that the two processes are not linked i.e. although the aged preparation studied in fig.1 showed an extensive degree of subunit cleavage, it contained only asymmetric forms (about 30% each of D (18.4 S), C (14.2 S) and A (9 S) forms). In addition as shown in fig.2, subunit cleavage was not greater in the more depolymerized forms of the enzyme, so that there was no obvious correlation between quaternary structure and subunit integrity.

Although the preparations used in fig.1 were not homogenous with respect to the polymerization state, the polypeptide bands appeared monodisperse, indicating a single kind of α chain in the three asymmetric forms D, C and A, as judged from the electrophoresis criterion. The same α chain was also found in the globular forms G, G′ and G″ (fig.2a).

The smaller DFP-labelled chains, which were found in aged preparations (fig.1 and 2) were clearly derived from the 90 000 subunit by intra-chain cuts that occurred during storage. At least three such bands appeared at 60 000 (α′) 30 000 (α″) and 15 000 (α‴) dalton in variable proportions. A series of degradative transformations seemed to occur as in the following scheme:

$$\alpha\ (90\,000) \rightarrow \alpha'\ (60\,000) + \beta'\ (30\,000)$$

Fragmentation did not prevent catalytic activity of the subunits, making their DFP labelling possible.

The unlabelled β′ fragment was visible in Coomassie Brilliant Blue stained gels as discussed below (fig.3 and 4). The α′ fragment might be further degraded, leading to α″ (30 000 dalton) and α‴ (15 000 dalton) DFP labelled peptides.

The breaks, occurring at apparently well defined locations on the polypeptide chain, may have resulted

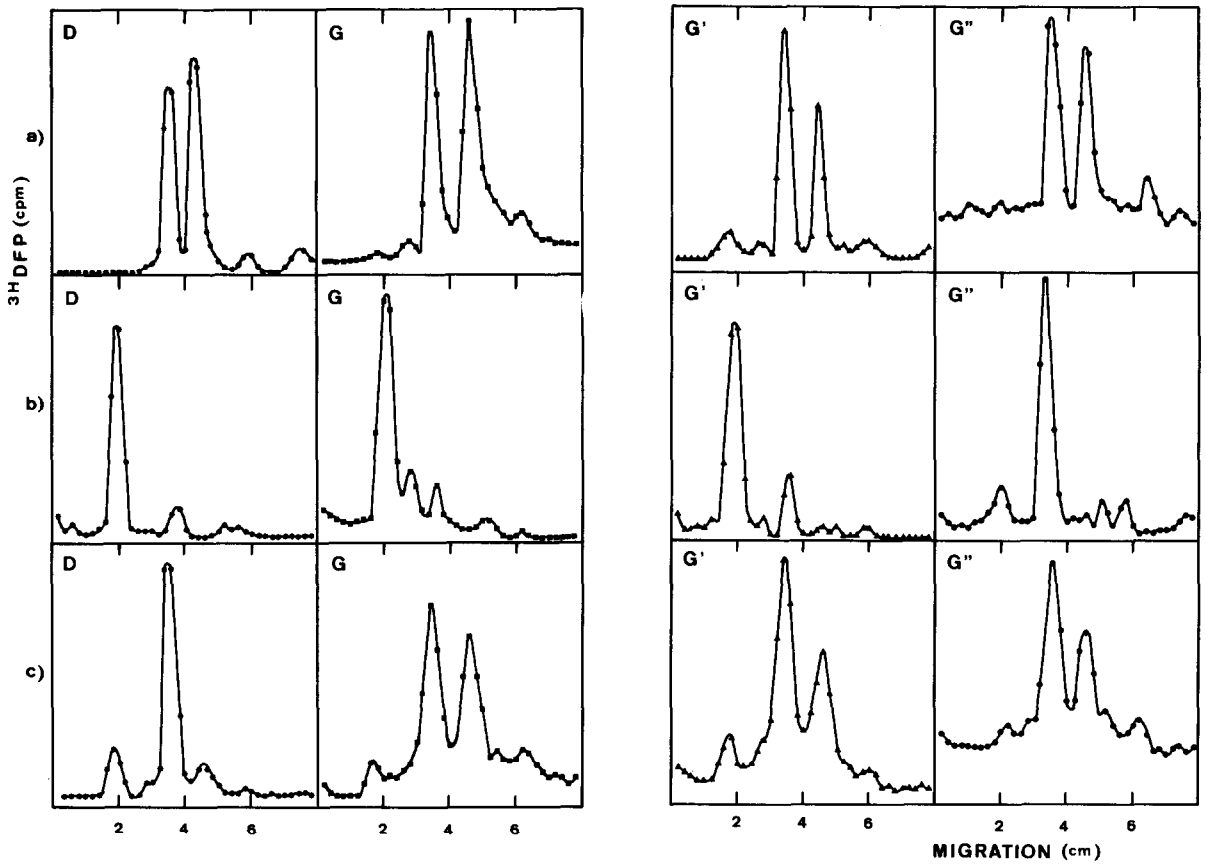

Fig.2. Comparison of SDS polyacrylamide gel electrophoresis patterns of the [^{3}H]DFP labelled asymmetric G, G′ and G″ forms of acetylcholinesterase. (×) Asymmetric, (■) G, (▲) G′, (●) G″. (a) Reduced enzymes, (b) non-reduced enzymes, (c) enzymes which had been reduced and alkylated prior to denaturation (such reduction did not alter activity or sedimentation). These enzymes were prepared from a single preparation of acetylcholinesterase which was labelled with DFP, and subsequently submitted to sonication and molecular sieve fractionation in order to obtain the globular forms.

from contamination of acetylcholinesterase preparations with proteases. However, this transformation also occurred in highly purified preparations and was blocked by DFP. Since acetylcholinesterase is known to hydrolyze amide bonds in acetylcholine analogs [13], an autolytic process is not unlikely.

3.2. *Intra- and inter-subunit disulfide bonds*

When SDS denaturation was performed without reduction the main band was not at 90 000, but at 180 000 dalton for all forms except the monomeric G″ (fig.2b). Disulfide bonds were apparently binding subunits into dimers and also preventing separation of the split fragments. The 180 000 dimer (α_2) thus appeared as a structural unit of acetylcholinesterase held together, at least in part, by inter-subunit disulfide bonds. As indicated by the small 90 000 dalton band a few of these bonds could however be spontaneously broken. Intra-subunit disulfide bonds also existed since the α' fragments were not liberated; this was particularly evident in the case of the G″ form.

In addition, a small band at 115 000 dalton could be seen in the electrophoresis patterns of unreduced G and G′. A small 60 000 (α') daltons band was also visible. This suggested that some α' fragments had been detached from the dimers by spontaneous reduction of some intra-subunit bonds, leaving an α monomer bound to their complementary 30 000 dalton β' chain.

The 115 000 dalton bands would thus correspond to an $\alpha\beta'$ association.

In some experiments, the enzymes were submitted to reduction and alkylation before their denaturation with SDS. After such an alkylation at least 90% of the activity was retained, and the sedimentation constants of the four D, G, G′ and G″ forms were unchanged. For the globular forms, the polypeptide patterns obtained with these preparations (fig.2c) exactly matched those obtained with preparations which had been denatured and reduced at the same time (fig.2a). For these molecules both types of disulfide bonds were therefore accessible to reduction in the native state, and were not necessary for maintaining either the quaternary structure (dimers) or the tertiary structure even though the subunit was largely fragmented into smaller peptides. By contrast with the globular forms, reduction of the asymmetric molecules before their denaturation did not yield the same polypeptide pattern: most of the DFP label was found in the 90 000 (α) dalton band, together with small amounts of the 180 000 (α_2) dalton dimer, and of the 60 000 (α') dalton fragment. Although dimers were mostly dissociated, the 60 000 (α') dalton chains were generally still linked with their complementary unlabelled β' fragments. The bonds linking the dimers thus appeared more readily accessible to reduction than the intra-subunit bridges in the asymmetric molecules.

3.3. *Protein and carbohydrate distribution*

Figure 3 shows the results of SDS electrophoresis of the D and G forms of acetylcholinesterase in both reduced and non-reduced states. The main protein bands in the gels of the non-reduced forms were the monomer and dimer whereas after reduction, only the 60 000 (α') and 30 000 dalton bands were visible: the α subunits were so extensively broken down in these preparations that they only appeared as a shoulder on the α' peak. The 30 000 dalton band was more intense compared with that in the [^{3}H]DFP profiles. All complementary unlabelled peptides (β') were thus probably located in this band in addition to the α'' fragments. The 115 000 dalton ($\alpha\beta'$) band was also visible in non-reduced patterns of both D and G forms. In the asymmetric form the high molecular weight bands appeared particularly numerous and conspicuous (some material even did not enter the gel). These polymers were almost totally dissociated after reduction.

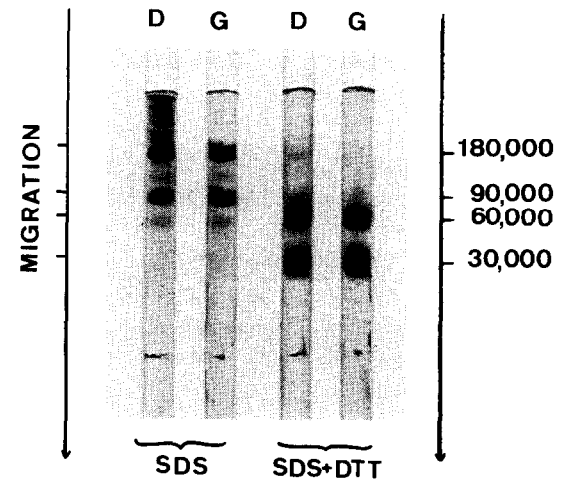

Fig.3. SDS polyacrylamide gels stained by Coomassie Brilliant Blue, showing polypeptide patterns for the D and G forms, in both non-reduced and reduced states (200 μg enzyme/gel). These preparations had been stored for one year, and showed extensive fragmentation.

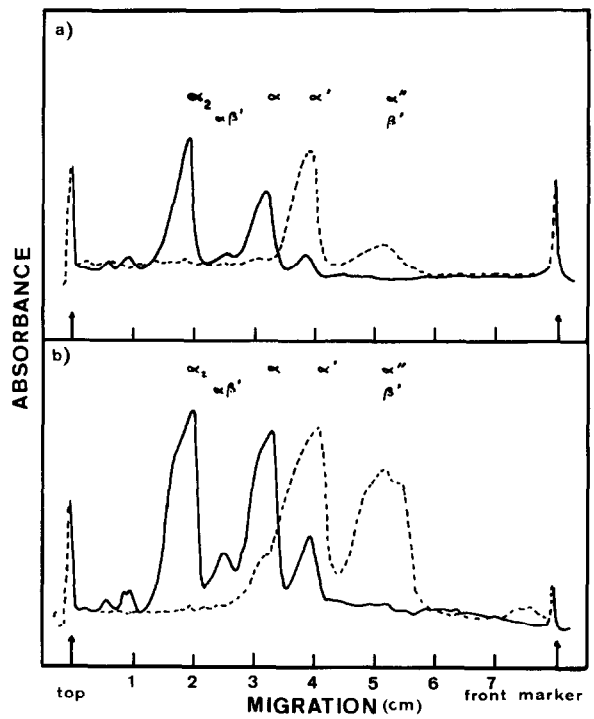

Fig.4. Densitometric tracings of (a) carbohydrate (periodic acid-Schiff) and (b) polypeptide (Coomassie Brilliant Blue) stained polyacrylamide gels for the G forms, in the non-reduced (——) and reduced (- - - - -) states (same preparations as in fig.3).

Figure 4 shows parallel densitometric tracings corresponding to reduced and non-reduced G acetylcholinesterase stained for polypeptides with Coomassie Brilliant Blue and for carbohydrates by the periodic acid-Schiff procedure. It was apparent that all polypeptide bands carried carbohydrate moieties. The ratios of integrated peaks, however, indicated that the relative carbohydrate content of the 30 000 dalton fragments (α'' and β') was about half that of the 60 000 daltons (α') bands. It thus appears that the β' part of the subunit carries much less carbohydrate residues than the α' section and it is therefore likely that the active serine bearing α' region is more exterior, in the tertiary structure of the subunit, than the β' region.

No specific band, either proteic or glycoproteic, could be assigned to the tail of the asymmetric forms. Electron microscopy [6] and molecular weight data [7] have indicated that the tail may consist of three 30 000 dalton chains. When the asymmetric forms were analyzed without reduction, no 30 000 dalton band was visible: it may be that the three tail chains were linked together by disulfide bridges and coïncided in the gel with the monomeric band or, alternately, that they were linked to the catalytic subunits via disulfide bridges. This would then explain the multiplicity of polymeric bands observed in the non-reduced D molecule (fig.3). It may also be that the tail subunits were fragmented into very small pieces, or that they did not penetrate the gels for configurational reasons, escaping detection in both cases.

4. Conclusion

Different patterns were found depending on the storage time of the enzyme: the original 90 000 dalton subunit (α), predominant in fresh preparations, gave rise to discrete fragments, possibly in part through an autolytic process. The active site serine was included mainly in a 60 000 dalton fragment (α'), but was also found in smaller 30 000 (α'') and 15 000 (α''') polypeptides. The complementary non-DFP-labelled peptides (β') were found in the 30 000 dalton range.

The molecular weight of 90 000 obtained for the subunit did not vary with SDS concentration in the gels (0.1% and 1.0%). This value may, of course, be biased because of the carbohydrate residues. Our values are close to those obtained by Dudai et al. for trypsinized (90 000–100 000 dalton) [15] and asymmetric acetylchlolinesterase (80 000 dalton) [16]. In the case of an '11 S' enzyme, which is probably equivalent to our G form, Rosenberry et al. [17] reported markedly smaller values (70 000, 50 000 and 20 000 dalton). However a close parallelism may be drawn between their results and our observations, regarding the fragmentation pattern of the subunit. Similar results were also recently presented by Morrod et al. who obtained values of 80 000, 55 000, 28 000 and 25 000 dalton.

In agreement with the last authors, we detected no definite correlation between subunit cleavage and the polymerization state of acetylcholinesterase: extensively degraded subunits were compatible with an intact quaternary structure for all forms, (including the asymmetric forms).

In non-reduced acetylcholinesterase (D, G and G' forms) the subunits were found associated as dimers (α_2, 180 000 dalton), indicating the existence of inter-subunit disulfide bridges. The single subunit of the monomeric G'' forms was not dissociated into its α' and β' fragments, showing that intra-subunit bridges also linked these two fragments within each subunit. These two classes of disulfide bonds could also be distinguished on the basis of their accessibility to reduction in the non-denatured enzyme: in the case of the asymmetric forms reduction produced only a 90 000 dalton band. Such inaccessibility of intra-subunit bonds of these molecules may result from their bulk or may be more specifically lined with the presence of a tail.

Dudai and Silman [18] have considered some of the possible dispositions of disulfide bridges between the subunits and their fragments. Our results demonstrate that both intra- and inter-subunit bridges do exist. From the occurrence of the 115 000 dalton bands, or $\alpha\beta'$ associations it can be further concluded that the subunit bridges link the β' fragment to the opposite subunit in each dimer. It is likely that subunits are associated into dimers with two-fold axial symmetry and linked via their β' sections, which, as discussed above, seemed to be deep-lying in the molecular structure*.

*It cannot be excluded at the moment that part or all of disulfide bridges might have arisen from oxidation during purification of the enzymes. This however would not invalidate the conclusions drawn regarding the arrangement of subunits in the molecules.

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