Chemical Modification of Acetylcholinesterase from Eel and Basal Ganglia: Effect on the Acetylcholinesterase and Aryl Acylamidase Activities†

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ABSTRACT: The effect of chemical modification on the acetylcholinesterase and the aryl acylamidase activities of purified acetylcholinesterase from electric eel and basal ganglia was investigated in the presence and absence of acetylcholine, the substrate of acetylcholinesterase, and 1,5-bis[4-(allyldimethylammonium)phenyl]pentan-3-one dibromide (BW284C51), a reversible competitive inhibitor of acetylcholinesterase. Trinitrobenzenesulfonic acid, pyridoxal phosphate, acetic anhydride, diethyl pyrocarbonate, and 2-hydroxy-5-nitrobenzyl bromide under specified conditions inactivated both acetylcholinesterase and aryl acylamidase in the absence of acetylcholine and BW284C51. Chemical modifications in the presence of acetylcholine and BW284C51 by all the above except diethyl pyrocarbonate selectively prevented the loss of acetylcholinesterase but not aryl acylamidase activity; modification by diethyl pyrocarbonate in the presence of acetylcholine and BW284C51 prevented the loss of both acetylcholinesterase and aryl acylamidase activities. Treatment with *N*-acetylimidazole resulted in the inactivation of acetylcholinesterase and the activation of aryl acylamidase. These changes in both the activities could be prevented by acetylcholine and BW284C51. Modification by phenylglyoxal, 2,4-pentanedione, or *N*-ethylmaleimide did not affect the enzyme activities. Indophenylacetate hydrolase activity followed a pattern similar to that of acetylcholinesterase in all the above modification studies. The results suggested essential lysine, tyrosine, tryptophan, and histidine residues for the active center of acetylcholinesterase and essential lysine, histidine, and tryptophan residues for the active center of aryl acylamidase. On the basis of the acetylcholine and BW284C51 protection studies, the active centers appeared to be situated at different loci with histidine and tyrosine as common residues.

Although acetylcholinesterase (AChE)[1] has been known for its esterase activity on acetylcholine (ACh) for a number of years, it is only recently that an acylamidase (Moore & Hess, 1975) or aryl acylamidase activity associated with it has been recognized (Fujimoto, 1976; Oommen & Balasubramanian, 1977; Naveh et al., 1981). This aryl acylamidase (AAA) activity results in the cleavage of the substrate *o*-nitroacetanilide to *o*-nitroaniline and acetate. The AChE and AAA activities from different sources such as basal ganglia, electric eel, erythrocyte membrane, and platelets have been identified with the same protein on the basis of their identical behavior on different affinity chromatographic purification procedures, on gel electrophoresis, on gel filtration, and in response to inhibitors and lipid requirements (George & Balasubramanian, 1980; Majumdar et al., 1982; Majumdar & Balasubramanian, 1982). An interesting feature of the AAA associated with AChE is its sensitivity to inhibition specifically by serotonin (Fujimoto, 1974, 1976; Oommen & Balasubramanian, 1977).

The bifunctional nature of AChE is analogous to the esterolytic and amidolytic activities exhibited by several known enzymes such as chymotrypsin, trypsin, carboxypeptidase A, elastase, and thrombin. In many of these enzymes, evidence is available for nonidentical active centers[2] for the esterolytic and amidolytic activities (Albizati & Hedrick, 1972; Simpson et al., 1963; Colletti-Previero et al., 1969).

We have undertaken a study of amino acid modification of AChE from electric eel and sheep basal ganglia for identifying the active centers of AChE and AAA. The involvement of serine, tryptophan, tyrosine, and histidine residues in either the esteratic site or the peripheral anionic site of AChE has been indicated by earlier workers [see review of Rosenberry (1975) and Blumberg & Silman (1978)]. The present work suggests in addition an essential lysine residue or residues in AChE. Furthermore, evidence for nonidentical but overlapping active centers for AChE and AAA is presented.

Materials and Methods

Proteases, 2,4,6-trinitrobenzenesulfonic acid (TNBS), *N*-acetylimidazole, diethyl pyrocarbonate (DPC), 2-hydroxy-5-nitrobenzyl bromide (ONBB), *N*-bromosuccinimide, phenylglyoxal, eserine sulfate, pyridoxal phosphate, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 1,5-bis[4-(allyldimethylammonium)phenyl]pentan-3-one dibromide (BW284C51), various amines, and choline derivatives were from Sigma Chemical Co. Indophenyl acetate (Sigma) was a kind gift from Dr. O. Lockridge, University of Michigan, Ann Arbor, MI. All other chemicals were either prepared or obtained as described earlier (Oommen & Balasubramanian, 1977; George & Balasubramanian, 1980).

Enzymes. (*m*-Carboxyphenyl)dimethylethylammonium chloride hydrochloride, a specific affinity ligand for AChE (Massoulié & Bon, 1976) attached through a six-carbon spacer arm to Sepharose 4B, was used for the purification of AChE. Highly purified AChE from electric eel (type VI S, Sigma Chemical Co.) and AChE of sheep basal ganglia were subjected to affinity chromatography as described earlier (George & Balasubramanian, 1980). The purified basal ganglia enzyme (2000–4000 units/mg of protein; 1 unit is defined as 1

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[1] Abbreviations: AChE, acetylcholinesterase; AAA, aryl acylamidase; TNBS, 2,4,6-trinitrobenzenesulfonic acid; DPC, diethyl pyrocarbonate; ONBB, 2-hydroxy-5-nitrobenzyl bromide; BW284C51, 1,5-bis[4-(allyldimethylammonium)phenyl]pentan-3-one dibromide; ACh, acetylcholine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); IPAase, indophenyl-acetate hydrolase; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

[2] The designation of active center refers to all those features of primary, secondary, and tertiary structure of the enzyme that are required for substrate binding, specificity, and hydrolysis of the substrate (Bethune et al., 1964).

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Table I: Experimental Conditions for Chemical Modification of AChE and AAA

protein-modifying reagent used	conditions of reaction maintained	ref
trinitrobenzene-sulfonic acid	the reaction mixture in 20 mM potassium phosphate buffer, pH 8, contained the enzyme and 1–6 mM trinitrobenzenesulfonic acid; the incubation was carried out at 37 °C for various time periods ranging from 5 to 60 min	Fields (1972)
pyridoxal phosphate	the reaction mixture in 20 mM potassium phosphate buffer, pH 6.2, containing the enzyme and 1–3 mM pyridoxal phosphate was incubated in the dark for 30 min at 37 °C; the mixture was then further incubated for 10 min in the presence of 30 mM sodium borohydride	Phillips et al. (1983)
N-acetylimidazole	the reaction mixture in 20 mM potassium phosphate buffer, pH 7.5, containing the enzyme and 1–3 mM *N*-acetylimidazole was incubated at 37 °C for 15 min	Riordan et al. (1965); Roskoski (1974)
diethyl pyrocarbonate	diethyl pyrocarbonate (1–3 mM) diluted in ethanol at 0 °C was incubated with the enzyme in 20 mM potassium phosphate buffer, pH 7, at 0–4 °C for 15 min [ethanol in the reaction mixture was kept 5% (v/v), which has no effect on enzyme activity]	Burstein et al. (1974)
2-hydroxy-5-nitrobenzyl bromide and *N*-bromosuccinimide	the reaction mixture in 20 mM potassium phosphate buffer, pH 5.9, contained the enzyme and 0.2–0.6 mM 2-hydroxy-5-nitrobenzyl bromide; incubation was at 0–4 °C for 2 min; for *N*-bromosuccinimide (0.2–0.4 μM), the pH of the reaction mixture was 5.9, and the reaction was carried out at 37 °C for 20 min	Barman & Koshland (1967); Horton & Koshland (1972); O'Brien & Test (1978)
phenylglyoxal and 2,4-pentanedione	the reaction mixture in 20 mM Hepes/NaOH, pH 7.6, containing the enzyme and 3–15 mM either phenylglyoxal or 2,4-pentanedione was incubated for 30 min at 37 °C	Chang & Huang (1981)
N-ethylmaleimide	the reaction mixture in 20 mM potassium phosphate buffer, pH 6.8, containing the enzyme and 3–15 mM *N*-ethylmaleimide was incubated at 37 °C for 30 min	Riordan & Vallee (1972a)

μmol of thiocholine formed per min) showed two minor protein bands in addition to the major bands corresponding to enzyme activities while the purified eel AChE (4000–6000 units/mg of protein) showed a single protein band corresponding to enzyme activity on analytical polyacrylamide gel electrophoresis (Davis, 1964) as described earlier (George & Balasubramanian, 1980).

Enzyme Assay and Protein Estimation. AChE was assayed with acetylthiocholine as substate according to Ellman et al. (1961) and AAA with *o*-nitroacetanilide as substrate according to the modified method of Hoagland & Graf (1971) as described previously (Oommen & Balasubramanian, 1979). The assay mixture (0.5 mL) for indophenyl acetate hydrolysis consisted of 100 mM potassium phosphate buffer, pH 8.0, 0.6 mM indophenyl acetate, and the enzyme. The absorbance of the indophenol liberated was measured at 625 nm (Kramer & Gamson, 1958). All the above assays were done at pH 8.0 except when the *N*-acetylimidazole-modified enzyme was used, which was assayed at pH 7.0 to avoid reversal of the modification at pH 8.0 (Riordan & Vallee, 1972b).

Protein was estimated according to Lowry et al. (1951) with crystalline bovine serum albumin as the standard or by absorption at 280 nm. All the spectrophotometric measurements were made in a Carl Zeiss PMQ II spectrophotometer.

Immunological Studies. Antiserum against electric eel AChE was raised by intradermal injection into a young rabbit of 43 μg of the purified eel AChE in 0.3 mL of 50 mM potassium phosphate buffer, pH 7.2/0.15 M NaCl mixed with 0.3 mL of Freund's complete adjuvant. A booster dose of 40 μg was injected on the 21st day. A week later, the rabbit was bled through the marginal vein of the ear and the serum collected.

Immunoprecipitation was done in 300 μL of a mixture containing the purified eel AChE (48 units in 150 μL of 50 mM potassium phosphate buffer, pH 7.2/0.15 M NaCl) and the antiserum. After being kept at 4 °C for 6 h, 3% poly(ethylene glycol) 6000 was added and this kept for a further 3 h at 4 °C, the mixture was centrifuged at 12000*g* for 30 min, and the supernatants and precipitates were assayed for AChE and AAA. Control mixture contained all the ingredients except the antiserum or contained the preimmune serum. The antiserum at the highest concentration used in immunoprecipitation studies had only less than 0.6% AChE or less than 3% AAA activity of the eel enzyme used.

Proteolytic Treatment. Eel enzyme (40 units of AChE in 200 μL) in 20 mM Tris-HCl buffer, pH 7.5, was incubated with trypsin (7–10 μg), chymotrypsin (10–12 μg), subtilisin (Carlsberg) (5 μg), or Pronase P (10 μg) for 10–30 min at 37 °C in different sets of experiments. The protease-treated enzyme samples were subjected to gel filtration on Sepharose 6B or polyacrylamide gel electrophoresis.

Chemical Modification. In general, chemical modification of the enzyme was performed by the addition of the protein-modifying reagent to the purified eel (15–30 units) or basal ganglia (15–30 units) AChE in 100–300 μL of reaction mixture at a given pH and incubation for a specified time at the indicated temperature (Table I). The mixture was diluted 10-fold with 20 mM potassium phosphate buffer, pH 7.0, to stop the reaction, and the excess reagent was removed by dialysis against 1000 volumes of the same buffer for 18 h with three changes. Following this, the enzyme activities were determined. Every experiment had a control that was subjected to the same treatment without the modifying reagents.

Protection Studies. Protection experiments were performed by preincubating the enzymes for 2 min with 15 mM ACh (substrate of AChE) or 20 μM BW284C51, a selective competitive reversible inhibitor of AChE (Austin & Berry, 1953; Rotundo & Fambrough, 1979; Vigny et al., 1978), followed by the addition of the protein-modifying reagent. The enzyme activities were determined after dilution and dialysis of the reaction mixture as described above.

Spectral Studies. The extent of trinitrophenylation (Okuyama & Satake, 1960) following the treatment with TNBS was determined by the method of Goldfarb (1966) with slight modification. The assay mixture after incubation was diluted to 0.9 mL by 20 mM potassium phosphate buffer, pH 8, 3 mM Na_2SO_3 was added to develop the color at 50 or 37 °C for 15 min, and the mixture was measured at different wavelengths ranging from 320 to 460 nm. O-Acetylation and carbethoxylation of the enzymes by *N*-acetylimidazole (Riordan et

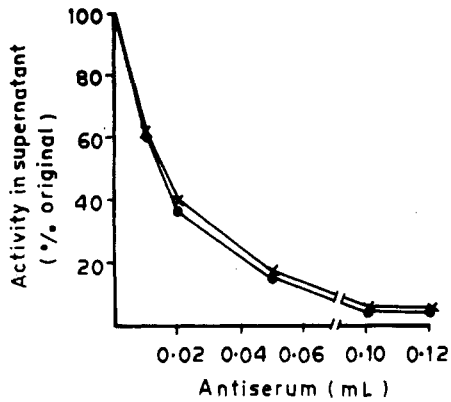

FIGURE 1: Immunoprecipitation of eel AChE (●) and AAA (×) by antiserum. Details of the methods are given in the text. The activities disappearing from the supernatant were recovered in the immunoprecipitates. A preimmune rabbit serum (0.15 mL) precipitated only <4% of AChE and AAA activities (not shown).

al., 1965) and DPC (Daron & Aull, 1982), respectively, were determined by UV absorption at 278 (for tyrosine) and 242 nm (for histidine).

Reversibility of Inactivated Enzyme. In certain experiments, the modified inactivated enzyme along with the native enzyme were incubated in the presence of 50 mM hydroxylamine, pH 7.5, for 40–75 min at 37 °C (Rice & Etzler, 1975). The mixture after dialysis was assayed for enzymatic activities.

Results

Identity of the Serotonin-Sensitive AAA with AChE. In earlier studies several lines of evidence have been provided for the identity of AChE and AAA in eel and basal ganglia (Fujimoto, 1976; George & Balasubramanian, 1980). We have also demonstrated a similar identity of AAA and pseudocholinesterase in human serum (George & Balasubramanian, 1981). As an additional criterion for the identity of the two activities with the same protein, immunoprecipitation of the eel enzyme was done (Figure 1). As seen from the figure, there was a coprecipitation of both AChE and AAA activities at different concentrations of antiserum used.

Treatment with Proteases. AAA and AChE activities of both electric eel and basal ganglia enzymes were lost at an identical rate when incubated with different amounts of each of the proteases trypsin, chymotrypsin, subtilisin, and Pronase P for different periods. The enzymes after limited proteolysis with each of the above proteases (which resulted in about 40–80% loss in activity) when subjected to either gel filtration or gel electrophoresis did not show a separation of the AAA and AChE activities (data not shown). These results suggested that the active centers of both these enzyme activities must be similar or close to each other if not identical.

Modification by TNBS. Treatment with TNBS, which reacts with amino groups, resulted in an identical irreversible inactivation up to 85–90% of both AAA and AChE activities from electric eel (lower inset of Figure 2). The time-dependent trinitrophenylation of lysine residues in the eel enzyme monitored at various wavelengths showed a peak at 345 nm and another peak at 420 nm, both of which increased with time (Figure 2) at 50 or 37 °C (upper inset of Figure 2). The increase in absorption at 420 nm due to trinitrophenylation was about 3 times higher at 50 °C as compared to 37 °C. For the basal ganglia enzyme also, a similar loss in AChE and AAA activities upon TNBS treatment and an increase in absorption at 420 nm with time due to trinitrophenylation were observed (data not shown). The inability of *N*-ethylmaleimide to inactivate AChE and AAA (see below) indicated that

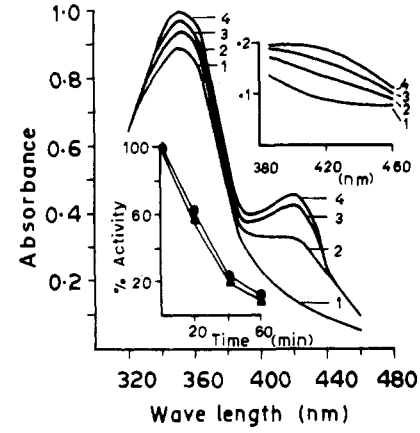

FIGURE 2: Time-dependent trinitrophenylation leading to the inactivation of eel AChE and AAA. Eel enzyme (40 units, AChE) samples in 0.3 mL of 20 mM potassium phosphate buffer, pH 8, were treated with 5.21 mM TNBS at 37 °C. Lower inset shows the loss of activity of AChE (●) and AAA (▲) at the indicated time interval. Curves in the main figure show the progression of the changes in absorbance (developed at 50 °C) in the region 320–460 nm of the trinitrophenylated enzyme determined as described under Materials and Methods. Upper inset shows changes in the absorbance developed at 37 °C. Enzyme incubated (1) 0, (2) 20, (3) 40, and (4) 60 min with TNBS.

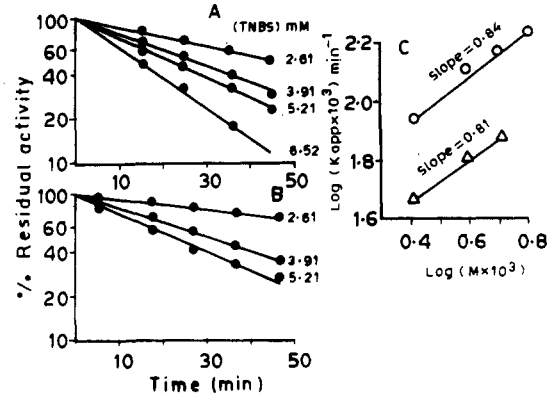

FIGURE 3: Semilogarithmic plot of time-dependent inactivation of eel AAA (A) and AChE (B) by TNBS. Enzyme (40 units, AChE) was incubated with different concentrations of TNBS in 20 mM potassium phosphate buffer, pH 8, for the indicated periods. Panel C shows the data plotted as log k_{app} of enzyme inactivation against log of TNBS concentrations for (○) AChE and (△) AAA.

TNBS inactivation does not involve cysteine SH groups (Fields, 1972). These observations suggested that lysine residues were essential for both the AChE and AAA activities.

Since the involvement of lysine residues in the active center of AChE has not been reported so far, it was studied in greater detail. Semilogarithmic plots of residual activity vs. time (using different concentrations of TNBS) were linear, indicating that the inactivation reaction followed pseudo-first-order kinetics for both AChE and AAA from eel and basal ganglia (Figures 3A,B and 4A,B). A plot of apparent first-order rate constant k_{app} (Levy et al., 1963) determined from the initial slope of the lines from Figures 3 and 4 vs. the corresponding concentrations of TNBS also gave a straight line that went through the origin (figure not shown), indicating that no reversible enzyme–reagent complex was formed prior to the inactivation (Kitz & Wilson, 1962). The reaction order with respect to TNBS concentration for the inactivation of AChE and AAA was determined from

$$k_{app} = k_2[M]^n$$

where n is the reaction order of TNBS (M) reacting with the enzyme residues to yield an inactive complex and k_2 is the

Table II: Effect of Protein-Modifying Agents in the Absence and Presence of ACh or BW284C51 on AChE, AAA, and IPAase Activities

protein-modifying agent	enzyme activity determined	% of original activity (100%) at different concentrations of protein modifying agent (mM)												
		electric eel							basal ganglia					
		0.2	0.4	0.6	1	2	3	5	0.2	0.4	0.6	1	2	3	5
TNBS	AChE				45		35	25				42		36	22
	IPAase				40		32	24				40		26	17
	AAA				35		24	18				48		37	19
TNBS + ACh	AChE				85		80	77				95		90	85
	IPAase				80		70	60				80		78	56
	AAA				40		25	17				45		36	21
TNBS + BW284C51	AChE				82		78	68				85		80	75
	IPAase				80		70	63				80		78	55
	AAA				42		26	20				50		42	28
pyridoxal phosphate	AChE				65	52	38					62	45	29	
	IPAase				63	50	33					60	50	33	
	AAA				65	54	39					59	42	30	
pyridoxal phosphate + ACh	AChE				85	80	76					90	87	75	
	IPAase				80	78	70					90	84	70	
	AAA				70	60	42					62	47	35	
pyridoxal phosphate + BW284C51	AChE				85	85	75					85	80	65	
	IPAase				80	80	60					85	84	70	
	AAA				70	55	42					60	50	32	
N-acetylimidazole	AChE				50	25	15					55	40	20	
	IPAase				52	27	20					54	39	20	
	AAA				300	329	341					227	281	318	
N-acetylimidazole + ACh	AChE				98	90	86					90	90	85	
	IPAase				82	73	70					90	90	85	
	AAA				100	98	98					100	100	100	
N-acetylimidazole + BW284C51	AChE				100	95	90					100	100	100	
	IPAase				82	73	70					90	90	85	
	AAA				100	98	95					105	100	100	
DPC	AChE				40	22	11					35	20	12	
	IPAase				50	28	12					40	25	13	
	AAA				50	30	18					35	22	10	
DPC + ACh	AChE				98	95	90					92	89	80	
	IPAase				80	75	68					75	69	65	
	AAA				90	82	70					90	80	75	
DPC + BW284C51	AChE				85	75	75					98	95	90	
	IPAase				75	70	67					76	70	65	
	AAA				84	80	72					90	90	75	
ONBB	AChE	55	42	30					75	54	40				
	IPAase	55	45	30					70	50	30				
	AAA	54	40	28					70	53	40				
ONBB + ACh	AChE	80	75	55					90	80	70				
	IPAase	80	75	65					90	75	65				
	AAA	55	40	30					70	55	42				
ONBB + BW284C51	AChE	80	78	60					90	80	75				
	IPAase	85	75	62					90	85	70				
	AAA	54	39	32					70	52	46				

second-order rate constant (Levy et al., 1963; Ramakrishna & Benjamin, 1981). Taking the logarithm of both sides and plotting log k_{app} against log [M] yields a line with a slope equal to n. These plots (Figures 3C and 4C) gave a value of approximately 1.0 for the reaction order n for both AChE and AAA, suggesting that at least one molecule of TNBS was required for inactivation of one active site of the enzyme.

Inactivation by TNBS and Pyridoxal Phosphate in the Presence of BW284C51 and ACh. The progressive inactivation of AChE, IPAase, and AAA with increasing concentrations of two lysine-modifying agents, viz., TNBS and pyridoxal phosphate, is shown in Table II. BW284C51 is a competitive reversible inhibitor of AChE and is known to inhibit both AChE and AAA to almost 98–100% at 10 μM (George & Balasubramanian, 1980). When 20 μM BW284C51 or 15 mM ACh (substrate of AChE) was included in the reaction mixture during treatment with TNBS or pyridoxal phosphate, the rate of inactivation of AChE and IPAase from both eel and basal ganglia was retarded without affecting the AAA inactivation (Table II). The results suggested that TNBS and pyridoxal phosphate acted on the lysine residue(s) at the active center of AChE and that a lysine residue(s) that could not be protected by BW284C51 or ACh was (were) responsible for inactivation of AAA. Acetic anhydride, another reagent for

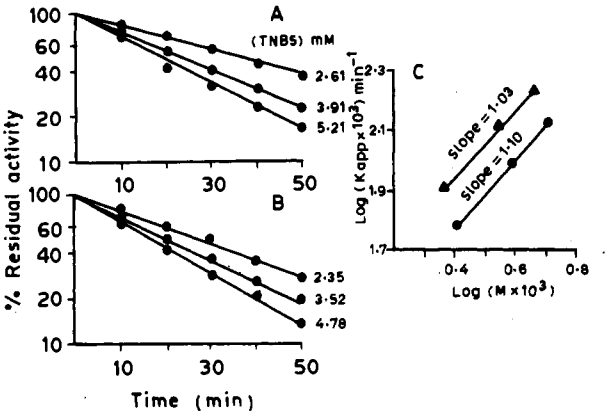

FIGURE 4: Semilogarithmic plot of time-dependent inactivation of basal ganglia AChE (A) and AAA (B). Details of experiments are as given for the eel enzyme under Figure 3. Panel C shows the data plotted as log k_{app} of enzyme inactivation against log of TNBS concentration for (●) AChE and (▲) AAA.

lysine NH_2 modification (Fraenkel-Conrat, 1957), also showed a similar inactivation pattern and protection by BW284C51 and ACh as above (data not shown).

Effect of N-Acetylimidazole. *N*-Acetylimidazole, which specifically modifies tyrosine, when used at increasing concentrations showed an inactivation of AChE and IPAase from both eel and basal ganglia to about 80% at 3 mM. However, there was a pronounced increase in AAA activity up to >300% of the original activity (Table II). BW284C51 and ACh could protect AChE and IPAase from loss of activity and AAA from gain in activity (Table II). Approximately 80% of the original AChE activity from 20% was restored to the inactivated enzyme from both eel and basal ganglia by 50 mM hydroxylamine at pH 7.5. Similarly, AAA activity was also reduced to 120% from >300% at the same concentration of hydroxylamine. Tyrosine acetylation by *N*-acetylimidazole was also supported by the significant fall in the UV absorption of the enzyme at 278 nm with progressive acetylation without appreciable change in absorption at 242 nm, characteristic for histidine (Riordan & Vallee, 1972b) (data not shown).

Treatment with DPC. DPC could inactivate AChE, IPAase, and AAA from eel and basal ganglia more than 80% at 3 mM (Table II). All three activities showed a similar inactivation pattern and were protected from inactivation when BW284C51 or ACh was included during the action of DPC (Table II). The UV absorption at 242 nm characteristic of *N*-carbethoxyhistidine was increased as the amino acid was modified, and there was no appreciable change at 278 nm, indicating that DPC did not react with tyrosine residues (data not shown; Muhlard et al., 1967). Hydroxylamine at 50 mM, pH 7.5, restored approximately 60% of the initial AAA and AChE activity that had been inactivated by 3 mM DPC. DPC has been shown to react with not only histidine but also lysine, tyrosine, and serine moieties (Melchior & Fahrney, 1970). The lysine and tyrosine modification by DPC has been reported to be irreversible by hydroxylamine (Burstein et al., 1974), and spontaneous decarbethoxylation accompanied by reactivation in 29 min has been shown in the case of serine hydroxyl group modification by DPC (Melchior & Fahrney, 1970). Under the conditions of the present experiments, it was observed that there was no spontaneous reactivation of the inactivated enzyme even after dialysis for 18 h at pH 7. This observation together with hydroxylamine reversal and the spectral studies led to the conclusion that the AChE, IPAase, and AAA inactivation was associated with essential histidine modification.

Tryptophan Modification. The more specific ONBB and less specific *N*-bromosuccinimide were used as tryptophan modifiers. They showed similar effects in the inactivation of AChE, IPAase, and AAA from both eel and basal ganglia (Table II). Though BW284C51 and ACh could partially protect AChE and IPAase, they could not protect AAA from inactivation (Table II).

Arginine and Cysteine Modification. Phenylglyoxal and 2,4-pentanedione react with the guanidine group of arginine residues at pH 7.5 (Chang & Huang, 1981), and *N*-ethylmaleimide alkylates the reactive –SH groups of cysteine at pH 7.0. However, these three amino acid modifiers up to 15 mM did not cause any significant inactivation of either AAA or AChE from both eel and basal ganglia, suggesting that probably arginine and cysteine residues are not located in the active center of AChE and AAA.

Discussion

The present study is an attempt to delineate the active centers of AChE and AAA in the AChE protein. It should be noted that, as per the definition of active center, covalent modifications at some distance from the active site causing conformational changes could also cause changes in enzyme activity.

The presence of serine, glutamic acid, tryptophan, tyrosine, and histidine at the active center of AChE has been indicated by earlier workers from kinetic studies [see review of Rosenberry (1975)] and from fluorescence and protein modification studies (Mooser et al., 1972; Roskoski, 1974; Blumberg & Silman, 1978). The present work shows an essential lysine residue(s) also involved in AChE activity. The amino acid modifiers used in these studies are well-known for their specificity of action. The following points rule out nonspecific inactivation of the enzymes and emphasize their specificity of action. (a) The inactivation can largely be prevented by BW284C51, a selective competitive inhibitor of AChE, or by ACh, the substrate of AChE. (b) Inactivation can be reversed by using hydroxylamine in the case of tyrosine and histidine modification. (c) Spectral changes corresponding to the modified amino acid residues could be detected in most of the cases. These spectral studies are valid at least for the eel enzyme, which was electrophoretically homogeneous. (d) Other arguments given under each amino acid modification under Results.

There was a parallel inactivation of both AChE and AAA by TNBS, pyridoxal phosphate, acetic anhydride, ONBB, and *N*-bromosuccinimide. Indophenyl acetate, a slow-reacting neutral substrate of AChE, was used in these studies to check whether different rate-limiting steps at a common site could be responsible for the differences between the esterase and amidase activities. In all the modification studies, IPAase activity followed a pattern similar to that of AChE. The inactivation of AChE and IPAase but not that of AAA by the above agents could be prevented by ACh and BW284C51. DPC inactivation of AChE, IPAase, and AAA could be prevented by ACh and BW284C51. *N*-Acetylimidazole, which modifies tyrosine, inactivated AChE and IPAase, while it activated AAA. This finding is similar to observations made by others (Simpson et al., 1963; Landaburu & Seegers, 1959) on the peptidase and esterase activities of carboxypeptidase A and thrombin. The observation that BW284C51 or ACh can prevent the chemical modification of tyrosine and the changes in AChE or AAA indicates that the amino acid modified is situated at the active center of AChE. Thus, Lysine, tyrosine, histidine, and tryptophan may be considered to be present at the active center of AChE and essential for catalytic activity. It is of interest to note that BW284C51 or ACh could not prevent the alterations in AAA activity resulting from modification of all but two (histidine and tyrosine) of the amino acids. These results suggest the following. (1) Essential histidine and tyrosine residues present at the active center of AChE are involved in the catalytic activity of both AAA and AChE. (2) Although lysine and tryptophan are essential for the catalytic activity of both AAA and AChE, their differential protection by BW284C51 and ACh suggests their separate localization. The overall picture emerging is one of nonidentical but overlapping active centers for AAA and AChE.

A number of purified enzymes, particularly peptidases such as trypsin, α-chymotrypsin, carboxypeptidase A, thrombin, and the amidase nicotinamide deamidase having esterase and amidase activities, have been shown to have nonidentical active centers or binding sites and different mechanisms for the two activities. Work on carboxypeptidase A has revealed that this enzyme has a complex catalytic system (Vallee et al., 1968),

and after acetylation of tyrosyl residues, the peptidase activity was abolished while esterase activity was increased 6-fold (Simpson et al., 1963). Landaburu & Seegers (1959) have shown that, during acetylation of thrombin, peptidase activity was completely lost but esterase activity was increased. Coletti-Previero et al. (1969) reported that formylation of several tryptophanyl residues in trypsin destroyed peptidase activity while only slightly affecting esterase activity. Studies on chymotrypsin (Epand et al., 1969) revealed a mechanistic difference in the catalysis of amide and ester substrates. In nicotinamide deamidase, if a single seryl residue was modified, both the esterase and amidase activities were lost, but with histidyl residue modification, only the amidase activity was abolished (Albizati & Hedrick, 1972). Recent investigations [for a review see Polgar & Halasz (1982)] of serine proteinases based on X-ray diffraction and other mechanistic studies indicate that the above conclusions may not be so simple as indicated with regard to the participation of the amino acids in enzyme catalysis. AChE is a complex protein with respect to the topography of its active center, and it is postulated to contain anionic, esteratic, and peripheral sites [see review of Rosenberry (1975)] involved in the catalytic activity. The present studies suggest that AChE has nonidentical overlapping active centers for its esterase and amidase activity. The validity of this conclusion must await further studies.

Registry No. AChE, 9000-81-1; AAA, 9025-18-7; IPAase, 91083-01-1; TNBS, 2508-19-2; ONBB, 772-33-8; DPC, 1609-47-8; ACh, 51-84-3; BW284C51, 402-40-4; pyridoxal phosphate, 54-47-7; acetic anhydride, 108-24-7; *N*-acetylimidazole, 2466-76-4; lysine, 56-87-1; tyrosine, 60-18-4; tryptophan, 73-22-3; histidine, 71-00-1.

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