polymerase N3 was not identical with the *M. orale* polymerase and (2) that, although the N3 activity may have been a consequence of mycoplasma contamination, the manner of its appearance and its potential significance remain unknown.

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Ligand-Induced Conformational Changes in Acetylcholinesterase Investigated with Fluorescent Phosphonates†

David J. Epstein, Harvey Alan Berman,* and Palmer Taylor

ABSTRACT: Steady-state kinetic studies have suggested that the catalytic activity of acetylcholinesterase is altered upon binding of certain ligands at a locus removed from the active center. In this study fluorescent phosphonates which conjugate with the active-center serine are employed to demonstrate directly that ligands which associate with the peripheral site alter active-site conformation. The fluorescent reagent [1-(dimethylamino)naphthalene-5-sulfonamido]pentyl methylphosphonofluoridate, when conjugated to the enzyme, proves to be a sensitive probe of active-site conformation. Displacement of propidium, a reversible fluorescent ligand of the peripheral site, was employed to assess peripheral-site occupation by a variety of enzyme inhibitors. Thus, occupation of the peripheral site and induced conformational changes of the active center can be compared directly. The transition-metal ions Zn^{2+} and Cu^{2+} are found to be inhibitors of acetylcholinesterase and show an unusually slow onset of inhibition. In the presence of Zn^{2+} the phosphonate-conjugated enzyme shows an enhanced fluorescence intensity where the emission maximum is shifted to shorter wavelengths; the change in fluorescence intensity occurs slowly, with a rate constant that is comparable to the rate of inhibition of the native enzyme. Displacement of propidium by Zn^{2+} or *d*-tubocurarine, a peripheral-site ligand, is found to occur much more rapidly than does the time course of enzyme inhibition or changes in fluorescence of the dansyl phosphonate. Thus, although occupation of the peripheral site is rapid, peripheral-site occupation by certain ligands induces a slow change in active-site conformation. Furthermore, the states induced by the different peripheral-site ligands can be distinguished spectroscopically, indicating nonequivalent conformational effects at the active center upon peripheral-site occupation.

The influence of quaternary inhibitors on steady-state catalysis by acetylcholinesterase (AchE)[1] suggests that such ligands alter enzyme activity by associating either with the active center or a peripheral anionic site (Changeux, 1966). Similar conclusions have been reached from the study of carbamylating agents which effectively act as hemisubstrates where the deacylation of acyl enzyme is sufficiently slow that it need not be considered in analysis of the kinetics of inhibition (Kitz et al., 1970; Rosenberry & Bernhard, 1971). The existence of a locus or loci physically distinct from the active center to which ligands such as *d*-tubocurarine and gallamine bind has been confirmed by studies with high-affinity reversible AchE inhibitors that manifest fluorescence changes upon binding to the enzyme. *N*-Methylacridinium binds exclusively to the enzyme active center with concomitant quenching of fluorescence emission of the acridinium moiety (Mooser et al., 1972), and studies employing this ligand have corroborated the presence of a *d*-tubocurarine binding site removed from the active center (Mooser & Sigman, 1974). Propidium, by contrast, which is competitive with *d*-tubocurarine and gallamine, is not displaced by active-center specific ligands (Taylor & Lappi, 1975). Thus, propidium, which exhibits enhanced fluorescence upon association with the enzyme, appears specific for the peripheral anionic site. Not only do active-center and peripheral-site ligands exhibit different modes of inhibition of substrate hydrolysis, but within the group of peripheral-site ligands characteristics of the inhibitory parameters vary substantially (Taylor & Lappi, 1975).

A particularly intriguing mode of AchE inhibition was recently uncovered by Pattison & Bernhard (1978), who observed that either *d*-tubocurarine in high concentrations or transition metals effect a slow conversion of the enzyme to an inactive state. The use of a fluorescent carbamylating agent enabled these workers to distinguish between inhibition of the initial rate of substrate association and the conversion to nonreactive enzyme. The slow onset of conversion and its slow reversion upon removal of ligand distinguish this inhibitory effect from the characteristic competitive and noncompetitive modes of inhibition examined previously.

To examine more directly the relationship between peripheral-site occupation and conformation at the active center, we have employed a fluorescent phosphonate, (dansyl-

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[1] Abbreviations used: AchE, acetylcholinesterase; M7C, *N*-methyl-7-(dimethylcarbamoxy)quinolinium iodide; M7H, *N*-methyl-7-hydroxyquinolinium iodide; DC_5MPF, [1-(dimethylamino)naphthalene-5-sulfonamido]pentyl methylphosphonofluoridate or (dansylamido)pentyl methylphosphonofluoridate; DC_5MP-AchE, (dansylamido)pentyl methylphosphonoacetylcholinesterase.

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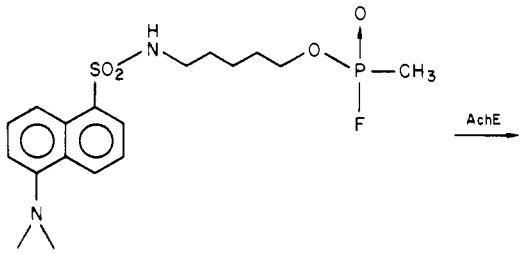
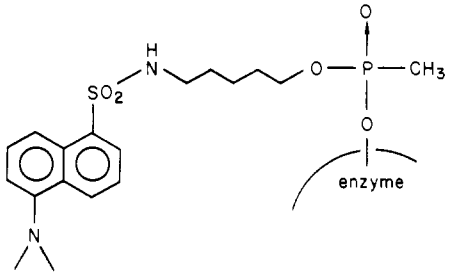
amido)pentyl methylphosphonofluoridate (I), which couples covalently to the active-site serine. The fluorescence properties



I



of this molecule are sensitive to changes in its immediate environment and hence provide a useful framework for examining the nature of conformational changes induced by peripheral-site ligands. With ligands that slowly convert AchE to an inactive form, kinetics of the loss of enzyme activity have been correlated with the corresponding spectroscopic changes in the conjugated fluorophore.

Experimental Procedures

Materials. The 11 S or lytic form of AchE was purified from *Torpedo californica* electroplax described previously (Taylor et al., 1974). Synthesis and structure verification of pyrenebutyl methylphosphonofluoridate have been described (Berman & Taylor, 1978). Similar methods were employed for synthesis and characterization of [1-(dimethylamino)-naphthalene-5-sulfamido]pentyl methylphosphonofluoridate (H. A. Berman, unpublished experiments). *d*-Tubocurarine chloride and acetylcholine chloride were purchased from Sigma, edrophonium chloride was a gift from Dr. W. E. Scott of Hoffmann-La Roche, and gallamine triethiodide was obtained from K & K Laboratories. *N*-Methyl-(7-dimethylcarbamoxy)quinolinium iodide (M7C) and *N*-methyl-7-hydroxyquinolinium iodide (M7H) were prepared by the method of Rosenberry & Bernhard (1971). All other compounds employed were reagent grade or the highest grade available.

Methods. Except where indicated, all experiments were conducted at 25 °C. Absorbance measurements were made on a Cary 16 spectrophotometer. Fluorescence spectra and kinetics of fluorescence changes with a half-life of more than 4 s were determined on a Farrand Mark I spectrofluorometer equipped with a corrected excitation unit, a temperature-controlled cell compartment, and a constant time base X–Y recorder. When recording kinetics of slow fluorescence changes, photodecomposition was minimized by removing the sample from the light path between measurements and employing a spectral bandwidth of less than 5 nm. Kinetics of fluorescence changes with a half-time of less than 4 s were recorded on a Durrum D_{110} stopped-flow spectrophotometer with a silvered fluorescence cuvette for enhanced sensitivity. The molar concentration of AchE active sites was determined by absorbance at 280 nm employing $E_{280}^{1\%} = 17.5$ and a molecular weight value of 80 000 or by M7C burst amplitudes (Rosenberry & Bernhard, 1971). AchE concentrations are reported as molarity of active sites.

Coupling of fluorescent inhibitors to the AchE active-site serine was accomplished by adding three- to fivefold molar excess of inhibitor to 5–10 μM AchE in 0.01 M Tris buffer, pH 7.4, containing 0.1 M NaCl and 0.04 M $MgCl_2$ at 4 °C. At intervals following inhibitor addition, the AchE activity of a small aliquot of the reaction mixture was determined. Coupling was considered complete when 99% of the enzyme activity was abolished, and this generally required less than 15 min. The labeled enzyme was then passed through a Sephadex G-25 column. The covalent fluorophore–AchE conjugate eluted in the void volume, while free inhibitor was found in the included volume. Labeled enzyme was then dialyzed overnight at 4 °C against two changes of buffer. Coupling of the pyrene fluorescent label is known to proceed stoichiometrically at the enzyme active site without extraneous labeling occurring at other sites (Berman & Taylor, 1978). The dansyl analogue appears to react in an identical fashion, although the low dansyl extinction coefficient precludes calculation of a precise stoichiometry by the usual absorbance analysis (Berman & Taylor, 1978).

The reaction of *N*-methyl-7-(dimethylcarbamoxy)quinolinium iodide (M7C) with AchE was conducted as described by Rosenberry & Bernhard (1971). M7C is a substrate for AchE, which upon hydrolysis liberates the fluorescent product M7H concomitant with formation of the dimethylcarbamyl–enzyme adduct. Reaction of M7C with AchE causes the development of an initial "burst" of fluorescence which is followed by a slow, linear increase in fluorescence. The latter arises from reaction of substrate with active enzyme, which is regenerated upon hydrolysis of the carbamylated enzyme. The burst amplitude is a measure of active-site concentration, while the burst rate is a measure of the intrinsic reactivity of active sites. Both parameters were analyzed as described previously (Rosenberry & Bernhard, 1971; Pattison & Bernhard, 1978). The concentration of M7H was determined from fluorescence measured at 510 nm upon excitation at 410 nm. The rate constants for induced changes in the burst amplitude were obtained from semilogarithmic plots of the burst amplitude for the M7C reaction measured at various times after addition of inhibitory ligand. Apparent dissociation constants were determined from plots of the fluorescence amplitudes at equilibrium vs. ligand concentrations.

All fluorescence determinations were made with the enzyme in 0.001 M Tris-HCl, pH 8, in 1.0-cm^2 cells containing 2 mL total volume. For titrations or kinetics involving dissociation of propidium, it was present in a concentration 2 to 3 times its dissociation constant. Emission spectra of DC_5MP-AchE fluorescence were measured with excitation at 290 and 340 nm. No difference in the wavelength for maximal emission could be detected with excitation at either wavelength; however, the emission intensity was greater upon excitation at 290 nm. The enhanced emission is a consequence of energy transfer between the protein tryptophanyl residues and the dansyl moiety. With excitation at 290 nm the ratio of dansyl to protein emission intensities reflects the stoichiometry of dansyl labeling. The rate constants obtained as well as the extent of propidium quenching were observed to be independent of the exciting wavelength.

Results

Fluorescent Phosphonate Conjugates of AchE as Probes of the Active Site. We have found previously that pyrenebutyl methylphosphonofluoridate when reacted with AchE forms an enzyme–phosphonate conjugate which has an apparent

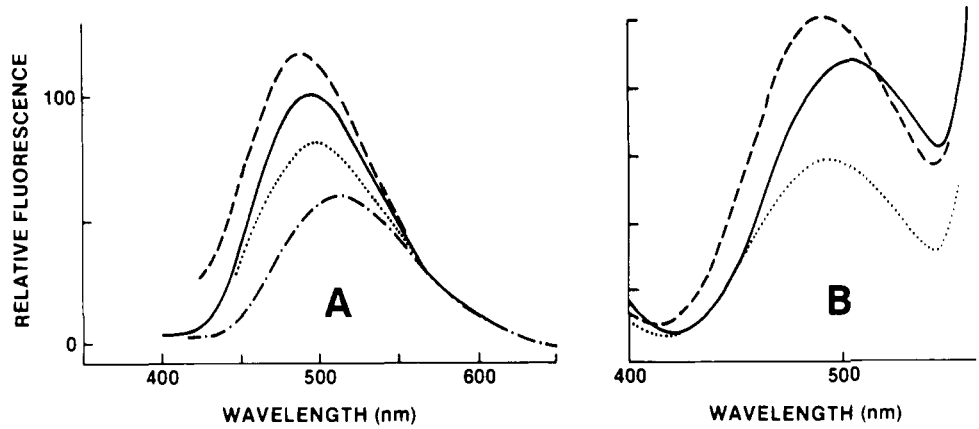


FIGURE 1: (A) Influence of exogenous ligands on the fluorescence emission spectra of (dansylamido)pentyl methylphosphonoacetylcholinesterase. Ligands were added to solutions of the enzyme (2.3×10^{-7} M) in (0.001 M) Tris-HCl buffer, pH 8. (---) $ZnCl_2$ (1×10^{-4} M); (···) *d*-tubocurarine (1.1×10^{-4} M); (-·-) gallamine (1.7×10^{-5} M); and (—) control. The excitation wavelength was 335 nm. (B) Influence of transition-metal ions on the fluorescence emission spectra of DC_5MP-AchE. $CuSO_4$ or $ZnCl_2$ was added to solutions of the enzyme (3×10^{-8} M) in (0.001 M) Tris-HCl buffer, pH 8. Fluorescence was excited at 290 nm, giving rise to a scatter signal at twice the excitation wavelength. (---) $ZnCl_2$ (3×10^{-5} M); (···) $CuSO_4$ (2×10^{-5} M); and (—) control.

stoichiometry of 1 phosphonate/80 000-dalton subunit. The kinetics of regeneration of active enzyme by oximes and the exclusion of fluorescent phosphonate labeling upon prior exposure of enzyme to diisopropyl fluorophosphate demonstrate that the fluorescent compounds react covalently with the active-site serine. Fluorescence of the bound pyrene moiety is found to be influenced by quenching through collisional (Stern–Volmer) and resonance energy transfer (Förster) mechanisms (Berman & Taylor, 1978). Fluorescence of the dansyl moiety is expected to serve as an indicator of altered active-site conformation since the emission spectrum of this fluorophore undergoes changes reflecting corresponding alterations in its local environment. Upon transfer of the dansyl moiety from water to solvents of decreasing dielectric constant, as in dioxane for example, the emission maximum of the dansyl moiety is shifted by 80 nm to shorter wavelengths and the quantum yield of fluorescence is enhanced sevenfold (Chen, 1967). The conjugate of (dansylamido)pentyl methylphosphonofluoridate and AchE, DC_5MP-AchE, shows a fluorescence emission maximum (502 nm) and an estimated quantum yield (0.78) that correspond closely to those found for the free dansyl moiety in dioxane (Chen, 1967) (Figure 1).

Effect of Peripheral-Site Occupation on DC_5MP-AchE Fluorescence. Ligands associating with AchE can be grouped into two classes: those ligands (e.g., edrophonium) which combine at the active center and show competitive inhibition of catalysis and those (e.g., *d*-tubocurarine, gallamine, and propidium) which bind at a remote locus or loci and display more complex inhibition behavior (Taylor & Lappi, 1975; Rosenberry & Bernhard, 1972; Roufagolis & Quist, 1972). Because of its fluorescence, propidium serves as a useful marker for peripheral-site occupation. As in the case for pyrenebutyl methylphosphono-AchE (Berman & Taylor, 1978), the absorption spectrum of propidium overlaps with the dansyl emission of DC_5MP-AchE and the association of propidium with the enzyme is expected to result in quenching of dansyl fluorescence by energy transfer between the emission and absorption dipoles of the dansyl moiety and propidium (Förster, 1959; Stryer, 1968). At saturating concentrations, propidium effects an 85% decrease in fluorescence intensity of DC_5MP-AchE. No shift in emission maximum can be discerned, as considered later (cf. Figure 4). Association between DC_5MP-AchE and *d*-tubocurarine or gallamine, ligands for the peripheral anionic site, is accompanied by a decreased fluorescence intensity and bathochromic shift in the dansyl emission spectrum; for *d*-tubocurarine the shift is slight (~4 nm), whereas for gallamine the shift is marked (~15 nm) (Figure 1). Neither gallamine nor *d*-tubocurarine possesses absorption spectra which overlap with the emission of the dansyl moiety; hence, dipolar energy transfer to the peripheral-site ligand is not responsible for the alteration in dansyl fluorescence. Thus, it seems likely that occupation of the peripheral site induces a change in active-site conformation which is reflected in the fluorescence of the dansyl moiety. Typically, a diminished quantum yield and a red-shifted emission spectrum are observed for the dansyl fluorophore when immersed in more polar solvents (Chen, 1967).

In the presence of Zn^{2+} the fluorescence intensity of the dansyl moiety of DC_5MP-AchE is enhanced ~20% while the emission maximum is shifted from 502 to 490 nm (Figure 1). The interaction of Zn^{2+} with DC_5MP-AchE, as determined from a plot of alteration in dansyl fluorescence vs. Zn^{2+} concentration, is characterized by an apparent dissociation constant of 6×10^{-7} M and is completely reversed in the presence of excess EDTA. Enhanced dansyl fluorescence with an attendant hypsochromic shift is consistent with the behavior seen for this fluorophore when in solvents of lower dielectric constant (Chen, 1967).

The transition metal Cu^{2+}, owing to its d–d transitions, differs from Zn^{2+} in possessing an absorption spectrum that overlaps with dansyl emission. Upon addition of Cu^{2+} to solutions of the dansyl-labeled enzyme, the hypsochromic shift characteristic of that induced by Zn^{2+} is observed but the fluorescence intensity is substantially diminished (Figure 1B). Thus, the dansyl fluorescence spectrum in the presence of Cu^{2+} likely reflects both Förster quenching as well as some change in the environment of the conjugated fluorophore. Only the latter would be reflected in a shift of the emission maximum.

The fluorescence spectrum of DC_5MP-AchE is unchanged in the presence of the alkaline earth cations Mg^{2+} and Ca^{2+}. Indeed, the presence of Mg^{2+} or Ca^{2+} antagonizes the alterations produced by Zn^{2+} on the fluorescence behavior of DC_5MP-AchE. Addition of the active-site selective ligand edrophonium to solutions of DC_5MP-AchE yields no change in dansyl fluorescence. This result is to be expected since edrophonium should not bind to the phosphorylated or phosphonylated enzyme (Suszkiw, 1973).

Kinetics of Ligand-Induced Alterations in Fluorescence of DC_5MP-AchE. If the distinctive slow conversion of AchE to

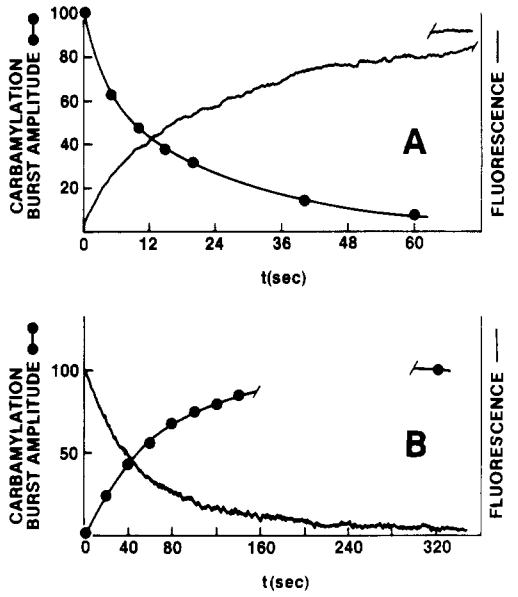


FIGURE 2: Kinetics of Zn^{2+} alteration in the AchE active-site conformation. (A) Kinetics of onset. (●) Kinetics measured by the decrease in M7C burst amplitude. $ZnCl_2$ (5×10^{-5} M) was added to AchE (7×10^{-8} M) at $t = 0$. At the indicated times, M7C (6.8×10^{-5} M) was added and the development of M7H fluorescence was monitored after 30 s (λ_{ex} = 410 nm; λ_{em} = 510 nm). (—) Kinetics measured by the change in quantum yield for the dansyl moiety. $ZnCl_2$ (5×10^{-5} M) was added to DC_5MP-AchE (2.6×10^{-8} M) at $t = 0$. Fluorescence emission was monitored at 500 nm with excitation at 290 nm. (B) Kinetics of reversion of the Zn-induced conformational change initiated by EDTA. (●) AchE (2×10^{-8} M) was mixed with $ZnCl_2$ (5×10^{-5} M) in low ionic strength buffer. After 5 min, M7C (2×10^{-5} M) was added with no consequent formation of M7H. After addition of EDTA (10^{-4} M), liberation of M7H was monitored by fluorescence at 510 nm with excitation at 410 nm. (—) $ZnCl_2$ (5×10^{-5} M) was added to a solution of DC_5MP-AchE (2.6×10^{-8} M) in low ionic strength buffer and incubated for 5 min. EDTA (10^{-4} M) was added, and DC_5MP-AchE fluorescence was monitored at 500 nm with excitation at 290 nm.

a nonactive state that is induced by Zn^{2+} or *d*-tubocurarine (Pattison & Bernhard, 1978) were reflected in the fluorescence of the conjugated phosphonates, similar kinetics for the two phenomena should be observed. The fluorescence enhancement in DC_5MP-AchE brought about by Zn^{2+} evolves slowly and is observed to exhibit first-order kinetics (Figure 2A) for which the rate constant is calculated to be 0.022 s^{-1}. This value is independent of Zn^{2+} concentration in excess of 10^{-5} M. The influence of Zn^{2+} on DC_5MP-AchE fluorescence is reversed by addition of EDTA in stoichiometric excess of the metal ion. The reversion to the original fluorescent state displays first-order kinetics (Figure 2B) with a calculated rate constant of 0.014 s^{-1}. This value was found to be independent of chelating ion concentration in excess of 1 equiv of Zn^{2+}.

The fluorescence changes of DC_5MP-AchE brought about by *d*-tubocurarine show kinetics which are distinct from those induced by Zn^{2+}. In the presence of *d*-tubocurarine, perturbation of the dansyl fluorescence occurs in a biphasic manner exhibiting a rapid first-order enhancement (k_1 = 3.5 s^{-1}) of low amplitude, as studied with stopped-flow fluorescence spectroscopy (not shown). This fast step is followed by a slower diminution (k_1 = 0.02 s^{-1}) in dansyl fluorescence (Figure 3). The rates characteristic of the slow and fast phases are observed to be independent of *d*-tubocurarine concentration in the range 0.5–1 $\times 10^{-4}$ M. It should be noted that the concentration of *d*-tubocurarine required to effect the change in dansyl fluorescence exceeds that required for propidium dissociation by *d*-tubocurarine (Taylor & Lappi, 1975). Fluorescence alterations in DC_5MP-AchE brought about by

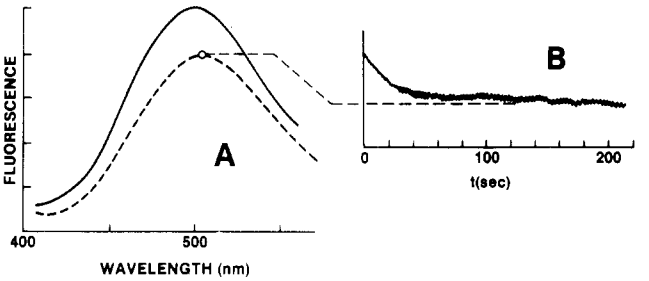


FIGURE 3: Influence of *d*-tubocurarine on the fluorescence of (dansylamido)pentyl methylphosphonoacetylcholinesterase. (A) Fluorescence emission spectra: (—) DC_5MP-AchE (10^{-7} M) in low ionic strength buffer; and (---) DC_5MP-AchE (10^{-7} M) in low ionic strength buffer following addition of *d*-tubocurarine (10^{-4} M). In the presence of high concentrations of *d*-tubocurarine (10^{-4} M) excitation at 290 nm introduces significant inner filter effects which diminish the intensity of the fluorescence signal. Hence, excitation was selected at 340 nm and λ_{em} = 500 nm. (B) Slow fluorescence decrease caused by *d*-tubocurarine. *d*-Tubocurarine (10^{-4} M) was added to DC_5MP-AchE (10^{-7} M), and the fluorescence emission of the dansyl moiety was monitored at 500 nm with excitation at 340 nm.

Table I: Kinetic and Equilibrium Parameters[a] for Zn^{2+}-Induced Changes in Acetylcholinesterase Conformation

	max rate of Zn-induced conversion, k_{app} (s^{-1})	EDTA reversal of Zn-induced conversion, k_{app} (s^{-1})	K_d (M)
redn of M7C burst amplitude	0.035[b] (0.02)[e]	0.014	2×10^{-7}[c]
enhancement of DC_5MP-AchE fluorescence	0.022	0.014[b]	6×10^{-7}[d]

[a] Measured by carbamylation kinetics and fluorescence of the AchE–phosphonate conjugate. [b] These rates show biphasic character and do not fit a single-exponential plot. The rates cited are for the dominant slow phase of reaction at 25 °C. [c] AchE at 1.5×10^{-8} M. [d] DC_5MP-AchE at 3×10^{-8} M. [e] Data of Pattison & Bernhard (1978).

gallamine are complete in less than a second; this rate was not investigated in stopped-flow experiments.

The influence of Zn^{2+} on the diminution of AchE activity as measured by hydrolysis of M7C (Pattison & Bernhard, 1978) as well as the data derived from analysis of DC_5MP-AchE fluorescence is summarized in Table I. Both kinetic and equilibrium constants for inhibition of enzyme activity show close correspondence with the data derived from fluorescence perturbation in DC_5MP-AchE. Further correspondence is seen from the study of the reactivation of enzyme activity with EDTA after Zn^{2+} treatment (cf. Figure 2B). The presence of Ca^{2+} and Mg^{2+} modifies AchE activity (Robaire & Kato, 1974) as well as antagonizes the Zn^{2+}-induced alteration in burst amplitude (Pattison & Bernhard, 1978). We observe that these cations diminish the Zn^{2+}-induced change in conformation measured by employing the active-site fluorescent phosphonate. In addition, *d*-tubocurarine has been observed to inactivate AchE in a biphasic manner for which the rate constant of the slow phase was calculated to be 0.03 ± 0.01 s^{-1} (Pattison & Bernhard, 1978), a value which is in agreement with the slow-phase rate characteristic of *d*-tubocurarine's effect on DC_5MP-AchE fluorescence, k_1 = 0.02 s^{-1}. Since the influence of exogenous ligands on the fluorescence spectrum of DC_5MP-AchE exhibits a temporal correspondence with changes in catalytic activity, the two

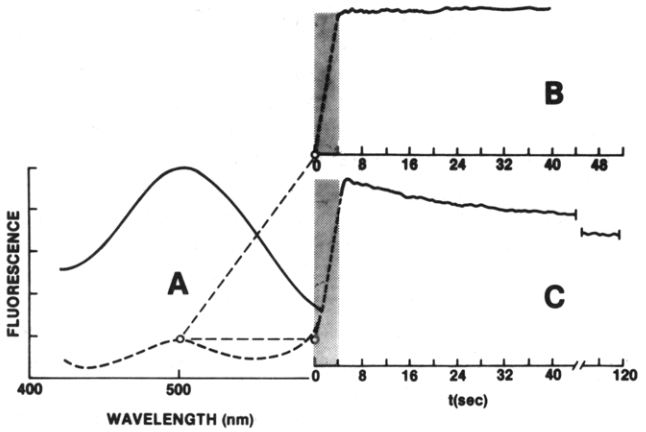


FIGURE 4: Displacement of propidium from DC_5MP-AchE by peripheral-site ligands. (A) (—) Fluorescence emission spectrum of DC_5MP-AchE (2.6×10^{-8} M) in low ionic strength buffer, λ_{ex} = 290 nm; and (---) fluorescence emission spectrum of DC_5MP-AchE (2.6×10^{-8} M) in the presence of propidium (1.5×10^{-6} M). (B) Propidium displacement by gallamine. At $t = 0$, gallamine (10^{-5} M) was added to DC_5MP-AchE (2.6×10^{-8} M) containing propidium (1.5×10^{-6} M), λ_{ex} = 290 nm and λ_{em} = 500 nm. (C) Propidium displacement by *d*-tubocurarine. At $t = 0$, *d*-tubocurarine (10^{-4} M) was added to a solution of DC_5MP-AchE (2.6×10^{-8} M) containing propidium (1.5×10^{-6} M). In the presence of high concentrations of *d*-tubocurarine (10^{-4} M) excitation at 290 nm introduces significant inner filter effects which diminish the intensity of the fluorescence signal. Hence, excitation was selected at 340 nm and λ_{em} = 500 nm.

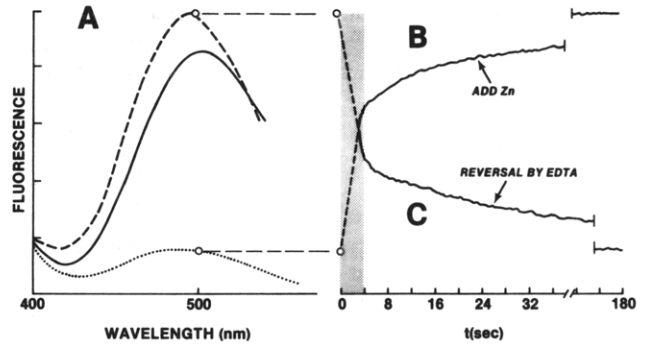


FIGURE 5: Influence of Zn^{2+} on the fluorescence properties of the DC_5MP-AchE–propidium complex. (A) Fluorescence emission spectra: (—) DC_5MP-AchE (2.6×10^{-8} M) in low ionic strength buffer; (···) spectrum obtained following addition of 3×10^{-7} M propidium; and (---) spectrum obtained following addition of $ZnCl_2$ (5×10^{-5} M) to the DC_5MP-AchE–propidium complex and allowing the system to equilibrate, λ_{ex} = 290 nm. (B) Kinetics of Zn^{2+}-induced changes in DC_5MP-AchE fluorescence. $ZnCl_2$ (5×10^{-5} M) was added to DC_5MP-AchE (2.6×10^{-8} M) previously equilibrated with 3×10^{-7} M propidium, and the fluorescence was monitored at 500 nm with excitation at 290 nm. (C) Kinetics of the reversal of the Zn^{2+}-induced changes in DC_5MP-AchE fluorescence. EDTA (10^{-4} M) was added, and the kinetics were monitored as described above.

phenomena likely reflect the same conformational events at the active center.

Examination of Peripheral-Site Occupation by Propidium Dissociation. Propidium association with the peripheral anionic site on DC_5MP-AchE can be examined directly by measuring the extent of energy transfer from the dansyl donor. Occupation of the peripheral site by ligands which displace propidium and hence alter the extent of energy transfer can be measured and compared with the slow kinetic changes observed in DC_5MP-AchE fluorescence.

Addition of gallamine (Figure 4B) and *d*-tubocurarine (Figure 4C), ligands competitive for the peripheral anionic site, to solutions of the DC_5MP-AchE–propidium complex results in a rapid increase in dansyl fluorescence of large amplitude attributable to propidium displacement. Superimposed on this fast phase is a slower phase for which the time course and final state achieved are characteristic of the influence of the displacing ligand seen in the absence of propidium. Hence, after the initial displacement of propidium by *d*-tubocurarine (Figure 4C), for example, a slow diminution of fluorescence is observed and occurs with a time course (k_1 = 0.02 s^{-1}) resembling the influence of the ligand on enzyme catalysis (k_1 = 0.03 ± 0.01 s^{-1}).

Addition of Zn^{2+} (5×10^{-5} M) to solutions of DC_5MP-AchE which have been equilibrated with propidium results in a rapid increase in dansyl fluorescence due to displacement of propidium and is followed by the slower fluorescence changes characteristic of the effect of Zn^{2+} (Figure 5B). Chelation of Zn^{2+} upon addition of EDTA allows propidium quenching to be rapidly reestablished as seen by the immediate large decrease in dansyl fluorescence (Figure 5C). Superimposed on this phase is a slower decrease in fluorescence which follows a time course resembling that observed for these reactants in the absence of propidium (cf. Figure 2B). Thus, displacement of propidium by Zn^{2+} or *d*-tubocurarine demonstrates that the kinetics of ligand association and dissociation are rapid in comparison with the slower changes that ensue after ligand occupation.

Discussion

Conjugation of DC_5MPF with AchE to form DC_5MP-AchE provides a spectroscopic probe for which fluorescence changes at the active center correlate remarkably well with AchE activity toward M7C. The covalent nature of phosphonate conjugates obviates consideration of reversible equilibria of the dansyl ligand as responsible for the fluorescence changes. In the presence of Zn^{2+} ion, DC_5MP-AchE exhibits a fluorescence spectrum in which the emission maximum occurs at shorter wavelengths. Such a hypsochromic shift, which is reminiscent of those observed when the dansyl moiety is transferred to environments of lower dielectric constant, reflects an interaction of the fluorophore with a conformationally altered enzyme rather than a direct interaction between metal ion and fluorophore. The absence of Zn^{2+}-induced spectral changes with pyrenebutyl methylphosphono-AchE (not shown) and the capacity of Zn^{2+} to displace propidium argue against direct metal interaction with the dansyl moiety at the active center.

Spectral changes of DC_5MP-AchE that occur in the presence of peripheral-site ligands provide support for the allosteric model of AchE where binding to AchE at the peripheral site alters active-site conformation. The present results, furthermore, suggest that occupation of the AchE peripheral site can induce more than a single conformation at the active center. Such a conclusion is reasonable when considered with knowledge of the diverse structures of the peripheral-site ligands and their disparate influences on enzyme catalysis (Taylor & Lappi, 1975).

Association and dissociation of propidium with DC_5MP-AchE results in large changes in the dansyl fluorescence as a result of Förster energy transfer. Peripheral-site occupation by other ligands, as measured by displacement of propidium, is seen to result from rapid ligand association which precedes the slower changes in dansyl fluorescence. The contrasting influences of *d*-tubocurarine, gallamine, and Zn^{2+} on the fluorescence of DC_5MP-AchE reflect different active-site conformations induced by the various peripheral-site ligands. Although *d*-tubocurarine and Zn^{2+} induce slow conformational changes in the enzyme, the resulting conformations are spectroscopically distinct (Figure 1 and Table I).

Proposed Scheme for the Conversion of AchE to the

Nonreactive Species. The measurement of active-site conformation by employing the fluorescent phosphonates concomitant with that of peripheral-site occupation as measured through ligand competition with propidium allows for an initial proposal of the mechanism of the induced change in state. Peripheral-site ligand association occurs within the mixing time of reactants as ascertained by displacement of propidium following the addition of metal or *d*-tubocurarine to solutions of DC_5MP-AchE; similarly, sequestration of free metal from the enzyme and reassociation of propidium reveal that ligand dissociation from the peripheral site is rapid. In contrast, the change in fluorescence of the conjugated dansyl moiety that occurs following metal or *d*-tubocurarine association exhibits a slow rate; subsequent reversion to the original fluorescent state upon removal of the ligand is also a slow process (Figure 2B). A minimal mechanism which considers rapid binding and dissociation steps that are linked to slow changes in conformation is the two-state scheme

$$\begin{array}{ccc} E + L & \rightleftharpoons & EL \\ \uparrow\downarrow & & \uparrow\downarrow \\ E' + L & \rightleftharpoons & E'L \end{array}$$

where E and E′ denote the two enzyme states. Pattison & Bernhard (1978), from their studies of enzyme carbamylation, have also suggested that two enzyme states predetermine ligand complexation. The overall kinetics we observe appear consistent with this scheme, where ligand association and dissociation are rapid steps and the EL $\rightleftharpoons$ E′L (at high ligand concentration) and E $\rightleftharpoons$ E′ conversions, consequently, are characterized by the sum of their rate constants, which are 0.022 and 0.014 s^{-1}, respectively. The association and dissociation rate constants for propidium binding to AchE have been demonstrated to be rapid, 1.2×10^9 M^{-1} s^{-1} and 764 s^{-1}, respectively (Bolger & Taylor, 1979). In the two-state scheme, conversion to E′L should be dependent on concentration up to a limiting value whereas reversion to the original state following ligand removal should be independent of L (Janin, 1973).

It is noted that although Zn^{2+} and *d*-tubocurarine induce conversions characterized by similar rate constants, the final states as revealed by the emission spectra of the active-site dansyl phosphonates appear spectroscopically distinct. Yet, the similarity of conversion rates for the two types of ligands suggests common mechanistic features in this isomerization.

In the two-state scheme, we assume that the peripheral-site ligands bind at a single class of sites. Owing to its high affinity, it is possible to demonstrate that propidium binds to a single site on each 80 000-dalton subunit of the tetramer. For the metals and *d*-tubocurarine a similar stoichiometry cannot be inferred from the dissociation experiments, and, in fact, higher concentrations of *d*-tubocurarine and gallamine are required for inducing the slow conversion in state than are necessary to simply dissociate propidium (Taylor & Lappi, 1975). Thus, binding at multiple sites may be required to induce the slow change in conformation. Ligands which induce a conversion to the unreactive enzyme may do so by an initial rapid association of ligand to the peripheral site followed by a slower isomerization of the complex or an association of a second equivalent of metal. However, since metal dissociation is rapid relative to isomerization to the original enzyme state, a slow conversion between E′ and E must remain as an essential component of the mechanistic scheme.

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