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Choline Acetyltransferase and Acetylcholinesterase: Evidence for Essential Histidine Residues†

Robert Roskoski, Jr.

ABSTRACT: Choline acetyltransferase (EC 2.3.1.6) catalyzes the biosynthesis of acetylcholine according to the following chemical equation: acetyl coenzyme A + choline ⇌ acetylcholine + coenzyme A. Ethoxyformic anhydride inactivates the enzyme prepared from bovine brain. Acetyl coenzyme A and coenzyme A, but not choline or acetylcholine, substantially protect against inactivation. The enzyme is reactivated by hydroxylamine treatment. The apparent pK_a of the reactive group is about 6.5. Ethoxyformic anhydride also inactivates, and hydroxylamine reactivates, the partially purified electric eel acetylcholinesterase (EC 3.1.1.7). High concentrations of acetylcholine substantially protect against inactivation. The apparent pK_a of the reactive group is about 6.1. Inhibition by ethoxyformylation which is reversed by hydroxylamine treatment provides evidence that histidine plays a role in the choline acetyltransferase and acetylcholinesterase reactions.

Acetylcholine is an established neurotransmitter at the vertebrate neuromuscular junction and a probable, but not proven, transmitter in the vertebrate central nervous system (*cf.* Iverson, 1970). Choline acetyltransferase (EC 2.3.1.6) catalyzes the following reversible reaction: acetyl coenzyme A + choline ⇌ acetylcholine + coenzyme A. Several studies support the notion of an essential enzymic sulfhydryl group. For example, thiol reagents inhibit choline acetyltransferase from squid head ganglia (Reisberg, 1954), primate placenta (Schuberth, 1966), torpedo (Morris, 1967), and mammalian brain (Potter *et al.,* 1968; Chao and Wolfgram, 1973; Roskoski, 1974a). Experiments with the bovine brain transferase suggest that an active site -SH reacts with acetyl coenzyme A to form an acetyl-thioenzyme intermediate (Roskoski, 1973, 1974a). This alleged thio ester intermediate, isolated by Sephadex gel filtration, further reacts with choline to form acetylcholine.

Thio ester intermediates are also associated with the glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) and papain (EC 3.4.4.10) reactions (Harris *et al.,* 1963; Lowe and Williams, 1965). Crystallographic structural analysis of the lobster muscle dehydrogenase (Buehner *et al.,* 1973, 1974) and papain (Drenth *et al.,* 1968) reveal a histidine residue in close proximity to the active site -SH. The interaction of the imidazole with the sulfhydryl and with the substrates may be important in the catalytic mechanism of these enzymes. These findings prompted a study of the effects of bromoacetyl coenzyme A and bromoacetylcholine, possible alkylating reagents, on choline acetyltransferase. These studies failed to implicate histidine; instead, the enzyme was inhibited by bromoacetylation of the active site sulfhydryl (Roskoski, 1974b).

In the present studies ethoxyformic anhydride inactivation and reversal by hydroxylamine treatment implicate histidine in the choline acetyltransferase and acetylcholinesterase (EC 3.1.1.7) enzyme reactions. Furthermore, both enzymes are inhibited by *N*-acetylimidazole and their activity spontaneously returns to control values within 1 hr. These results are consistent with the hypothesis that choline acetyltransferase and acetylcholinesterase are inhibited by chemical modification of enzymic histidine residues.

Experimental Section

Materials. Ethoxyformic anhydride and *N*-acetylimidazole were purchased from Sigma Chemical Co. Electric eel acetylcholinesterase (1058 units mg^{-1}) was a product of Worthington Biochemical Corp. Acetylthiocholine chloride was purchased from Pfaltz and Bauer, Inc. Decamethonium bromide was purchased from K and K Laboratories, Inc., and indoxyphenyl acetate, Calbiochem.

Methodology for Chemical Modification. For reaction with choline acetyltransferase, ethoxyformic anhydride, dissolved in absolute ethanol, was added to give the specified concentration of inhibitor. Ethoxyformic anhydride and *N*-acetylimidazole were dissolved in acetonitrile for the other experiments. These solutions were prepared immediately before use. A 1-μl solution of inhibitor was added to 100 μl of enzyme solution to initiate the reaction unless specified otherwise. Solvent alone was added to the control samples.

Acetylcholinesterase Assay. The spectrophotometric

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TABLE I: Ethoxyformic Anhydride Inhibition of Choline Acetyltransferase.[a]

Concn (μM)	Enzyme Activity (pmol/5 min)	Concn (μM)	Enzyme Activity (pmol/5 min)
0	94.2	50	7.5
10	64.1	100	3.8
25	17.9	1000	0

[a] The enzyme extract (20 μg of protein) was incubated 5 min at 37° in buffer C (50 mM potassium phosphate–100 mM KCl–0.1 mM EDTA (pH 7.4)) with the specified concentration of reagent in 100 μl. Then 10-μl aliquots were assayed for choline acetyltransferase activity as previously described (Roskoski, 1973).

TABLE II: Effect of Substrates on Ethoxyformic Anhydride Inhibition of Choline Acetyltransferase.[a]

Addition	Concn	Enzyme Activity (% Control)
None		29
Acetyl coenzyme A	15 μM	94
Coenzyme A	15 μM	62
Acetylcholine	2 mM	28
Choline	2, 10 mM	31

[a] The experiment was carried out as described in Table I except that the enzyme was preincubated 5 min at 37° with the specified substrate prior to the addition of ethoxyformic anhydride (25 μM final). The control value was 88 pmol/5 min. The apparent K_m values were previously reported: acetyl coenzyme A, 15 μM; choline, 0.75 mM; coenzyme A, 20–200 μM; acetylcholine 1–5 mM (Roskoski, 1974a).

procedure of Ellman and coworkers (1961) was used. Aliquots of enzyme (to 20 μl) were added to 300 μl of 0.1 M potassium phosphate (pH 7.0), 2 mM acetylthiocholine, and 0.25 mM 5,5′-dithiobis(2-nitrobenzoic acid). The absorbance changes at 412 nm (1-cm path length) were followed on a Beckman 25 spectrophotometer. With indoxylacetate and *p*-nitrophenyl acetate as substrates, assays were carried out using the methodology of O'Brien (1969) except that the volumes were scaled down to 300 μl. Esterase assays were performed at ambient temperature.

Preparation of the bovine brain choline acetyltransferase and the radiochemical enzyme assay were described previously (Roskoski, 1973). Sources of other materials are previously documented (Roskoski, 1973, 1974a).

Results

General Characteristics of Ethoxyformic Anhydride Inhibition of Choline Acetyltransferase. Ethoxyformic anhydride proved to be a potent inhibitor of transferase activity (Table I). A 50 μM concentration inhibits activity 92% under standard experimental conditions (37°, 5 min, pH 7.4). Although the reaction conditions differ, concentrations used for the ethoxyformylation of creatine kinase (Pradel and Kassab, 1968), pepsin, and pancreatic RNase (Melchoir and Fahrney, 1970) are three to five orders of magnitude greater than those reported here. The inactivation is proportional to the reagent concentration and exhibits good pseudo-first-order kinetics during the 5-min reaction. Since the inhibitor hydrolyzes with a $t_{1/2}$ of about 27 min at pH 7 (Melchoir and Fahrney, 1970), these kinetics do not obtain during long incubations.

The effects of the substrates on the inactivation are shown in Table II. Acetyl coenzyme A and coenzyme A substantially protect against ethoxyformic anhydride inhibition. On the other hand, choline and acetylcholine do not protect.

Hydroxylamine Reversal of Ethoxyformic Anhydride Inactivation. To identify the amino acid residue associated with ethoxyformic anhydride inhibition, the effect of hydroxylamine on enzyme inactivated with 75 μM ethoxyformic anhydride was tested. Treatment of the inhibited transferase with 20 mM hydroxylamine for 5 and 30 min, followed by dialysis for 1 hr to remove NH_2OH, reactivates the enzyme 72 and 96%, respectively. Similar treatment with 100 mM NH_2OH for 5 min reactivates the enzyme 98%. These results are consistent with the hypothesis that enzyme inhibition is associated with ethoxyformylation of an enzymic histidine residue (Melchoir and Fahrney, 1970; Burstein *et al.*, 1974).

Determination of the pK_a of the Reactive Residue. The pH dependence of the ethoxyformic anhydride inhibition of the choline acetyltransferase was measured after a 5-min incubation. The apparent first-order rate constants (K_{app}) were calculated using the methodology of Burstein and coworkers (1974) (Table III). A pK_a of 6.5 ± 0.3 was calculated from the slope of such a plot using the data given in Table IV. The experiment was performed in quadruplicate.

Ethoxyformic Anhydride Inhibition of Acetylcholinesterase. Because of the evidence supporting the contention of essential histidine residues in acetylcholinesterase (*cf.* Cohen and Oosterbaan, 1963), ethoxyformic anhydride inactivation of the electric eel enzyme was tested. Incubation with 0.35 mM ethoxyformic anhydride for 5 min at 23° (pH 7.0) inhibits enzyme activity 50%. Similar results are observed using the neutral substrates indoxyl acetate and *p*-nitrophenyl acetate. Inactivation is proportional to reagent concentration, and good pseudo-first-order kinetics obtain during the 5-min reaction.

The rate of acetylcholinesterase inhibition was measured in the presence of several substrates and inhibitors. Compounds which bind to the substrate anionic site (Froede and Wilson, 1971) including choline (40 mM), thiocholine (40 mM), and tetramethylammonium ion (40 mM) failed to protect the enzyme against ethoxyformic anhydride inactivation. Decamethonium (2×10^{-5} M; $K_i = 10^{-5}$ M) and *d*-tubocurarine (8×10^{-5} M; $K_i = 3 \times 10^{-5}$ M), compounds which bind to a postulated peripheral anionic site, distinct from the substrate anionic site (Krupka, 1966; Mooser and Sigman, 1974), also failed to alter the rate of enzyme inhibition. Acetylcholine (40 mM) and acetylthiocholine (40 mM), on the other hand, protected more than 90% against enzyme inactivation.

To establish the identity of the residue associated with inactivation, hydroxylamine reversibility was tested. Incubation of 1.7 μg of acetylcholinesterase (100 μl) with ethoxyformic anhydride (0.50 mM) for 5 min decreases activity to

TABLE III: pH Dependence of Ethoxyformic Anhydride Inhibition of Choline Acetyltransferase.[a]

pH	K_{app} (min^{-1})	pH	K_{app} (min^{-1})
5.7	0.019	7.2	0.079
6.2	0.041	7.7	0.086
6.7	0.062		

[a] The ethoxyformic anhydride treatment (20 μM) was carried out as described in Table I except that 10 mM phosphate was used at the specified pH. To adjust the pH after the 5-min incubation, aliquots (20 μl) of 0.3 M potassium phosphate (pH 7.4) were added to the solution, and transferase activity was measured in 10-μl samples (Roskoski, 1973). The rate constants and pK_a were calculated by the procedure of Burstein and coworkers (1974).

TABLE IV: pH Dependence of Ethoxyformic Anhydride Inhibition of Acetylcholine Esterase.[a]

pH	K_{app} (min^{-1})	pH	K_{app} (min^{-1})
6.2	0.211	7.2	0.460
6.7	0.335	7.7	0.517
7.0	0.420		

[a] Acetylcholinesterase (80 μg in 100 μl of 5 mM potassium phosphate–100 mM KCl) was incubated with 0.75 mM ethoxyformic anhydride for 2.5 min at 23° at the specified pH. Then 20-μl aliquots were assayed as described in the Experimental Section. The rate constants and pK_a were determined by the procedure of Burstein *et al.* (1974).

30% of control. Aliquots of the enzyme were incubated with 20 mM NH_2OH for 5 and 30 min. Enzyme activity was 60 and 92% of the control after this treatment. Dilution of the enzyme reaction mixture 50-fold prior to assay prevents significant hydroxylaminolysis of acetylthiocholine thereby eliminating adventitious thiocholine generation. The pH dependence on the rate of inactivation was measured (Table IV). From these data, using the methodology of Burstein *et al.* (1974), the pK_a of the reactive group is 6.1 ± 0.3.

N-Acetylimidazole Inhibition of Choline Acetyltransferase and Acetylcholinesterase. Reaction of *N*-acetylimidazole with polypeptidic histidine and cysteine produces adducts which undergo spontaneous hydrolytic regeneration. Dilute hydroxylamine treatment, however, is necessary to cleave *O*-acetyltyrosine formed during the *N*-acetylimidazole reaction (Pontremoli and coworkers, 1966).

N-Acetylimidazole (1.0 mM) treatment of choline acetyltransferase for 10 min at 23° (pH 6.7) inactivates the enzyme 40%. Enzyme activity spontaneously returns to control values within 1 hr. Acetyl coenzyme A (10 μM) fully protects against inactivation. Similarly, after *N*-acetylimidazole (0.25 mM) inhibition of acetylcholinesterase (23°, 10 min, pH 7.0), enzyme activity spontaneously reactivates from 52 to 96% within 1 hr. Acetylcholine (40 mM) almost completely protects against this inactivation. The failure of Ellman's and other thiol reagents to inactivate acetylcholinesterase suggests that *N*-acetylimidazole inhibition is unrelated to cysteine modification with spontaneous reactivation. Although an active site thiol has been postulated for choline acetyltransferase, acetylation of this group leads to a chemically competent enzyme which undergoes deacetylation in the presence of acceptor choline (Roskoski, 1973; 1974b). This deacetylation, which involves a single turnover, is assumed to occur rapidly. Therefore, the *N*-acetylimidazole inhibition of choline acetyltransferase probably involves a residue other than the active site -SH group. Spontaneous reactivation is consistent with the hypothesis that histidine is the chemically modified residue in these enzymes.

Discussion

Ethoxyformic anhydride inhibits choline acetyltransferase and acetylcholinesterase. Reactivation by hydroxylamine suggests that histidine is the chemically modified residue (Melchoir and Fahrney, 1970). Guanidino, α- and ϵ-amino, (Melchoir and Fahrney, 1970), and phenolate groups (Burstein *et al.*, 1974) are not regenerated by hydroxylamine treatment under the conditions of the present experiments. Although these functional groups may react with ethoxyformic anhydride, hydroxylamine reversal indicates that such chemical modification is not inhibitory. The reactive sulfhydryl groups of arginine and creatine kinase (Pradel and Kassab, 1968) and glyceraldehyde-3-phosphate dehydrogenase (Ovádi and Keleti, 1970) are not modified by ethoxyformylation. Although acetylcholine protects against thiol reagent inactivation of choline acetyltransferase (1974a), it fails to protect against ethoxyformic anhydride inactivation, indicating that modification of the active site sulfhydryl is not the basis of the latter inhibition. The characteristics of the ethoxyformylation of the active site serine hydroxyl of α-chymotrypsin (Melchoir and Fahrney, 1970) differ substantially from the type of inhibition observed for acetylcholinesterase reported here. For example, the rate of spontaneous hydrolysis and reactivation at pH 7 of α-chymotrypsin and acetylcholinesterase are 29 min and about 48 hr, respectively. Secondly, hydroxylamine reversal of the inhibited chymotrypsin requires a few seconds and that of acetylcholinesterase, 45 min. These experiments support the notion that inhibition of the acetylcholinesterase is associated with histidine, and not serine, modification.

The pseudo-first-order kinetics of inhibition suggests that a modification of a single residue is inhibitory. Experiments with highly purified enzymes, however, are required to rigorously establish this hypothesis (Burstein and coworkers, 1974). The apparent pK_a of each reactive residue (6.5 for choline acetyltransferase and 6.1 for acetylcholinesterase) is reasonable for an enzymic histidine (Tanford and Hauenstein, 1956). Because enzymes of only 10% purity were used in the present experiments, spectral confirmation of histidine modification was unfeasible (Burstein *et al.*, 1974). *N*-Acetylimidazole inhibition and spontaneous reactivation provide additional indirect evidence for the involvement of an enzymic histidine.

The inhibition of enzyme activity by chemical modification of histidine does not establish that the imidazole is involved in the chemistry of the catalytic process. The modification may, for example, alter the tertiary structure of the enzyme. That the reactive histidine forms a part of the active site of choline acetyltransferase is indicated by the finding that acetyl coenzyme A and coenzyme A substantially protect against inhibition. In the case of acetylcholinester-

ase, high concentrations of acetylcholine also protect. Substances which bind to the substrate anionic site (choline, tetramethylammonium ion) and to the peripheral anionic site (decamethonium and *d*-tubocurarine), on the other hand, do not alter the rate of inactivation. That the residue modified by ethoxyformylation is unrelated to binding cationic substrates is also substantiated by the finding that the rate of enzymic hydrolysis by uncharged substrates (*p*-nitrophenyl acetate and indoxyl acetate) is decreased to the same extent as that of acetylthiocholine. This is in contrast to the bovine erythrocyte acetylcholinesterase inhibition by aziridinium substances where acetylcholine hydrolysis is inhibited 93% and *p*-nitrophenyl acetate hydrolysis is inhibited only 48% (O'Brien, 1969).

The acetylcholinesterase reaction proceeds by a two-step chemical mechanism (*cf.* Froede and Wilson, 1971). The active site serine –OH reacts with acetylcholine to form an acetyl–enzyme intermediate. Water then reacts with the intermediate to form acetate and the regenerated enzyme. Histidine has previously been implicated in the enzyme mechanism (*cf.* Cohen and Oosterbaan, 1963). The evidence supporting this contention is largely based on pH dependence of the enzyme reaction with substrate (Mounter *et al.*, 1957; Krupka, 1966) and inhibitors such as 2,4-dinitrofluorobenzene and diazo compounds (Mounter *et al.*, 1957). The present experiments provide additional evidence for a role of histidine in the acetylcholinesterase reaction.

In addition to possible structural and binding roles, histidine may play two other, and not mutually exclusive, roles in the acyl transfer reactions of choline acetyltransferase and acetylcholinesterase. First, it may enhance the reactivity of the respective active site nucleophiles (–SH in the former and –OH in the latter). Second, it may function as a general acid-base catalyst by donating or accepting protons from substrates or fragments thereof. Although transient acetylimidazoles have not been exhaustively excluded from the catalytic mechanisms (Cohen and Oosterbaan, 1963), there is little, if any, direct support for this contention. In the case of choline acetyltransferase, the alleged acetyl–enzyme intermediate is isolable by Sephadex gel filtration. The bond between the acetyl group and enzyme is stable in 10% trichloroacetic acid (90°, 20 min), but is readily cleaved by dilute alkali (pH 10) (Roskoski, 1973). This is not consistent with an acetylimidazole bond, which is unstable in acid and alkali, and even undergoes hydrolysis at pH 7 with a $t_{1/2}$ of 2 hr (Jencks and Carriuolo, 1959). The postulated acetyl–enzyme bond is cleaved by performic acid oxidation and these chemical characteristics are those of a thio ester (Roskoski, 1973). Further experiments are required to show that the rate constants for the formation and further reaction of the postulated thio ester intermediate are adequate to account for the observed rate of the enzyme reaction.

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