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The Turnover Numbers of Acetylcholinesterase Forms

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SUMMARY

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The turnover numbers of different acetylcholinesterase forms from electric eel, including the 18 S, 14 S, and 8 S asymmetrical forms and the 11 S lytic form, were measured using an extraordinarily active phosphorylating inhibitor, 7-(diethoxyphosphinyloxy)-*N*-methylquinolinium ion, as a titrating agent for the concentration of active sites. The 18 S and 14 S forms were about equally active. The 8 S and 11 S forms were about 35% more active than the 18 S and 14 S forms.

INTRODUCTION

Acetylcholinesterases isolated from different tissues exhibit a number of multiple molecular forms that may differ in molecular weight, Stokes radius, sedimentation coefficient, glycoprotein composition, and isoelectric properties. For example, acetylcholinesterase forms of differing molecular weights have been observed following extraction from bovine caudate nucleus (1), rat diaphragm (2), ciliary muscle of the human eye (3), and eel electric organ (4). In the case of the enzyme isolated from the electric organ of *Electrophorus electricus*, older methods of purification often involved prolonged storage under toluene and consequent lipid extraction and autolysis. This solubilization procedure results in the appearance of predominantly one molecular weight form, having a sedimentation coefficient of approximately 11 S. However, if the eel enzyme is isolated without using proteolytic solubilization procedures, three highly asymmetrical forms, with sedimentation coefficients of approximately 18 S, 14 S, and 8 S, are obtained (4, 5).

Although the general importance of acetylcholinesterase in cholinergic neurotransmission is well established, the possible physiological role of the multiple molecular weight forms remains unclear. Recently differences in molecular species distribution of acetylcholinesterase were observed in rat skeletal muscle following denervation (6). Differences in the distribution of acetylcholinesterase molecular weight forms between normal and dystrophic muscles have also been reported (7). Hall (2), in studying the multiple forms of acetylcholinesterase (16 S, 10 S, and 4 S) and their distribution in end plate and non-end plate regions of rat diaphragm muscle, concluded that the 16 S form was specifically associated with the end plate region of muscle and might correspond to the end plate enzyme. The 4 S and 10 S enzyme forms were not as localized and may serve other roles in the cholinergic system. The above findings suggest that different molecular weight acetylcholinesterase forms may play different roles in both normal and abnormal

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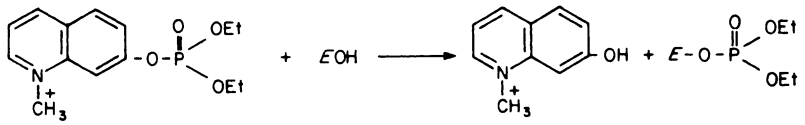
cholinergic neurotransmission processes. Moreover, the results provide a basis for studying the catalytic and structural features of different acetylcholinesterase forms in order to better define and understand their individual enzymatic properties.

In the present investigation the three asymmetrical forms of eel acetylcholinesterase as well as the 11 S form were examined. The rate of acetylthiocholine hydrolysis per catalytic center (activity per site or turnover number) was determined for each of these enzyme species.

To evaluate the activity per site we had to assay the activity of an enzyme solution under specified conditions of temperature, pH, and composition of the assay medium. We also had to measure the concentration of active sites in the enzyme solution, i.e., the normality of the enzyme solution. To do this we used the extremely potent phosphorylating agent 7-(diethoxyphosphinyloxy)-*N*-methylquinolinium fluorosulfonate as an irreversible inhibitor of acetylcholinesterase. The reaction, where *E*OH



is the enzyme, with OH representing the active serine, is extremely rapid ($k_i \approx 2.5 \times 10^8$ M^{-1} min^{-}) and goes to completion ($K_{eq} = 10^6$) (8, 9). Two methods were used for titrating the enzyme to measure the concentration of active sites. In the method of residual activity (10), quantities of DEPQ7+ [2] less than the concentration of active sites of the enzyme solution are added to the enzyme solution and the reaction is allowed to go to completion (11). The enzyme normality is twice the concentration of DEPQ7+ that produces 50% inhibition of the enzymatic activity.

In the second method excess DEPQ7+ is added to the enzyme solution and the concentration of 7-hydroxy-*N*-methylquinolinium ion that is produced by the reaction is measured fluorometrically (11). This compound is intensely fluorescent. The concentration of the phenol is the same as the normality of the enzyme. This method is the same as the method of Rosenberry and Bernhard (12), except that DEPQ7+ is used rather than the *N*,*N*-dimethylcarbamoyl derivative of 7-hydroxy-*N*-methylquinolinium ion. The values for activity per site for the enzyme forms represent a basic catalytic property and serve in the characterization of each acetylcholinesterase species.

METHODS

Materials. Acetylthiocholine, 5,5'-dithiobis(2-nitrobenzoic acid), and bovine serum albumin were purchased from Sigma Chemical Company. 7-(Diethoxyphosphinyloxy)-*N*-methylquinolinium fluorosulfonate and *N*-methyl-7-hydroxyquinolinium were synthesized as previously described (11). Twice-crystallized trypsin was obtained from Worthington Biochemical Corporation. Sodium phosphate (monobasic) was obtained from Fisher Scientific Company, and the sucrose used for density centrifugation was ultrapure special enzyme grade purchased from Schwarz/Mann. Some eel acetylcholinesterase used in these experiments was obtained commercially from Worthington and Sigma.

Isolation of acetylcholinesterase forms. Isolation and purification of the asymmetrical eel acetylcholinesterase forms followed the procedure of Ashani and Wilson (13) as modified by Voss *et al.*[3] This method involves solubilization of the eel enzyme in 1 M NaCl, followed by covalent affinity chromatography using the irreversible phosphorylator 2-aminoethyl-*p*-nitrophenyl methylphosphonate as the affinity ligand. The enzyme was removed from the affinity gel by elution with pyridine-2-aldoxime and dimethyl sulfoxide.

[2] The abbreviation used is: DEPQ7+, 7-(diethoxyphosphinyloxy)-*N*-methylquinolinium fluorosulfonate.

[3] F. Voss, W. Soucie, and I. B. Wilson, manuscript in preparation.

Separation of acetylcholinesterase forms was accomplished by a 5–20% sucrose gradient centrifugation in 1 M NaCl–0.05 M phosphate buffer, pH 7.4. The 18 S, 14 S, and 8 S peaks were well separated from each other. We used the peak fractions for this work. Very little enzyme was required. These samples were dialyzed to remove sucrose and recentrifuged separately to check that each form remained true.

Activity measurements. Acetylcholinesterase activity was measured at 25° in 0.05 M phosphate buffer (pH 7.43), 0.001 M acetylthiocholine, and 0.00033 M 5,5'-dithiobis(2-nitrobenzoic acid), with and without 0.4 M NaCl (14). The asymmetrical enzyme forms aggregate in low salt concentrations but do not aggregate in 0.4 M NaCl–0.05 M phosphate buffer.

Activity per site (11). The normality of the enzyme solutions was obtained mostly by residual activity. From 0 to 25 μl of freshly prepared DEPQ7$^+$ standard solutio, 72.2 nM in 0.05 M phosphate buffer, pH 7.4, were added to 0.1-ml portions of enzyme solution, 10–20 nN, and 5 μl were used for assay in 3 ml of assay medium. The results of one such determination are shown in Fig. 1.

We used a Perkin-Elmer MPF-2A recording spectrofluorometer for the leaving group assay. Excitation was at 400 nm, and emission was measured at 500 nm. The enzyme concentration was about 5 nN, and that of DEPQ7$^+$ was 20 nM. A calibration curve was obtained using standardized concentrations of 7-hydroxy-*N*-methylquinolinium iodide (12).

RESULTS

A typical plot of the data for the method of residual activity is given in Fig. 1 for the 18 S species. The linearity of the plot indicates either that the rate constant for reaction with DEPQ7$^+$ must be the same for all sites or that the hydrolytic activity is the same for all sites, or both.

The mean values for the activity per site, acetylthiocholine molecules hydrolyzed per minute per site, along with the standard errors of the mean, are presented in Table 1 for the different molecular weight forms of acetylcholinesterase. The 18 S and 14 S forms had about the same turnover numbers; the 14 S form appeared to be a small percentage more active per site, but the errors were too large to make this close a distinction. The 8 S form was distinctly more active per site, about 30% more active than the 18 S form. The 11 S preparations varied in activity per site,

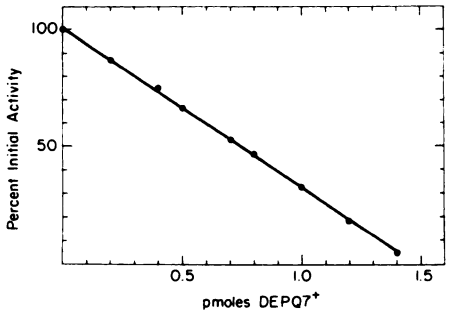


FIG. 1. *Residual enzyme activity as a function of DEPQ7$^+$ concentration*

DEPQ7$^+$ was added to 0.1 ml of 18 S acetylcholinesterase solution. The x intercept is 1.49 pmoles, corresponding to 14.9 nN enzyme.

TABLE 1

Turnover numbers of acetylcholinesterase forms

Activity was measured in 0.5 M phosphate buffer, pH 7.43, at 25°, using 0.001 M acetylthiocholine and 0.00033 M 5,5'-dithiobis(2-nitrobenzoic acid), with and without 0.4 M NaCl. The numbers in parentheses are the numbers of experiments. R indicates the method of residual activity, and F indicates the method of fluorescence.

Enzyme species	High salt	Low salt
	10^5 min^{-1}	
Worthington 11 S	6.26 ± 0.23 (4) R	6.96 ± 0.23 (6) R
Sigma 11 S	5.99 ± 0.06 (3) R	
Worthington 11 S		7.27 ± 0.15 (4) F
Tryptic 11 S (from 18 S)	6.83 ± 0.03 (2) R	
8 S	6.88 ± 0.06 (3) R	
14 S	5.52 ± 0.02 (2) R	
	5.44 ± 0.14 (5) F	
18 S	5.22 ± 0.23 (8) R	

depending upon the source. An 11 S form that was prepared by treating purified 18 S enzyme with trypsin in this laboratory had a turnover number about the same as the 8 S form, and somewhat higher than other 11 S forms. During the course of the trypsinization of the 18 S form, the total activity of the enzyme solution increased by about 15%, even though the number of active sites decreased by about 15%.

The values given for the lower salt concentration with Worthington 11 S enzyme were determined in completely separate experiments. Approximately 12% higher values of the turnover number were obtained in lower salt, but the uncertainty in the size of the increased value is large. However, we measured the activity of an enzyme sample in "low-salt" and "high-salt" assay mixtures and found a ratio of 1.10. It is not known whether this same salt effect applies to the other enzyme forms, because the 18 S and 14 S forms aggregate in low salt. An assay in low-salt medium would involve uncertainty as to whether the enzyme had aggregated.

DISCUSSION

The significance of acetylcholinesterase in synaptic function relates to its role in terminating the action of the neurotransmitter acetylcholine. Much of the evidence supportive of this role has been obtained from experiments analyzing the actions of anticholinesterases on the end plate potential and miniature end plate potential. Synaptic geometry and synaptic localization of acetylcholinesterase are factors that may be significant in determining the time courses of cholinergic events (15).

The 14 S and 8 S forms have been regarded as degradative forms produced during solubilization of the native 18 S species. However, more recent studies have raised the question whether all three forms may exist as "native" forms in neural tissue. Recent experimental evidence has indicated differences in synaptic localization of different acetylcholinesterase enzyme forms. The understanding of possible physiological consequences of differing distributions of acetylcholinesterase forms and their synaptic location may depend upon defining the catalytic and structural features that distinguish one molecular weight form from another. Of particular interest may be differences in the substrate turnover number or the activity per site.

Our results do not indicate much difference in the turnover numbers of the 18 S and 14 S forms, but the 8 S form, which was obtained only in small amounts, was distinctly more active. However, it is not at all certain that these observations with the enzyme from electric eel can be generalized to other tissues, and it is possible that larger differences may be found in other tissues. It would seem important to repeat these measurements with other tissues.

Other features of interest in this study have to do with comparisons between the asymmetrical 18 S enzyme form and the globular 11 S species, as well as the trypsin-catalyzed conversion of 18 S to 11 S. Reported values for the specific activity (activity per milligram) of purified 11 S enzyme assayed under different conditions have ranged from about 10 to 12.5 mmoles of acetylcholine hydrolyzed per milligram per minute (16, 17, 18). The corresponding specific activity so far obtained for the native 18 S form is considerably lower, as anticipated, since the 18 S form, in contrast to the 11 S enzyme, contains a tail component in addition to the catalytic subunits. However, the difference between the specific activities of the purified 18 S and 11 S forms is greater than can be accounted for by the presence of the noncatalytic 18 S "tail" alone, and it has been suggested that the lower specific activity for the 18 S form compared with the 11 S species may indicate that preparations of the 18 S species may not be as pure as the 11 S form. We now see that there is another reason why the specific activity of the 18 S species should be lower than the 11 S form: the activity per site is lower; i.e., the inherent catalytic ability is less.

Since the 11 S form results from the proteolysis of the asymmetrical forms, it may be heterogeneous. We observed distinct differences in the activity per site of the various 11 S preparations. The obser-

vation that the actual activity of an 18 S enzyme solution increased when it was converted to the 11 S form shows that the relatively low turnover number of the 18 S enzyme is not the result of denaturation of the 18 S enzyme but an improvement in its catalytic activity by partial proteolysis.

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