THE SUBUNITS OF AN ACETYLCHOLINESTERASE PREPARATION PURIFIED FROM TRYPSIN-TREATED ELECTRIC EEL TISSUE

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1. Introduction

The molecular properties of acetylcholinesterase (AchE, EC 3.1.1.7) are of particular interest because of its role in nervous transmission [1]. The enzyme has been purified from the electric organ of the electric eel in a form with a sedimentation coefficient of about 11 S, and some of the properties of the purified species have been reported [2, 3]. More recently Massoulié and Rieger [4] have shown that AchE is present in extracts of electric organ tissue in three principal isozymic forms which can be distinguished by their sedimentation coefficients (about 8 S, 14 S and 18 S). A component with a sedimentation coefficient of 11 S is present only in very small amounts in extracts of fresh tissue, but almost all AchE activity appears in this form after treatment of tissue extracts with trypsin [4]. It is thus possible that the purified enzyme described in the literature is obtained as a consequence of autolysis or other processes occurring during purification.

We have recently described the preparation of a resin suitable for purification of AchE by affinity chromatography [5]. By preceding affinity chromatography with tryptic digestion of the tissue extract we have now developed a simple procedure for purifying the 11 S form of AchE in high yield [6]. The purified enzyme migrated as one band on disc gel electrophoresis. Previous observations have indicated that the 11 S purified AchE is composed of subunits with a molecular weight of about 64,000 or less [3, 7, 8]. On examining the 11 S preparation that we obtained by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) and β-mercaptoethanol, we observed two major polypeptide components which appeared to have molecular weights of about 88,000 and 64,000. It was, therefore, decided to label the active site of the purified enzyme with radioactive diisopropylfluorophosphate (DFP) and to study the migration of the labelled enzyme on SDS-acrylamide gels, in order to see in which of the two components the label appeared. In parallel, similar experiments were performed utilising the total extract from electric organ tissue.

2. Materials and methods

A purified preparation of AchE with a sedimentation coefficient of 11.4 S was prepared by tryptic digestion of a salt extract of toluene-treated electric organ tissue, followed by gel filtration on Sephadex G-100, absorption of the enzyme peak obtained on the affinity column previously described [5], and elution with the enzyme inhibitor decamethonium. The detailed procedure will be described elsewhere [6]. This preparation had a specific activity of 7600–8100 units per mg protein, assuming $E^{1\%}_{280}$ = 16.5 [6], one unit of enzyme hydrolyzing 1 μmole acetylcholine per min when assayed titrimetrically in a reaction mixture containing 2.5 mM acetylcholine, 0.1 M NaCl, 0.02 M $MgCl_2$, 0.01% gelatin, pH 7.0, at 25°.

The mixture of isozymes from fresh electric organ tissue was prepared by homogenizing the tissue in 2.5 volumes of cold 1 M NaCl–0.01 M phosphate,

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pH 7.0, and taking the supernatant after centrifugation at 10,000 *g* for 20 min.

The AchE preparations were radioactively labelled with H^3-DFP (New England Nuclear) according to the procedure of Froede and Wilson [7]. Reactivation of labelled enzyme with pyridine 2-aldoxime methiodide (2-PAM) was performed, 60 min after treatment with DFP, by diluting an aliquot of the enzyme solution with an equal volume of 2×10^{-3} M 2-PAM in 0.02 M phosphate, pH 8.0. After incubation for 150 min, the enzyme solution was dialyzed against 5×10^{-4} M 2-PAM in the same buffer for 3 hr, and then overnight against 0.2 M NaCl–0.01 M phosphate, pH 7.4.

Sucrose gradient centrifugation was performed as described by Martin and Ames [9]. Disc gel electrophoresis on 7.5% acrylamide gels at pH 8.3 was performed according to Davis [10]. SDS-acrylamide gel electrophoresis and molecular weight calibration were routinely performed according to Shapiro et al. [11]. The conditions of Lenard [12] were also employed. When labelled enzyme was electrophoresed, the gels were sliced into 1 mm sections, and incubated with shaking for 16 hr at 37° in 0.2 ml NCS (Nuclear Chicago) prior to addition of scintillation fluid.

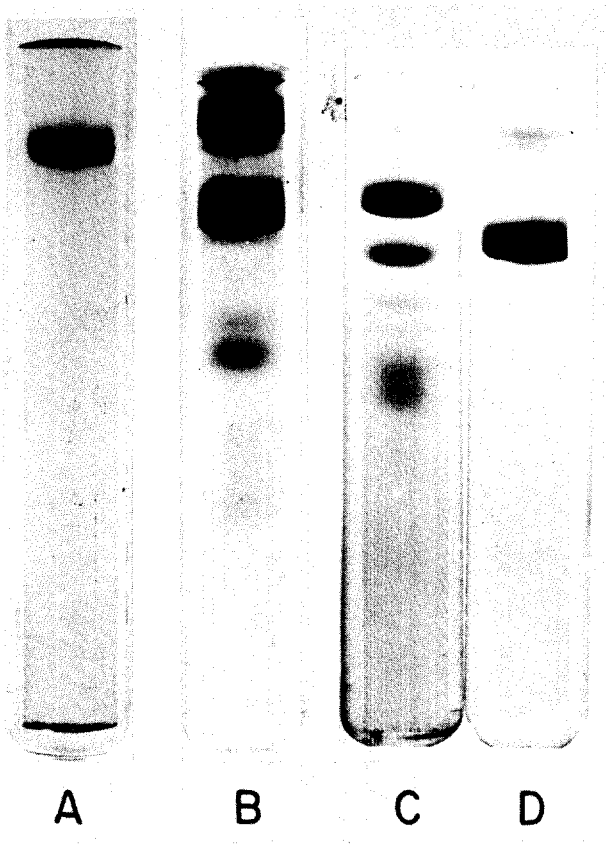

Fig. 1. Acrylamide gel electrophoresis of purified AchE from trypsin-treated tissue of the electric organ. (A) Electrophoresis in 7.5% acrylamide, Tris-glycine buffer, pH 8.3. The protein was stained with Coomassie blue. (B) Electrophoresis of the same AchE preparation in the presence of SDS. Denaturation was performed by heating the sample in 0.5% SDS, 0.01 M phosphate, pH 7.2, at 100° for 3 min, and the 7.5% acrylamide gel contained 0.1% SDS in 0.075 M phosphate, pH 7.2. The gels were stained with Coomassie blue and molecular weights were estimated, using bovine serum albumin, trypsin and lysozyme as markers. (C) Electrophoresis of the same AchE preparation; conditions were as for (B) except that 0.01 M mercaptoethanol was present both during denaturation and electrophoresis. (D) Electrophoresis of bovine serum albumin under the same conditions used for (C) and run in parallel.

3. Results

Fig. 1A shows that the purified 11.4 S isozyme migrates as one major band on acrylamide gels. However, in the presence of SDS, two main components are observed with molecular weights of 90,000 ± 9000 and 160,000 ± 20,000 (fig. 1B). When electrophoresis is performed with SDS in the presence of β-mercaptoethanol, two major components are also observed, with molecular weights of 88,000 ± 9000 and 64,000 ± 6000 (fig. 1C). The molecular weights and relative intensities of the bands obtained in the presence of SDS and mercaptoethanol did not vary even when the extreme conditions described by Lenard were employed [12], or when the AchE was pretreated with 6 M guanidine hydrochloride and mercaptoethanol, and subsequently treated with iodoacetamide.

When the purified enzyme was labelled with H^3-DFP and examined on sucrose gradients (fig. 2A), the radioactivity peak moved with a sedimentation coefficient of 11.4 S, similar to the value obtained for unlabelled enzyme. Electrophoresis of the labelled enzyme on SDS-acrylamide gels both in the presence and absence of mercaptoethanol revealed, in each case, two main peaks of radioactivity (figs. 3A and 4A).

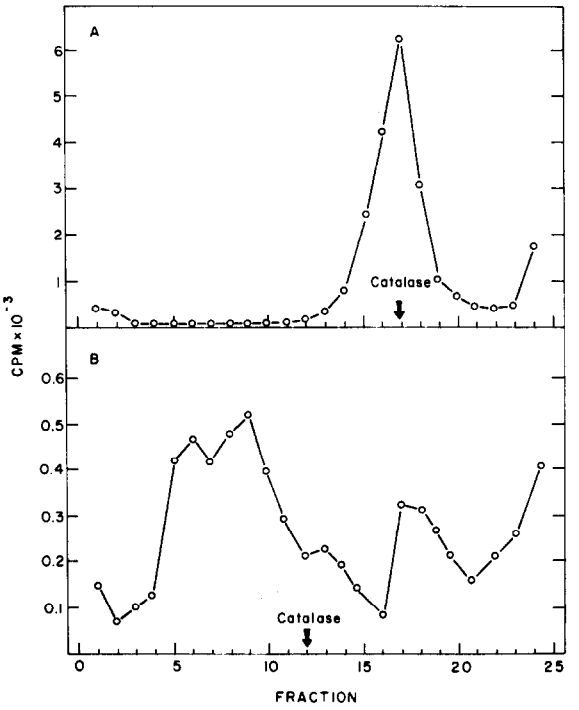

Fig. 2. Sucrose gradient centrifugation of H^3-DFP-labelled AchE preparations. (A) Purified AchE, in 0.1 M NaCl–0.01 M phosphate, pH 7.0. (B) Total extract of electric organ tissue in 1.0 M NaCl–0.01 M phosphate, pH 7.0. In each experiment 4.1 ml of a 5–20% linear sucrose gradient was layered on a 0.6 ml cushion of 50% sucrose, 0.15 ml solution of the enzyme sample was layered on top and centrifugations were performed at 4° in a SW 39 rotor, at 38,000 rpm, for 5½ hr (A) or 8½ hr (B). The marker used was catalase, which was located by its extinction at 407 nm.

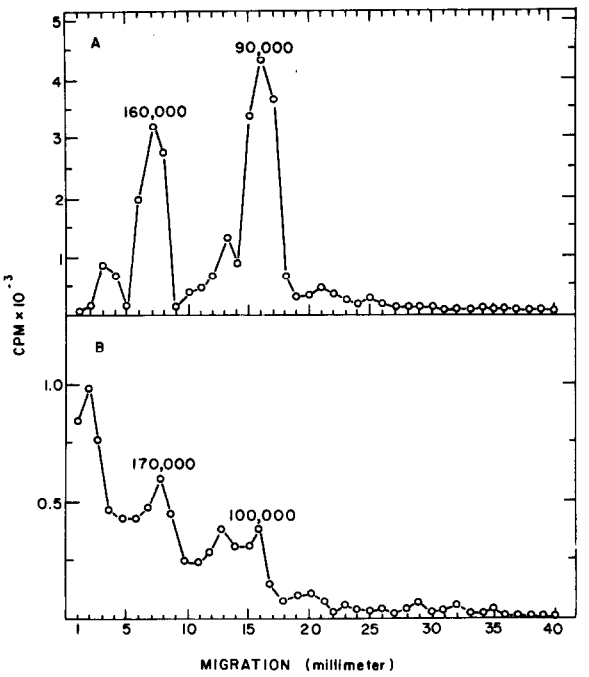

Fig. 3. SDS-acrylamide gel electrophoresis of H^3-DFP-labelled AchE samples. Denaturation, electrophoresis and calibration were as described for fig. 1B. The gels were sliced and radioactivity measured as described under Materials and methods. (A) Purified AchE from trypsin-treated tissue. (B) Total extract (in 1.0 M NaCl) from electric organ tissue.

The molecular weights estimated for these peaks were the same as for those observed by staining for protein. If the preparation of H^3-DFP-labelled AchE was treated with 2-PAM, under conditions where the 2-PAM would be expected to remove the diisopropylphosphoryl group specifically from cholinesterases [13], more than 90% of the counts were removed from the protein.

Extracts of fresh electric organ tissue, when centrifuged on sucrose gradients, yielded three main peaks of AchE activity, as described by Massoulié [4]. When such extracts were labelled with H^3-DFP, sucrose gradient centrifugation yielded a distribution of radioactivity (fig. 2B) similar to the distribution of enzymic activity for the unlabelled enzyme. When this labelled preparation was treated with SDS in the absence or presence of mercaptoethanol, acrylamide gel electrophoresis yielded the radioactivity patterns shown in figs. 3B and 4B, respectively.

4. Discussion

The fact that essentially all the radioactivity could be removed from an H^3-DFP-labelled preparation of the purified AchE by treatment with 2-PAM under conditions which would be expected to reactivate only AchE [13], provides strong support for the view that both polypeptide components observed on SDS-acrylamide gels contain active sites

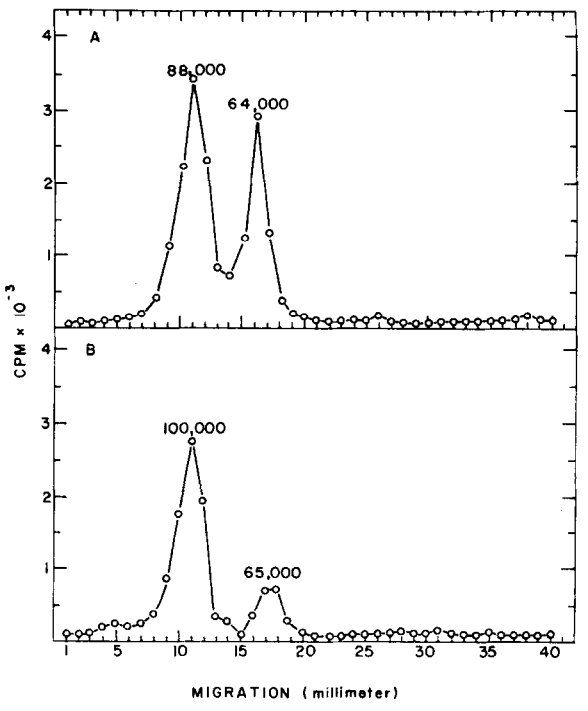

Fig. 4. SDS-acrylamide gel electrophoresis of H^3-DFP-labelled AchE, in the presence of 0.01 M β-mercaptoethanol. Denaturation, electrophoresis and calibration were performed as described for fig. 1C. (A) Purified AchE from trypsin-treated tissue. (B) Total extract (in 1.0 M NaCl) from electric organ tissue.

of the enzyme. The similarity between AchE distribution and H^3-DFP distribution on sucrose gradient also supports the view that only AchE is being labelled. However, our present data do not exclude the possibility that the sites labelled by DFP are not all equivalent.

Previous authors who have investigated the molecular weights of the subunits of various purified preparations of AchE, have reported values of 64,000 or less for these subunits [3, 7, 8], even though the values that they reported for the sedimentation coefficients of the intact enzyme were not markedly different from those which we observed. However, our results clearly indicate the presence of a polypeptide chain with a molecular weight above 80,000. Although it is possible that electrophoresis on SDS-acrylamide gels does not reflect accurately the correct values for molecular weights of the subunits, numerous workers have studied a wide variety of proteins using this system, and discrepancies from known values for molecular weights are usually very small (see for example [11, 14, 15]). It is also possible that incomplete dissociation into subunits occurred. Available evidence indicates that the extreme conditions of Lenard [12], which yielded results no different from those obtained using the conditions of Shapiro et al. [11], should cause complete dissociation; moreover, even reduction in 6 M guanidine followed by treatment with iodoacetamide did not change the results. It should be borne in mind that our method of preparation, utilizing tryptic digestion, may well have yielded a preparation different from that tested by other authors. Sedimentation velocity measurements may not have been sufficiently sensitive to detect the difference, particularly since it is possible that the preparation may be a mixture of species containing the different subunits in differing ratios.

In fig. 3B, the major labelled species has a molecular weight of about 100,000. This suggests that the major polypeptide chain in the native enzyme is indeed heavier than the major component in the purified enzyme preparations studied by previous authors. Our observations thus seem to indicate that conversion to the 11 S species involves autolytic or tryptic digestion of at least some of the active site-containing polypeptide chains.

The differences observed in distribution of radioactivity and of protein staining, depending on whether AchE samples were electrophoresed on SDS-acrylamide gels in the presence or absence of mercaptoethanol, are in general agreement with the observations of Froede and Wilson [7], indicating that intersubunit disulfide bridges are involved in maintaining the quaternary structure of the enzyme.

The resutls that we have presented raise many questions as to the relationship between native AchE and the various purified preparations. We are presently involved in studying more accurately the molecular weights of the enzyme and of its subunits, the modes of conversion of the isozymic species, and the arrangement of the disulfide bridges connecting the subunits.

Acknowledgements

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