The rate of thermal inactivation of *Torpedo* acetylcholinesterase is not reduced in the C231S mutant

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Abstract The rate of thermal inactivation of *Torpedo* AChE at pH 8.5 was increased by the sulfhydryl reagent 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). At 30°C or 37°C, inactivation rates with 0.3 mM DTNB increased about 5-fold for the wild-type enzyme and for two site-specific mutants, D72S and V129R. The reversible active site inhibitor, ambenonium, completely stabilized the wild type enzyme and partially stabilized the D72S mutant. However, ambenonium did not protect against the destabilization introduced by DTNB, which still accelerated inactivation of D72S 5-fold. When the only free sulfhydryl group in AChE was removed by replacing cysteine 231 with serine, increased rates of thermal inactivation were observed. The inactivation rate incrased by a factor of 2 to 3 for the single mutant (C231S) and by a factor of 5 for the double mutant V129R/C231S. Even in the C231S mutants, DTNB still had an additional effect. It increased the inactivation rate for C231S and V129R/C231 by a factor of about 1.5 to 3 beyond the rates seen in the absence of DTNB. Therefore, at least part of the destabilization seen with DTNB in enzymes that retain C231 does not involve reaction of DTNB with C231.

Key words: Acetylcholinesterase; Thermal inactivation; Disulfide reagent; Site-specific mutagenesis

1. Introduction

Molecular modeling based on the three-dimensional structure of acetylcholinesterase (AChE) from the electric ray *Torpedo* [1] has predicted a number of residues to be involved in the catalytic mechanism of this enzyme, and site-specific mutagenesis has supported several of these predictions [2–4]. However, a number of questions about the dynamic nature of the catalytic process and the role of a peripheral site at the surface of the deep active site gorge remain to be explored. It is attractive to construct the new mutants required to investigate these questions in *Torpedo* AChE itself and thus to avoid any uncertainties introduced by computer imposition of the *Torpedo* AChE structure on the sequences of AChEs from other species. A problem with this approach is that *Torpedo* AChE is quite susceptible to thermal inactivation, and mammalian cells expressing this AChE require culturing at 27°C to allow significant accumulation of active enzyme [5]. The problem is compounded in site-specific *Torpedo* AChE mutants. Many of these, as illustrated here with D72S and V129R, are less stable than the wild type enzyme. In an effort to identify specific residues that might be involved in the instability of *Torpedo* AChE, we have focused on the inactivation of the enzyme by sulfhydryl reagents targeted to residue C231.

The activity of *Torpedo* AChE has long been known to be sensitive to sulfhydryl alkylating reagents [6–8]. In contrast, AChEs from mammals or the electric eel *Electrophorus* are insensitive to these reagents [9–11]. This difference in sulfhydryl sensitivity is consistent with known AChE sequences. All AChE and butyrylcholinesterase (BChE) sequences contain three conserved intrasubunit disulfide bonds within the catalytic subunits (see [4]) and one or two additional cysteine residues near the C-terminus that are involved in intersubunit disulfide linkage [12–14]. *Torpedo* AChE differs from the mammalian and *Electrophorus* enzymes in containing one more cysteine residue (C231) that is not involved in a disulfide bond in the assembled enzyme and that reacts with sulfhydryl alkylating agents [12,15]. In contrast, mammalian and *Electrophorus* AChEs do not incorporate radioalkylating agents prior to disulfide reduction [13,14,16,17]. Steinberg et al. [8] also showed that 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), a chromogenic disulfide used to titrate the thiocholine hydrolysis product in the widely used assay of AChE developed by Ellman et al. [18], and other disulfide reagents can inactivate *Torpedo* AChE by reacting stoichiometrically to form a mixed disulfide that presumably involves C231 [19].

dIn this report we examine the effects of DTNB on the stability of wild type and mutant *Torpedo* AChEs and describe the consequences of mutating C231 to C231S on the enzyme stability. Part of the inactivating effect of DTNB remains in this mutant, indicating that DTNB has destabilizing effects on *Torpedo* AChE unrelated to mixed disulfide formation with C231.

2. Materials and methods

The pEF-Bos vector [20] expressing the H subunit of *Torpedo* AChE was mutagenized with oligonucleotide primers, and COS-7 cells were transfected as described [5,21]. After incubation first for 24–48 h at 37°C and then for 48 h at 27°C, the cells (10^7 per dish) were washed and extracted at 4°C with 400 μl of 50 mM Tris-HCl, 40 mM $MgCl_2$, 1% Triton X-100, and proteolysis inhibitors (0.1 mg/ml bacitracin, 25 U/ml aprotinin, 1 mM benzamidine, 10 mM EDTA, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin), and the supernatants obtained following centrifugation at 10,000 × *g* were stored at −80°C until use [5].

The conventional assay of AChE activity by the speectrophotometric procedure of Ellman et al. [18] was modified as follows. In Assay 1, an aliquot corresponding to 10 μl of extract containing AChE (5–50 munits) was added to 1.0 ml of pH 8.5 buffer (20 mM Tris-HCl (pH 8.5), 0.1% Triton X-100) with or without DTNB (0.3 mM). After incubation for the indicated time at 37°C (unless otherwise noted), DTNB was added to those samples in which it had been omitted. The reaction was initiated by adding acetylthiocholine (10 μl, to a final concentration of 0.75 mM) and measuring the change in absorbance at 412 nm for 1 min on a Cary 210 spectrophotometer. To measure AChE activities in incubation mixtures containing AChE inhibitors, Assay 2 was intro-

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duced to include an inhibitor dilution step. In Assay 2, an aliquot corresponding to 10 μl of AChE extract was mixed with inhibitor and pH 8.5 buffer with or without 0.3 mM DTNB (0.3 mM) in a total of 55 μl. After incubation for the indicated time at 37°C, 940 μl of 0.75 mM acetylthiocholine and 0.3 mM DTNB in pH 8.5 buffer were added (the same final concentrations as in Assay 1). Rates of inactivation in the concentrated incubation mixture in Assay 2 were an order of magnitude lower t han those in Assay 1 even in the absence of added inhibitor, indicating that components in the cell extracts, probably Mg^{+2}, remain high enough in concentration to exert a stabilizing effect. Enzyme activities are expressed in milliunits (munits), defined as 1 nmol of acetylthiocholine hydrolyzed per minute under the assay conditions and calculated from $\Delta\varepsilon_{412} = 14.15\ nM^{-1} \cdot cn^{-1}$ for thiolate dianion produced in the assay [22].

Inactivation rates were calculated assuming a simple first-order inactivation of AChE activity according to Eqn. 1.

$$v = v_{initial}\, e^{-kt} \quad (1)$$

Observed velocities v were corrected for minor background substrate hydrolysis (< 5% of $v_{initial}$) which remained after complete inactivation of AChE activity.

3. Results and discussion

The conventional Ellman assay of AChE activity utilizes acetylthiocholine as substrate and DTNB as the indicator of the thiocholine hydrolysis product. Preliminary assays of site-specific mutants of *Torpedo* AChE expressed in COS cells revealed that some mutants were unstable in this conventional assay at pH 7.0 and 25°C, although wild type *Torpedo* AChE showed no loss of activity. To compare the stabilities of the wild type and mutant AChEs, a modified assay was introduced in which the enzyme was preincubated in a Tris-HCl buffer (20 mM, pH 8.5) at 37°C with or without DTNB prior to activity determination. As shown in Fig. 1, inactivation of the wild type AChE was observed under these conditions, and the rate of inactivation increased about 5-fold when DTNB was included in the incubation mixture (Table 1). The D72S mutant was less stable than wild type AChE in the absence of DTNB, and it also was inactivated more rapidly than the wild type when DTNB was present (Fig. 1 and Table 1).

As reviewed in section 1, the increased rate of inactivation of wild t ype AChE in the presence of DTNB in Fig. 1 could indicate formation of a mixed disulfide involving C231 and 5-thio-(2-nitrobenzoic acid). To test whether inactivation rates were reduced when a cationic inhibitor was bound to the AChE active site, incubations were conducted in the presence of ambenonium. This specific AChE inhibitor has high affinity for the human AChE active site with a competitive inhibition constant K_i = 0.1 nM [23]. When 2 nM ambenonium was added to incubations of *Torpedo* AChE at pH 8.5 and 37°C, the inactivation rates of the wild type enzyme became insignificant and those of D72S were decreased substantially (Fig. 2 and Table 1). Those observations are consistent with the stabilization of AChE by active site ligands reported previously (e.g. [16]). However, the inactivation rate of D72S in the presence of 0.3 mM DTNB was still about 5 times higher than in its absence (Fig. 2), a difference essentially identical to that for wild type enzyme in the absence of ambenonium in Fig. 1. Thus ambenonium did not protect against the additional destabilization induced by DTNB, an observation that contrasts with a previous report that the inactivation of *Torpedo* AChE by alkylating agents could be inhibited by cationic ligands bound to the

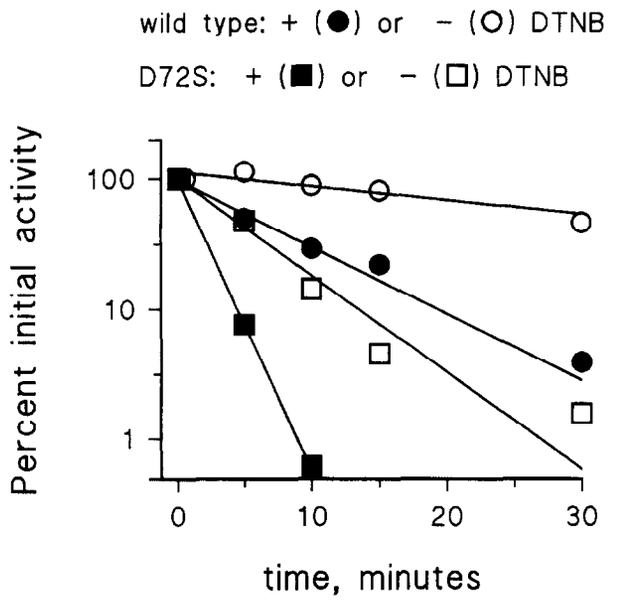

Fig. 1. Inactivation of wild type *Torpedo* AChE and the D72S mutant by incubation in pH 8.5 buffer at 37°C. Samples of wild type AChE with (●) or without (○) 0.3 mM DTNB and D72S with (■) or without (□) 0.3 mM DTNB were incubated for the indicated times prior to measuring AChE activity as outlined in Assay 1 in section 2. Lines were calculated assuming a simple first order inactivation of AChE during the incubation (see eq. 1). Initial activities corresponded to 8 munit of wild type and 29 munit of D72S per assay.

AChE active or peripheral sites [8]. Other active site ligands also can stabilize *Torpedo* AChE activity. For example, inactivation of wild type AChE in the absence of DTNB at 37°C was about four times faster in pH 7.0 sodium phosphate buffer than in pH 8.5 Tris-HCl buffer (data not shown), consistent with some AChE stabilization by cationic Tris bound to the active site.

The examine directly whether the increased rates of inactivation in the presence of DTNB in Figs. 1 and 2 could result from a mixed disulfide involving C231 and 5-thio-(2-nitrobenzoic acid), the site-specific mutant C231S was prepared. This muta-

Table 1
Rates of inactivation of wild type and mutant torpedo AChEs in the presence and absence of DTNB

Mutant	T (°C)	k_{-DTNB} (min^{-1})	k_{+DTNB} (min^{-1})	$\frac{k_{+DTNB}}{k_{-DTNB}}$
wild type[a]	37	0.022 ± 0.003	0.112 ± 0.007	5.2
D72S		0.164 ± 0.025	> 0.5	> 3
C231S		0.083 ± 0.016	0.183 ± 0.005	2.2
wild type[a]	30	0.0016 ± 0.0005	0.0067 ± 0.0020	4.2
C231S		0.0029 ± 0.0015	0.0086 ± 0.0015	3.0
V129R		0.035 ± 0.010	0.154 ± 0.021	4.4
V129R/C231S		0.20 ± 0.05	0.32 ± 0.06	1.6
	Plus 2 nM ambenonium			
wild type	37	< 0.001	< 0.001	
D72S		0.0030 ± 0.0005	0.0156 ± 0.0014	5.2

Inactivation rates in the absence (k_{-DTNB}) or presence (k_{+DTNB}) were measured as outlined in section 2. Assay 1 was used in the absence of ambenonium and Assay 2, in its presence. The temperature (T) refers to the incubation temperature prior to assay.
[a]Average of duplicate measurements.

tion eliminates any possibility that DTNB could form a covalent linkage with a free sulfhydryl group on *Torpedo* AChE. We found that replacement of cysteine 231 by serine increased the rate of thermal inactivation even in the absence of DTNB by a factor of 2 to 3 (Fig. 3). Addition of 0.3 mM DTNB gave a slight further increase in the rate of inactivation of the C231S mutant to a value that was comparable to that of the wild type enzyme in the presence of DTNB (see Table 1). Although small, the further destabilization of C231S by DTNB indicates that at least part of the destabilization of wild type enzyme by DTNB does not involve reaction of DNB with C231.

These points were reinforced by comparison of V129R and V129R/C231S mutants. These mutants were too unstable for good quantitation at 37°C, so the incubation temperatures were reduced to 30°C in pH 8.5 bufrer. As shown in Table 1, conversion of C231 to C231S in the double mutant V129R/C231S increased the rate of inactivation about 5-fold. DTNB still had a destabilizing effect on this double mutant, as in C231S, increasing the inactivation rate by a factor of 1.5 relative to the rate in the absence of DTNB. Therefore, it is again apparent that DTNB induces a small destabilization which cannot result from its reaction with the free cysteine residue.

Destabilization of *Torpedo* AChE by DTNB indicates a reduction in the activation energy for AChE denaturation in the presence of DTNB. According to classical transition state theory, the extent of this reduction is $\Delta G^{\ddagger}_{DTNB}$ and is given by Eqn. 2.

$$\ln(k_{+DTNB}/k_{-DTNB}) = \Delta G^{\ddagger}_{DTNB}/RT \quad (2)$$

For the AChEs with C231 i nTable 1, the average ratio for k_{+DTNB}/k_{-DTNB} is about 5 and thus corresponds to a $\Delta G^{\ddagger}_{DTNB}$ of about 1.0 kcal/mole. Part of this destabilization may involve formation of a mixed disulfide involving C231 and 5-thio-

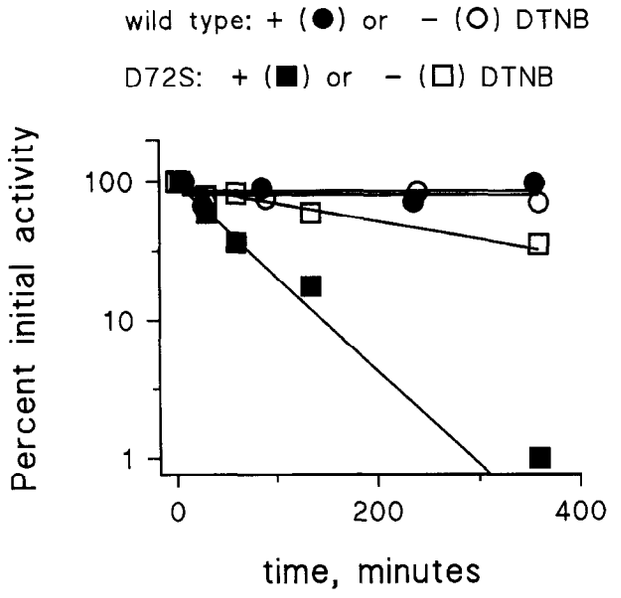

Fig. 2. Ambenonium binding at the active site protects against *Torpedo* AChE inactivation but does not block the destabilization induced by DTNB. Samples of wild type AChE with (●) or without (○) 0.3 mM DTNB and D72S with (■) or without (□) 0.3 mM DTNB were incubated with 2 nM ambenonium in pH 8.5 buffer at 37°C for the indicated times prior to measuring AChE activity as outlined in Assay 2 in section 2. Lines were calculated as in Fig. 1. Initial activities corresponded to 8 munit of wild type and 15 munit of D72S per assay.

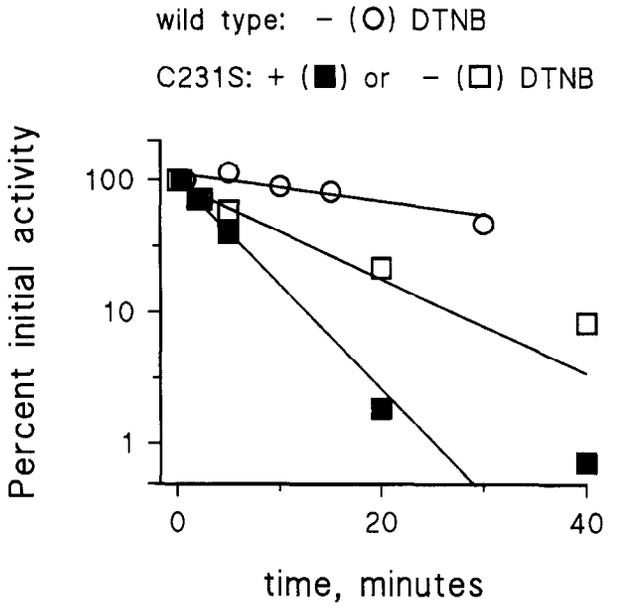

Fig. 3. The mutation C231S destabilizes *Torpedo* AChE and partially but not completely eliminates further destabilization by DTNB. The points for wild type AChE without DTNB (○) were from Fig. 1. Samples of C231S with (■) or without (□) 0.3 mM DTNB were incubated in pH 8.5 buffer at 37°C and analyzed as in Fig. 1. Initial activity corresponded to 16 munit of C231S per assay.

(2-nitrobenzoic acid), but ratios of k_{+DTNB}/k_{-DTNB} still range from 1.6 to 3.0 for the C231S mutants in Table 1 and indicate that between 0.3 and 0.7 kcal/mole of this destabilization cannot involve this mixed disulfide. The molecular basis of this additional destabilization is not clear.

Even though the C231S mutation did not stabilize wild type AChE or double mutants in the presence of DTNB and actually destabilized them in the absence of DTNB, introduction of this mutation into double mutants has been observed to result in higher recoveries of AChE activity in transfected COS cell extracts (data not shown). This result may indicate that C231 can participate in inappropriate disulfide bond formation during AChE folding and that the C231S mutation can eliminate this nonproductive pathway.

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