Biochimica et Biophysica Acta, 350 (1974) 95–99

BBA 67192

PUROMYCIN AS AN INHIBITOR OF ACETYLCHOLINESTERASE

D. R. MOSS, D. E. MOSS and D. FAHRNEY*

Departments of Biochemistry and of Psychology, Colorado State University, Fort Collins, Colo. 80521 (U.S.A.)

(Received December 10th, 1973)

SUMMARY

A kinetic analysis of the interaction of puromycin, an inhibitor of protein synthesis, with bovine red cell acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) shows that puromycin is a reversible mixed inhibitor of this enzyme. Puromycin inhibition is similar to that of the pachycurares, such as *d*-tubocurarine, and is quite distinct from inhibition by the leptocurares, such as decamethonium. The Hill coefficient of the interaction of puromycin with bovine red cell membrane-bound acetylcholinesterase at 100 μM substrate is 0.87 which indicates that puromycin causes negative cooperativity in the interaction with this enzyme. The apparent $I_{0.5}$ for puromycin inhibition of bovine red cell enzyme at 100 μM substrate is 126 μM.

INTRODUCTION

Puromycin is an inhibitor of protein synthesis [1] that is widely used to determine the importance of protein synthesis in various biological responses. For example, puromycin has been used to determine if protein synthesis is necessary for a "spontaneous increase" in acetylcholinesterase activity observed in the presence of increased substrate [2] and it has also been used extensively to examine a possible role of protein synthesis in memory processes [3]. The results of the experiments reported here which demonstrate that puromycin is a pachycurare-like inhibitor of acetylcholinesterase are important to the interpretation of the results obtained with puromycin in these earlier studies.

Curare-like compounds are classified as lepto-, and pachycurares. Leptocurares, typified by decamethonium, act like acetylcholine insofar as they produce depolarization of the synaptic membrane. The pachycurares, typified by *d*-tubocurarine, block acetylcholine-induced depolarization and are thought to compete with acetylcholine for its postsynaptic receptor [4]. In this regard, it is interesting that concurrent with the experiments reported here, Wulff [5] characterized puromycin as a *d*-tubocurarine-like pachycurare because puromycin blocked the postjunctional

* Reprint requests: D. Fahrney, Department of Biochemistry, Colorado State University, Fort Collins, Colo. 80521, U.S.A.

response to acetylcholine in the frog sartorius muscle preparation. Of more direct interest to us, however, is the fact that, in addition to direct effects on membrane permeability, lepto- and pachycurare compounds have very different effects on acetylcholinesterase [4]. The leptocurares appear to be classical competitive inhibitors. On the other hand, the pachycurares show nonclassical behavior that supports the contention that they are bound at an allosteric site on acetylcholinesterase and, in some cases, at the catalytic site [4]. Our results which show that puromycin is a *d*-tubocurarine-like inhibitor of acetylcholinesterase extend the results obtained by Wulff [5].

EXPERIMENTAL PROCEDURE

Enzyme

Bovine and human red cell membrane-bound acetylcholinesterase samples were prepared from freshly drawn bovine and human blood by the method of Burger et al. [6]. Ca^{2+} (1 mM) was used during hemolysis to stabilize the membrane-bound enzyme. A suspension of red cell "ghosts" prepared by this method served as a source of acetylcholinesterase activity.

Enzyme assay

The activity of acetylcholinesterase was assayed by the procedure of Ellman et al. [7] at pH 7.0, 25 °C. All assays were run in triplicate using a Beckman Kintrac VII recording spectrophotometer. The change in absorbance at 412 nm was linear for several min with a recorder range of 0.1 absorbance units. The 3.0-ml asay medium contained 2.6 ml of 0.1 M phosphate (sodium) buffer, pH 7.0, 0.1 ml of enzyme solution, 0.1 ml of 0.01 M 5′,5′-dithio-bis-(2-nitrobenzoic acid), and 0.1 ml inhibitor solution. The cuvettes were allowed to equilibrate for 10 min with continuous stirring in the spectrophotometer at 25 °C. The reaction was started by the addition of 0.1 ml of acetylthiocholine solution. All solutions were in 0.1 M phosphate (sodium) buffer, pH 7.0, except that substrate solutions were prepared in distilled water. Enzyme solutions were stored at 4 °C, assayed regularly, and discarded at the first indication of a change in the value of K_m. Enzyme solutions were not retained longer than one week.

Michaelis constants (K_m) were determined by the statistical method of Wilkinson [8] using initial velocities obtained over a substrate concentration range from 25 to 250 μM. The Michaelis constants for acetylcholinesterase with acetylthiocholine substrate under the above assay conditions were 77 μM (bovine) and 54 μM (human) with standard errors of 6–8 %.

Materials

Acetylthiocholine and *d*-tubocurarine chloride pentahydrate were purchased from Sigma. Puromycin dihydrochloride was obtained from Sigma and nutritional Biochemicals. The 5′,5′-dithio-bis-(2-nitrobenzoic acid) was from Pierce Chemical.

RESULTS

The use of the Hill equation, particularly in the form presented by Loftfield and Eigner [9], is helpful in clarifying the behavior of reversible enzyme inhibitors.

These workers have shown that both classical and nonclassical inhibition can be interpreted by the Hill equation:

$$\log\left[\left(\frac{v_0}{v_i}\right) - 1\right] = n_H \log[I] + \text{constant}$$

where v_0 is the uninhibited velocity, v_i is the inhibited velocity, and [I] is the inhibitor concentration. A plot of $\log\left[\left(\frac{v_0}{v_i}\right) - 1\right]$ against log[I] gives a line with slope n_H, where n_H is defined as the Hill coefficient. The Hill coefficient is a measure of the degree of cooperativity between interacting subunits of the enzyme. A Hill coefficient of 1.0 is indicative of competitive inhibition in which no interactions between binding sites exist. Hill plots of the data for decamethonium with membrane-bound acetylcholinesterase of human and bovine red cells at 100 μM substrate are presented in Fig. 1; straight lines fitted to these data by least squares linear regression have Hill coefficients of 0.96 (human) and 0.95 (bovine). By this analysis, decamethonium appears to be a competitive inhibitor. Also shown in Fig. 1 are Hill plots for the

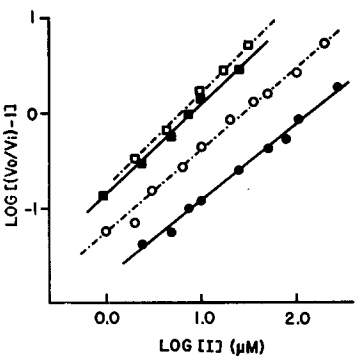

Fig. 1. Hill plot of human and bovine red cell acetylcholinesterase interaction with representative curares at 100 μM substrate. Human enzyme is represented by open symbols, bovine enzyme by filled symbols, decamethonium by squares, and *d*-tubocurarine is represented by circles. Human red cell acetylcholinesterase interaction with decamethonium has a Hill coefficient of 0.96 and with *d*-tubocurarine 0.87. Bovine interaction with decamethonium has a Hill coefficient of 0.95 and with *d*-tubocurarine 0.83.

interaction of *d*-tubocurarine with acetylcholinesterase. In contrast to the results with decamethonium, n_H shows a greater deviation from a value of 1.0 with Hill coefficients of 0.87 (human) and 0.83 (bovine). Values of n_H less than 1.0 are considered diagnostic of negative cooperativity; that is, the existence of two or more interacting sites on the enzyme such that the binding of one inhibitor molecule makes it more difficult for the next to bind [10]. Thus, this analysis indicates that *d*-tubocurarine interacts with acetylcholinesterase in such a way that it causes negative cooperativity.

The above results provide an excellent basis for comparison and analysis of the results with puromycin in the bovine red cell enzyme. Our results contradict the report by Burkhalter [2] which states that puromycin does not inhibit acetylcholinesterase. As demonstrated in Fig. 2, the Hill plot of puromycin inhibition of acetylcholinesterase is linear over a concentration range of 25 to 1500 μM at 100 μM

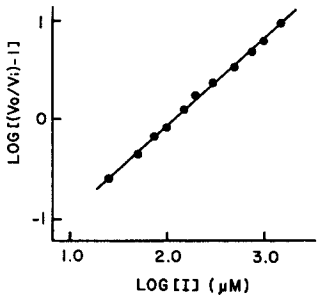

Fig. 2. Hill plot of bovine red cell acetylcholinesterase interaction with puromycin at 100 μM substrate; Hill coefficient is 0.87. The inhibitor concentration that corresponds to a value of 0.0 on the log $[(v_0/v_i) - 1]$ axis is the apparent $I_{0.5}$ value, the concentration at which $v_i = v_0/2$ at the specified substrate concentration. The apparent $I_{0.5}$ value is 126 μM puromycin at 100 μM substrate.

substrate. The Hill coefficient of puromycin inhibition as calculated from these data is 0.87. Thus, puromycin appears to interact with acetylcholinesterase in a way that causes negative cooperativity and, in this regard, puromycin inhibition resembles that of a pachycurare. The apparent $I_{0.5}$ determined from Fig. 2 is 126 μM puromycin at 100 μM substrate. The interaction of puromycin with the bovine red cell enzyme was also analyzed with a Dixon plot (Fig. 3). The usefulness of this type of analysis is discussed by Schlamowitz et al. [11]. In a Dixon plot, $1/v$ is plotted against [I], where v is the initial velocity in the presence of the inhibitor concentration [I]. The intersection of the lines formed by use of two or more substrate concentrations should be at $-I_{0.5}$. An intersection on the absicca indicates noncompetitive inhibition. An intersection at $1/V$ indicates competitive inhibition, and an intersection between the absicca and $1/V$ indicates mixed inhibition. As shown in Fig. 3, the inhibition of bovine acetylcholinesterase by puromycin at 25, 50, and 100 μM substrate did not result in a clear intersection. The range of $I_{0.5}$ observed at different substrate concentrations as determined from Fig. 3 gives a range of 67 to 115 μM puromycin. A duplicate experiment gave a range of 70 to 95 μM puromycin. Both the Hill plot analysis and the Dixon plot analysis indicate nonclassical mixed inhibition of acetylcholinesterase by puromycin.

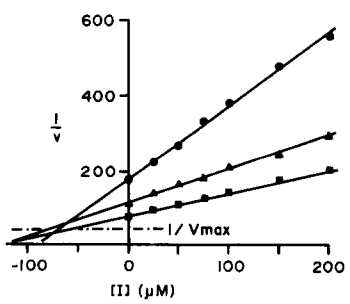

Fig. 3. Dixon plot of bovine red cell acetylcholinesterase interaction with puromycin. Circles represent the interaction the inhibition of the enzyme at 25 μM substrate, triangles at 50 μM substrate, and squares at 100 μM substrate. Experimental error for every point was such that the symbol used to plot the point included the error. These lines cannot be drawn so that a single intersection is formed while the lines remain within the experimental error.

Puromycin inhibition of acetylcholinesterase appears to be completely reversible. Incubation of the enzyme in the presence of 10^{-4} M puromycin at 4 °C and at 25 °C did not result in any detectable inhibition of the enzyme when the puromycin was removed from the incubation mixture. The 4 °C incubation was continued for up to 24 h.

DISCUSSION

Our results which show that puromycin is an inhibitor of acetylcholinesterase are in contrast to those obtained by Burkhalter [2]. There are, however, some important differences in procedure that could account for the contradiction. For example, one important difference could be that Burkhalter assayed the enzyme at pH 8.0 while we assayed the enzyme at pH 7.0. In one experiment we found bovine red cell acetylcholinesterase was inhibited 26% by 75 μM puromycin at pH 8.0 and 40% by 75 μM puromycin at pH 7.0. Burkhalter did not assay for inhibition of acetylcholinesterase at puromycin concentrations that exceeded 50 μM and so he may not have observed any effect.

The finding that puromycin is an inhibitor of acetylcholinesterase may be important to the interpretation of experiments in which puromycin is used to interfere with memory. It is consistent with the cholinergic hypothesis of memory as proposed by Deutsch [12] for puromycin to interfere with memory either through its effect on the acetylcholine receptor as reported by Wulff [5] or because of its effect on acetylcholinesterase as reported here. Regardless of the exact mechanism by which puromycin causes amnesia for learned responses, it is important to recognize that it is an inhibitor of acetylcholinesterase.

ACKNOWLEDGEMENTS

This work was supported in part by the U.S. Public Health Service Research Grant GM 17848. D.R.M. acknowledges the support of the U.S. Army.

REFERENCES

1 Yarmolinsky, M. B. and de la Haba, G. (1959) Proc. Natl. Acad. Sci. U.S. 45, 1721–1725
2 Burkhalter, A. (1963) Nature 199, 598–599
3 Barondes, S. H. (1970) Int. Rev. Neurobiol. 12, 177–205
4 Changeux, J. P. (1966) Mol. Pharmol. 2, 369–392
5 Wulff, V. J. (1973) Pharmacol. Biochem. Behav. 1, 177–182
6 Burger, S. P., Fujii, T. and Hanahan, D. J. (1968) Biochemistry 7, 3682–3699
7 Ellman, G. L., Courtney, K. D., Andres, V. and Featherstone, R. M. (1961) Biochem. Pharmacol. 7, 88–95
8 Wilkinson, G. N. (1961) Biochem. J. 80, 324–332
9 Loftfield, R. B. and Eigner, E. A. (1969) Science 164, 305–308
10 Koshland, Jr, D. E. (1970) in The Enzymes (Boyer, P. D., ed.), pp. 342–396, 3rd edn, Vol. I, Academic Press, New York
11 Schlamowitz, M., Shaw, A. and Jackson, W. T. (1969) Texas Rep. Biol. Med. 27, 483–488
12 Deutsch, J. A. (1971) Science 174, 788–794