Acetylcholinesterase: Inhibition by Tetranitromethane and Arsenite

**BINDING OF ARSENITE BY TYROSINE RESIDUES**

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Tetranitromethane inhibits acetylcholinesterase with respect to the hydrolysis of both acetylthiocholine and indophenyl acetate. The loss of activity with indophenyl acetate, a poor substrate, is preceded by an increase in enzyme activity. Only 12 of the 21 tyrosine residues/monomer of enzyme are susceptible to nitration. Loss of activity with respect to indophenyl acetate occurs well after no further nitration of tyrosines occurs and must be due to the modification of other residues. Incubation of the enzyme with arsenite before nitration results in the nitration of only 10 tyrosines. This experiment reveals that the structural basis for the binding of arsenite is the formation of a diester with two tyrosine residues.

TNM$^1$ has been used as an agent for the modification of tyrosine residues in enzymes (1–5). Nitration of tyrosine at the 3-position is the modification seen under mild conditions (1), but modification of tryptophan, methionine, and cysteine has also been reported (6–10). The reaction of TNM with acetylcholinesterase, EC 3.1.1.7, has been shown to cause loss of enzyme activity toward ATCh as substrate but not toward the poor substrate IPA (5). It was also found that 2-PAM accelerated the loss of enzyme activity (5).

Arsenite (arsenous acid at pH < 9.2) is a well known and potent inhibitor of enzymes that contain lipoic acid; the mechanism of inhibition is the formation of a cyclic dithioarsenic diester (11).

It is remarkable that acetylcholinesterase is also inhibited readily by arsenite (12–14) even though this enzyme contains no lipoic acid and no sulphydryl groups (15). The inhibition is quasi-reversible in the sense that removal of free arsenite restores activity only very slowly at neutral pH, with a first order rate constant of $1.9 \times 10^{-2}$ min$^{-1}$ (10). Inhibition is also slow with a second order rate constant of $140 \text{ M}^{-1}\text{ min}^{-1}$ so that with $10^{-3} \text{ M} \text{ arsenite}$ the half-time for the reaction is about 5 min. The dissociation constant is $1.5 \times 10^{-4}$ which is comparable to the value for lipoic acid containing enzymes. A number of compounds, including 2-PAM, very greatly increase the rate of inhibition and dissociation with little change in the dissociation constant (14).

The structural basis for the binding of arsenite is unknown. We might speculate that a diester or triester is formed with adventitiously located hydroxyl groups (14), and diesters are formed spontaneously between arsenite and simple compounds that contain neighboring hydroxyl groups (16, 17). One of the most stable complexes is formed with $a$-catechol which raises the possibility that the tyrosines of acetylcholinesterase might be involved. However, this complex is very much weaker than the arsenite complex with acetylcholinesterase. Although simple diols form more stable complexes with borate than with arsenite, borate does not inhibit the enzyme nor interfere with its inhibition by arsenite (14, 18). Even so, the formation of a diester (or triester) appeared to be the most attractive possibility. We, therefore, decided to investigate whether one or more tyrosines are esterified by arsenite by studying the effect of arsenite on the nitration of the tyrosine residues of the enzyme. We can predict that if a diester should be formed, the two tyrosines involved will not be nitrated by TNM because only free phenols can be nitrated at a measurable rate. The reason for this is that there must be a high electron density at the ortho position, and this can be obtained only in the phenolate anion. The phenolate anion is the actual reactant (1, 19). Thus by measuring the number of tyrosines that are nitrated in the presence and absence of arsenite, we can tell whether an arsenite ester is formed. We found that 12 of the 21 tyrosines/catalytic subunit were nitrated by TNM but only 10 in the presence of arsenite demonstrating that diester is formed with arsenite and two tyrosine residues.

**METHODS AND RESULTS**

Our measurements of the inhibition of acetylcholinesterase by TNM are essentially in agreement with previous work of others (5). Our rate of inhibition with respect to ATCh hydrolysis is rather faster, and we have noted an initial rise in activity toward IPA (Fig. 1). Activity toward IPA does not actually decrease until considerable time has elapsed so that 50% inhibition occurs at 160 min as compared to 30 min for ATCh hydrolysis. As reported by Fuchs et al. (5) we find that 2-PAM greatly increases the rate of ATCh inhibition; 0.1 and 0.5 mM 2-PAM increased the rate by a factor of 20. At higher concentrations of 2-PAM the increase in rate was less.

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Fig. 1. Activity of acetylcholinesterase toward ATCh and IPA as substrates during nitration. Samples of 2 \times 10^{-4} \text{ M} acetylcholinesterase were inhibited by 10 mM TNM at pH 8.0, 25 °C, 50 mM glycylglycine, 50 mM NaCl. Activity of the enzyme at various times during nitration was measured. \( \Delta \), ATCh as substrate; \( \times \), IPA as substrate. All points are from duplicate experiments except for IPA at 5, 30, and 160 min which are single measurements.

Fig. 2. Loss of activity and tyrosine residues during nitration with and without arsenite preincubation. Samples of 2 \times 10^{-4} \text{ M} acetylcholinesterase were treated with TNM at pH 8.0, 25 °C, 50 mM glycylglycine, 50 mM NaCl. Aliquots were taken over a time course and both activity, using ATCh as substrate, and tyrosines were determined, as described under “Methods.” \( \Delta \), enzyme activity; \( \bigcirc \), enzyme activity with incubation in 5 mM arsenite prior to TNM treatment; \( \bigcirc \), tyrosine residues; \( \bigcirc \), tyrosine residues of enzyme incubated with 5 mM arsenite prior to TNM treatment; \( \bigcirc \), enzyme activity of acetylcholinesterase incubated with 5 \times 10^{-4} \text{ M} arsenite and then diluted into 10 mM TNM. TNM was 10 mM without arsenite and 15 mM in the presence of 5 mM arsenite. All points are duplicates.

Inhibition progressed. There were initially 21 tyrosines/monomer, in agreement with 19, 25, and 26 reported in the literature as calculated from the amino acid composition using 80,000 as the subunit molecular weight (29–31).

Our method of calculation is as follows. The enzyme preparation contained 1200 units/ml assayed according to Gordon et al. (32) as described under “Methods.” The turnover number under these conditions is \( 6.0 \times 10^{6} \text{ min}^{-1} \), so that the enzyme concentration (in terms of catalytic sites) is \( 2.00 \times 10^{-8} \text{ M} \). Six samples of 0.05 ml yielded 2.09 ± 0.016 nmol of tyrosine. Therefore, there are 21 tyrosine residues/active site.

The number of tyrosines decreased as TNM inhibition progressed and leveled off at 9 residues, indicating 12 residues are susceptible to nitration (Fig. 2). At this time, 90 min, the enzyme still has 17% activity toward ATCh and 90% activity toward IPA. Further loss in enzyme activity must be due to the nitration or possibly the oxidation of residues other than tyrosine.

To determine the effect of arsenite on the nitration of tyrosine residues we used 5 mM arsenite and increased TNM from 10 to 15 mM to compensate for the slow reaction of TNM and arsenite. Actually, the solubility of TNM is only 50 times into 10 mM TNM. The rate of loss of enzyme activity with incubation in 10^{-10} \text{ M} acetylcholinesterase were treated with TNM at pH 8.0, 25 °C, 50 mM glycylglycine, 50 mM NaCl. All points are from duplicate experiments except for IPA at 5, 30, and 160 min which are single measurements.

Arsenite does slow the rate of inhibition of the enzyme by TNM, about 8-fold (Fig. 2). In order to assay the arsenite-enzyme, the enzyme was freed from arsenite and TNM by dilution into 2-PAM and ascorbic acid at pH 7.0. A similar experiment was done without such a large concentration of arsenite present; the enzyme was reacted with 5 \times 10^{-4} \text{ M} arsenite to inhibit the enzyme almost completely and then diluted 50 times into 10 mM TNM. The rate of loss of enzyme activity was about the same.

Our most important observation was that arsenite protects about 2 tyrosines from nitration. At 60 min there are 1.9 more tyrosines when arsenite is present than when there is no arsenite. This difference slides down to 1.5 residues 2 h later. A decrease in the number of tyrosines protected was expected because as discussed above we anticipated that there would be dissociation of arsenite from the enzyme.

We decided to repeat these experiments on a much shorter time scale by having 0.1 mM 2-PAM present. The rate of loss of ATCh hydrolysis is greatly increased and so too is the rate of loss of tyrosines, but again the number of tyrosines levels off after 8 min and remains constant at 9 residues up to at least 2 h (Fig. 3). The number of susceptible tyrosines is unchanged. In the presence of 5 mM arsenite the number of tyrosines levels off at 11 residues. Although the experiment is neater in the presence of 2-PAM and the results are more clear-cut, the conclusion is the same; arsenite protects two tyrosines by forming a diester with these residues.

In the presence of 2-PAM, arsenite does not protect the ATCh-hydrolyzing activity of the enzyme much, if at all, against inhibition by TNM, and this result is different from the effect of arsenite in the absence of 2-PAM.

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prior to TNM treatment. TNM was 10 mM in the absence of arsenite with incubation in 5 mM arsenite prior to TNM treatment; 0, tyrosine described under "Methods."

The activities of acetylcholinesterase treated with 10 mM TNM in the absence and presence of arsenite, respectively. All points were duplicates except tyrosine ± arsenite at 15 min; these were quadruplicates. The average values for tyrosine residues/subunit after leveling off had occurred (10, 12, and 15 min) were 9.11 ± 0.07 and 11.0 ± 0.04 in the absence and presence of arsenite, respectively.

stable. We measured the arsenite dissociation constant ($K_a$) with untreated enzyme, enzyme that had been nitrated for 180 min, and enzyme that had been nitrated for 180 min with 5 mM arsenite present, using ATCh and IPA as substrates and arsenite as an inhibitor of the remaining enzyme activity. The dissociation constants were the same with both substrates, 1.0 × 10^{-6}, 2.6 × 10^{-6}, and 1.2 × 10^{-5} M (Fig. 4). Thus the "fully" nitrated enzyme still forms a complex with arsenite but it is 2.6 times weaker. The enzyme that has all but two of the susceptible tyrosines nitrated has about the same dissociation constant. Thus the ability of the enzyme to form a diester is little affected by the nitration of the other tyrosines or by modification of other residues.

The proportionality of $E'/E$ (arsenite-enzyme/active enzyme) with arsenite concentration shows that the stoichiometry of the reaction of arsenite and enzyme is 1:1.

We have previously shown that fluoride and arsenite binding are very highly anticooperative and possibly mutually exclusive (18). We, therefore, nitrated the enzyme in the presence of 0.1 mM fluoride and 0.1 mM 2-PAM and measured the number of tyrosines after 5 and 120 min. There were 9 tyrosines, just as before. Thus, fluoride does not protect any tyrosines from nitration.

**DISCUSSION**

The most important result of this study is the discovery that the formation of a diester with two tyrosines is the structural basis for the binding of arsenite by acetylcholinesterase. The formation of these covalent bonds explains the slow reaction of the enzyme with arsenite and the slow dissociation of arsenite. It is not clear why binding is so strong. Perhaps a triester is formed with a third hydroxyl group not derived from tyrosine. Or perhaps the electron-rich arsenic atom forms a hydrogen bond. Another possibility is that the spatial orientation of the two tyrosines is perfect for the formation of an arsenite diester. Parallel stacked tyrosines would be one such orientation.

Our finding that not all the tyrosines but only 12 of 21 are susceptible to nitration is consistent with the nitration of other proteins (1–4) and in general with the modification of residues by selective or partially selective reagents. Again, an enhancement of activity such as we observe with IPA after nitration of acetylcholinesterase is unusual but not rare when proteins are modified. For example, acetylation or iodination of tyrosine in carboxypeptidase inhibits peptidase activity almost completely but increases esterase activity 6-fold (33, 34). Also alkylation of acetylcholinesterase inhibits its hydrolysis of acetylthiocholine by 70% but doubles its activity toward IPA (35). Inhibition of acetylcholinesterase with a diazonium salt also increases activity toward IPA (36).

Our finding that fluoride does not affect the nitration of tyrosine is evidence against the idea that fluoride might form...
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A hydrogen bond with these 2 residues because if it did, we should expect the ionization of the phenols to be decreased and, therefore, the rate of nitrination to be much slower.

REFERENCES


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Supplementary Material to Acetylcholinesterase, Stability to Thermolabile and Argonate Staining of Acetylcholines by Tyrosine Residues

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Methods

Enzyme purification.

Acetylcholinesterase from the electric organ of Electrophorus electricus (E.C. 3.1.1.7) was prepared from tissue in the tyrosylated state with a specific activity of 28000 U/mg protein. The enzyme was extracted in 0.5M potassium phosphate buffer (pH 7.4), and dialyzed against buffer (48 h) to remove phosphate. The enzyme was stored at -10°C.

NADH measurements were performed under standard conditions with 1 nmol of NADH at 22°C. The enzyme was run in triplicate, and the average was taken.

