Biochimica et Biophysica Acta, 616 (1980) 384–388

BBA Report

BBA 61383

AGING OF SOMAN-INHIBITED ACETYLCHOLINESTERASE

PH-RATE PROFILES AND TEMPERATURE DEPENDENCE IN ABSENCE AND IN PRESENCE OF EFFECTORS

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(Received June 20th, 1980)

Key words: Acetylcholinesterase; pH-rate profile; Temperature dependence; Effector; Soman inhibition

Summary

The influence of pH and temperature upon the dealkylation (aging) of soman-inhibited acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) was investigated. The pH-rate profile, as measured at 5°C, shows a maximum aging rate at pH 5.0 and indicates the involvement of two ionizable groups with pK_a values of 6.0 and 4.5. The pH-rate profile was not changed essentially by the two effector compounds, semicarbazide and HH54. The activation energy of aging at pH 7.0 was determined as to $6.12 \cdot 10^4$ J/mol; it was lowered by the accelerator of aging semicarbazide to $5.28 \cdot 10^4$ J/mol and increased to $9.42 \cdot 10^4$ J/mol by the retarding compound HH54.

Acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) can be phosphonylated by certain organophosphates to give the corresponding 'phosphonylated' enzyme derivatives, which are enzymatically inactive; inhibition of acetylcholinesterase by soman (*O*-[1,2,2-trimethylpropyl]methylphosphonofluoridate) thus leads to *O*-[1,2,2-trimethylpropyl]methylphosphonylated acetylcholinesterase [1].

The phosphonyl residue can be removed from the enzyme by pyridinium-aldoximes under recovery of the enzymatic activity ('reactivation') [2]. Medication with reactivating oximes is the basis for the causal therapy of organophosphate poisoning.

Soman-inhibited acetylcholinesterase, however, undergoes a rapid consecutive reaction consisting of a dealkylation of the trimethylpropyl moiety which is called aging. The aged enzyme is no longer reactivatable by oximes. The fraction of aged enzyme and the time course of aging can be determined via the loss of reactivatability of the inhibited enzyme.

Aging occurs, as well, with other phosphonylated derivatives of acetylcholinesterase, but it proceeds extremely fast with the soman-inhibited enzyme. This is generally considered to be the reason for the non-satisfactory effect of oxime treatment in soman poisoning [2].

Compounds which retard aging would be of great interest for therapeutic purposes, because they could prolong the time available to reactivate the enzyme by simultaneously applied oxime. In some previous papers, several compounds have been reported to influence the aging of soman-inhibited acetylcholinesterase [3—8]. With regard to their mode of action, as well as to their possible therapeutic relevance, it seemed worthwhile to assimilate some knowledge about the influence of pH and temperature upon the aging process in the absence and presence of such effectors.

Two compounds were selected as representatives for both retarders and accelerators of aging [8]. Because of the high velocity of the reaction at room and body temperature, the pH-rate profile was investigated only at 5°C, the dependence on temperature was determined at pH 7.0.

The compounds (3,4-bispyridinium-1,1-dimethyl)urea diiodide (HH54) [9] and 2-hydroxyiminomethyl-1-methyl-pyridinium iodide [10] were synthesized according to the cited literature. Acetylcholinesterase from bovine erythrocytes was purchased from Sigma, München, and contained $6.9 \cdot 10^{-12}$ mol of active sites per mg, as determined by titration with soman [11]. The purity of soman was 97%. For calibration of the glass-calomel electrode at different temperatures, three appropriate standard buffers (Ingold) were used.

A solution of 20 mg acetylcholinesterase in 1 ml 155 mM NaCl was cooled to 0°C and adjusted to pH 9.0 by addition of 1 M NaOH. To 0.5 ml, containing $6.9 \cdot 10^{-11}$ mol acetylcholinesterase, $13.8 \cdot 10^{-11}$ mol of soman in 4 μl ethanol were added. One-half of the soman, i.e. an amount equivalent to the moles of acetylcholinesterase present, is considered to consist of the two fast reacting isomers [2]. After 20 min the enzyme had become inhibited by more than 99% as controlled by a 20 μl sample.

The aging experiments were started immediately after the 20 min inhibition period; the solution was mixed with 4.5 ml 155 mM NaCl, thermostated to the desired temperature and adjusted to the respective pH. During the aging reaction, a constant pH was maintained by means of a pH-stat titration arrangement. In cases when the influence of semicarbazide or HH54 was to be tested, these compounds were included into the 155 mM NaCl solution; their final concentrations being 1.25 and 2 mM, respectively.

After appropriate time intervals, samples (0.1—1.0 ml) were transferred from the aging mixture to test tubes containing 1.0 ml 100 mM 2-hydroxyiminomethyl-1-methylpyridinium iodide (reactivating agent) in 155 mM NaCl. The volume of the reactivation mixture was filled up to 2.0 ml with 155 mM NaCl. After adjustment to pH 8.0 the solution was kept at 25°C overnight (16—24 h).

1.8 ml of the reactivation mixture were used to determine the enzymatic

activity by pH-stat titration at pH 7.0, 25°C, with 20 mM NaOH in presence of 55 mM acetylcholine chloride as a substrate.

The plots of the logarithms of the maximal reactivatabilities vs. aging time yielded straight lines, from which the first-order rate constants of aging were calculated.

The linearity of these plots suggests, that the aging rates of the two phosphonylated enzyme derivatives (originating from the fast reacting isomers of soman) in the reaction mixture must be very similar. This corresponds to the finding of Keijer and Wolring, that under their conditions both rate constants differ by only about 10% [12].

Several preliminary checks and control assays served to ensure a proper function of the experimental arrangement. (1) All activity data were corrected for the rate of spontaneous hydrolysis of the substrate, which was not influenced by the reactivating agent or the effectors. (2) When the aging experiment was performed at pH 9.0 and 0°C, the rate constant of aging resulted as $0.1 \cdot 10^{-2}$ min^{-1}, indicating that after the 20 min inhibition period under these conditions, maximally 2.5% of the phosphonylated enzyme will have aged. (3) The recovery of the enzymatic activity was studied by inhibiting and reactivating the enzyme in the usual way, but omitting the aging procedure. After applying the necessary corrections, the recovery of enzymatic activity was found to be 82%. Control experiments showed, that the 18% loss of activity could not be caused by an inactivation of the enzyme by the 4 μl ethanol (solvent for soman) nor by the reactivating agent (during the reactivation period). Extrapolation of the linear plots of the logarithms of maximal reactivatability vs. aging time to aging time zero showed, that there were no significant additional losses of recovery of the enzymatic activity during the aging periods, under the different conditions of pH and temperature. (4) The enzyme preparation as purchased from Sigma contained the additives gelatine, NaCl and sodium phosphate. The ionic strength of sodium phosphate solutions changes with varying pH, and the aging rate depends on the ionic strength [2]. In order to avoid interference from this side, we used 155 mM NaCl as the reaction medium; thus, the ionic strength was increased by more than 20-times that value originating from the sodium phosphate, the final concentration of which was approx. 0.5 mM in the aging experiments. (5) In the enzyme assays by pH-stat titration, the reactivating agent and the effectors were still present. Their possible inhibitory effect was taken into account. Inhibition by semicarbazide proved to be negligible, inhibition by the reactivating agent amounted to 25%, whereas HH54 in the respective concentration inhibited the enzyme by 89%. (6) In the presence of HH54 the reactivation proceeded significantly slower but still fast enough to reach its endpoint within 24 h. (7) Because preliminary investigations revealed, that the acceleration of aging by semicarbazide is maximal with an effector concentration of 1.25 mM (Fig. 1), this concentration was applied in all later experiments. (8) With respect to the rapid increase of the aging rate with temperature the pH-rate profile was determined only at 5°C, where the rate constants were in the well measurable range from 0.0019—0.37 min^{-1}.

As Fig. 2 shows, aging in the absence of any effector reaches its maximum rate constant (k_{max}) at pH 5.0 and $k_{max}/2$ at pH 6.0 and 4.5, respectively. These figures are in good agreement with the data reported by Keijer et al.

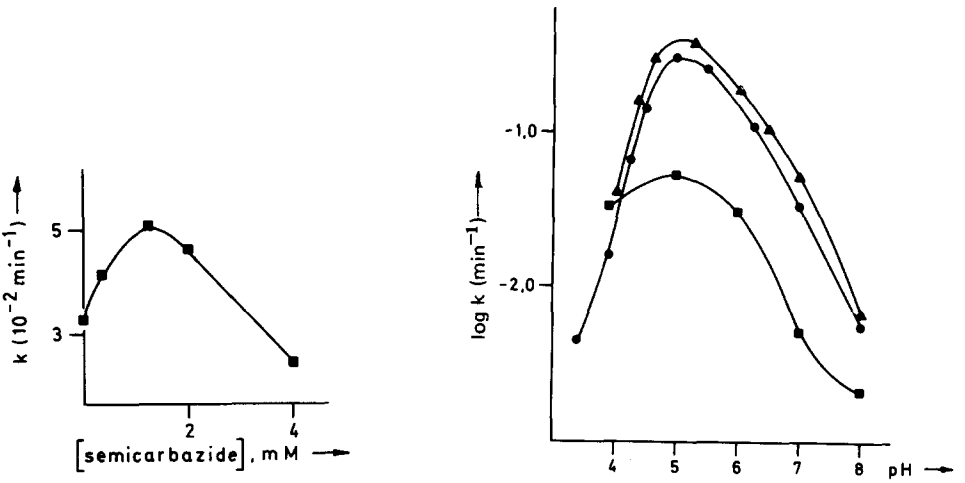

Fig. 1. Aging rate constants of soman-inhibited acetylcholinesterase in the presence of different concentrations of semicarbazide at pH 7.0, 5°C in 155 mM NaCl.

Fig. 2. pH-rate profiles of aging of soman-inhibited acetylcholinesterase at 5°C in 155 mM NaCl; in the presence of 1.25 mM semicarbazide (▲———▲), 2.0 mM 3,4′-bispyridinium-1,1′-dimethyl)urea diiodide (HH54) (■———■) and in the absence of any effector (○———○).

[13] about the aging of cycloheptylmethylphosphonylated acetylcholinesterase in the pH range of 4—8. At 25°C and an ionic strength of 0.100 their figures are: k_{max} at pH 4.75, $k_{max}/2$ at pH 5.84 and approx. 4.2; (the latter value was extrapolated from the experimental data given in the paper).

The conformity of the results from two different phosphonylated derivatives of acetylcholinesterase suggests, that in both cases, identical ionizable groups

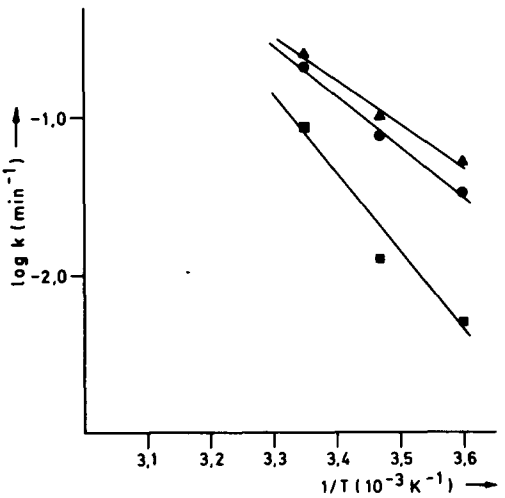

Fig. 3. Arrhenius plots of the rate constants of aging of soman-inhibited acetylcholinesterase at pH 7.0 in 155 mM NaCl; in the presence of 1.25 mM semicarbazide (▲———▲), 2.0 mM (3,4′-bispyridinium-1,1′-dimethyl)urea diiodide) (HH54) (■———■) and in the absence of any effector (○———○).

with pK_a values of around 6 and 4 are involved in the aging process, and that the reactions may follow the same mechanism.

In presence of semicarbazide, k_{max} is achieved at pH 5.0, $k_{max}/2$ at pH 6.0 and 4.5, respectively. Thus, the mechanism does not seem to be changed by this effector. With some reservations, arising from the high inhibitory effect mentioned above, the same conclusion can be drawn for the effect of HH54 in the range of pH 5—8 with k_{max} at pH 5.0 and $k_{max}/2$ at pH 6.1.

The temperature dependence of aging has been measured at pH 7.0 and 5, 15 and 25°C. Assuming a linear relationship between $\log k$ and $1/T$, the following activation energies were calculated from the Arrhenius plots (Fig. 3): non-influenced aging $6.12 \cdot 10^4$ J ($s = 0.34 \cdot 10^4$) per mol; aging in presence of 1.25 mM semicarbazide $5.28 \cdot 10^4$ J ($s = 0.38 \cdot 10^4$) per mol; aging in presence of 2 mM HH54 $9.42 \cdot 10^4$ J ($s = 0.71 \cdot 10^4$) per mol.

With increasing temperature, the influence of the effectors, especially the retarding compound HH54, becomes much smaller. So it seems rather unlikely, that in vivo HH54 could bring about a supporting effect in the oxime therapy of soman poisoning via retardation of aging.

References

1 Aldridge, W.N. and Reiner, E. (1972) Enzyme Inhibitors as Substrates; Frontiers of Biology, Vol. 26, pp. 8—37, North Holland Publishing Co., Amsterdam
2 Aldridge, W.N. and Reiner, E. (1972) Enzyme Inhibitors as Substrates; Frontiers of Biology, Vol. 26, pp. 53—91, North Holland Publishing Co., Amsterdam
3 Berry, W.K. and Davies, D.R. (1966) Biochem. J. 100, 572—576
4 Patocka, J. and Baigar, J. (1974) Cesk. Pharm. 23, 25—27
5 Crone, H.D. (1974) Biochem. Pharmacol. 23, 460—463
6 Sterri, S.H. (1977) Biochem. Pharmacol. 26, 656—658
7 Schoene, K. and Wulf, R. (1972) Arzneim. Forsch. (Drug Res.) 22, 1802
8 Schoene, K. (1978) Biochim. Biophys. Acta 525, 468
9 Hohler, W. (1973) Thesis, Freiburg/Br.
10 Ginsburg, S. and Wilson, J.B. (1975) J. Am. Chem. Soc. 79, 481
11 Schoene, K. (1971) Biochem. Pharmacol. 20, 2527—2529
12 Keijer, J.H. and Wolring, G.Z. (1969) Biochim. Biophys. Acta 185, 465—468
13 Keijer, J.H., Wolring, G.Z. and de Jong, L.P.A. (1974) Biochim. Biophys. Acta 334, 146