The Number of Catalytic Sites in Acetylcholinesterase

By W. LEUZINGER
Battelle Institute, Geneva, Switzerland

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By using two methods of titration, the number of active sites in acetylcholinesterase was determined. Either stepwise inhibition of the enzyme by an irreversible inhibitor, namely di-isopropyl phosphorofluoridate, or direct measurement of the concentration of active sites by titration with *o*-nitrophenyl dimethylcarbamate yielded an equivalent weight of approx. 130 000 for an active site in acetylcholinesterase. This indicates two sites per molecule, since the native enzyme has a molecular weight of 260 000.

The ability to prepare pure acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) on a large scale (Leuzinger & Baker, 1967) and to crystallize it (Leuzinger, Baker & Cauvin, 1968) have made it possible to initiate meaningful investigations into the physical and chemical properties of the enzyme. The molecular weight of acetylcholinesterase has been determined by sedimentation equilibrium and found to be 260 000 (Leuzinger, Goldberg & Cauvin, 1969). Evidence has been presented that the enzyme splits in the presence of guanidine hydrochloride and mercaptoethanol into four subunits, each having one-fourth of the molecular weight of the native enzyme (Leuzinger *et al.* 1969). The examination of the C-terminal amino acids revealed two categories of polypeptide chains (Leuzinger *et al.* 1969). Analysis of these results suggests that the enzyme is a dimer, each protomer consisting of two non-identical polypeptide chains, which are, however, approximately equal in mass. These results also made it necessary to reinvestigate the number of active sites. Previously on the basis of kinetic studies it had been suggested that acetylcholinesterase had an equivalent weight per active site of 50 000–240 000, which would imply one to four sites per molecule of native enzyme (Michel & Krop, 1951; Lawler, 1961; Wilson & Harrison, 1961; Kremzner & Wilson, 1964).

EXPERIMENTAL

Materials. *o*-Nitrophenyl dimethylcarbamate was purchased from British Drug Houses Ltd., Poole, Dorset, U.K. and recrystallized once from ether–heptane solution. Its melting point was determined as 57.0°C.

o-Nitrophenol was obtained from Matheson, Coleman and Bell Co., East Rutherford, N.J., U.S.A., recrystallized twice from ethanol and dried over P₂O₅. Its melting point was determined as 45.0°C.

Di-isopropyl phosphorofluoridate was purchased from Aldrich Chemical Corp., Milwaukee, Wis., U.S.A. For better handling, it was diluted in propane-1,2-diol. All buffers and salts used were of reagent grade.

Enzyme. Crystalline and electrophoretically pure acetylcholinesterase was used, prepared as described by Leuzinger & Baker (1967) from electric tissue of *Electrophorus electricus*, with a specific activity of 730 mmol of acetylcholine hydrolysed/h per mg of protein.

Methods. The enzyme activity was measured by titrating the acetic acid released with 0.01 M-NaOH with an automatic titrator at pH 8. The assay medium consisted of 0.02 M-MgCl₂, 0.05 M-NaCl, 0.01% recrystallized bovine albumin and 2.7 mM-acetylcholine. The enzyme concentrations were measured spectrophotometrically at 280 nm by taking a value of 19.0 for $E_{280}^{1\%}$ (M. Goldberg, personal communication).

RESULTS

Titration of acetylcholinesterase with di-isopropyl phosphorofluoridate. Alkyl phosphates such as di-isopropyl phosphorofluoridate are potent irreversible inhibitors of acetylcholinesterase as well as of many other hydrolytic enzymes. The catalytic area of the enzyme is subdivided into two spatially and functionally separated groups, an anionic site reacting by coulombic and van der Waals' forces with the quaternary nitrogen atom of the substrate, and an esteratic site at which the actual hydrolysis occurs (Wilson, 1960). The process is a two-step reaction. First, an intermediate acyl-enzyme is formed, and in a second step the acetylated enzyme reacts rapidly with water to give acetate and free enzyme. In the reaction with di-isopropyl phosphorofluoridate a phosphorylated instead of an acetylated enzyme is formed, which is unable to react with water. The degree of inhibition depends primarily on the concentration of the enzyme (Augustinsson & Nachmansohn, 1949). Therefore one can expect a

Fig. 1. Stepwise inhibition of acetylcholinesterase by di-isopropyl phosphorofluoridate. Experimental details are given in the text.

stoichiometric relation between enzyme and di-isopropyl phosphorofluoridate, as long as the concentration of the enzyme is well established.

For the titration experiment a 1% enzyme solution in 0.02M-sodium phosphate buffer, pH 7.0, was mixed with different amounts of di-isopropyl phosphorofluoridate at 10°C, and after 30 min the remaining activity was determined by pH-stat. The average values for five experiments are presented in Fig. 1, where activity is plotted against mol of inhibitor/mol of enzyme. Enzyme activity initially decreases as a linear function of inhibitor concentration, reaching 88.2% inhibition at a concentration corresponding to 2 mol of inhibitor/mol of enzyme. Further decrease of activity by addition of more di-isopropyl phosphorofluoridate does not produce a significant increase in inhibition. Therefore one can conclude that two sites for di-isopropyl phosphorofluoridate are present in a molecule of acetylcholinesterase of mol.wt. 260 000.

Similar experiments yielding the same result were carried out with ^{32}P -labelled di-isopropyl phosphorofluoridate (The Radiochemical Centre, Amersham, Bucks., U.K.; sp. radioactivity 63.5 mCi/mmol). A possible error in this method is that the inhibitor may phosphorylate other groups in addition to the active complex. To discount this possibility in one experiment the native enzyme was incubated first with unlabelled di-isopropyl phosphorofluoridate in the presence of acetylcholine and reactivated afterwards with pyridine-2-aldoxime methiodide (Fluka AG, Buchs, Switzerland). Practically the same result was obtained, indicating that there is little non-specific binding of di-isopropyl phosphorofluoridate to pure acetylcholinesterase under the experimental conditions.

Titration of acetylcholinesterase with *o*-nitrophenyl dimethylcarbamate. Another method that we have used to determine independently the number of active sites in acetylcholinesterase is to use a poor

Fig. 2. Liberation of *o*-nitrophenol as a function of time. Experimental details are given in the text.

substrate rather than an inhibitor. This method has been employed by Bender *et al.* (1966) with different hydrolytic enzymes.

For acetylcholinesterase *o*-nitrophenyl dimethylcarbamate seems to be an appropriate substrate. The enzyme was dialysed against 15 mM-sodium phosphate buffer, pH 8, and subsequently centrifuged for 15 min at 45 000g. Then 1.2 ml of an enzyme solution (2.15 mg/ml) was incubated at 23°C in a quartz cell of 10 mm path length with 0.04 ml of 3.1 mM-*o*-nitrophenyl dimethylcarbamate (in water-acetonitrile, 1:1, v/v) which gave a final concentration of 0.1 mM-*o*-nitrophenyl dimethylcarbamate.

The absolute standard in these determinations is the small organic titrant rather than the enzyme, which has to be the absolute standard in a rate-assay procedure. In view of the great difficulties in defining a 100% pure and active enzyme as a standard, the above method is obviously of great advantage, especially when a stoichiometric reaction can be followed readily and accurately, which in this case means the liberation of *o*-nitrophenol, represented in the general eqn. (1) as P_1 :

The conversion of enzyme E into acyl-enzyme ES' and liberation of P_1 can be considered a stoichiometric reaction with respect to an individual active site. This process can be observed before the regeneration of the enzyme occurs and therefore a direct measure of the enzyme concentration can be made.

In Fig. 2, the liberation of *o*-nitrophenol indicated by the change in E_{415} is plotted against time. The initial pre-steady-state of 1–2 min after the addition of the titrant to the enzyme solution is followed by a steady-state reaction. By extrapolation of the linear portion of the curve to zero time the initial rise in E_{415} is obtained. This value, corrected

according to the characteristic absorption of *o*-nitrophenyl methylcarbamate, can be related to the molar extinction of *o*-nitrophenol under the reaction conditions ($\epsilon_{415} = 2.72 \times 10^4$). On the other hand, the resulting concentration of *o*-nitrophenol can be now associated with the equivalent weight for an active site, as long as the protein concentration and the enzyme activity is known. In three experiments with different enzyme preparations the values 130 000, 142 000 and 133 000 were found.

DISCUSSION

The finding of two active sites for acetylcholinesterase per 260 000 mol.wt. units (i.e. about 130 000 per site) is consistent with the proposed $\alpha_2\beta_2$ dimer structure (Leuzinger *et al.* 1969). The presence of a twofold symmetry in the number of sites is further supported by labelling experiments with *p*-trimethylammoniumbenzene diazonium fluoroborate and by equilibrium-dialysis experiments with decamethonium, a bis-quaternary competitive inhibitor of the enzyme (W. Leuzinger, unpublished work). It is noteworthy that Serlin & Fluke (1956) determined the minimum molecular weight to be 110 000, close to the value given above, by taking a completely different approach. They exposed

enzyme preparations to electron, proton and α -particle bombardment. In their method, the rate of inactivation is related to the radiation dose.

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