Biophysical Chemistry 10 (1979) 147–151

INHIBITION OF ACETYLCHOLINESTERASE BY TX-100

D.B. MILLAR, J.P. CHRISTOPHER and W.H. BISHOP
Environmental Biosciences Department, Naval Medical Research Institute, Bethesda, Maryland 20014, USA

Received 26 January 1979

At high detergent concentrations, approximately the equivalent of 2 micelles of TX-100 reversibly bind to acetylcholinesterase and fully inhibit the enzyme. This result suggests that the appropriate lipid environment might regulate this neuronal enzyme's function.

1. Introduction

The etiology of the High Pressure Neurological Syndrome is not understood, but the general opinion is that pressue sensitive sites in neural tissue (membranes) are involved. This is consistent with the idea of lipid–protein interactions being altered by hyperbaric environments [1]. To investigate this possibility, knowledge of the lipid binding characteristics of essential neural proteins is required as a first step. We have recently reported that eel acetylcholinesterase (E.C.3.1.1.7) binds little TX-100 when exposed to the low detergent levels used in tests designed to distinguish integral from nonintegral membrane proteins [1]. At such levels, there was no detectable effect of detergent on enzyme activity.

Here, we present evidence that acetylcholinesterase activity can be almost completely suppressed by high (20%) levels of TX-100. This loss of activity is reversible, does not appear to be a cooperative phenomenon and occurs well beyond the CMC of TX-100 suggesting micellar participation.

2. Methods

E. Electricus 11S acetylcholinesterase was prepared by the method of Christopher et al. [2]. Enzymatic activity was assayed at 20°C by Ellman's method [3] in 0.1 M NaCl, 0.1 M NaP, pH 8.0. The desired level of *TX-100 in the assay* was arrived at by substituting for assay buffer the appropriate volume of TX-100 dissolved in buffer constituents to a final concentration as above. Sedimentation velocity experiments were done at 20°C at a protein concentration of 1.5 mg/ml in a Beckman-Spinco Model E analytical ultracentrifuge. *Apparent* diffusion coefficients were calculated from these sedimentation velocity experiments according to Schachman [4]:

$$D_{app} = (1/4\pi t)(A/H_{max})^2(1 - \omega^2 \cdot St)$$

where A is the area under the Schlieren curve estimated by the trapezoidal rule, H_{max} is the peak height, t is time corrected for time to full speed, and the other symbols have their usual meanings. Molecular weight was calculated from S and D values using the Svedberg equation [5], assuming $\bar{V}$, the partial specific volume of the native, uncomplexed enzyme to be 0.731.

3. Results and discussion

At levels of detergent greater than 1%, enzyme activity was not initially linear with time. However, even at the highest concentration used (~20%), linearity was achieved after six minutes of reaction had occurred. Activity decrease was calculated from the linear portion of such curves and was reproducible within a few percent. Fig. 1 shows the effect of TX-100 on the activity of acetylcholinesterase. It is

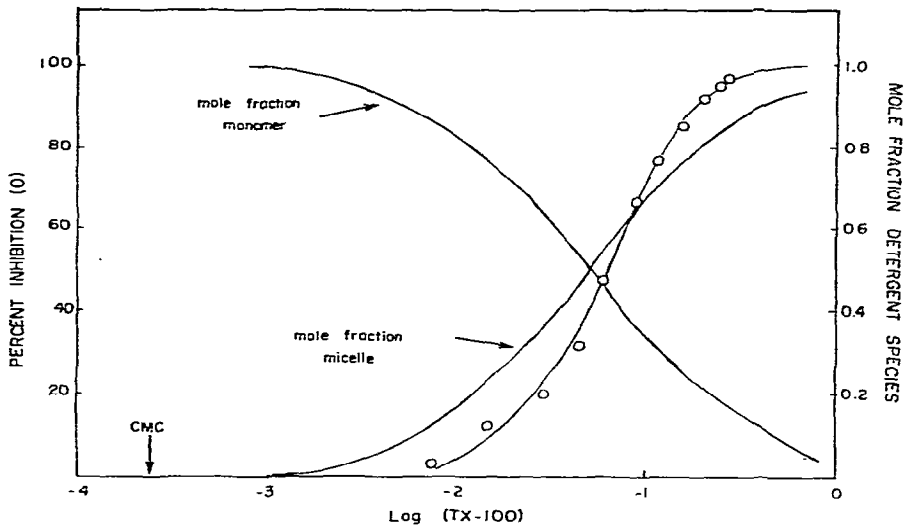

Fig. 1. Inhibition of acetylcholinesterase activity by TX-100. (o) percent inhibition; the line connecting the experimental points is calculated from parameters derived assuming inhibition occurs by micelle enzyme interaction using the appropriate forms of eq. (12). The calculated mole fraction of micelle and monomer are also presented. TX-100 concentration is expressed as monomers. Details in text.

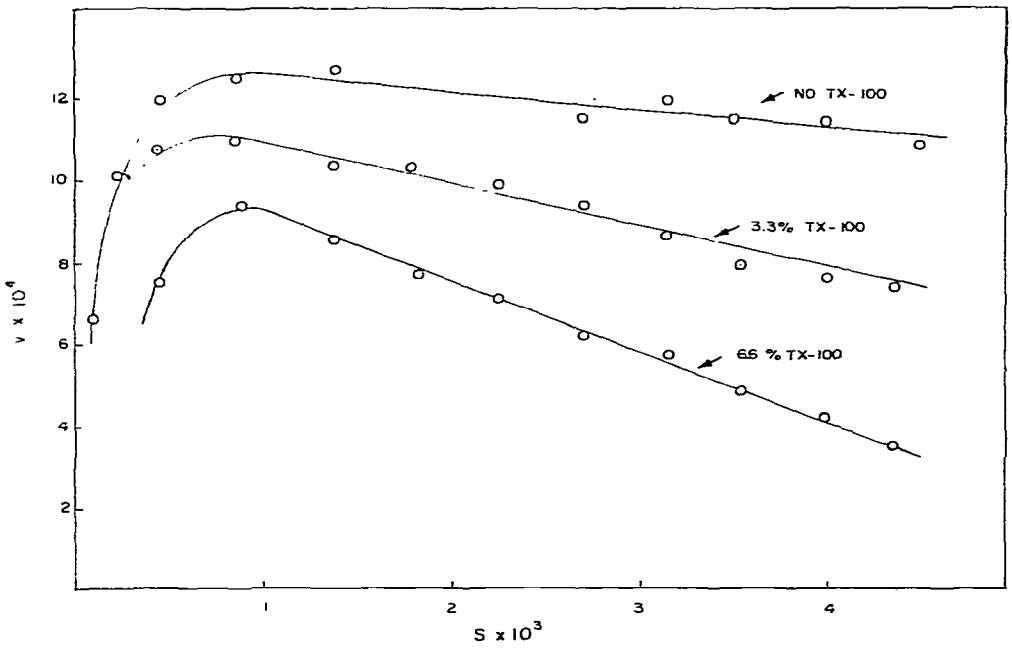

Fig. 2. Substrate inhibition of acetylcholinesterase enzyme activity at various detergent levels. v units, ML^{-1} min^{-1}; S units, ML^{-1}.

apparent that no inhibition takes place until the TX-100 concentration increases well beyond the CMC. The possibility that substrate (acetylthiocholine) or Ellman reagent are sequestered by the hydrophobic interior of the TX-100 micelle and that this is the cause of the apparent inactivation is eliminated by the experiments discussed below. That TX-100 seems to alter the substrate inhibition of acetyl cholinesterase in a manner not consistent with the sequestration hypothesis is shown in fig. 2. Note that with increasing TX-100 concentration, the slope of the linear portion of the curve steepens, suggesting altered interaction of substrate with binding site. Further, if only sequestration did occur, then increasing TX-100 levels at high substrate concentration should relieve inhibition. It is seen that this is not so. The following experiment also indicates that it is interaction between TX-100 and the enzyme that is responsible for the apparent inhibition. Enzyme was incubated at 20°C for 60 min in 10% TX-100, 0.01 M NaP, pH 7.5. At the end of this time, aliquots were removed and activity measured either of two ways: (a) by adding an aliquot directly to assay medium, or (b) by first diluting an aliquot 100-fold with assay buffer and then assaying the dilution. No TX-100 was present in either assay buffer or assay medium. Assaying by method (a) resulted in continuously curving lines of increasing activity which achieved linearity after 5 min. If the sample was diluted into assay buffer (method (b)) and incubated 5 min and then the activity measured, linear assays resulted with values equal to those obtained with no dilution and 5 min incubation in the assay buffer (in these experiments, appropriate alteration in the size of the assay samples were made). For experiments of this kind, about 87–91% of the activity was recovered whereas in the control incubation (no TX-100), 100% of the activity was recovered. These experiments suggest that sequestration is not contributory but that interaction of detergent with enzyme leads to altered enzyme activity and that this alteration is largely reversible.

The Triton X-100 monomer-micelle association is now fairly well described in the recent literature [6,7]. but what may not be generally recognized is that on a *molar* basis the concentration of monomer and micelle can be approximately equivalent even at detergent concentrations well above the CMC. Fig. 1

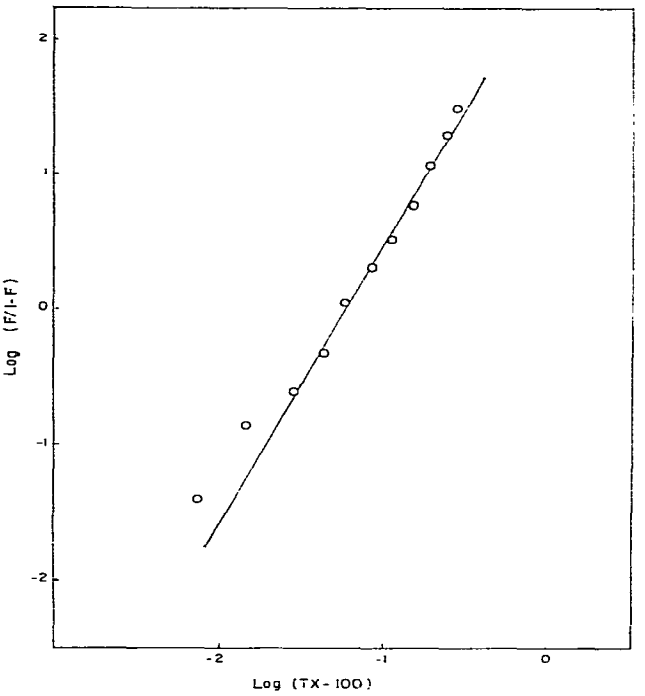

Fig. 3. Plot of $\log\{F/(1-F)\}$ versus log(TX-100) to determine, n, the interaction parameter. Details in text.

also shows the mole fraction of each species versus total detergent (expressed as monomer) for TX-100 in 0.01 M NaP, pH 7.0. The curves presented are calculated from the equilibrium association constants given by Biaselle and Millar [6]. In these calculations it is assumed that the change in ionic strength does not significantly change the association mechanism for this non-ionic detergent. As will be seen below, this distribution of species presents some interpretive embarrassment. If one ignored the presence of the two physically distinct forms of the detergent, an analytical treatment could be obtained as follows: if detergent is $\gg$ than enzyme (detergent/enzyme molar ratio = 10^4–10^6 in the present experiments) and if F_∞ is the fraction of enzyme inhibited at infinite detergent (1.0 in our experiments, see fig. 1), and F is the fraction inhibited at detergent concentration C_t (where C_t is the *total* molar detergent concentration expressed as monomers) one arrives at: $\log(F/F_\infty - F) = n \log C_t + \log K_a$. A plot of $\log(F/F_\infty - F)$ versus $\log C_t$ will have a slope of n, the number of moles of detergent

bound to a mole of enzyme. At the 50% inhibition point, $\log K_a$ (K_a is the association constant for detergent to enzyme) is equal to $-n \log C_t$. Such a plot for our data is shown in fig. 3. We derive a value of 2.04 for n and a K_a of 3.7×10^2. The obvious difficulty here is that we simply do not know what the physical reality of n is since it is derived from the clearly erroneous assumption of uniformity of detergent species. The analytical ambiguity concealed in the above primitive treatment is explored in the following presentation.

The simplest system of reactions which involves the least number of assumptions and remains tractable, follows. No claim for generality is made here; the equilibria considered only represent a superposition of two possible limiting situations. These are: 1) the enzyme molecule P interacts with n_1 molecules of detergent monomer, M, to yield the inactive complex PM_{n_1}, and 2) enzyme molecule P interacts with n_2 molecules of detergent micelle, D, to form inactive complex PD_{n_2}. It is assumed that the detergent consists of only two species, micelles of aggregation number b and monomers. The equilibrium constants and conservation relations for these processes are here defined, with C_t representing total molar concentration of detergent again expressed as monomer and $(P)_t$ the total molar concentration of enzyme:

$$P + n_1 M \rightleftharpoons PM_{n_1}, \qquad {}^1K_a = (PM_{n_1})/(P)(M)^{n_1}, \quad (1)$$

$$P + n_2 D \rightleftharpoons PD_{n_2}, \qquad {}^2K_a = (PD_{n_2})/(P)(D)^{n_2}, \quad (2)$$

$$bM \rightleftharpoons D, \qquad K_m = (D)/(M)^b, \quad (3)$$

$$C_t = b(D) + (M), \quad (4)$$

$$(P)_t = (P) + (PM_{n_1}) + (PD_{n_2}). \quad (5)$$

In what follows, F_∞ will be again taken as unity; that is, the fraction of enzyme inhibited at infinite detergent concentration is unity. In the situation where both types of inhibition can occur the fraction F of enzyme inhibited at finite detergent concentration may be expressed:

$$F = \{(PM_{n_1}) + (PD_{n_2})\}/(P)_t \quad (6)$$

From eqs. (1) and (2):

$$F = \frac{1}{(P)_t}\{{}^1K_a(P)(M)^{n_1} + {}^2K_a(P)(D)^{n_2}\}, \quad (7)$$

$$F = \frac{(P)}{(P)_t}\{{}^1K_a(M)^{n_1} + {}^2K_a(D)^{n_2}\}. \quad (8)$$

Using eq. (5),

$$\frac{F}{1-F} = {}^1K_a(M)^{n_1} + {}^2K_a(D)^{n_2}. \quad (9)$$

In the range of detergent concentration where inhibition is observed, a very good approximation to (D) is, from eq. (4),

$$(D) = C_t/b, \quad (10)$$

Since, from eq. (3),

$$(M) = \{(D)/K_m\}^{1/b} = \{C_t/bK_m\}^{1/b}. \quad (11)$$

Then eq. (9) becomes,

$$\frac{F}{1-F} = \left(\frac{{}^1K_a}{(bK_m)^{n_1/b}}\right)C_t^{n_1/b} + \left(\frac{{}^2K_a}{b^{n_2}}\right)C_t^{n_2}. \quad (12)$$

In one limit where ${}^2K_a$ is zero (micelle and enzyme do not interact), the plot $\log\{F/(1-F)\}$ versus $\log C_t$ is a line of slope n_1/b and ordinate intercept $\log\{{}^1K_a/(bK_m)^{n_1/b}\}$. In the other limit where monomer M and enzyme do not interact, ${}^1K_a$ is zero, the same plot has slope n_2 and intercept $\log\{{}^2K_a/b^{n_2}\}$.

In an intermediate situation where ${}^1K_a$ and ${}^2K_a$ are such that both types of interaction are significant and, for simplicity, that $n_1/b \approx n_2 = n$, then the equation becomes:

$$\log\left(\frac{F}{1-F}\right) = n \log(C_t) + \log\left[\left(\frac{1}{b^n}\right)\left({}^2K_a + {}^1K_a/K_m^n\right)\right]. \quad (13)$$

It is apparent that in this case, n is mechanistically complex as is the intercept and thus the situation is analytically uninformative.

With our experimental data, the first theoretical possibility leads to an $n_1/b = 2.04$. Since we know b ($b = 125$) n_1 would equal 255 or nearly the equivalent of two bound micelles. In the case of the second limiting condition, the value $n = 2.04$ interprets as ~2 micelles binding to the enzyme. This intriguing identity of values can be shown for theoretical model systems to be a consequence of the linkage between monomer and micelle fundamentally expressed in eq. (3) and the fact that the enzyme is at near vanishing concentration compared to detergent. While the present treatment

does not normally allow an explicit choice of binding mechanism, it is superior to the first, spare treatment given above in that reasonable speculation about the physical significance of n can be made. Additionally, if information about the detergent association is available, more meaningful interpretation is possible. Thus, fig. 1 shows monomer mole fraction declining and micelle mole fraction increasing as inhibition occurs. If monomer were the inhibiting species, one might expect some inhibition to occur below the CMC as the TX-100 enzyme ratio is of the order of 10^5. This result is consistent with the conception that it is micelles which are the cause of enzyme inhibition. For this mechanism, the ${}^2K_a$ value is of the order of 5.6×10^6.

In either case, it may be calculated from the data of fig. 3 that at 10% TX-100 and enzyme at 1.5 mg/ml and assuming that $n = 2$, the enzyme is about 85% inhibited or has an average value of 1.7 equivalent micelles/mole. The molecular weight of such a complex is 4.4×10^5. Calculating [5,6] the $\overline{V}$ of such a complex gives a value of 0.786. With values of S and D estimated from sedimentation velocity experiments at 20°C in 10% TX-100 to be 4.20 S and 1.12 F respectively, we can compute a value of apparent complex MW of 4.3×10^5. The close agreement tends to support the interpretations advanced above. The interaction of acetylcholinesterase with TX-100 at very high detergent levels is of interest in terms of the topology of the enzyme and its membrane location. It is clearly not an integral membrane protein, as defined by accepted criteria [1], but if there were some physical means to insert the enzyme near high concentrations of lipids, the well recognized pressure disruption of lipid organization might be expected to play a role in the alteration of this neural enzyme's activity.

The conclusion reached in earlier work [1] was that it seemed unlikely, in situations of normal membrane architecture, that the enzyme was deeply embedded in the lipid bilayer. With reference to the High Pressure Neurological Syndrome, the present results suggest that, in addition to neurological dysfunction from direct pressure effects on membrane structure, other effects resulting from interaction between non-integral membrane proteins and the pressure disrupted lipid regions might also contribute.

Acknowledgement

Naval Medical Research and Development Command, Research Work Unit No. ZF5152.014.9025. The opinions and assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the view of the Navy Department or the naval service at large.

References

[1] D.B. Millar, J.P. Christopher and D.O. Burrough, Biophys. Chem. 9 (1978) 9.
[2] J.P. Christopher, L. Kurlansik, D.B. Millar and C. Chignell, Biochim. Biophys. Acta 425 (1978) 112.
[3] G.L. Ellman, K.D. Courtney, V. Andres and R.M. Featherstone, Biochem. Pharmacol. 7 (1961) 88.
[4] H.K. Schachman, in: Methods in enzymology, eds. S.P. Colowick and N.O. Kaplan (Academic Press, New York, 1957) p. 32.
[5] T. Svedberg and K. Pedersen, The ultracentrifuge (The Clarendon Press, Oxford, 1940, reprinted by the Johnson Reprint Corporation, New York, 1959) p. 62.
[6] C.J. Biaselle and D.B. Millar, Biophys. Chem. 3 (1975) 355.
[7] S. Yedgar, Y. Barenholz and V.G. Cooper, Biophys. Biochim. Acta 363 (1974) 98.