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SPONTANEOUS REACTIVATION OF ACETYLCHOLINESTERASE INHIBITED BY DIISOPROPYLFLUOROPHOSPHATE *

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When electric eel acetylcholinesterase is inhibited by diisopropylfluorophosphate at 0°C most of the enzyme is irreversibly inactivated. However, 0.13–0.18% of the initial activity returns spontaneously after the unbound inhibitor is removed or when the inhibited enzyme is diluted into a large excess of competing substrate. A subsequent inhibition-reactivation cycle results in an essentially complete return of activity with minimal aging. The extent of aging increases substantially when inhibition and reactivation are performed at temperatures above 0°C. The free energy of activation for spontaneous reactivation was determined to be 20.1 kcal · mol^{-1}. This large free energy of activation indicates that the reactivation process is a typical dephosphorylation reaction. The computer program used in determining the rate constants and the final extent of reactivation may be widely applicable in similar kinetic studies.

Introduction

We have previously described a highly unusual situation in which acetylcholinesterase (acetylcholine-acetylhydrolase, EC 3.1.1.7) inactivated by a variety of irreversible inhibitors is observed to reactivate spontaneously [1,2]. More particularly, repeated cycles of inhibition followed by reactivation reveal a component of electric eel acetylcholinesterase which preferentially reactivates and which is relatively resistant to aging.

The present study describes a method for efficiently inhibiting the bulk of the acetylcholinesterase activity while preserving activity of the spontaneously reactivating species. Thermodynamic parameters for reactivation of the resulting preparations indicate that the reactivation process is a typical dephosphorylation reaction. The computer program that we used may be widely applicable in studies that require the fitting of a semilogarithmic function to the experimental data.

Materials and Methods

General. Acetylcholinesterase from *Electrophorus electricus* was purchased from Worthington Biochemicals, Inc., at a specific activity of 4000 I.U./mg. The lyophylized preparations were dissolved in MOPS at a concentration of 2.0 mg protein/ml. DFP was from Sigma.

Enzyme activity was assayed by a modification of the technique of Ellman et al. [3] in which we used 0.67 mM acetylthiocholine and 0.67 mM 5,5′-dithio-

* Supplementary data to this article are deposited with, and can be obtained from, Elsevier/North-Holland Biomedical Press B.V., BBA Data Deposition, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. Reference should be made to No. BBA/DD/178/69305/660/91–95. The supplementary information includes a Fortran IV program to fit the reactivation data to the exponential rate equation.

Abbreviations: MOPS, 0.01 M 3-(*N*-morpholino)propanesulfonic acid buffer (pH 7.60); DFP, diisopropylfluorophosphate.

bis(2-nitrobenzoic acid) in MOPS instead of in phosphate buffer. Preliminary experiments showed that, because of competition by substrate, at least 0.05 ml of a reaction mixture containing $5 \cdot 10^{-4}$ M DFP could be diluted directly into this assay system with complete quenching of further acetylcholinesterase inhibition. Absorbance measurements were performed at 412 nm using a recorder and spectrophotometer equipped with a temperature-controlled sample compartment (Gilford Instrument Laboratories, Inc., Oberlin, OH).

Inhibition of acetylcholinesterase by DFP. Sufficient $5 \cdot 10^{-3}$ M DFP was mixed with 0.2–0.3 ml enzyme stock (0.5–1.0 mg protein/ml) to make the final concentration of inhibitor $5 \cdot 10^{-4}$ M. After incubation for 18 h at 0°C, the entire sample was applied to the top of a 1.5 ml column of Sephadex G-75 (medium) packed in a Pasteur pipette and eluted with MOPS. Fractions of 0.2 ml vol. were collected and assayed for enzyme activity after incubation at 37°C for 2 h. Pooled fractions having peak activity were stored at 4°C until used. Enzyme irreversibly inhibited under these conditions was designated acetylcholinesterase I. The activity present after one such inhibition-reactivation cycle was designated acetylcholinesterase II and was employed in studies of the reactivation kinetics.

Aliquots of the stock acetylcholinesterase II solution, usually 0.1 ml, were incubated with $5 \cdot 10^{-4}$ M DFP at the desired temperature for 1.5 h. The entire system was then quickly washed into a cuvette with 3.0 ml assay mixture and absorbance changes were recorded for at least 60 min at the desired temperature. An initial enzyme activity was chosen such that total absorbance change did not exceed the linear range of the assay.

Kinetics of spontaneous reactivation of acetylcholinesterase II following DFP inhibition. Aliquots of acetylcholinesterase II were inhibited with DFP at 0°C and assayed at various temperatures as described above. Acetylcholinesterase II that had not been subjected to a second inhibition was assayed as a control at each temperature. Enzyme activity was determined at 5-min intervals by manually constructing tangents to the observed absorbance vs. time curve, very much as described by Hart and O'Brien [5] in their analysis of inhibition kinetics. The maximum possible extent of reactivation was assumed to result in an activity equal to that of the control acetylcholinesterase II that had not been subjected to a second cycle of DFP inhibition.

A Fortran IV program (see footnote, p. 1) was used to fit the reactivation data to the exponential rate equation.

$$V = V_{INF}[1 - e^{k_{obs}(t - t_0)}] \quad (1)$$

where V is percent reactivation, V_{INF} is percent reactivation at infinite time, k_{obs} is the calculated first-order rate constant and t_0 is the time when percent reactivation is equal to zero. An iterative least-squares procedure is used to fit (V, t) data and the best values for V_{INF}, k_{obs} and t_0 are obtained.

The free energy of activation for spontaneous reactivation, E_a, was determined by use of the Arrhenius equation.

$$\ln k_{obs} = -\frac{E_a}{RT} + \ln A \quad (2)$$

The entropy change, $S^{\ddagger}$, and enthalpy change, $H^{\ddagger}$, for the transition state were obtained from the Arrhenius plot and the relationship,

$$\ln \frac{k_{obs}}{T} = \ln \frac{R}{Nh} + \frac{S^{\ddagger}}{R} - \frac{H^{\ddagger}}{RT} \quad (3)$$

where R, N and h are the conventional constants.

Results

Effect of inhibition temperature on spontaneous reactivation. After overnight exposure to diisopropylfluorophosphate at 0°C the acetylcholinesterase activity was essentially nil but increased progressively until, after 1 h in the assay system, it had reached 0.13–0.18% of the initial uninhibited level. This result was highly reproducible, the final extent of reactivation being somewhat higher than we previously reported [2] because the present procedure eliminates contaminating acetylcholinesterase I more efficiently while it prevents irreversible inhibition of acetylcholinesterase II. Similar extents of reactivation were obtained following either direct dilution into the assay system or removal of the inhibitor by gel filtration and incubation at 37°C. Furthermore, the rate and extent of reactivation were not affected by the individual components of the reaction system.

TABLE I

ACTIVITY OF ACETYLCHOLINESTERASE II FOLLOWING INHIBITION WITH DIISOPROPYLFLUOROPHOSPHATE AT VARIOUS TEMPERATURES AND SPONTANEOUS REACTIVATION AT 37°C

Inhibition temperature (°C)	(*a*) Activity after inhibition (% of initial)	(*b*) Activity after reactivation (% of initial)	% Reactivation $\frac{(b)-(a)}{\text{control}-(a)} \times 100$
Uninhibited control *	100.0	104.0	-
0	6.2	90.0	89.3
25	2.5	31.2	29.4
37	10.4	13.4	3.4

* Initial activity is 0.920 A_{412}/min at 25°C.

Enzyme treated in this manner was used to examine the relationship between inhibition temperature and ability to reactivate spontaneously. Table I shows that acetylcholinesterase II was extensively inhibited at all of the temperatures used. This is as expected. However, the ability of the enzyme to reactivate is strongly dependent upon the temperature at which inhibition was performed. The extent of reactivation after inhibition at 25°C is quite similar to that which we previously observed when both inhibition and reactivation were performed at ambient temperature.

Temperature dependence of reactivation. If acetylcholinesterase II that had been inhibited a second time at 0°C was diluted into the assay system, it reac-

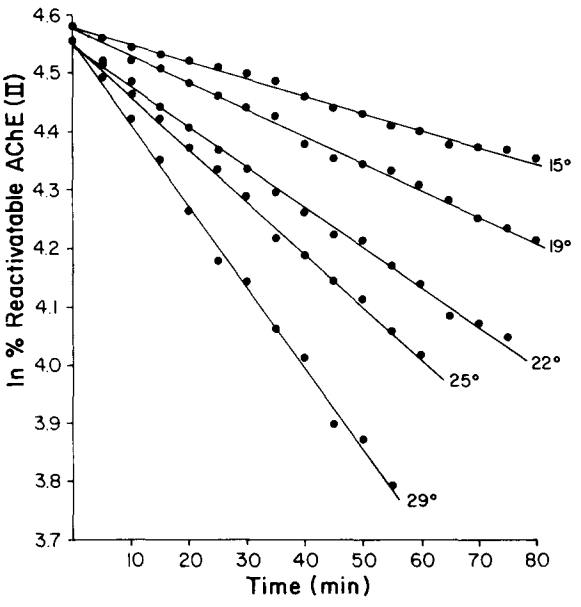

Fig. 1. Spontaneous reactivation of DFP-inhibited acetylcholinesterase II at various temperatures. Total reactivatable enzyme activity was determined by extrapolation of the activity data to infinite time.

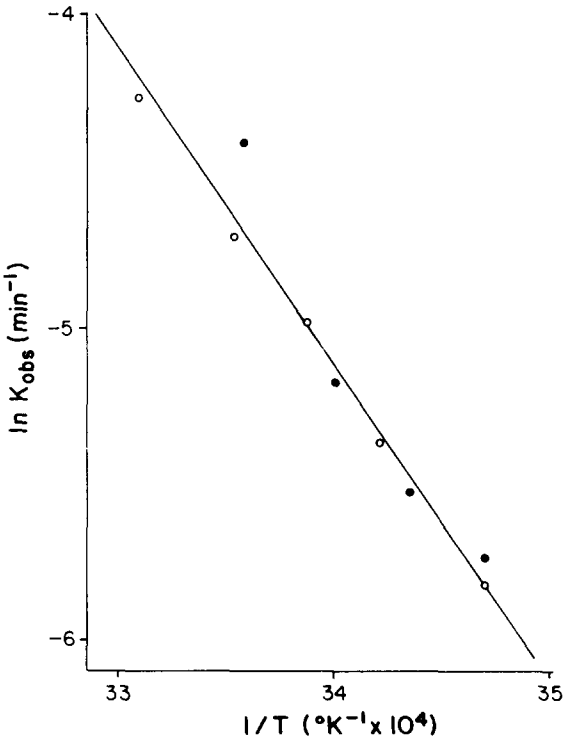

Fig. 2. Arrhenius plot of rate constants for spontaneous reactivation of acetylcholinesterase II. Data from two separate experiments are plotted; open circles are derived from the data presented in Fig. 1.

tivated as shown in Fig. 1. Using the equation $100(V_0 - V_t)/V_0$ to calculated percent reactivatable acetylcholinesterase II, it was found that the plots of ln% reactivatable acetylcholinesterase II vs. time were linear; with correlation coefficients of at least 0.998. Apparent first-order rate constants were obtained from these slopes, e.g., at 25°C the value of k_{obs} was 0.0112 min^{-1}. Two separate experiments yielded the data shown in the form of an Arrhenius plot in Fig. 2. The plot is essentially linear over the temperature range used (correlation coefficient of 0.979) and an activation energy of 20.1 kcal · mol^{-1} was calculated from it. Similarly, application of Eqn. 3 showed that $S^{\ddagger}$ is −70.0 cal · mol^{-1} and that $H^{\ddagger}$ is 19.7 cal · K^{-1} · mol^{-1}. The mean extent of reactivation at V_{INF} was 93.0% with a standard deviation of 18.0%. This degree of precision was possible only because the computer program we used allowed reliable extrapolation of the data to infinite time.

Discussion

Even though previous work appeared to demonstrate spontaneous reactivation of acetylcholinesterase that had been inhibited by any one of several irreversible inhibitors, return of enzyme activity could, conceivably, have been due to dissociation of a non-covalent inhibitor-enzyme complex. The present study shows that the activation energy for the spontaneous reactivation process is 20.0 kcal · mol^{-1}. When this value is compared with that of 14.4 kcal · mol^{-1} obtained by Aldrich [6] for the reactivation of erythrocyte acetylcholinesterase inhibited by dimethyl *p*-nitrophenyl phosphate, it becomes clear that the reactivation process involves hydrolysis of a covalent bond.

The computer program used for fitting curves to the experimental data is adapted from one described by Lieske et al. [7]. The approach is more general than that of Guggenheim [8] because it does not require that the data points be spaced at constant time intervals. The present version of this program has two other advantages over those previously reported. First, it is considerably simplified and is translated into the more widely used Fortran IV. Second, it permits extrapolation of the extent of reactivation to infinite time on the basis of data obtained from less than one half-life . The present study makes this demand on the program because the reactivation rate is so slow at the lowest temperatures that reactivation reaches less than 30% of completion by the time the reaction system becomes unusable.

The ability reliably to extrapolate the extent of reactivation to infinite time revealed that, throughout the temperature range employed, reactivation of acetylcholinesterase II was nearly complete. Although our previous data [1,2] indicated that acetylcholinesterase II inhibited by a number of organophosphates must be relatively resistant to aging, it is surprising that the DFP-inhibited enzyme fails to undergo this reaction to an appreciable extent. The present study exploits the well known temperature dependence of the aging process [9]. Complete inhibition is achieved at low temperature and then acetylcholinesterase II is reactivated in the absence of free inhibitor at 37°C while, at the same time, acetylcholinesterase I is allowed to age. This strategy is important because even though reactivation of acetylcholinesterase II is nearly complete at 25 or 37°C, Table I shows that when it is inhibited at these temperatures and, presumably, subjected to multiple cycles of inhibition and reactivation, there is considerable irreversible loss of activity.

Various changes in the physical environment of the phosphorylated enzyme [10], as well as exposure to certain bispyridinium salts [11], have been shown to decrease the rate of aging by a factor of about two to three relative to the rate of reactivation. Thus, the present observations on the rapidly reactivating species describe a more extreme situation than any previously observed. This refractoriness to aging may be related to the reduced rate constant for inhibition [2] that is also characteristic of acetylcholinesterase II, but determination of the exact mechanism will require complete purification of the enzyme so that the active site can be described in detail.

Acknowledgements

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References

1 Hovanec, J.W., Broomfield, C.A., Steinberg, G.M., Lanks, K.W. and Lieske, C.N. (1977) Biochim. Biophys. Acta 483, 312–319

2 Lanks, K.W., Lieske, C.N. and Papirmeister, B. (1977) Biochim. Biophys. Acta 483, 320–330

3 Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88–95

4 Lanks, K.W. and Sklar, G.S. (1976) Res. Commun. Chem. Pathol. Pharmacol. 14, 269–275

5 Hart, G.J. and O'Brien, R.D. (1973) Biochemistry 12, 2940–2945

6 Aldridge, W.N. (1953) Biochem. J. 54, 442–448

7 Horton, G.L., Lowe, J.R. and Lieske, C.N. (1977) Anal. Biochem. 78, 213–228

8 Guggenheim, E.A. (1926) Philos. Mag. 2, 538

9 Harris, L.W., Fleischer, J.H., Clark, J. and Cliff, W.F. (1966) Science 154, 404–407

10 Aldridge, W.N. and Reiner, E. (1972) Enzyme Inhibitors as Substrates, Frontiers of Biology, Vol. 26, p. 79. North Holland Publishing Co., Amsterdam

11 Schoene, K. (1978) Biochim. Biophys. Acta 525, 468–471