THE INTERACTION OF THE FLUORESCENT DYE 1-ANILINO-8-NAPHTHALENE SULFONIC ACID WITH HORSE SERUM BUTYRYLCHOLINESTERASE

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Summary

The binding of 1-anilino-8-naphthalene sulfonic acid (ANS) to horse serum butyrylcholinesterase is accompanied by a marked increase in the fluorescence quantum yield of the dye. When the ANS ligand binds to the enzyme in the presence of acetylcholine an apparent "uncompetitive" mode of inhibition is demonstrated. This type of inhibition may, however, be artifactual since the ANS molecule will also bind to the enzyme in the absence of substrate. Binding of the dye to the protein has been characterized by fluorescent titrations and by fluorescence polarization.

The use of fluorescence and fluorescence techniques to study the binding of various ligands to proteins has been well established. The binding of a fluorescent dye to a protein moiety has been utilized to characterize both substrate binding sites and to study the binding of effector molecules to allosteric proteins (1). In this regard the fluorescent dye 1-anilino-8-naphthalene sulfonic acid (ANS) has been utilized by various investigators in studying the nature of hydrophobic or non-polar binding sites on many protein-containing structures (2,3). The binding of 1-anilino-8-naphthalene sulfonate to hydrophobic areas is most usually characterized by a large increase in the fluorescence quantum yield of the bound versus the free dye, along with the

development of fluorescence polarization.

This report describes the interaction of the magnesium salt of 1-anilino-8-naphthalene sulfonic acid with the enzyme butyryl-cholinesterase (acetylcholine acetyl-hydrolase EC 3.1.1.8) derived from horse serum.

The binding of magnesium ANS to butyrylcholinesterase has been studied by several techniques. The functioning of ANS as an inhibitor of butyrylcholinesterase was studied at 26° utilizing a potentiometric method that has previously been described (4). The titrant was 0.01 N sodium hydroxide and the acetylcholine concentration was varied from 7.7×10^{-3}M to 2.2×10^{-3}M. Reactions were initiated by the addition of substrate and initial velocities were determined.

Fluorescence studies were performed on a Farrand MK-I Spectrofluorometer equipped with quartz Polacoat polarizing filters. By employing the general methods developed by Klotz (5) a determination of the number of dye binding sites per mole of enzyme was carried out as well as the determination of the association constant of the dye-protein complex. These experiments were conducted at pH 7.4 in 0.1 M sodium phosphate buffer. The excitation wavelength was 382 mμ and the emission monochromator was set at 474 millimicrons. Polarization spectra of the ANS-butyrylcholinesterase complex were obtained by the method of Chen and Bowman (6). Emission was observed at 470 millimicrons.

The data obtained from the inhibition studies (Fig. 1), when plotted according to Lineweaver and Burk (7), demonstrated that ANS apparently functions as an "uncompetitive" inhibitor of the enzyme. The uncompetitive kinetics seen with this compound is difficult to explain since by definition the inhibitor should only

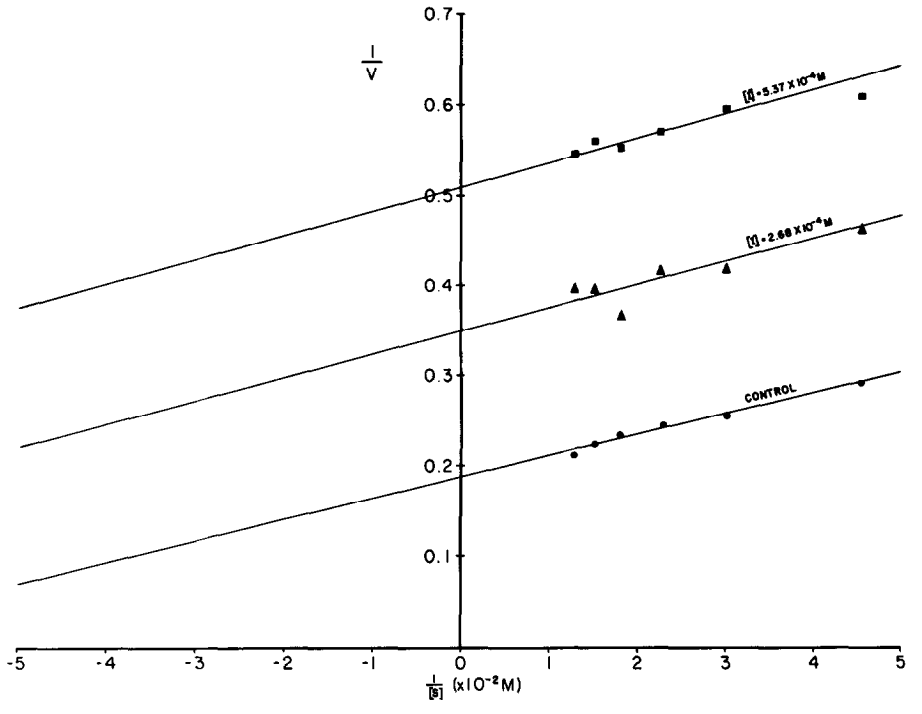

Fig. 1 - Apparent uncompetitive inhibition of horse serum butyrylcholinesterase by 1-anilino-8-naphthalene sulfonic acid. Initial velocity measurements are expressed on the vertical axis as μmoles acetylcholine hydrolyzed per milligram protein. Substrate concentrations are expressed as molar concentrations on the horizontal axis. Inhibitor concentrations are as indicated in the figure.

bind to the protein after the enzyme-substrate complex is formed. Fluorescent titration data is presented in Fig. 2 in the form of a Klotz plot and clearly indicates the binding of ANS to butyrylcholinesterase in the absence of acetylcholine. Therefore the uncompetitive kinetics seen in Fig. 1 may be artifactual. We have also found in the course of our investigations that sodium dodecyl sulfate, another hydrophobic anion, likewise inhibits butyrylcholinesterase in a manner which is difficult to describe using Lineweaver - Burk kinetics. From the Klotz plot an ANS-protein association constant (K_A) of $3.0 \times 10^5 M^{-1}$ can be derived. Data from

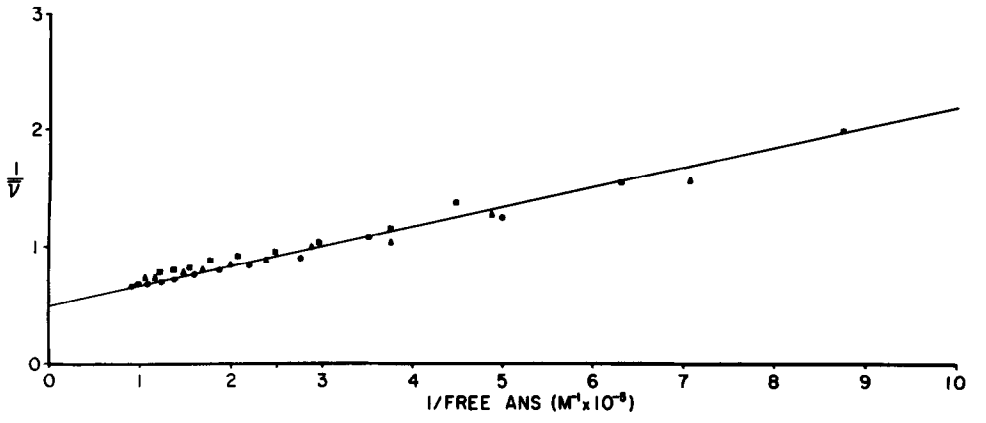

Fig. 2 - Evaluation of the binding of ANS to butyrylcholinesterase. Here the y intercept is the reciprocal of the number of dye binding sites and the x intercept is equal to $-1/K_A$. $\bar{\nu}$ is the number of moles of ANS, bound/mole of protein. Enzyme concentrations of 1.25×10^{-6}M (■), 2.5×10^{-6}M (▲) and 3.75×10^{-6}M (●) were used for the determinations.

the plot also indicates that 2 moles of ANS are bound per mole of enzyme. Although a molecular weight of 200,000 was assumed for these calculations it should be pointed out that a wide range of molecular weights have been reported (8). Therefore, depending on the molecular weight chosen, the number of binding sites determined for the ANS molecule may vary from about 2.0 to 4.0. Further analysis of the dye-protein interaction is indicated in the polarization spectrum shown in Fig. 3. Both the enhancement of fluorescence and polarization of fluorescence are abolished on the addition of 5M guanidine to the ANS-butyrylcholinesterase complex.

A preliminary evaluation of these results indicates the existence of two selective ANS binding sites on butyrylcholinesterase. As reflected by the dye-protein association constant these sites show a relatively high affinity for the dye molecule. Preliminary data on the binding of ANS to the enzyme acetylcholinesterase yields almost identical kinetics. However, unlike butyrylcholinesterase an inter-

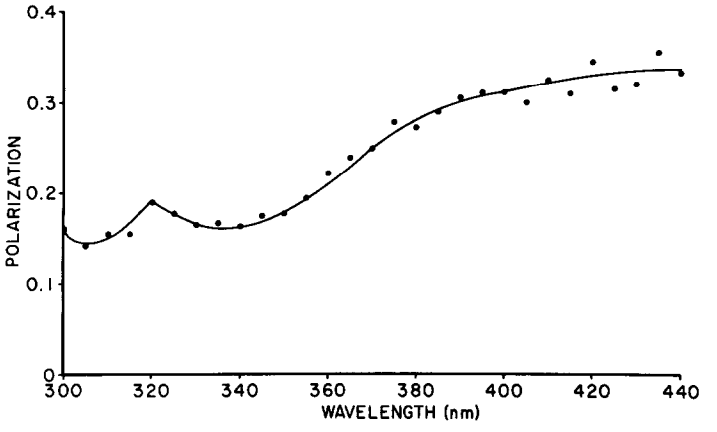

Fig. 3 - Polarization of fluorescence spectrum of the ANS-butyrylcholinesterase complex in 0.1M Na phosphate buffer (pH 7.4). Enzyme concentration is 5×10^{-6}M and the ANS concentration is 5×10^{-5}M. Even though free dye is present, its contribution to the polarization spectrum is minimal. Grating correction factors were applied to the spectrum according to Chen and Bowman (6). Emission was observed at 470 millimicrons.

action of ANS with the acetyl enzyme initiates an irreversible denaturation process. This phenomena is currently being studied further.

Even though one usually thinks of inhibitors of cholinesterases as being positively charged quaternary nitrogen containing compounds, Heilbronn (9) recently reported on the inhibition of serum cholinesterase by the negatively charged fluoride ion. However, no definitive explanation has been offered concerning the mechanism of this inhibition. Therefore, we are currently investigating the possibility of a relationship between the inhibition of this enzyme by the fluoride ion and by the ANS ion.

A more complete analysis of these data is presently being carried out and a detailed interpretation of our findings will be presented elsewhere.

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