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DANSYLARGININE *N*-(3-ETHYL-1,5-PENTANEDIYL)AMIDE

A POTENT AND SELECTIVE FLUORESCENT INHIBITOR OF BUTYRYLCHOLINESTERASE

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Abstract—Interactions between dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide (DAPA) and the cholinesterases were examined by the techniques of enzyme kinetics and fluorescence spectroscopy. When tested with partially purified enzyme preparations, DAPA was a potent inhibitor of butyrylcholinesterase ($IC_{50} = 2 \times 10^{-7}$ M) but not of acetylcholinesterase ($IC_{50} = 4 \times 10^{-4}$ M). For a detailed study of the effects of DAPA on butyrylcholinesterase (BuChE), the enzyme was purified to homogeneity from horse serum, with the aid of affinity chromatography on *N*-methyl acridinium. The kinetics of the inhibition of purified BuChE by DAPA were complex, having both competitive and non-competitive features, and it was not possible to estimate K_i unambiguously. Spectroscopic measurements showed that the fluorescence of the dansyl moiety was strongly affected by the binding to BuChE. With excitation at 330 nm, total fluorescence emission from bound DAPA (at 450 nm and above) was 21-fold greater than from free DAPA. In a titration experiment, this enhancement of fluorescence intensity was used to calculate that each monomer of BuChE has two apparently independent DAPA-binding sites with a K_d of 4.5×10^{-7} M. Further measurements showed that the fluorescence emission of bound DAPA was markedly blue-shifted (to 502 nm from 570 nm in free solution) and that the fluorescence lifetime of this form was greatly prolonged (to 24 nsec from 2.7 nsec). These observations indicate that the high affinity binding sites on BuChE lock DAPA in a highly non-polar environment.

Active-site-directed fluorescent probes have been used to gain information about the physical and chemical nature of the loci at which substrates and inhibitors bind to enzymes [1–3]. This approach has been applied with particular success to the serine proteases and to a related family of enzymes, the cholinesterases [4–6].

Recently a new fluorescent inhibitor of thrombin was described by Okamoto *et al.* [7, 8]. This compound, dansylarginine *N*-(3-ethyl-1,5-pentanediyl)-amide (DAPA)† has also been synthesized by Nesheim *et al.* [9], who used it to study the active site of thrombin. Most serine proteases other than thrombin, including trypsin, reptilase, plasmin and factor X_a, are little affected by DAPA [7–9]. However, since Nagano *et al.* [10] stated that DAPA would inhibit "pseudocholinesterase", we decided to determine whether the fluorescence of the ligand could be used to characterize its interaction with this enzyme. Described below are results indicating that DAPA does inhibit horse serum butyrylcholinesterase (EC 3.1.1.8), in a potent and selective manner, and exhibits major changes in fluorescence properties on binding to the protein.

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† Abbreviations: DAPA, dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide; BuChE, butyrylcholinesterase (EC 3.1.1.8); AChE, acetylcholinesterase (EC 3.1.1.7); SDS, sodium dodecylsulfate; and POPOP, 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene.

MATERIALS AND METHODS

Horse serum (frozen, non-sterile, type 2) was purchased from Pel-Freez Biologicals (Rogers, AR). Bio Gel A 1.5M, acrylamide, bisacrylamide (*N*,*N*′-methylenebisacrylamide), TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine), and ammonium persulfate were from Bio-Rad Laboratories (Richmond, CA). DEAE Sephadex A-25 and CNBr-activated Sepharose 4B were from Pharmacia Fine Chemicals (Piscataway, NJ). BW284C51 [1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide] was purchased from the Burroughs Wellcome Co. (Research Triangle Park, NC). Ethopropazine (10-[2-diethylaminopropyl]phenothiazine) hydrochloride and choline chloride were from the Sigma Chemical Co. (St. Louis, MO), as were partially purified horse serum BuChE and bovine erythrocyte AChE.

Starting materials for the synthesis of the cholinesterase affinity ligand were 9-chloroacridine (Eastman, Rochester, NY) recrystallized from benzene; propanediamine, *N*-benzyloxycarboxyl-ε-amino caproic acid, *N*-hydroxysuccinimide (recrystallized from hot ethylacetate) and dicyclohexylcarbodiimide, all from the Aldrich Chemical Co. (Milwaukee, WI).

DAPA was the gift of Drs. M. E. Nesheim and K. G. Mann of the Hematology Research Section, Mayo Foundation.

BuChE assay

For estimations of the total content and specific

activity of BuChE, the method of Ellman *et al.* [11] was used, with acetylthiocholine replaced by butyrylthiocholine as substrate (final concentration 1 mM). Optical density was determined as a function of time with a Gilford model 250 spectrophotometer in 1 ml, 1 cm light-path cuvettes, at 23°. The reaction was initiated by the addition of 50 μl of substrate to 950 μl of enzyme-preparation buffered to pH 8.0 with 0.1 M sodium phosphate. Thirty seconds were allowed for equilibration of temperature in the sample chamber; the linear increase in absorbance was then measured for at least 60 sec. The dilution of enzyme was chosen to give a reaction rate between 0.02 and 0.1 O.D. per min. Enzyme activity was calculated in units of μmoles of substrate hydrolyzed per min.

For experiments on enzyme-kinetics (carried out at low protein concentration), a sensitive radiometric assay was employed. This assay, based on the organic solvent extraction of [^{14}C]butyrate liberated during hydrolysis of [^{14}C]butyrylcholine at 37° (New England Nuclear Corp., Boston, MA), was performed as previously described [12].

Protein determinations

Protein was measured by the dye-binding assay of Bradford [13], using Bio-Rad Coomassie Brilliant Blue G-250 dye-concentrate, with bovine serum albumin as a reference standard.

Enzyme purification

Stage 1: *Ammonium sulfate fractionation and acid precipitation.* The first stage of the purification followed the procedure of Main *et al.* [14]. Four hundred milliliters of horse serum (Pel-Freez Biologicals) was made 33.6% saturated in ammonium sulfate by the addition of 80 g or 200 g per liter of the solid salt over a 30 min period with constant stirring at room temperature. Stirring was continued for an additional 30 min, and the precipitate was removed by centrifugation at 4200 *g* for 30 min at room temperature. The supernatant fraction (SI) was slowly acidified to pH 3.0 by the addition of 2.5 M H_2SO_4. The precipitate was collected as above and discarded, while the supernatant fraction (SII) was neutralized to pH 7.8 by the addition of 5 M NaOH.

A second fractionation was performed by the addition of 130 *g* ammonium sulfate/l of neutralized SII supernatant fraction (54% of saturation). The precipitate was removed by centrifugation for 40 min and the supernatant fraction (SIII) was retained.

The cholinesterase activity was precipitated by the addition of 69 g/l ammonium sulfate to SIII, bringing the final ammonium sulfate concentration to 64% of saturation. The precipitate was allowed to form for 1 hr and was then centrifuged for 45 min at 5000 *g* at room temperature. The pellet was resuspended in 50 mM sodium phosphate buffer, pH 8.0 (from here on referred to as phosphate buffer). The resuspended pellet was then dialyzed overnight against water and, for a subsequent 24 hr, against phosphate buffer. At the end of stage 1, the specific activity of BuChE had been increased by a factor of 180, and the overall yield was 18% (Table 1).

Stage 2: *Gel filtration.* The sample was applied to a Bio Gel A 1.5 M column (5 × 72 cm) equilibrated with phosphate buffer. The same buffer was used for elution, and 8 ml fractions were collected for assay of BuChE activity and total protein. The fractions containing cholinesterase with maximal specific activity (collected between elution volumes of 500–650 ml and 900–1000 ml) were combined and concentrated to 3 ml using an Amicon XM50 filter. This stage increased the specific activity of BuChE about 7-fold, with an apparent slight increase in total activity, possibly owing to further removal of inhibitory ammonium ions (Table 1).

Stage 3: *Ion exchange chromatography.* The sample from stage 2 was applied to a DEAE Sephadex A-25 ion exchange column (1 g resin) and rinsed with 30 mM choline chloride in phosphate buffer. It was anticipated that all of the BuChE would bind [14]. However, 75% of the applied BuChE activity was found in the column effluent and rinse, while much of the contaminating protein was retained on the column. Therefore, the breakthrough fractions were combined, concentrated to ~ 3 ml on an XM50 membrane, and dialyzed overnight against phos-

Table 1. Summary of purification of horse serum BuChE

Purification step		Vol. (ml)	Units (total)	Protein (mg)	Sp. act. (units/mg)	Yield (%)	Purification
Serum (starting material)		400	1,220	29,050	0.042		
Stage 1	1st $(NH_4)_2SO_4$ (33% supernatant)	400	871	28,100	0.031	71	
	Acid precipitation	340	450	440	1.02	37	24
	2nd $(NH_4)_2SO_4$ (54% supernatant)	360	390	110	3.52	32	84
	3rd $(NH_4)_2SO_4$ (64% precipitate)	7.5	220	28	7.72	18	180
Stage 2	Gel filtration and concentration (Bio Gel A 1.5 M)	3.0	350	6.00	58.1	29	1,380
Stage 3	Ion exchange and concentration (DEAE Sephadex A-25)	2.5	260	0.96	272	21	6,480
Stage 4	Affinity chromatography and concentration (*N*-methyl acridinium)	3.0	260	0.17	1,540	21	36,700

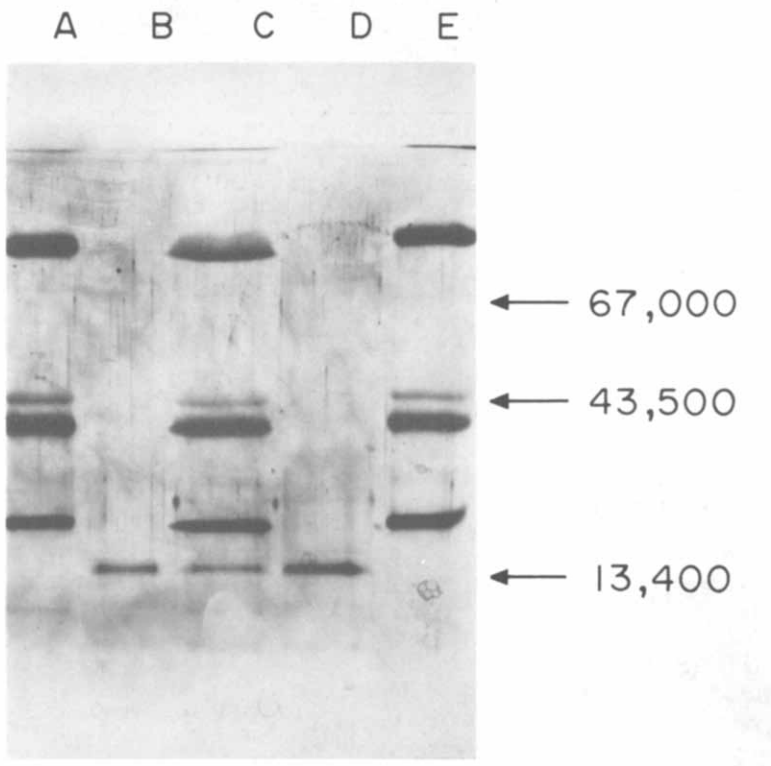

Fig. 1. SDS slab-gel electrophoresis of affinity-purified horse serum BuChE. Acrylamide concentration was 10%. The gels were run for 2.5 hr at 20 mA and were silver-stained by the method of Oakley *et al.* [17]. Key: (A) molecular weight standards (67,000, 43,500 and 13,400 daltons); (B) 1.25 μg of purified BuChE; (C) 0.5 μg of BuChE plus molecular weight standards; (D) 0.625 μg of BuChE; and (E) molecular weight standards.

phate buffer before continuing with the purification procedure.

Stage 4: *Affinity chromatography*. An affinity column (1.5 × 4 cm) was used, consisting of 10-methyl-9[N^{β}(ε-aminocaproyl)β-aminopropylamino]-acridinium-Sepharose 4B, synthesized and prepared according to the method of Webb and Clark [15]. The column was equilibrated with phosphate buffer, and the sample from the preceding stage was applied in a total volume of 3 ml. The column was rinsed with 10 column-volumes of phosphate buffer. None of the applied BuChE activity appeared in the effluent. The column was eluted with a 100 ml linear choline chloride gradient (0–1 M), and 2 ml fractions were collected and assayed for protein and enzyme activity. Fractions with maximal specific BuChE activity (eluting between 0.05 and 0.2 M choline chloride) were pooled, concentrated to ~ 3 ml on an XM50 membrane, and dialyzed overnight against phosphate buffer. The result of stage 4 was a further 5.7-fold purification, at virtually 100% yield, to a final specific activity 36,000 times greater than that of the starting material. The overall yield of the 4 stage purification procedure was 21% (Table 1).

Purity of BuChE

The enzyme obtained from our purification procedure was judged to be free of AChE for two reasons: (1) it was completely inactivated by the specific BuChE-inhibitor, ethopropazine, at a concentration of 10^{-4} M; and (2) it was largely unaffected by the specific AChE-inhibitor, BW284C51, at a concentration of 10^{-6} M.

The specific activity of the pure BuChE appeared to be somewhat greater than that of the electrophoretically homogenous BuChE of Main *et al.* [14]. However, since our protein determinations were calibrated with bovine serum albumin, they provided only a relative estimate of protein content. The true specific activity of our final product could, therefore, have been closer to previously reported values.

Purity and molecular weight of our BuChE preparation were tested by SDS gel electrophoresis [16]. Silver staining of the gels [17] revealed only one major band (Fig. 1). Although only 1.5 μg was loaded, the maximal concentration of impurities could not have been greater than 20% and probably was considerably smaller. The molecular weight estimated for BuChE from its migration on both 7.5% and 10% acrylamide gels [calibrated with standards ranging from 43,500 to 220,000 daltons (data not shown) and from 13,400 to 67,000 daltons respectively] was 85,400.

Fluorescence determinations

Fluorescence measurements were performed in a 0.3 cm^2 quartz cuvette (0.2 ml total volume) in an SLM 4800 subnanosecond fluorometer (SLM Instruments, Champaign Urbana, IL) equipped with EMI 9813QA photomultiplier tubes. The sample chamber

was thermostatically controlled at 25°. To control for variations in light output from the lamp, fluorescence readings were taken as ratios of intensities recorded from the sample and from a reference cuvette containing a solution of POPOP in ethanol. For measurement of fluorescence spectra and intensities, the excitation light at 330 nm was selected by use of a monochromator (2 nm slit width) and a Corning 7-54 filter placed in the excitation path. For emission spectra the excitation beam was vertically polarized and the emission observed through a polarizer oriented to the vertical. The technical spectra were subsequently corrected for wavelength variation in instrument response through use of correction factors generated for this instrument with an NBS standard lamp. For measurement of emission intensities, a Schott KV 450 filter (which passes all emissions at wavelength > 450 nm so that Rayleigh and Raman scattering are eliminated) was employed in lieu of a monochromator. Fluorescence lifetimes were measured by the phase-modulation technique of Spencer and Weber [18]. Modulation frequencies of 6 and 18 MHz were used. Vertically polarized excitation at 330 nm was employed and the emission observed through a Schott KV 418 filter. A polarizer in the emission path was oriented to 54.7° to eliminate the effects of Brownian rotation on the measurement of the fluorescence lifetimes. The phase (τ_ϕ) and modulation (τ_m) data obtained at the two frequencies were subjected to analysis of heterogeneous lifetimes by the method of Weber [19] to extract the lifetimes of the bound and free DAPA.

Least squares non-linear regression analysis

Enzyme-kinetics data were analyzed by the method of Wilkinson [20], using an iterative unweighted least squares approximation to the hyperbolic Michaelis–Menten equation. The calculations were performed on a Hewlett–Packard model 85 microcomputer using a locally written BASIC program (listing available on request) to obtain estimates of apparent K_m and V_{max}, with associated standard errors.

Fluorescence data were interpreted in terms of the binding equations developed below (see Results). These equations were fitted by means of a generalized iterative least squares non-linear regression program adapted for the HP 85 microcomputer by Peck and Barrett [21]. For the calculations, the molar concentration of active enzyme sites was determined by dividing the concentration of enzyme protein (in mg/ml) by the estimated molecular weight of the active BuChE monomer (85,400).

RESULTS

Kinetics of BuChE inhibition

DAPA was first tested in a wide range of concentrations for the ability to inhibit partially purified, commercial preparations of the cholinesterases. With [^{14}C]acetylcholine and [^{14}C]butyrylcholine as substrates (final concentration 1 mM), the IC_{50} concentrations of DAPA were found to be $4 \pm 0.5 \times 10^{-4}$ M against AChE and $2 \pm 0.1 \times 10^{-7}$ M against BuChE. DAPA is thus a highly selective inhibitor of the latter enzyme.

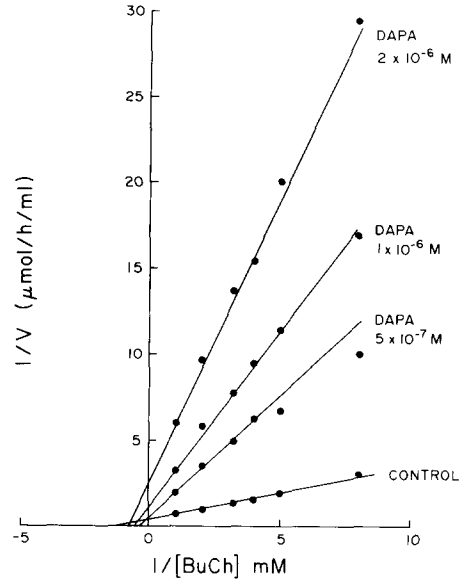

Fig. 2. BuChE activity at various substrate concentrations in the presence or absence of DAPA. Apparent K_m and V_{max} were calculated by direct iterative fit to the Michaelis–Menten hyperbola. The best-fitting lines are shown on a double-reciprocal plot, for the sake of illustration.

For a more comprehensive analysis of the interaction between DAPA and BuChE, the enzyme was isolated from horse serum and purified to virtual homogeneity. The final stage of this purification was aided by affinity chromatography on *N*-methyl acridinium (see Materials and Methods).

In the absence of inhibitor, purified BuChE exhibited essentially "Michaelis–Menten" kinetics

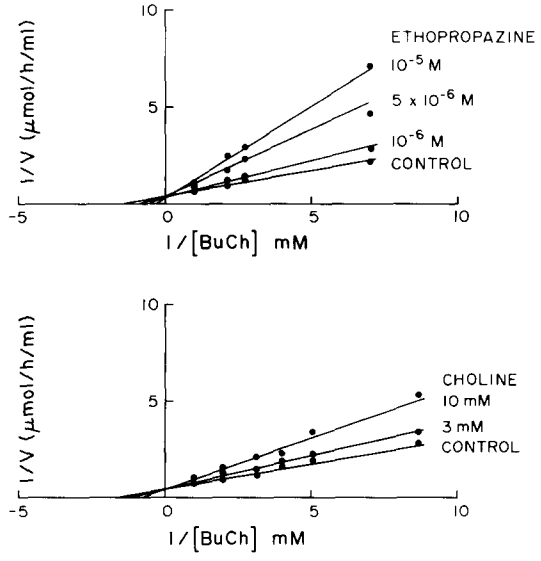

Fig. 3. BuChE activity at various substrate concentrations in the presence or absence of choline chloride or ethopropazine hydrochloride. Best-fitting lines were generated as in the preceding figure.

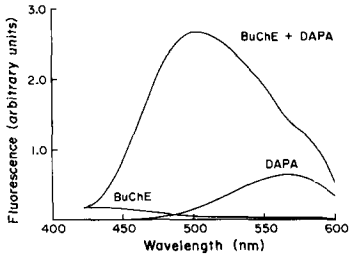

Fig. 4. Effect of binding to BuChE on the fluorescence of DAPA. Corrected emission spectra are shown for purified horse serum BuChE (0.36 μM) and for DAPA (0.5 μM) in the presence and absence of this enzyme. Excitation in all cases was at 330 nm. The spectra are truncated at 600 nm because the correction factors at longer wavelengths were too large to be reliable.

over the range of substrate concentrations from 0.1 to 1 mM (Fig. 2). Higher concentrations led to slight substrate activation (data not shown). The apparent K_m for butyrylcholine determined by direct, iterative, least squares fit to the hyperbola was 0.78 ± 0.06 mM, while the V_{max} was 2.7 ± 0.1 (in arbitrary units).

Further experiments showed that DAPA is a "mixed type" inhibitor of BuChE. A low concentration of DAPA (0.5 μM) more than doubled the apparent K_m (to 2.2 ± 0.6 mM), but also reduced V_{max} by one-third (to 1.7 ± 0.4). Higher concentrations of DAPA had more effect on V_{max}, but, surprisingly, less effect on the K_m. For example, in the presence of DAPA at 2 μM, the V_{max} of BuChE was 0.39 ± 0.04 and the K_m was 1.3 ± 0.19 mM. As a result, the best-fitting lines did not intersect at a common point on a double-reciprocal plot (Fig. 2) or on a Hofstee plot (not shown). This pattern of effects is not interpretable in terms of any simple kinetic model. Nevertheless, it was highly reproducible (obtained in three independent experiments).

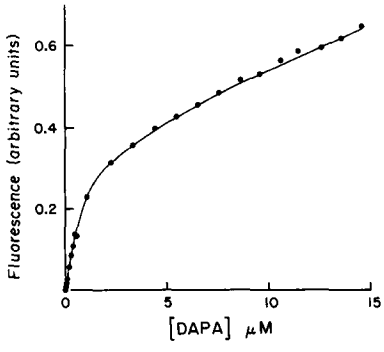

Fig. 5. Titration of the fluorescence of BuChE-DAPA. Total fluorescence emission was recorded, using a KV 450 filter and excitation at 330 nm. The fluorescence of the system containing BuChE (average concentration 0.35 μM) and DAPA (from 0 to 15 μM) is shown, after subtraction of the background fluorescence and scattering from enzyme. The solid line was fitted by iterative non-linear regression techniques, using the binding equation developed in Results.

By contrast, two other agents inhibited the purified horse serum BuChE in a simple competitive manner. Thus, choline (K_i = 9.5 mM) and ethopropazine (K_i = 4.2 μM) were both without effect on V_{max} when added in concentrations that substantially increased the apparent K_m (Fig. 3). These results showed that the complex kinetics of BuChE in the presence of DAPA were not the result of impurities in the enzyme preparation nor of artifacts in the assay for enzyme activity.

Although we could not make an unambiguous estimate of the K_i for DAPA versus BuChE, it seems likely that the binding of this ligand had a dissociation constant of less than 1 μM. To obtain more evidence on this point, we used fluorescence spectroscopy to examine the interaction between enzyme and inhibitor.

Fluorescence spectroscopy

Since DAPA in aqueous solution absorbs light maximally at 330 nm [9], this wavelength was used throughout for excitation. Emission spectra for DAPA in the presence and absence of BuChE are shown in Fig. 4. Addition of the protein caused a marked blue shift in the emission maximum (from 570 to 502 nm) as well as a large increase in total fluorescence intensity. No such effects were seen when an equivalent concentration of bovine serum albumin was substituted for BuChE (data not shown). The small shoulder near 580 nm in the fluorescence spectrum of DAPA + BuChE was not apparent in the technical (uncorrected) spectrum. It presumably represents a contribution from free DAPA which would otherwise have gone unnoticed because of the relatively poor sensitivity of the EMI phototube at this wavelength.

A determination of fluorescence lifetime was made with 0.7 μM DAPA in the presence of 0.22 μM BuChE. It was found that τ_ϕ was 12.1 ± 14 nsec, while τ_m was 20.7 ± 0.1 nsec. Calculations showed that 85% of the fluorescence was attributable to a species with a lifetime of 24 nsec and 15% to a species with a lifetime of 2.7 nsec. The latter value is close to what would be expected for this fluorophore in aqueous solution [9]. Thus, it appears that the fluorescence lifetime of DAPA is increased about 9-fold by binding to BuChE.

We did not have enough purified BuChE to provide a saturating concentration of DAPA-binding sites and, therefore, could not directly estimate the relative fluorescence intensity of bound and free DAPA. An indirect estimate of this parameter and of the dissociation constant of the DAPA-BuChE complex was made by titrating increasing concentrations of DAPA against a single, subsaturating concentration of enzyme (0.36 μM).

The fluorescence emission, measured with a 450 nm high-pass filter, was taken to represent variable contributions from free and bound DAPA. A standard curve for DAPA in buffer with no added protein indicated that the fluorescence intensity of the free ligand was 2.2 ± 0.1 F.U. (arbitrary units) per μM. This component can be recognized as the limiting linear slope in Fig. 5, which shows the total fluorescence of the DAPA–BuChE mixture, as a function of DAPA concentration.

Calculation of binding parameters

The fluorescence of bound DAPA (F_b) may be estimated by subtracting the linear component from the raw values for total fluorescence. The data may then be analyzed in terms of the following equations:

$$K_d = \frac{[D]\cdot[P]}{[DP]} = \frac{[D]\cdot[1-y]}{y} \quad (1)$$

where $[D]$ and $[P]$ are the concentrations of free DAPA and enzyme (monomer), $[DP]$ is the concentration of bound DAPA, and y is the fractional occupancy of binding sites.

$$[D] = [D_0] - y\cdot n\cdot[P_0] \quad (2)$$

where $[D_0]$ and $[P_0]$ are the concentrations of total drug and enzyme, and n is the number of binding sites per enzyme-monomer. Therefore,

$$K_d = ([D_0] - y\cdot n\cdot[P_0])\cdot(1-y)/y \quad (3)$$

In this equation, y can be replaced by F_b/F_{max}, which is the fluorescence of DAPA actually bound, as a proportion of the maximal fluorescence from bound DAPA when all binding sites are occupied:

$$K_d = ([D_0] - (F_b/F_{max})\cdot n\cdot[P_0])\cdot(F_{max} - F_b)/F_b \quad (4)$$

Equation 4 can be solved graphically after a linearizing transformation. However, it is preferable to use iterative non-linear regression techniques to obtain the unweighted, least squares fit of the dependent variable, F_b, to the independent variable, $[D_0]$:

$$F_b = F_{max}\cdot\frac{(K_d + [D_0] + n\cdot[P_0]) - \sqrt{(K_d + [D_0] + n[P_0])^2 - 4n\cdot[P_0][D_0]}}{2n\cdot[P_0]} \quad (5)$$

The computer-generated curve fitting equation 5 to the measurements of DAPA fluorescence is shown in Fig. 5. The excellence of the fit is apparent ($r^2 = 0.9994$). According to this analysis, the best estimate of K_d is 0.45 ± 0.07 μM, the best estimate of n is 2.0 ± 0.3 sites/monomer, and the best estimate of maximal fluorescence is 3.32 ± 0.03 F.U. (in the same arbitrary units used to express the fluorescence of DAPA in free solution). The only one of these parameters that is sensitive to error in the estimate of enzyme concentration is n, which varies in a reciprocal manner (i.e. a 25% overestimate of BuChE concentration yields a 20% underestimate of n). Assuming that the concentration of BuChE monomer was actually 0.36 μM, we may conclude that the maximum concentration of BuChE–DAPA complex would have been 0.72 μM. The fluorescence intensity of bound DAPA in micromolar concentration would, therefore, have been $3.32/0.72 = 4.67$ F.U., or 21-fold greater than that of free DAPA.

Using these figures, one can calculate that the fractional occupancy of binding sites at a DAPA concentration of 0.7 μM and a BuChE concentration of 0.23 μM (the conditions under which fluorescence lifetime was measured) would be 0.49. Assuming a 21-fold enhancement of fluorescence intensity on binding, one predicts that 90% of the flurescence would be from bound DAPA and 10% from free DAPA. This prediction is in reasonable agreement with the results of the fluorescence-lifetime experiment.

In a final experiment, undertaken to examine the relation between the DAPA-binding site(s) and the active site of BuChE, we tested the ability of choline to displace bound DAPA. The concentrations of BuChE and DAPA were held constant (0.44 and 0.5 μM, respectively), while that of choline chloride was varied from 30 to 700 mM (i.e. from 3 to 70 times its K_i). A concentration-dependent reduction of DAPA fluorescence was observed (Fig. 6). However, it proved impossible to fit these results by an equation based on the calculated K_d and n for DAPA binding, unless the K_d for choline was taken to be on the order of 120 mM. Even then, the computer-generated curve failed to follow the sigmoidal shape of the fluorescence data (Fig. 6). It was concluded that DAPA was not binding solely to the anionic site normally occupied by choline.

DISCUSSION

Purification of horse serum BuChE in good yield from a small amount of starting material was aided by affinity chromatography on *N*-methyl acridinium. Although this ligand was developed for the isolation of AChE [15], it is also known to interact strongly with serum cholinesterase ($K_i = 5.3 \times 10^{-8}$ M) [5]. The availability of purified enzyme was essential to the present study, for the kinetic constants determined with crude enzyme were unreliable, and the fluorescence of DAPA was obscured by large emissions from contaminating substances.

Our results show that DAPA is a potent and selective inhibitor of horse serum BuChE, with a far greater affinity for this enzyme than for AChE. This degree of specificity is not surprising since DAPA readily distinguishes thrombin from the closely related serine proteases involved in blood clotting [9].

The complex kinetics of BuChE in the presence of DAPA are not unexpected since there are multiple ways in which such an inhibitor might act. For example, the guanidino group of DAPA might occupy the anionic site that is thought to be responsible for the initial binding of substrate [22, 23]. This would be manifested as a competitive component of inhibition. In addition, like many other anticholinesterases, DAPA might bind to the same anionic site on the acylated intermediate of BuChE (i.e. after hydrolysis and liberation of the choline moiety, but before regeneration of the serine hydroxyl at the esteratic site). If such binding affected the rate of regeneration, as is likely [22], inhibition of BuChE by DAPA would also display a non-competitive component. A final level of complexity is offered by the possibility of binding to allosteric sites [24]. At pres-

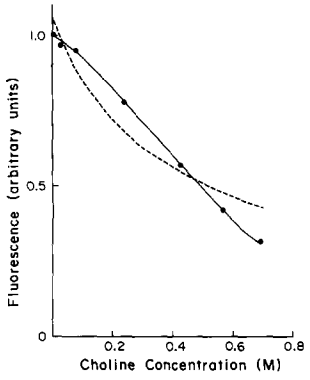

Fig. 6. Competition of choline for DAPA-binding sites on BuChE. The concentrations of enzyme and DAPA were held at 0.44 and 0.5 μM throughout. That of choline was raised in a stepwise fashion by replacing increasingly large aliquots of the mixture with a solution containing enzyme and fluorophore (0.44 and 0.5 μM) plus choline chloride (2 M). Total fluorescence was recorded using a KV 450 filter and excitation at 330 nm. The solid line was fitted by eye to the data points. The dashed line represents the best iterative fit to the binding equation, assuming two DAPA-binding sites with a K_d of 4.5×10^{-7} M for each enzyme active site.

ent, the observed inhibitory effects of DAPA on BuChE defy explicit quantitative analysis. However, it seems likely that DAPA binds in part at the anionic site or in its immediate vicinity, thereby preventing the binding of substrate, and retarding the deacylation of the esteratic site.

Our fluorescence-titration data indicate that the DAPA–BuChE complex has a dissociation constant of about 4.5×10^{-7} M. This binding is slightly firmer than that manifested by another fluorescent active-site-directed probe of BuChE, 1-(5-dimethylaminonaphthalene-1-sulfamide)-3-*N*,*N*-dimethylaminopropane [5] and is only one order of magnitude weaker than the binding of DAPA to thrombin [9]. It is interesting to note that there may be two DAPA-binding sites per active site of BuChE. If a locus other than the anionic site were able to affect the catalytic function of this enzyme, its occupation by DAPA could help account for the complex kinetics of inhibition. However, estimates of *n* are notoriously unreliable, and the existence of a second site for DAPA binding must be confirmed by independent experiments.

The alterations in fluorescence properties which occurred when DAPA bound to BuChE were uniformly greater than those accompanying binding to thrombin [9]. Thus, the increase in fluorescence intensity was 21-fold (vs 3-fold), the bathochromic or "blue" shift was 68 nm (vs 23 nm), and the increase in fluorescence lifetime was up to 9-fold (vs 2.2-fold). The marked blue shift, the enhanced fluorescence intensity, and the increased fluorescence lifetime all suggest that bound DAPA is largely protected from dynamic quenching effects of components in the surrounding solution, primarily of solvent. In these respects the DAPA-binding site on BuChE differs from that on thrombin only by being even more isolated from solvent and from polar side chains. The heterogeneity in the fluorescence lifetime could be due to differences in the micro-environments of the two (putative) binding sites for DAPA. Alternatively, if the two binding sites are essentially equivalent, then the heterogeneity in τ is simply to the presence of bound and free species of the probe. The lifetime of the free probe is attenuated because of the polarity of the solvent and probably also because of intramolecular quenching by the guanidino group of the arginyl residue.

Additional studies on the interaction between DAPA and BuChE are warranted. Such investigation may shed further light on the nature of the anionic site and may also provide a basis for evaluating the possibility that cholinesterases possess allosteric sites.

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