SPECTRAL EVIDENCE FOR THE PRESENCE OF TRYPTOPHAN IN THE BINDING SITE OF ACETYLCHOLINESTERASE*

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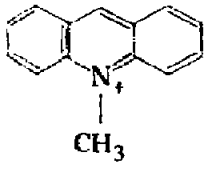
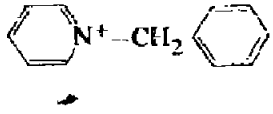
1. Introduction

Quaternary derivatives of pyridine and acridine are good inhibitors of acetylcholinesterase (AChE; EC 3.1.1.7) [1–3]. Spectral changes of either the AChE or the aromatic amine in the enzyme–inhibitor complex can yield information on the residues located at the binding site of the enzyme. Mooser et al. [2] have shown that the binding of fluorescent quaternary aromatic amines of the types mentioned above is accompanied by strong quenching of their fluorescence. In the following, we present spectral data indicating that tryptophan is involved in the binding site on AChE for the quaternary aromatic amines *N*-methylacridinium (MA^+) and *N*-benzylpyridinium (BP^+).



MA^+



BP^+

Three independent lines of evidence will be cited: i) Binding of BP^+ to AChE is accompanied by marked quenching of the fluorescence of the enzyme. ii) Binding of MA^+ is accompanied by the appearance of a characteristic charge-transfer band. iii) Indole derivatives act as strong quenchers of MA^+.

* This paper is dedicated to the memory of Professor Arieh Berger.

2. Materials and methods

Purified 11 S AChE was prepared as described previously [4]. MA^+ and BP^+ were prepared by quaternization of acridine and pyridine with dimethyl sulfate and benzyl chloride, respectively; both compounds were separated and crystallized from ethanol–water as their perchlorate derivatives, and were chromatographically and analytically pure.

AChE activity assays were performed either titrimetrically using acetylcholine as substrate [5], at pH 7.0 and 25°, or spectrophotometrically using acetylcholine [6], at pH 8.0 and 25°. Using the first procedure, inhibition constants were determined by the Dixon method [7], plotting the reciprocal of the initial velocity against the inhibitor concentration. When the second method was employed, inhibition constants were determined from Lineweaver–Burke plots.

Fluorescence intensities and fluorescence spectra were measured as described elsewhere [8]. Absorption spectra were recorded using a Cary 16 spectrophotometer. Tryptophan was determined colorimetrically according to Spies and Chambers [9], and spectrophotometrically, according to Beaven and Holiday [10].

3. Results and discussion

The fluorescence spectrum of AChE was recorded at pH 7.0, using an excitation wavelength of 290 nm. The emission band showed a maximum at 337 nm which is typical of the tryptophan fluorescence observed in proteins [11]. The quantum yield of emission was determined by comparing the corrected

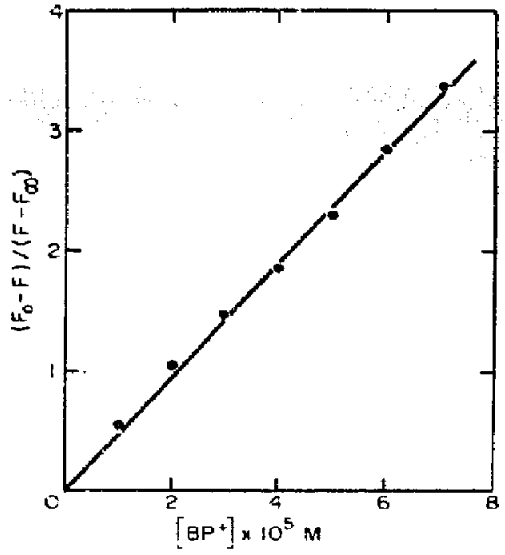


Fig. 1. Quenching of acetylcholinesterase (AChE) fluorescence by *N*-benzyl-pyridinium perchlorate (BP^+). A solution of AChE (A_{280} = 0.13) in 0.01 M phosphate–0.1 M NaCl, pH 7.0, was excited at 290 nm, and the bulk fluorescence was detected through a saturated potassium biphthalate solution used as a filter. Aliquots of BP^+ were added to yield final conc. up to 10^{-3} M. The results are presented as $(F_0-F)/(F-F_\infty)$ vs. $[BP^+]$ (see text).

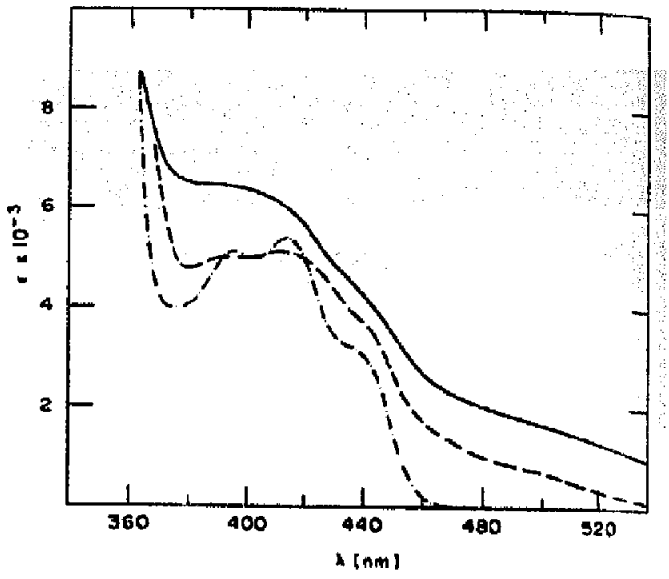


Fig. 2. Absorption spectra of *N*-methylacridinium perchlorate (MA^+) in the absence and presence of the sodium salt of L-acetyltryptophan (ALT^-) and of acetylcholinesterase (AChE) (–·–·–) 10^{-4} M MA^+ in 1:1, v/v, methanol–water. (——) Mixture of 10^{-4} M MA^+ and 0.4 M ALT^- in 1:1, v/v, methanol–water. (– – –) 10^{-5} M MA^+ in the presence of AChE (2 mg/ml) in 0.01 M phosphate–0.1 M NaCl, pH 7.0. The curves display the spectra as the molar extinction coefficient (ϵ).

area under the emission band with that of an aqueous solution of tryptophan with the same optical density at 290 nm, using the method of Parker and Rees [12]. Taking into account the range of published values of 13–20% for the quantum yield of tryptophan fluorescence [13, 14], the corresponding quantum yield for the enzyme is 10 ± 2%.

N-benzylpyridinium (BP^+) was found to be a good competitive inhibitor of AChE, with K_I values of 1.0×10^{-5} M and 1.5×10^{-5} M determined for the inhibition of AChE action on acetylcholine and acetylthiocholine, respectively, by the methods outlined above. Concomitantly, the fluorescence intensity of AChE was recorded in the presence of increasing amounts of BP^+. At the excitation wavelength of 290 nm almost selective excitation of tryptophyl residues is achieved without absorption by BP^+. The fluorescence intensity of AChE markedly decreases upon addition of the inhibitor, and it levels off at 58% of its initial value at 10^{-3} M BP^+. Since the concentration of BP^+ was much greater than that of the enzyme, we could derive K_{diss} according to the following equation:

$$\frac{F_0 - F}{F - F_\infty} = \frac{[BP^+]}{K_{diss}} \qquad (1)$$

where F_0, F and F_∞ are the fluorescence intensities in the presence of BP^+ at concentrations of 0, $[BP^+]$ and 10^{-3} M, respectively. The recorded fluorescence intensities are plotted as $(F_0 - F)/(F - F_\infty)$ vs. $[BP^+]$ in fig. 1. The slope of the straight line yields a value of $K_{diss} = 2.1 \times 10^{-5}$ M, which is in fair agreement with the value of K_I obtained in the competitive inhibition measurements.

The tryptophan content of AChE was determined both colorimetrically [9] and spectrophotometrically [10], amino acid analysis being performed in parallel on aliquots of the same stock solution. The colorimetric method yielded a value of 3.5 ± 0.5 tryptophan residues per 100 amino acid residues, and the spectrophotometric method, 3.3 ± 0.5 residues per 100 amino acid residues. Both values are considerably higher than those reported previously [15, 16]. However, which-

ever value is employed there appears to be a large number of tryptophan residues per active site (10–20), the exact value depending not only on the tryptophan analysis, but also on the subunit molecular weight and number of active sites (see, for example, [2, 4 and 16]). The marked quenching observed suggests, therefore, either that the quantum yield of the tryptophan in the active site is unusually high, or that there is very efficient energy transfer to this tryptophan. Preliminary experiments indicate that the first explanation is more tenable, since no change in fluorescence emission maximum (337 nm) occurred on quenching with the pyridinium ligand, nor was any increase in the degree of fluorescence polarization observed.

Pyridinium compounds quench the fluorescence of tryptophan derivatives by a charge-transfer interaction between the indole side chain, which acts as an electron donor, and the pyridinium nucleus, which acts as an electron acceptor [17]. In principle, a charge-transfer interaction should be accompanied by a typical new absorption band [18]. However, the tryptophan–pyridinium charge-transfer band is masked by the absorption band of the protein [17, 19] and is, therefore, practically undetectable. MA^+ is a much stronger inhibitor of AChE than BP^+, possessing a K_i of $2-3 \times 10^{-7}$ M [2, 3]. Since it is also a stronger electron acceptor than BP^+ [20], the charge-transfer band for its interaction with indole would be expected to be in the visible region [18]. To test this possibility we studied the absorption spectrum of MA^+ in the presence of AChE and of the sodium salt of acetyl-L-tryptophan (ALT^-). The latter compound was chosen as a possible model for the anionic site which is believed to serve as the binding site of quaternary substrates and inhibitors by AChE [1]. Fig. 2 shows the absorption spectrum of MA^+ in the absence and presence of 0.4 M ALT^-, in the region of 340–500 nm where ALT^- is completely transparent. The figure clearly shows the existence of a new absorption band in the MA^+–ALT^- mixture. From the absorption which emerges at 470 nm, in a series of solutions containing 10^{-4} M MA^+ and 0.05–0.4 M ALT^- in 1:1, v/v, methanol–water, we have determined a dissociation constant of 0.16 M for a 1:1 complex of MA^+ and ALT^-. Accordingly, the spectrum shown in fig. 2 is that of a mixture of 71% MA^+–ALT^- and 29% free MA^+. Fig. 2 also shows the absorption spectrum of 10^{-4} M MA^+ in the presence of 2 mg per ml AChE, i.e. at a concentration of active sites of at least 2×10^{-5} M (see [2, 4 and 16]). Under such conditions the ligand should be essentially fully bound, and was indeed observed to be practically void of fluorescence. The spectrum of the AChE–MA^+ complex is similar in shape to that of the ALT^-–MA^+ complex. The lower extinction coefficient of the AChE–MA^+ complex may result from a restricted geometry of interaction. When 10^{-2} M decamethonium chloride, which is also a strong competitive inhibitor of AChE, is added to the MA^+–AChE mixture, the absorption spectrum is found to return to that of free MA^+, as expected [21].

In order to strengthen the case for involvement of tryptophan in the interaction between AChE and MA^+, we studied the effect of both ALT^- and indole on the fluorescence yield of MA^+. Both compounds are indeed strong quenchers of MA^+. Their quenching efficiency was determined according to the Stern–Volmer equation

$$F_0/F = 1 + K[Q] \qquad (2)$$

where F_0 and F are the fluorescence intensities in the absence and in the presence of a quencher at a concentration [Q], and K is a constant [22]. Solutions containing 10^{-6} M MA^+ and increasing amounts of indole or ALT^- of up to 5×10^{-3} M were excited at 358 nm and their emission intensities determined at 485 nm. The results were plotted as F_0/F vs. [Q] and yielded straight lines with slopes of $K = 550\ M^{-1}$ and $470\ M^{-1}$ for indole and ALT^-, respectively. These high K values indicate that both compounds are efficient quenchers of excited MA^+.

The substantial contribution of charge-transfer interaction between the indole in the binding site of AChE and the quaternary amine, indicates that the distance between the two species is less than ~ 5 Å, and that the rings are facing each other [23]. More precise elucidation of the role, location and number of tryptophan residues involved will entail additional work both by spectroscopic techniques and by chemical labelling and modification.

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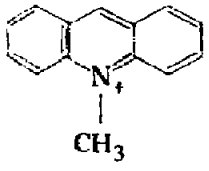
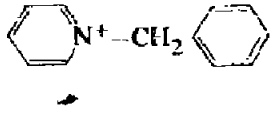


MA^+



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