MOLECULAR PHARMACOLOGY, 13, 621-624

Nuclear Magnetic Resonance Studies of the Enzymatic Hydrolysis of Acetylcholine: a Critical Comment

K. S. KRISHNAN AND P. BALARAM

Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India

(Received December 22, 1976)
(Accepted February 24, 1977)

SUMMARY

KRISHNAN, K. S. & BALARAM, P. (1977) Nuclear magnetic resonance studies of the enzymatic hydrolysis of acetylcholine: a critical comment. *Mol. Pharmacol.*, 13, 621-624.

Studies at 100 MHz of the hydrolysis of acetylcholine by brain membrane-bound acetylcholinesterase show that the *N*-methyl resonance acquires a doublet character as the hydrolysis proceeds. These results provide a simple explanation for the time-dependent NMR line broadening observed earlier for the interaction of acetylcholine with purified acetylcholinesterase.

INTRODUCTION

Time-dependent NMR line broadening of the trimethylammonium protons of acetylcholine on binding to acetylcholinesterases was reported by Kato (1). In those studies, carried out at 60 MHz, the change in linewidth of the *N*-methyl signal at half-maximal amplitude ($\Delta\nu_{NCH_3}$) as a function of time was used to study binding of the substrate to the enzyme. The amplitude of the acetate resonance was used to monitor the rate of hydrolysis. These measurements were later extended to study the effects of inhibitors on acetylcholinesterase activity (2). Fisher has pointed out that such time-dependent NMR line broadening should be interpreted with caution (3). We report in this communication results of NMR experiments at 100 MHz on the hydrolysis of acetylcholine by membrane-bound acetylcholinesterase, which suggest a reinterpretation of the data published earlier by Kato.

This is contribution 94 from the Molecular Biophysics Unit, Indian Institute of Science. This work was supported by the Department of Science and Technology-Science and Engineering Research Council and the University Grants Commission.

MATERIALS AND METHODS

The membrane fractions were isolated from monkey brains by the procedure described earlier (4). Membrane samples were dialyzed for 36 hr against frequent changes of distilled water at 4°, and then lyophilized. The average acetylcholinesterase activity in the membrane fractions, assayed by the procedure of Ellman *et al.* (5), was 42 μmoles of acetylthiocholine hydrolyzed per gram of protein per minute (4). Acetylcholine chloride (Sigma) solutions (2.5 mg/ml) were prepared in unbuffered D_2O at pH 7. Weighed amounts of freeze-dried membranes were added directly to the substrate solutions in D_2O. NMR spectra were recorded on a Varian HA-100 spectrometer at a probe temperature of 28°. Slow sweep rates with high filtering were used. Figure 1 shows spectra recorded at a sweep rate of 0.5 Hz/sec. Figure 2 shows spectra recorded at a sweep rate of 0.05 Hz/sec with a low-pass filter

ISSN 0026-895X

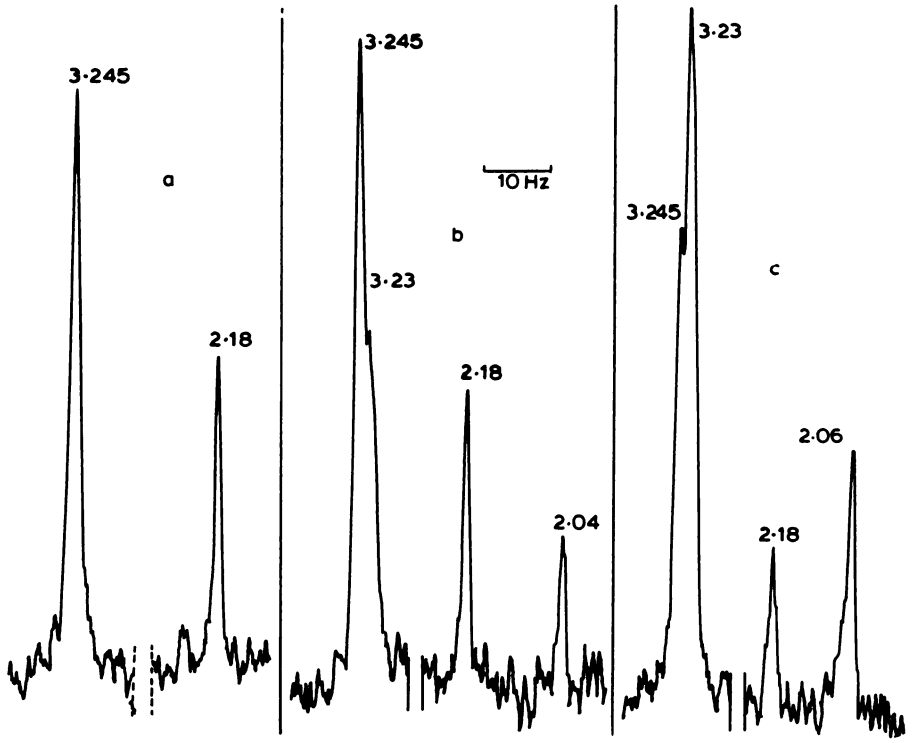

FIG. 1. *^1H NMR spectra at 100 MHz of acetylcholine chloride in the presence of monkey brain membranes as a function of time*

The concentration of acetylcholine chloride was 2.5 mg/ml of D_2O. Spectra were recorded immediately after the addition of freeze-dried membranes (3 mg, dry weight) (a) or at 1 hr (b) or 26 hr (c).

used to minimize noise. Chemical shifts (δ) are expressed as parts per million downfield from the internal standard, 2,2'-dimethylsilapentane-5-sulfonate.

RESULTS AND DISCUSSION

The ^1H NMR spectrum of acetylcholine has peaks at 3.25 ppm, due to the $-N(CH_3)_3$ group, and at 2.18 ppm, due to the CH_3-CO- protons (Fig. 1a). Addition of 3 mg of freeze-dried membrane fractions to a solution of 2.5 mg/ml of acetylcholine in D_2O did not result in any line broadening. Enzymatic hydrolysis is detectable, as evidenced by the appearance of the acetate methyl resonance at 2.03 ppm after 1 hr at 28°. There is also an apparent broadening of the $N(CH_3)_3$ protons. At high resolution this may be seen to result from the appearance of a new resonance 1.6–1.7 Hz (at 100 MHz) upfield from the original $N(CH_3)_3$ signal. The new resonance may be assigned to the *N*-methyl protons of choline, formed by the hydrolysis of acetylcholine. Figure 2 shows clearly that the intensities of this closely spaced doublet interchange with time at a rate corresponding to the appearance of the acetate signal.

The linewidths ($\Delta\nu_{1/2}$) of the signals in the presence of membranes are: acetylcholine CH_3-CO-, 0.9–1.0 Hz; $N(CH_3)_3$, 1.5 Hz; choline $N(CH_3)_3$, 1.6 Hz; acetate, 0.5–0.6 Hz. The choline linewidths are obtained by decomposition of the spectra in Fig. 2 and correspond to values obtained for freely tumbling small molecules in solution. The lack of any line broadening accompanying immobilization by binding could be due either to the small fraction of bound acetylcholine at these concentrations or to slow exchange between bound and free species. Attempts to observe line broadening at lower substrate concentrations (0.5 mg/ml) and higher membrane

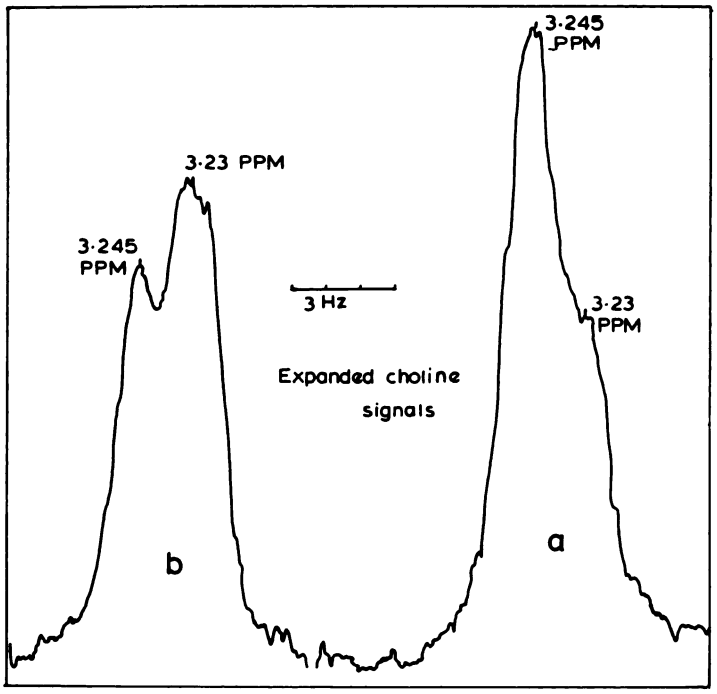

FIG. 2. *Choline methyl signals of spectra in Fig. 1 expanded to a sweep width of 50 Hz*
a. At 1 hr. b. At 26 hr.

levels (15 mg/ml) were unsuccessful. In experiments using DSS[1] as the internal standard, marked broadening of the DSS methyl resonances was observed at these membrane concentrations, suggestive of strong binding of the anionic DSS molecules.

The results presented above suggest a very simple explanation of the time-dependent line broadening observed for the interaction of acetylcholine with purified acetylcholinesterase (1, 2). The data in Figs. 2 and 3 of Kato (1) clearly show that this broadening parallels the disappearance of the acetyl resonance as hydrolysis proceeds. It has further been noted that the widths of the acetyl and acetate resonances are unaffected and that the *N*-methyl resonance of choline chloride is also unchanged by addition of enzyme. We suggest that, as hydrolysis proceeds, the *N*-methyl resonance acquires a doublet character as the result of separate signals from acetylcholine (substrate) and free choline (product). These lines are 1.6 Hz apart at 100 MHz and have widths of 1.5 Hz. It should be noted that there is a slight increase in linewidth on addition of membrane suspensions to acetylcholine solutions (1.2–1.5 Hz). This is probably due to macroscopic inhomogeneities, since the acetyl resonance also increases from 0.7 to 1.0 Hz. These results show that no detectable line broadening occurs on binding, suggesting slow exchange between free and complexed acetylcholine. If initial acylation of an active site residue is involved, slow exchange is plausible. At the concentrations used in this study, as well as those of Kato (1, 2), the signals due to bound species are not detectable under the conditions of the NMR experiments. The earlier experiments (1, 2) were carried out at 60 MHz under conditions of poorer resolution. The two *N*-methyl peaks would be 1.0 Hz apart, and intrinsic linewidths of 1–1.5 Hz would lead to a broader resonance of 2–2.5 Hz. This broadening would of course be time-dependent and parallel the course of hydrolysis. At the high enzyme concentrations used, hydrolysis is very rapid, requiring spectra to be recorded at high

[1] The abbreviation used is: DSS, 2,2'-dimethylsilapentane-5-sulfonate.

sweep rates (1 Hz/sec). This also limits resolution. The experiments reported in this communication were carried out over many hours, since the total enzyme levels were low. The sweep rates used in Figs. 1 and 2 (0.5 and 0.05 Hz/sec) allow higher resolution. It is therefore likely that the noncovalent binding of acetylcholine to acetylcholinesterase has yet to be unambiguously detected by equilibrium NMR methods.

ACKNOWLEDGMENT

We thank the Department of Organic Chemistry, Indian Institute of Science, for use of the HA-100 spectrometer.

REFERENCES

1. Kato, G. (1969) *Mol. Pharmacol.*, 5, 148–155.
2. Kato, G. (1972) *Mol. Pharmacol.*, 8, 582–588.
3. Fisher, J. J. (1973) in *A Guide to Molecular Pharmacology-Toxicology*, (Featherstone, R. M., ed.), p. 583, Marcel Dekker, New York.
4. Krishnan, K. S. & Balaram, P. (1976) *Exp. Cell Res.*, 101, 299–306.
5. Ellman, G. L., Courtney, D. K., Andreas, V. & Featherstone, R. M. (1961) *Biochem. Pharmacol.*, 7, 88–95.