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Chemical modification of the bifunctional human serum pseudocholinesterase

Effect on the pseudocholinesterase and aryl acylamidase activities

Rathanam BOOPATHY and Aiylam S. BALASUBRAMANIAN

Neurochemistry Laboratory, Department of Neurological Sciences, Christian Medical College and Hospital, Vellore

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The effect of chemical modification on the pseudocholinesterase and aryl acylamidase activities of purified human serum pseudocholinesterase was examined in the absence and presence of butyrylcholine iodide, the substrate of pseudocholinesterase. Modification by 2-hydroxy-5-nitrobenzyl bromide, *N*-bromosuccinimide, diethylpyrocarbonate and trinitrobenzenesulfonic acid caused a parallel inactivation of both pseudocholinesterase and aryl acylamidase activities that could be prevented by butyrylcholine iodide. With phenylglyoxal and 2,4-pentanedione as modifiers there was a selective activation of pseudocholinesterase alone with no effect on aryl acylamidase. This activation could be prevented by butyrylcholine iodide. *N*-Ethylmaleimide and *p*-hydroxymercuribenzoate when used for modification did not have any effect on the enzyme activities. The results suggested essential tryptophan, lysine and histidine residues at a common catalytic site for pseudocholinesterase and aryl acylamidase and an arginine residue (or residues) exclusively for pseudocholinesterase.

The use of *N*-acetylimidazole, tetranitromethane and acetic anhydride as modifiers indicated a biphasic change in both pseudocholinesterase and aryl acylamidase activities. At low concentrations of the modifiers a stimulation in activities and at high concentrations an inactivation was observed. Butyrylcholine iodide or propionylcholine chloride selectively protected the inactivation phase without affecting the activation phase. Protection by the substrates at the inactivation phase resulted in not only a reversal of the enzyme inactivation but also an activation. Spectral studies and hydroxylamine treatment showed that tyrosine residues were modified during the activation phase. The results suggested that the modified tyrosine residues responsible for the activation were not involved in the active site of pseudocholinesterase or aryl acylamidase and that they were more amenable for modification in comparison to the residues responsible for inactivation.

Two reversible inhibitors of pseudocholinesterase, namely ethopropazine and imipramine, were used as protectors during modification. Unlike the substrate butyrylcholine iodide, these inhibitors could not protect against the inactivation resulting from modification by 2-hydroxy-5-nitrobenzyl bromide, *N*-bromosuccinimide and trinitrobenzenesulfonic acid. But they could protect against the activation of pseudocholinesterase and aryl acylamidase by low concentrations of *N*-acetylimidazole and acetic anhydride thereby suggesting that the binding site of these inhibitors involves the non-active-site tyrosine residues.

Although the physiological role of pseudocholinesterases (PsChE) has been obscure for many years, recent studies indicate that they may have some definite functions in the mammalian system. The possibility of PsChE acting as a post-translational precursor of acetylcholinesterase has been suggested by Koelle et al. [1]. Human serum PsChE has been demonstrated to act as a peptidase on substance P [2]. A soluble form of cholinesterase from bovine fetal serum has been shown to have peptidase activity on enkephalins and several other peptides [3]. Histochemical studies on the visual pathway have suggested that PsChE or its endogenous substrate may be a neuroactive substance in the primate brain [4].

Human serum is a well known source of PsChE [5]. We [6], as well as others [7], have shown that an aryl acylamidase (AAA) activity is exhibited by human serum PsChE. Both the PsChE and AAA activities have been identified with the same protein on the basis of their identical behaviour on column chromatographic and affinity chromatographic procedures, gel filtration, gel electrophoresis, their coprecipitation by anti-PsChE antibody and identical response to potential cholinesterase inhibitors [6]. An interesting aspect of the AAA associated with human serum PsChE was its specific inhibition by serotonin and activation by tyramine [6, 7].

The bifunctional nature of PsChE exhibiting an AAA activity is reminiscent of the amidolytic and esterolytic activities exhibited by several purified proteases like chymotrypsin, trypsin, carboxypeptidase A, elastase and thrombin.

Correspondence to A. S. Balasubramanian, Neurochemistry Laboratory, Christian Medical College and Hospital, Vellore, India 632004

Trivial names. Ethopropazine, 10-(2-diethylaminopropyl) phenothiazine; imipramine, *N*-(γ-dimethylaminopropyl) iminodibenzyl.

Abbreviations. PsChE, pseudocholinesterase; AAA, aryl acylamidase; BCI, butyrylcholine iodide; HNBB, 2-hydroxy-5-nitrobenzyl bromide; $(NO_2)_3PhSO_3$, 2,4,6-trinitrobenzenesulfonic acid; SDS, sodium dodecyl sulfate.

Enzymes. Pseudocholinesterase or butyrylocholinesterase or cholinesterase or acylcholine acylhydrolase (EC 3.1.1.8); aryl acylamidase or arylacylamide amidohydrolase (EC 3.5.1.13); acetylcholinesterase or acetylcholine acetylhydrolase or true cholinesterase (EC 3.1.1.7).

Table 1. *Experimental conditions for chemical modification of PsChE and AAA*

Protein modifying reagent used	Conditions of reaction for modification	References
HNBB and *N*-bromosuccinimide	the reaction mixture in 20 mM sodium acetate buffer, pH 5.0 containing the enzyme and 0.5–10 mM HNBB was incubated at 0–4 °C for 3 min; in the case of *N*-bromosuccinimide the reaction was carried out at 37 °C for 15 min with 2.5–25 μM modifier	[32, 33]
Diethylpyrocarbonate	diethylpyrocarbonate, 0.5–10 mM in ethanol, was incubated with enzyme in 20 mM potassium phosphate buffer, pH 7.0 at 22 °C for 15 min (ethanol in the reaction mixture was kept at 5% v/v which had no effect on enzyme activity)	[15, 23]
$(NO_2)_3PhSO_3$ and acetic anhydride	the reaction mixture in 20 mM potassium phosphate buffer, pH 8.0, containing the enzyme and 1–20 mM $(NO_2)_3PhSO_3$ was incubated at 37 °C for 30 min; for acetic anhydride (1–15 mM) the pH of the reaction mixture was 7.0 in 100 mM phosphate buffer and the reaction was carried out at 37 °C for 20 min	[16, 34]
Phenylglyoxal and 2,4-pentanedione	the reaction mixture in 20 mM Hepes/NaOH, pH 7.5, containing the enzyme and 1–20 mM phenylglyoxal or 0.5–10 mM 2,4-pentanedione was incubated for 20 min at 37 °C	[35]
N-Acetylimidazole and tetranitromethane	the reaction mixture in 20 mM potassium phosphate buffer, pH 7.0, containing the enzyme and 10–300 mM *N*-acetylimidazole, was incubated at 37 °C for 20 min; in the case of tetranitromethane the reaction was carried out in 20 mM Tris HCl buffer, pH 8.0, containing the enzymes and 0.021–4.2 mM tetranitromethane in ethanol (5% v/v) at 37 °C for 30 min	[16, 27–29]
N-Ethylmaleimide and *p*-hydroxymercuribenzoate	the reaction mixture in 20 mM potassium phosphate buffer, pH 7.0, containing the enzyme and 0.1–20 mM *N*-ethylmaleimide in ethanol (5% v/v) was incubated at 37 °C for 30 min; for *p*-hydroxymercuribenzoate, the reaction was in 20 mM Tris/HCl, pH 8.0, containing the enzyme and 0.2–2.0 mM *p*-hydroxymercuribenzoate at 37 °C for 30 min	[30]

Some of these proteases have been shown to have non-identical active centers for their esterolytic and amidolytic activities [8–12]. Moreover acetylcholinesterase from electric eel and basal ganglia has been suggested to have non-identical partially overlapping active centers for its esterase and acyl amidase activities [13]. In view of these findings and the reported protease activity attributed to serum PsChE [2], it was of interest to find out which amino acids are involved in the active centers of PsChE and AAA activities. In the present study we have used chemical modification of the purified human serum PsChE as a means to this end. Evidence for non-identical but overlapping active centers is presented. Moreover the stimulation of PsChE and AAA activities by modification of a class of tyrosine residues present at a site different from the active site has been demonstrated.

MATERIALS AND METHODS

Materials

2-Hydroxy-5-nitrobenzyl bromide (HNBB), *N*-bromosuccinimide, diethylpyrocarbonate, 2,4,6-trinitrobenzenesulfonic acid [$(NO_2)_3PhSO_3$], phenylglyoxal, 2,4-pentanedione, *N*-acetylimidazole, tetranitromethane, *N*-ethylmaleimide, *p*-hydroxymercuribenzoate, ethopropazine · HCl, imipramine · HCl and choline derivatives were purchased from Sigma Chemical Co., USA. All other chemicals were either prepared or obtained as in [6].

Purification of human serum PsChE and enzyme assays

PsChE from human serum was purified to homogeneity essentially as described earlier [6] with some modifications. The active fractions after DEAE-cellulose chromatography were pooled, dialyzed against 20 mM potassium phosphate buffer pH 7.0 and subjected to procainamide-Sepharose affinity chromatography as in [6]. The active fractions eluted with a 0.1–0.6 M gradient of NaCl in the same buffer were pooled, dialyzed against the buffer and rechromatographed on procainamide-Sepharose. The column after washing with the buffer was eluted with 50 mM procainamide in the buffer and the active fractions dialyzed exhaustively against the buffer. The purified enzyme was homogenous by polyacrylamide gel electrophoresis [6].

The assay conditions for serum PsChE and AAA were the same as described before [6] except that the pH of the assay was 7.0. Protein was estimated according to Lowry et al. [14].

Chemical modifications

Modification of the enzyme was done by incubating 4–5 μg of the purified enzyme with different concentrations of the modifying agents (total volume 0.5 ml) at a given pH for a specified time at the indicated temperature (Table 1). At the end of incubation, the reaction mixture was diluted twofold with 20 mM potassium phosphate buffer pH 7.0 (in the case of diethylpyrocarbonate treatment 1 mM imidazole and in the case of $(NO_2)_3PhSO_3$ treatment 0.5 mM sodium sulfite were included in the diluting buffer to terminate the modification), chilled in an ice bath and dialyzed at 4 °C against 1 l of 20 mM potassium phosphate buffer, pH 7.0 for 16–20 h with three changes. (In separate experiments it was verified that at 0–4 °C no detectable modification by *N*-acetylimidazole, tetranitromethane, phenylglyoxal and 2,4-pentanedione took place. But, a 108–116% activity was observed with acetic

anhydride at the higher concentrations of 7–15 mM.) Following this, the enzyme activities were determined. Every experiment had a control that was subjected to the same treatment without the modifying reagents. In those experiments where time-dependent modification was studied it was ensured that the control (unmodified) enzyme activities did not change significantly with the time of incubation.

Protection studies

Protection experiments were performed by preincubating the purified enzyme at ambient temperature for 3 min with 50 mM of butyrylcholine iodide (BCI) (substrate of PsChE) followed by the addition of the protein-modifying reagent in a total volume of 0.5 ml. (BCI formed a yellow colour or brown precipitate with high concentrations of tetranitromethane as modifier and hence propionylcholine chloride was used under these conditions.) Protection studies were also carried out with the selective reversible inhibitors of PsChE namely ethopropazine (75 μM) and imipramine (100 μM) in a similar way. Dialysis was done as described above.

Spectral studies

Carbethoxylation of histidine by diethylpyrocarbonate [15] and O-acetylation by acetic anhydride or *N*-acetylimidazole of tyrosine residues [16] were determined by ultraviolet absorption at 242 nm (for histidine) and 278 nm (for tyrosine). The extent of trinitrophenylation following the treatment with $(NO_2)_3PhSO_3$ was determined by the method of Goldfarb [17] with slight modification. The modified enzyme mixture, after incubation was diluted to 1.0 ml with 20 mM phosphate buffer, pH 8.0 containing 0.5 mM sodium sulfite to develop the colour and the incubation was continued for a further period of 5 min at 37 °C and the resultant mixture was measured at different wavelengths within 400–450 nm. The trinitrophenylated complex showed a characteristic peak at 420 nm. All spectrophotometric measurements were made in an LKB Ultrospec spectrophotometer.

Hydroxylamine treatment

Acetylated or carbethoxylated enzymes were incubated with hydroxylamine (NH_2OH) upto 1 M at pH 7.0–8.0 (pH adjusted with ammonia) in 0.5 ml at 37 °C for 1 h. The loss of enzyme activity was < 10% when the native enzyme was incubated with 1 M NH_2OH. Dialysis for removal of excess NH_2OH was done against 20 mM phosphate buffer, pH 7.0.

SDS polyacrylamide gel electrophoresis

Dialysed and concentrated samples of modified or unmodified enzymes with 1% SDS were prepared by boiling at 100 °C for 3 min without 2-mercaptoethanol. In one case, the unmodified enzyme was prepared in a similar way with added 5% 2-mercaptoethanol. All the samples were subjected to SDS gel electrophoresis in 10% gel according to Laemmli [18]. Standard marker proteins used to determine the molecular masses were immunoglobulin G (IgG) unreduced (M_r 150000), bovine serum albumin (M_r 68000), IgG heavy chain (M_r 50000), ovalbumiun (M_r 45000) and IgG light chain (M_r 23500).

RESULTS

Modifications were carried out by the following reagents. HNBB and *N*-bromosuccinimide for modification of tryptophan residues; diethylpyrocarbonate for histidine; phenylglyoxal and 2,4-pentanedione for arginine; *N*-acetylimidazole and tetranitromethane for tyrosine; $(NO_2)_3PhSO_3$ and acetic anhydride for lysine.

Modification by HNBB and N-*bromosuccinimide*

N-Bromosuccinimide and the more specific HNBB were used as reagents for modification of tryptophan residues. Both these reagents caused the inactivation of PsChE and AAA activities (Table 2). There was about 60% loss in both activities at 10 mM HNBB and about 85% loss at 25 μM *N*-bromosuccinimide. There was a parallel loss in both PsChE and AAA activities at the different concentrations of HNBB and *N*-bromosuccinimide used (Table 2). The presence of 50 mM BCI as protector prevented the loss in both PsChE and AAA activities to a significant extent (Table 2).

Modification by diethylpyrocarbonate

This reagent has been used for the modification of histidine [15, 23]. With increasing concentrations of diethylpyrocarbonate there was a loss in activity of both PsChE and AAA which was about 60–70% at 10 mM of the modifier (Table 2). In the presence of BCI there was almost a 100% protection of both the activities against inactivation at the different concentrations of the modifier used (Table 2).

Diethylpyrocarbonate modification of histidine has been shown to be reversible by NH_2OH [15, 23]. When the enzyme inactivated using 10 mM diethylpyrocarbonate was treated with 0.5 M NH_2OH at pH 7.5 as given under Methods the activity was restored to 80% of the original. The enzyme inactivated at lower concentrations of the modifier were fully restored to the original activity upon NH_2OH treatment. The possible involvement of lysine, tyrosine and serine in the modification by diethylpyrocarbonate [24] was excluded on the following bases. (a) Lysine and tyrosine modification by diethylpyrocarbonate is shown to be irreversible by NH_2OH [23]. (b) Spontaneous decarbethoxylation accompanied by reactivation in 29 min has been shown in the case of serine hydroxyl group modification by diethylpyrocarbonate [24]; in the present experiment there was no reactivation of the inactivated enzyme even after dialysis for 16–20 h at pH 7.0. (c) The ultraviolet absorption at 242 nm characteristic of *N*-carbethoxyhistidine was increased as the amino acid was modified and there was no significant change at 278 nm excluding the involvement of tyrosine residues [15, 25] (and data not shown).

Modification by $(NO_2)_3PhSO_3$

Exposure of the enzyme to different concentrations of $(NO_2)_3PhSO_3$ which reacts with amino groups resulted in the inactivation of both PsChE and AAA activities (Table 2). At 20 mM $(NO_2)_3PhSO_3$ 60–70% of both activities were lost. There was a complete protection from loss of activities when the modification was carried out in the presence of BCI (Table 2).

Fig. 1 shows the time-dependent inactivation by 10 mM $(NO_2)_3PhSO_3$ of PsChE and AAA activities and the spectral changes associated with it. There was a progressive decrease

Table 2. *Effect of protein modifying agents in the absence and presence of butyrylcholine iodide on PsChE and AAA activities*

Modifying agent	Activity of																			
	PsChE with modifier at (mM)									AAA with modifier at (mM)										
	0	0.0025	0.005	0.0125	0.025	0.5	1.0	5.0	10	20	0	0.0025	0.005	0.0125	0.025	0.5	1.0	5.0	10	20
	%																			
HNBB	100	–	–	–	–	76	65	49	42	–	100	–	–	–	–	75	64	46	42	–
HNBB + BCI	100	–	–	–	–	93	85	72	72	–	100	–	–	–	–	88	84	73	76	–
N-bromo-succinimide	100	84	67	42	15	–	–	–	–	–	100	82	63	37	12	–	–	–	–	–
N-bromosuccin-imide + BCI	100	96	94	92	90	–	–	–	–	–	100	93	97	94	91	–	–	–	–	–
Diethylpyro-carbonate	100	–	–	–	–	100	98	59	38	–	100	–	–	–	–	99	88	46	27	–
Diethylpyro-carbonate + BCI	100	–	–	–	–	100	98	102	114	–	100	–	–	–	–	98	96	98	100	–
$(NO_2)_3PhSO_3$	100	–	–	–	–	–	89	59	48	41	100	–	–	–	–	–	68	45	41	30
$(NO_2)_3PhSO_3$ + BCI	100	–	–	–	–	–	97	102	98	96	100	–	–	–	–	–	98	100	98	96

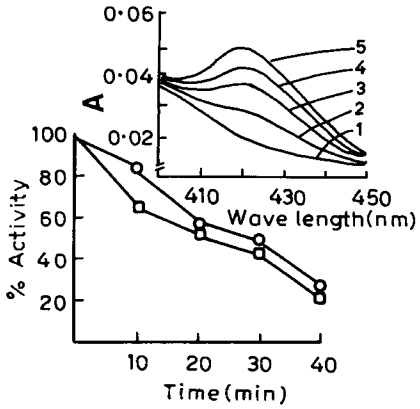

Fig. 1. *Time-dependent inactivation of PsChE (○) and AAA (□) by modification with 10 mM $(NO_2)_3PhSO_3$.* Modification of the enzyme was carried out for the indicated time interval. Details of the modification procedure and assay are given under Methods. There was no change in control enzyme activity (100%) up to 40 min of incubation at 37 °C. The inset in the figure shows the changes in the absorbance of the modified enzyme in the region of 400–450 nm, determined as described under Methods for the time intervals of (1) 0, (2) 10 min, (3) 20 min, (4) 30 min and (5) 40 min of treatment with $(NO_2)_3PhSO_3$

in the activities with time. The time-dependent trinitrophenylation of $-NH_2$ groups in the modified enzyme, monitored in the range of 400–450 nm showed a peak at 420 nm which increased with the time of treatment (inset of Fig. 1).

Treatment of the $(NO_2)_3PhSO_3$-modified enzyme with NH_2OH upto 1 M at pH 8.0 did not restore the lost activity to any significant extent suggesting that tyrosine groups were not involved in the inactivation by $(NO_2)_3PhSO_3$. Moreover spectral studies of the $(NO_2)_3PhSO_3$-modified enzyme did not show any change in absorbance at 278 nm (characteristic of tyrosine). The involvement of cysteine $-SH$ groups in the modification by $(NO_2)_3PhSO_3$ resulting in inactivation was ruled out by the observation that *N*-ethylmaleimide upto 20 mM and *p*-hydroxymercuribenzoate upto 2 mM (both $-SH$ group modifying agents) did not have any effect on the enzyme activities. Assuming the non-involvement of an N-terminal amino acid, these results indicated that $(NO_2)_3PhSO_3$ modified the lysine-NH_2 groups in the enzyme resulting in the inactivation of PsChE and AAA.

Results using acetic anhydride, another $-NH_2$ group modifying agent will be described below.

Modification by phenylglyoxal and 2,4-pentanedione

These two specific modifiers interact with the guanido group of arginine residues. The results obtained on treatment of serum PsChE with these two reagents at different concentrations are shown in Fig. 2. Unlike the parallel inactivation of both PsChE and AAA, resulting from the modifications described so far, modification by phenylglyoxal and 2,4-pentanedione caused a progressive activation of PsChE alone without affecting AAA to any significant extent. At 20 mM of phenylglyoxal, PsChE activity was 195% of the control and at 10 mM of 2,4-pentanedione it was 180%. BCI as protector could completely reverse this activation (Fig. 2). The time-dependent modification by phenylglyoxal and 2,4-pentanedione further corroborated their effect in the activation of PsChE without affecting AAA (Fig. 3). With 5 mM of each of these modifiers, PsChE activity rose up to 160–170% at 40 min. BCI could completely protect PsChE activity from

activation by both the modifiers (Fig. 3). Taken together, these results suggested that arginine modification altered only the PsChE activity (without affecting AAA) and that this alteration could be prevented by BCI.

Modification by N-*acetylimidazole and tetranitromethane*

Both *N*-acetylimidazole and tetranitromethane have been used as modifying agents selectively for tyrosine [26–28]. The changes in the activity of PsChE and AAA resulting from modification by these agents at different concentrations are shown in Fig. 4. Both enzyme activities showed a similar pattern of changes. At 10 mM and 25 mM *N*-acetylimidazole there was an increase in activity (about 135% of original activity at 25 mM). At 50–100 mM the activity was about 100–90% of the original and above 100 mM there was a fall in activity (Fig. 4A). The inset in Fig. 4A shows the change in absorbance at 278 nm resulting from modification by

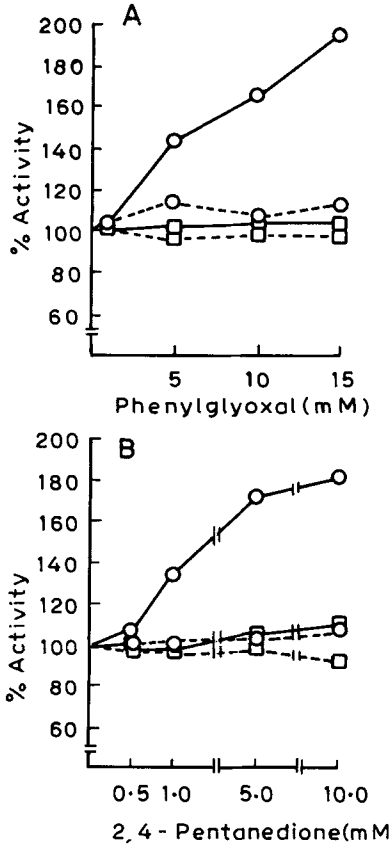

Fig. 2. *Effect of modification with increasing concentrations of phenylglyoxal (A) and 2,4-pentanedione (B) on PsChE (○) and AAA (□) activity.* The modification was carried out in the absence (——) or presence (----) of BCI as protector. Details of the modification procedure and assay are given under Methods. The control enzyme activity at zero concentration of the modifier is taken as 100%

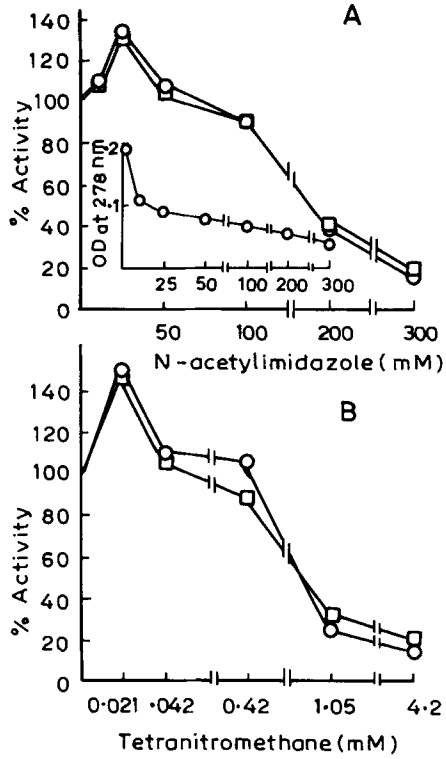

Fig. 4. *Changes in the activity of PsChE (○) and AAA (□) by modification of the enzyme with* N-*acetylimidazole (A) and tetranitromethane (B) at different concentrations.* The enzyme was incubated at 37°C with the modifying agent at the respective concentration for 20 min (with *N*-acetylimidazole) or 30 min (with tetranitromethane). The curve in the inset of A shows the changes in absorbance at 278 nm of the modified enzyme samples. Details of the modification procedure and assay of enzymes are given under Methods. The control enzyme activity at zero concentration of modifier is taken as 100%

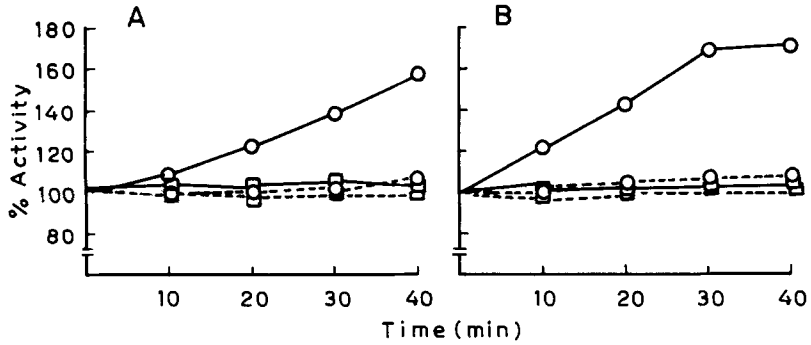

Fig. 3. *Changes in activities of PsChE (○) and AAA (□) as a function of time of modification with 5 mM phenylglyoxal (A) or 5 mM 2,4-pentanedione (B) in the absence (——) or presence (----) of BCI as protector.* Details of the modification and assay are given under Methods. There was no detectable change in control enzyme activity (100%) up to 40 min of incubation at 37°C

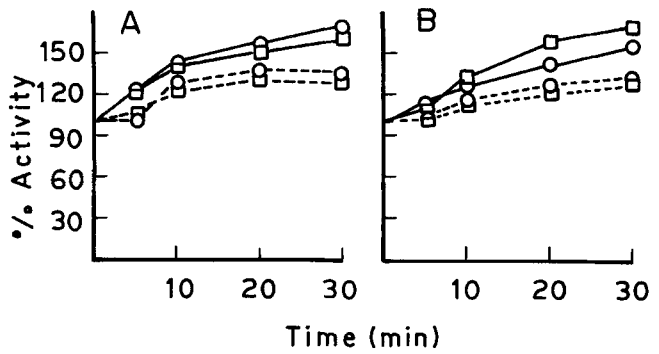

Fig. 5. *The activation of PsChE (○) and AAA (□) as a function of time by modification with 25 mM* N*-acetylimidazole (A) or 0.021 mM tetranitromethane (B) in the absence (——) or presence (– – –) of BCI as protector.* The enzyme was incubated for the desired period at 37°C for modification. Details of the modification procedure and assay are given under Methods. There was no change in control enzyme activity (100%) up to 30 min of incubation at 37°C

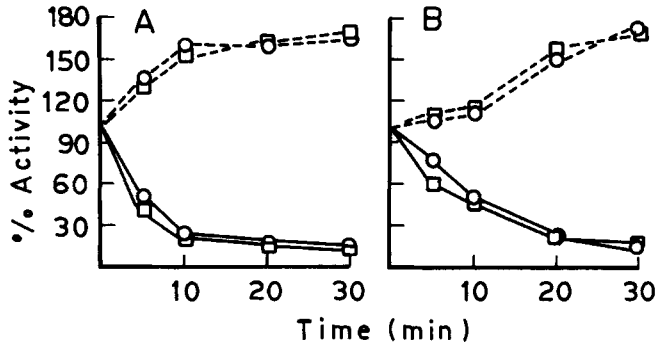

Fig. 6. *Changes in the activity of PsChE (○) and AAA (□) as a function of time by modification with 200 mM* N*-acetylimidazole (A) or 4.2 mM tetranitromethane (B) in the absence (——) or presence (– – –) of BCI (A) and propionylcholine chloride (B) as protector.* The enzyme was incubated for the desired period at 37°C for modification. Details of the modification procedure and assay are given under Methods. There was no change in control enzyme activity (100%) up to 30 min of incubation at 37°C

N-acetylimidazole at the different concentrations. Similar activity changes were observed when tetranitromethane was used for modification. At 0.021 mM there was an activation up to 150%, between 0.042 and 0.42 mM, the changes were minimal and above 0.42 mM there was a progressive fall in activity (Fig. 4B). These patterns of concentration-dependent changes in activity were confirmed in several batches of the purified serum PsChE. The results suggested that at lower concentrations of *N*-acetylimidazole or tetranitromethane the amino acids modified caused an increase in activity whereas at higher concentrations, the modified amino acids caused a decrease.

The two phases of the activity changes became more apparent when modification was carried out with a low or high concentration of these two modifiers for different periods (Figs. 5 and 6). Fig. 5 shows the changes in activity when modification was carried out with 25 mM *N*-acetylimidazole for different periods upto 30 min in the absence or presence of BCI as a protector. There was a continuous increase in activity of both PsChE and AAA upto about 165% of original at 30 min in the absence of BCI. In the presence of BCI as protector during modification, there was a small reduction in the enhancement of activity but the enhancement could not be abolished by the protector. Similar results were shown when modification by 0.21 mM tetranitromethane for different periods up to 30 min in the absence or presence of BCI was carried out (Fig. 5B).

Fig. 6A shows the changes in activity when modification was carried out with a high concentration (200 mM) of *N*-acetylimidazole for different periods in the absence and presence of BCI as protector. There was a continuous fall in both PsChE and AAA activities up to 15% of original at 30 min in the absence of the protector. Modification in the presence of the protector however resulted in not only a complete reversal of the fall in activity but also an enhancement in activity upto 165% at 30 min similar to observations made when modification was carried out with a low concentration (25 mM) of *N*-acetylimidazole as shown in Fig. 5A. Fig. 6B shows the activity changes when a high concentration (4.2 mM) of tetranitromethane was used for modification for different periods, either in the presence or absence of the protector; the results were similar to those shown in Fig. 6A.

The nearly identical pattern of changes in enzyme activities by *N*-acetylimidazole and tetranitromethane suggested that both the agents modified tyrosine residues. Spectral studies indicated a progressive fall in absorption at 278 nm (characteristic for tyrosine) after modification of the enzyme with *N*-acetylimidazole at different concentrations (10–300 mM, inset of Fig. 4A). It has been shown that tyrosine modified by *N*-acetylimidazole is deacetylated in the presence of NH_2OH at alkaline pH [29]. We found that the activation of PsChE and AAA after modification by 25 mM *N*-acetylimidazole was completely reversible on incubation either with 0.8 M NH_2OH at pH 7.0 or with 0.2 M NH_2OH at pH 8.0. On the other hand, the inactivated PsChE and AAA obtained by the modification of the enzyme with 200 mM *N*-acetylimidazole could be reversed only to about 40–50% by incubation with 1 M NH_2OH at pH 8.0. Whether this partial reversibility by NH_2OH is characteristic of the 'buried' nature of the tyrosine residues involved [29] or whether any amino acids other than tyrosine (such as lysine; see below for acetylation by acetic anhydride) are also modified at the higher concentration of *N*-acetylimidazole cannot be conclusively stated. However, the possibility of the activity changes due to histidine modification by *N*-acetylimidazole was excluded because acetylation of this amino acid is spontaneously reversed [26, 28]. The involvement of –SH groups in the modification by tetranitromethane [27] resulting in activity changes was also excluded because *N*-ethylmaleimide upto 20 mM and *p*-hydroxymercuribenzoate upto 2 mM did not influence PsChE or AAA activities [30].

The results shown in Fig. 5 indicated that the tyrosine residues modified by the low concentrations of *N*-acetylimidazole (25 mM) or tetranitromethane (0.021 mM) had the following features. (a) Their modification resulted in an activation of the PsChE and AAA activities. (b) They appeared to be a class of tyrosine residues which were more amenable to modification as compared to the residues whose modification needed a higher concentration of the modifiers and resulted in the inactivation of the enzyme. (c) They did not appear to be at the active site of the enzyme because of the relatively poor protection offered by the substrate BCI against modification; on the other hand, the amino acids responsible for the inactivation phase of the enzyme at higher

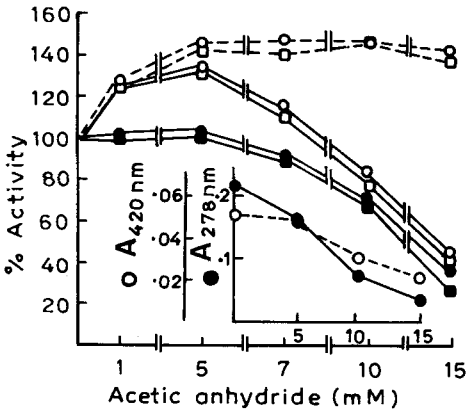

Fig. 7. *Effect of increasing concentrations of acetic anhydride modifier on PsChE (○) and AAA (□) activites in the absence (——) or presence (----) of BCI.* (●——● PsChE and (■——■) AAA activities of the modified enzyme after treatment with 0.2 M NH_2OH at pH 7.5. The inset shows the absorbance of the modified enzyme (●) at 278 nm (for tyrosine) and (○) 420 nm (for free $-NH_2$ groups) after modification by acetic anhydride at different concentrations. The details of modification, assay and NH_2OH treatment are given under Methods. The control enzyme activity at zero concentration of the modifier is taken as 100%

concentrations of *N*-acetylimidazole and tetranitromethane could be completely protected by BCI or propionylcholine chloride. (d) The activation of PsChE and AAA observed while protecting modification at higher concentrations of *N*-acetylimidazole and tetranitromethane (Fig. 6) could be due to the availability of the modifying agents exclusively for the more amenable class of tyrosine residues which were only poorly protected by BCI. This also corroborated the view that the more amenable class of tyrosine residues responsible for activation of PsChE and AAA were not at the active site.

Modification by acetic anhydride

Acetic anhydride is known to acetylate the lysine $-NH_2$ groups and tyrosine hydroxyl groups in proteins [9, 16, 26]. Fig. 7 shows the effect of modification on the enzyme when different concentrations of acetic anhydride were used. There was an activation of both PsChE and AAA to about 140% of original activity at 5 mM acetic anhydride followed by a decline in activity and progressive inactivation at higher concentrations of acetic anhydride (Fig. 7). The results suggested that at lower concentrations of acetic anhydride there was an activation of both PsChE and AAA and at higher concentrations an inactivation, a phenomenon similar to that observed when *N*-acetylimidazole and tetranitromethane were used for modification of tyrosine residues (see Fig. 4). In order to differentiate tyrosine residues and $-NH_2$ residues involved in acetylation, the modified enzyme obtained at each of the different concentrations of acetic anhydride was treated with NH_2OH (0.2 M at pH 7.5 or 1 M NH_2OH at pH 8.0). The results shown in Fig. 7 indicates that the activation of the enzyme at lower concentrations of acetic anhydride was reversed by NH_2OH but the inactivation was unaffected suggesting that acetylation of tyrosine residues was responsible for the activation whereas acetylation of $-NH_2$ groups (and possibly 'buried' tyrosine residues) was responsible for the inactivation. The available free $-NH_2$ groups after acetic anhydride modification was estimated by trinitrophenylation with $(NO_2)_3PhSO_3$ and measuring the absorbance at 420 nm (inset of Fig. 7). As shown in the inset, up to 5 mM acetic anhydride the decrease in 420-nm absorbance was insignificant but above 5 mM there was a progressive decline in absorbance. Moreover the changes in absorbance at 278 nm of the modified enzyme shown in the inset supported the acetylation of tyrosine residues. When BCI was used as a protector during modification, the activation of the enzyme alone was seen at all concentrations of acetic anhydride including the higher concentrations (Fig. 7). This observation was similar to the effect of BCI as protector during modification of tyrosine residues by *N*-acetylimidazole (see Figs. 5 and 6). Taken together these results indicated that tyrosine residues present at a site different from the active site, and more amenable for acetylation, were responsible for the activation. With BCI as protector, all the available acetic anhydride was used exclusively for modification of these tyrosine residues because of the selective protection by BCI of the active-site lysine (and possibly tyrosine) residues.

Use of ethopropazine and imipramine as protecting agents

Ethopropazine is a selective reversible inhibitor of PsChE [19, 21, 22]. Yet another reversible inhibitor is imipramine [20, 22]. Although conclusive evidence is lacking, it is suggested that ethopropazine may be acting as a non-competitive inhibitor [21, 22] and imipramine as an allosteric competitive inhibitor [20, 22]. We used both these inhibitors as protectors during the various chemical modification experiments at different concentrations of the modifier. Neither of these inhibitors (75 μM ethopropazine and 100 μM imipramine) showed any protective effect against inactivation of PsChE and AAA when modifications were carried out with HNBB, *N*-bromosuccinimide and $(NO_2)_3PhSO_3$. This was in contrast to the protection offered by BCI in the above modification procedures. On the other hand, the activation of PsChE resulting from modification of arginine residues by phenylglyoxal and 2,4-pentanedione could be protected by both ethopropazine and imipramine (data not shown), similar to observations made with BCI as protector. With diethylpyrocarbonate as modifier, ethopropazine could protect both PsChE and AAA from inactivation while imipramine was ineffective as a protector (data not shown). These results suggested different binding sites for BCI, ethopropazine and imipramine but that a modifiable arginine residue or residues may be common amongst them.

The results obtained on the use of ethopropazine and imipramine as protectors during modification by *N*-acetylimidazole are shown in Fig. 8. It has already been pointed out (Fig. 5) that at low concentration of *N*-acetylimidazole (25 mM) there was an activation of PsChE and AAA and that this may be due to modification of a class of tyrosine residues not protectable by BCI and therefore not present at the active site. With ethopropazine and imipramine as protectors the activation was abolished and instead inactivation of PsChE and AAA was observed (Fig. 8A). These results indicate that the non-active-site tyrosine residues were protected from modification by ethopropazine and imipramine and therefore all the *N*-acetylimidazole was exclusively utilized for the modification of the active-site tyrosine (and possibly lysine) residues resulting in the inactivation of the enzyme. These

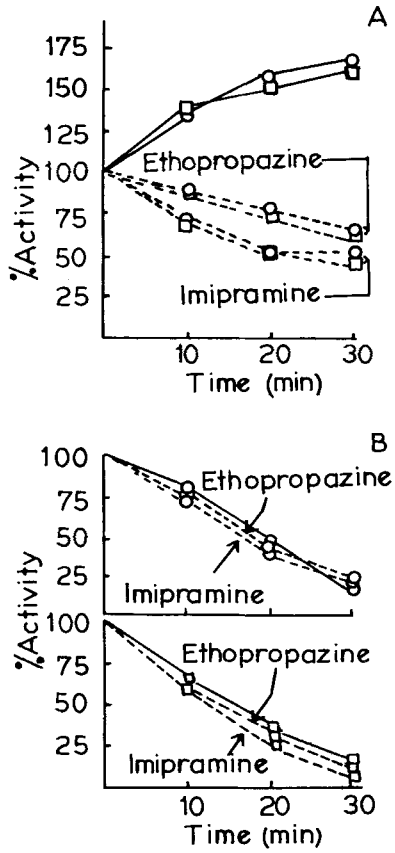

Fig. 8. *Changes in activity of PsChE (○) and AAA (□) as a function of time by modification with (A) 25 mM or (B) 200 mM* N*-acetylimidazole in the absence (———) and the presence (– – – –) of ethopropazine or imipramine as protectors during modification.* Details of the protection procedure and assay are given under Methods. There was no change in control enzyme activity (100%) up to 30 min of incubation

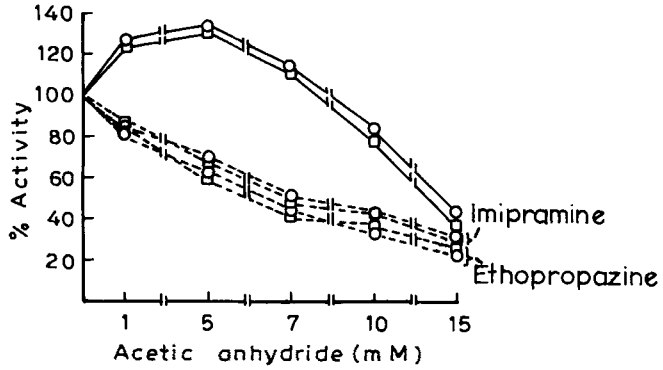

Fig. 9. *Effect of increasing amounts of acetic anhydride as modifier on PsChE (○) and AAA (□) activities in the absence (———) or presence (– – – –) of ethopropazine or imipramine as protectors during modification.* Details of the protection procedure and assay are given under Methods. The control enzyme activity at zero concentration of the modifier is taken as 100%

conclusions were further confirmed when it was found that ethopropazine and imipramine could not protect against the inactivation of PsChE and AAA at high concentration (200 mM) of *N*-acetylimidazole (Fig. 8B). These results indicated that ethopropazine and imipramine were capable of selectively protecting the non-active-site tyrosine residues from acetylation by *N*-acetylimidazole.

A further line of evidence to suggest that ethopropazine and imipramine bind to the non-active-site tyrosine residues rather than the active-site tyrosine residues came from modification studies using acetic anhydride. Fig. 9 shows modification by acetic anhydride at different concentrations in the absence and pressure of ethopropazine and imipramine. In the presence of either ethopropazine or imipramine a progressive fall in both PsChE and AAA activities was observed, suggesting the selective protection of the non-active-site tyrosine residues by the inhibitors from acetylation and the consequent availability of the modifier exclusively for the acetylation of the active-site tyrosine (and possibly lysine) residues.

Rates of PsChE activity changes by the various modifiers

The rates of activity changes were studied at three or four time intervals at three different concentrations of each of the following modifying agents: *N*-bromosuccinimide, diethylpyrocarbonate, *N*-acetylimidazole, $(NO_2)_3PhSO_3$ and phenylglyoxal. Semilogarithmic plots of residual activity against time were linear indicating pseudo-first-order rate kinetics up to 70–80% inactivation by *N*-bromosuccinimide, diethylpyrocarbonate, $(NO_2)_3PhSO_3$ and high concentrations (above 200 mM) of *N*-acetylimidazole (data not shown). A slower rate of inactivation was observed beyond 70% inactivation in the case of *N*-bromosuccinimide and beyond 80% inactivation in the case of diethylpyrocarbonate. The rate of activation by phenylglyoxal and by low concentrations of *N*-acetylimidazole (below 20 mM) also followed a pseudo-first-order process up to about 170% activity in the case of phenylglyoxal and 140% activity in the case of *N*-acetylimidazole. A slower rate of increase beyond this limit was observed for each of these two modifiers.

SDS-gel electrophoresis of the modified and unmodified PsChE

SDS-gel electrophoresis of the unmodified enzyme after reduction by 2-mercaptoethanol showed the predominance of the monomeric form of PsChE (M_r 92000) (Fig. 10). Under non-reducing conditions, SDS gel electrophoresis gave both the monomer and the dimer (M_r 183000) [5, 31]. Samples of enzyme modified by the various reagents were subjected to SDS-gel electrophoresis under non-reducing conditions. All of them gave the dimer and monomer like the unmodified enzyme and there was no evidence of a complete conversion to the monomer subunit in any of the modified enzymes (Fig. 10).

DISCUSSION

The present studies were undertaken to delineate the active-site amino acid residues involved in the PsChE and AAA activities of the bifunctional PsChE enzyme from human serum. Either one specific reagent or more than one have been used for the modification of each amino acid in the purified enzyme. In all cases either spectral studies or other supporting evidence has been presented to exclude the possibility of the involvement of alternative amino acids during modification. BCI, the substrate of PsChE, has been used as a protective agent during modifications in order to identify whether the modified amino acid belongs to the active

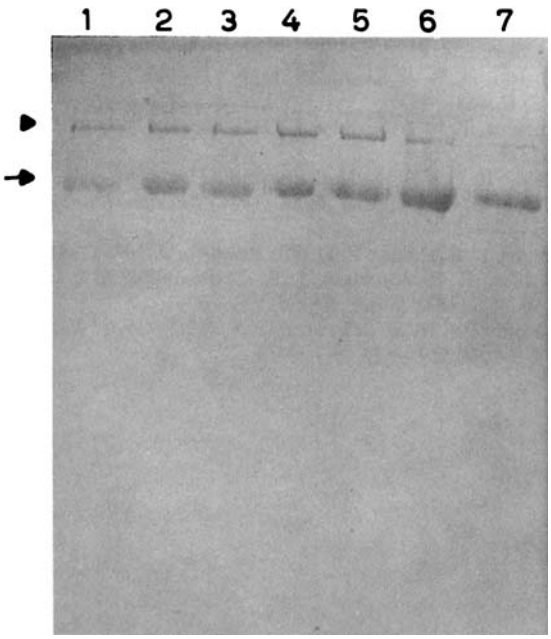

Fig. 10. *SDS slab gel electrophoresis of the modified and unmodified enzyme preparation (8 μg) in the presence of 1% SDS).* Lane 1–5 are the unreduced modified enzyme samples, modified by (1) 10 mM HNBB for 30 min, (2) 10 mM diethylpyrocarbonate for 15 min, (3) 20 mM $(NO_2)_3PhSO_3$ for 30 min, (4) 20 mM phenylglyoxal for 30 min and (5) 200 mM *N*-acetylimidazole for 20 min. Details of modification are given under Methods. Lane (6) is unmodified enzyme without 2-mercaptoethanol and lane (7) unmodified enzyme with 2-mercaptoethanol. Arrows ▶ and ⟶ respectively indicate the dimeric and monomeric form of the enzyme

site or not. It is to be noted that modification of amino acids in an enzyme may alter not only the binding and hydrolysis of the substrate but also result in conformational changes that in turn can influence the catalytic activity. Moreover the actual participation of the amino acids in catalysis may involve a more complex mechanism than that which is suggested by the amino acid modification studies alone [36]. Nevertheless, a chemical modification study of the kind described here involving several modifying agents for the different amino acids and results complementing each other provides valuable information on the amino acid residues involved in catalytic activity.

Of the four amino acids, *viz* tryptophan, histidine, lysine and arginine, modification of the first three resulted in the inactivation of both PsChE and AAA, suggesting that these amino acids may be present at the active site of both enzymes. BCI, the substrate for PsChE, could prevent both PsChE and AAA inactivation resulting from modification of these three amino acids, suggesting that tryptophan, lysine and histidine may be present at a common active site of PsChE and AAA. Arginine modification, however, resulted in the activation of PsChE (that could be prevented by BCI) without affecting AAA. This suggested that the modifiable arginine residue present at the active site of PsChE was not involved in the active site of AAA. Altough unusual, arginine modification resulting in the activation of an enzyme is known [37]. The collective results thus suggest non-identical but overlapping active sites for PsChE and AAA.

Tyrosine modification by three different reagents, namely *N*-acetylimidazole, tetranitromethane and acetic anhydride, provides evidence for the existence of one or more tyrosine residues that are readily susceptible to modification resulting in the activation of both PsChE and AAA and that do not seem to belong to the active site of PsChE and AAA. Experiments leading to such a conclusion are described in detail in Results. More interestingly, ethopropazine and imipramine, two reversible inhibitors of PsChE, could protect the non-active-site tyrosine residues against modification by *N*-acetylimidazole and acetic anhydride, indicating that the binding site of these inhibitors involves the non-active-site tyrosine residues.

Modification of tyrosine residues has been reported to result in the activation of the esterase activity of a number of proteases like chymotrypsin, trypsin, carboxypeptidase A, pepsin, elastase and thrombin [8–12]. This enhancement in activity has been attributed to the active-site tyrosine residues in carboxypeptidase A and secondary-site tyrosine residues in pepsin [12, 38]. It remains to be seen whether modification of the non-active-site tyrosine residues in human serum PsChE that results in an increase in the PsChE and AAA activity would also result in an enhancement of its reported protease activity.

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