THE SUBUNIT MOLECULAR WEIGHT OF ACETYLCHOLINESTERASE

David B.MILLAR and Melba A.GRAFIUS
Biochemistry Division, Naval Medical Research Institute, National Naval Medical Center, Bethesda, Md. 20014, USA

Received 30 October 1970

1. Introduction

The significance of the molecular structure and conformational changes of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) in the transmission of neural messages is being studied intensively and has recently been reviewed [1]. Knowledge of the *in vivo* cellular geometry of the enzyme is vital to an understanding of the various environmental forces which influence neural transmission. While the *in vivo* architecture of acetylcholinesterase is difficult to ascertain, we have been engaged in a study of the *in vitro* properties of the enzyme. These results [2–4] have led us to suggest that the enzyme probably exists in a polymeric form in the membrane structure. The purpose of this communication is to report investigations into the subunit molecular weight of the enzyme. Our results, giving a subunit molecular weight of 42,200 in neutral 5 M guanidine and 21,500 in acid 5 M guanidine are at variance with a previous estimate of 64,000 [5]. Our data indicate the native enzyme ($\sim$ 260,000 MW) to be at least a hexamer.

2. Materials and methods

Acetylcholinesterase was extracted from *Electrophorus electricus* by methods previously described [3] and partially purified by a method essentially identical to Lawler's [6]. The enzyme was finally passed through a column of Sepharose 2B using 0.015 M sodium phosphate, 0.1 M NaCl pH 7.1 as eluant. The enzyme activity was measured with a pH stat system in 1.5 mM sodium phosphate 0.1 M NaCl, 0.01 M $MgCl_2$, pH 7.4 buffer containing 2% albumin, and 3.3 mM acetylcholine chloride. The titrant was 0.01 N NaOH. Protein determination was by micro-Kjeldahl and was performed in the laboratory of Dr. William C.Alford, NIAMD, NIH, to whom we extend our appreciation. The specific activity of the preparation reported on here was 675. Using 750 [7] as the value for pure acetylcholinesterase, this preparation was 90% pure. Polyacrylamide disc gel electrophoresis using the nitro BT stain for enzyme activity [8] revealed one major band and 2 minor bands, one on either side of the major band. The major band accounted for 95% of the stain. Amidoschwartz staining showed the major band (corresponding to the major band of enzyme activity) and three minor bands, one of which did not correspond to an enzymatically active band. This band was less than 1% of the total protein.

Molecular weights were measured in the Spinco Model E analytical ultracentrifuge using interference optics. Yphantis meniscus depletion runs [9] using a layering cell to hasten equilibrium [10] were used throughout. Molecular weights were computed from the variation of fringe height with distance as described before [10], except that data were computer processed with a Mark I Advanced Basic program using essentially the same format and algorithms as before. Viscosity and densities of the guanidine solutions were experimentally determined. For the other buffers, the values were extracted from the critical

The opinions or assertions herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large. From the Bureau of Medicine and Surgery, Navy Department, Research Task MR005.06.0006.

tables. $\bar{v}$ of acetylcholinesterase was taken to be 0.731 mg/g in both aqueous solutions and in 5 M guanidine [5]. All solutions were vigorously dialysed overnight against 2 changes of buffer.

Sucrose gradient density sedimentation was conducted as previously described [3].

3. Results

Fig. 1 shows a sucrose gradient sedimentation analysis of the enzyme preparation used by us. It is clearly quite homogeneous with no trace of other enzymically active sedimenting material. The S value of the enzyme estimated by means of a catalase marker is 11.1, in good agreement with previous values [11, 12]. The molecular weight calculated for this S value, assuming sphericity, is 212,000.

3.1. *Native acetylcholinesterase molecular weight*

The molecular weight of the enzyme was determined by meniscus depletion runs at 8900 and 11,000 rpm in 7 mm solution columns. Table 1 shows the results of three experiments. The average molecular weight is 259,000, which is in good agreement with other reports [5]. In these runs we did not notice any polydispersity with respect to molecular weight. With other preparations not reported on here, we have observed lower molecular weight values near the meniscus. The value of 259,200, compared with that calculated from the sedimentation coefficient, indicates the enzyme to be slightly asymmetric.

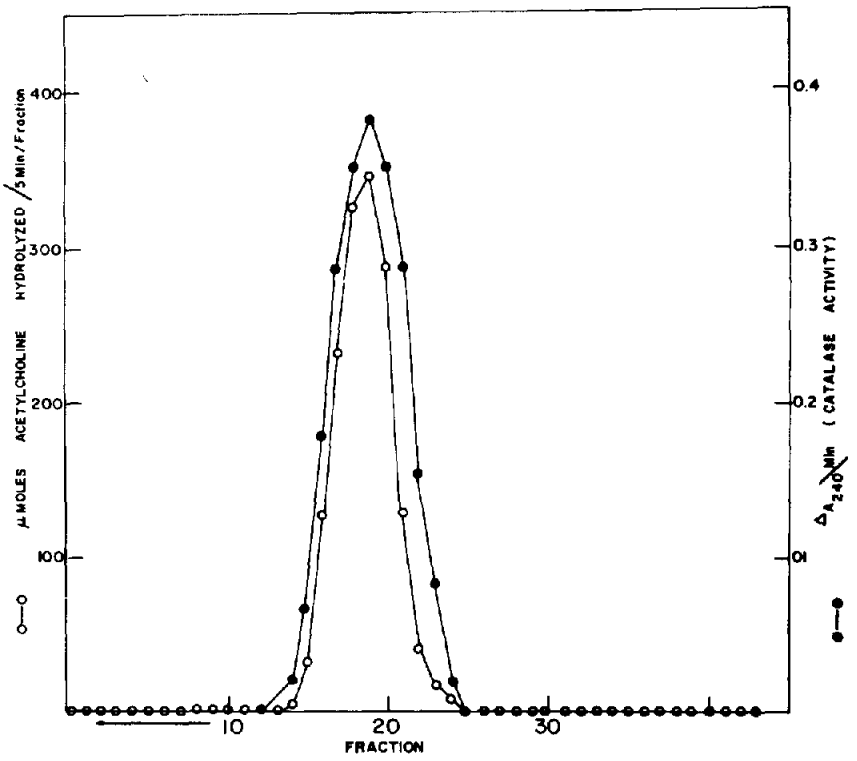

Fig. 1. Sucrose gradient sedimentation of highly purified acetylcholinesterase. Centrifuged at 35,300 rpm for 16 hr; 7 drops per fraction; sample and sucrose in 0.015 M sodium phosphate, 0.1 M NaCl buffer, pH 7.1; protein concentration 0.988 mg/ml; specific activity 675. ○—○, acetylcholinesterase activity; ●—●, bovine liver catalase activity. It is clear that the purification procedure has removed any other faster sedimenting enzymatically active species which have been reported to occur in acetylcholinesterase preparations [2–4].

Table 1
Molecular weight of acetylcholinesterase.

Run	Weight average molecular weight
1	266,200
2	259,000
3*	252,500
Average	259,200

Solvent: 0.1 M NaCl, 0.015 M $Na_2H\,PO_4$, pH 7.1; T = 20°.

* Plus 0.005 M dithiothreitol. Note that inclusion of this reagent made essentially no difference to the molecular weight.

3.2. *Subunit molecular weight*

Fig. 2 shows two estimations of the molecular weight of acetylcholinesterase in 5 M guanidine, 0.01 M dithiothreitol, pH 7.0. The average value of the two runs is 42,200. Leuzinger et al. [5] have reported 64,500 for a similar experiment. Froede and Wilson give an estimate of 49,000. Since we have found that neutral guanidine does not always completely disrupt intermolecular forces, we prepared samples of the enzyme in 5 M guanidine but at pH 2, in 0.015 M NaH_2PO_4, 0.1 M NaCl, 0.01 M dithiothreitol. The results are shown in fig. 2. It is clear that a further dissociation takes place to subunits of the order of 21,500. This value is obtained by linear extrapolation of the data and is subjected to some uncertainty but is compatible with the amino acid minimum molecular weight [7].

4. Discussion

We have assumed that any disulfide bridges in the protein are in an intrasubunit configuration [7]. We have done so on the basis of the observations of Froede and Wilson [13] that low concentrations of sodium dodecylsulfate (no mercaptoethanol) give rise to similar subunit distributions as does guanidine plus mercaptoethanol.

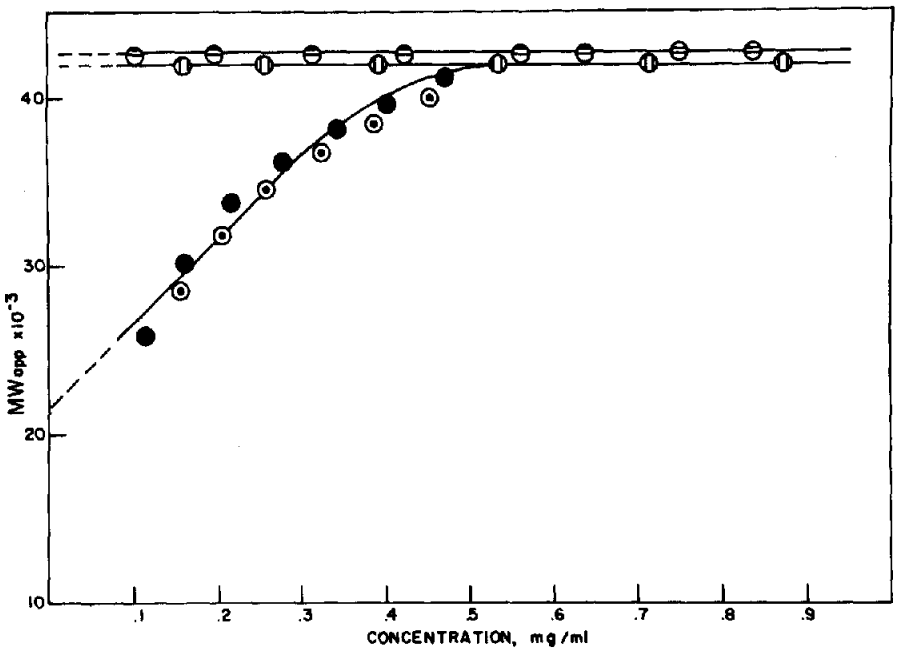

Fig. 2. Molecular weight of acetylcholinesterase subunits in 5 M guanidine. Centrifugations were carried out in 7 mm solution columns at 33,450 rpm, 20°. •, ⊙, in 5 M guanidine, 0.01 M dithiothreitol, 0.015 M NaH_2PO_4, 0.1 M NaCl, pH 2.0. The MW concentration dependence at pH 2 may indicate a strong association process between monomer and higher n-mers. ○, ○, 5 M guanidine, 0.01 M dithiothreitol, pH 7.0.

An amino acid analysis of acetylcholinesterase has been reported [7] as well as an end group analysis [5]. Two different carboxyl-terminal amino acids were found, suggesting two different polypeptide chains. The minimum molecular weight computable from the amino acid analysis is about 24,000, although this figure is not absolutely rigid since it is computed on the assumption of internal homogenity (only one chain).

Leuzinger et al. [5] have reported that acetylcholinesterase in 6 M guanidine has a molecular weight of 64,000 as measured by sedimentation equilibrium. They also reported concentration dependence of the molecular weight in guanidine. Our data encompasses quite low concentrations but does not show any such behaviour. Further, between their molecular weight and ours in guanidine, the difference is about equivalent to one chain of 22,000. If there had been a dissociation due to experimental conditions, we would have detected the presence of this low molecular weight piece in our pH 7 guanidine experiments.

Our value of about 21,500 for the subunit in acid-guanidine is compatible with the amino acid analysis minimum molecular weight. It thus appears possible that our 42,200 MW piece is a dimer of 2 chains of about 21,500 each (assuming molecular weight identity).

We cannot, however, rule out the possibility that in acid guanidine a sensitive peptide bond in the 42,200 MW subunit is cleaved, giving rise to the 21,500 MW piece. Therefore, we will discuss the enzyme in terms of the 42,200 MW piece. Leuzinger et al. [5] suggest from their studies that the native enzyme is an $\alpha_2\beta_2$ structure, the subunit molecular weight being 64,000. However, our results would indicate that there are 6 subunits of 42,200 MW. They also cite from unpublished studies that there are only 2 active sites per 260,000 MW. Kremzner and Wilson [11] and Froede and Wilson [13] have presented evidence, based upon activity inhibition studies, that there are 4 active sites in the native enzyme (MW 230,000). Further, the data of Michel and Krop [14] on the inhibition of acetylcholinesterase enzymatic activity by DFP could easily be recalculated using the current specific activity value of 750 for pure enzyme [7] to give a DFP binding equivalent weight of 43,500. This number permits 6 binding sites per molecule of acetylcholinesterase of 260,000 MW. It might be that not all sites are equivalent [15] and, therefore, not detectable by most procedures previously employed. However, if the number of active sites is indeed less than the number of subunits per mole of native enzyme, it is reasonable to assume that some of the polypeptide chains perform functions other than those catalyzing the hydrolysis of acetylcholine, possibly directly regulating membrane permeability.

References

[1] D.Nachmansohn, Science 168 (1970) 1059.
[2] M.A.Grafius and D.B.Millar, Biochim. Biophys. Acta 110 (1965) 540.
[3] M.A.Grafius and D.B.Millar, Biochemistry 6 (1967) 1034.
[4] M.A.Grafius and B.D.Millar, Arch. Biochem. Biophys. 126 (1968) 707.
[5] W.Leuzinger, M.Goldberg and E.Cauvin, J. Mol. Biol. 40 (1969) 217.
[6] H.C.Lawler, J. Biol. Chem. 238 (1963) 132.
[7] W.Leuzinger and A.L.Baker, Proc. Natl. Acad. Sci. U.S. 57 (1967) 446.
[8] J.Uriel, Ann. Inst. Pasteur 101 (1961) 104.
[9] D.A.Yphantis, Biochemistry 3 (1969) 297.
[10] D.B.Millar, V.Frattali and G.E.Willick, Biochemistry 8 (1969) 2416.
[11] L.T.Kremzner and I.B.Wilson, Biochemistry 3 (1964) 1902.
[12] J.Massoulie, F.Rieger and S.Tsuji, European J. Biochem. 14 (1970) 430.
[13] H.C.Froede and I.B.Wilson, Israel J. Med. Sci. 6 (1970) 179.
[14] H.O.Michel and S.Krop, J. Biol. Chem. 190 (1951) 119.
[15] C.C.Winlund and M.J.Chamberlin, Biochem. Biophys. Res. Commun. 40 (1970) 43.