Bioorganic & Medicinal Chemistry Letters, Vol.2, No 12, pp 1735-1740, 1992
Printed in Great Britain

0960-894X/92 $5.00 + .00

REVERSIBLE MODULATION OF SERINE PROTEASE ACTIVITY BY PHOSPHONATE ESTERS

Ildiko M. Kovach[a]* and Linda McKay[b]

[a]The Catholic University of America, Department of Chemistry, Washington D.C. 20064
[b]The University of Kansas, Center for Biomedical Research, 2099 Constant Avenue Lawrence Kansas 66046

(*Received* 11 *September* 1992)

Abstract: Temporary modification of serine hydrolase activity with 4-nitrophenyl phenacyl and 4-nitrophenacyl methylphosphonates occurs with self-catalyzed intramolecular reactivation of chymotrypsin, trypsin, thrombin and plasmin.

Transient modification of serine protease activity is of great medical interest since many disease states can be corrected by diminishing the activity of a serine protease involved in the impaired biochemical function.

A variety of organophosphorus compounds that act as irreversible inhibitors of serine hydrolase enzymes have been studied in recent years in this laboratory.[1] Irreversible inhibition of vital enzymes such as acetylcholinesterase (AChE)[2] and other serine enzymes,[1] however, entails toxic effects[3] and thus has only marginal use in medicine. The advantageous properties[1b] of phosphonate esters, particularly those with a good leaving group, can be harnessed by the introduction of ligands into P that can provide anchimeric assistance to dephosphonylation i.e. enzyme reactivation.

Our choice of the functional group to build into P was phenacyl, which has a carbonyl β to the alkoxy substituent in phosphorus and which is known[4,5,6] to provide an excellent nucleophile in the hydrated form for adjacent attack. Hydrolytic reactions of phenacyl-substituted phosphonate esters were found[6,7,8] to be rapid due to the indicated intramolecular reaction, yet, AChE was reversibly inhibited by in-situ generation of 4-nitrophenyl phenacyl methylphosphonate in a preliminary study.[6]

EXPERIMENTAL METHODS

Synthesis of 4-nitrophenyl phenacyl methylphosphonate (PMN) and 4-nitrophenyl 4-nitrophenacyl methylphosphonate (NPMN) The synthesis and purification of these compounds was carried out according to Lieske et al..[7] Analytical purity was 98% for PMN and 97% for NPMN based on basic hydrolysis to 4-nitrophenol (400 nm). The melting points were 112-114°C for PMN and 146-147 °C for NPMN. Other properties also agreed with the earlier report.

Kinetics of Dephosphonylation.

Inactivation of the enzymes (Sigma Chemical Co.) was effected by the introduction of racemic PMN or NPMN from 0.05 - 0.30 M methanolic stock solutions in less than 30% of the total volume of 50-200 μL incubation mixture at pH 7 - 8 at 25.0 °C. Bovine pancreatic α-chymotrypsin (EC 3.4.21.1, Type VII) and porcine pancreatic trypsin (3.4.21.4, Type II) in 60 - 200 nmol quantities were inhibited by 8 - 10 fold excess of the inhibitor. Human plasma thrombin (EC 3.4.21.5) and bovine plasmin (fibrinolysin 3.4.21.7) in 0.2 - 10 pmol quantities were inhibited by up to 1000 fold excess of the inhibitors. Excess inhibitor was allowed to hydrolyze in 10 - 30 minutes. In control runs, the adducts were purified by chromatography on Sephadex-25 G support until all 4-nitrophenol was removed. The 50-200 μL incubation mixture was diluted typically to 2.0 ml volume with the appropriate buffer for reactivation.

Complete inactivation of the enzymes was verified by two techniques, by spectroscopic monitoring of the sudden release of stoichiometric amounts of 4-nitrophenol and by simultaneous monitoring of the loss of enzyme activity. Recovering enzyme activity was monitored in each case by drawing aliquots from the reactivation mixture and titrating for enzyme activity. Active site titrants were 4-methylumbelliferyl 4'-trimethylammoniumcinnamate chloride (MUTMAC) for chymotrypsin, 4-methylumbelliferyl 4'-guanidino benzoate (MUGB) for trypsin and thrombin and N-p--tosyl-gly-pro-lys-4-nitroanilide in 0.55 mM aqueous solutions for plasmin.

Due to a small loss of enzyme activity during lengthy reactivation times with chymotrypsin and trypsin, data points at each time coordinate were corrected. The correction term was calculated from the apparent rate constant obtained from the exponential decay curve for a control run under identical conditions to the respective kinetic run, but in the absence of inhibitor. Corrected active site concentrations were fitted to an exponential time dependence using ENZFITTER.[9]

The solvent isotope effects were measured under completely identical conditions in water and heavy water. Buffer solutions were made in water and heavy water by dissolving the same weights of the buffer components.

RESULTS AND DISCUSSION

Although the PMNs are subject to fairly rapid intramolecular decomposition at pHs above neutral,[7] their phosphonylation of the serine hydrolases seems to be much more facile than their hydrolysis, with rate constants $> 20\ M^{-1}\ s^{-1}$ for PMN and $> 200\ M^{-1}\ s^{-1}$ for NPMN. In all dephosphonylation reactions, simple first order kinetic behavior was observed indicating the

presence of a homogeneous species, which is consistent with an expected enantioselectivity of at least 10.[14,36] The presence of the 4-nitrophenol product of phosphonylation did not influence the reactivation kinetics.

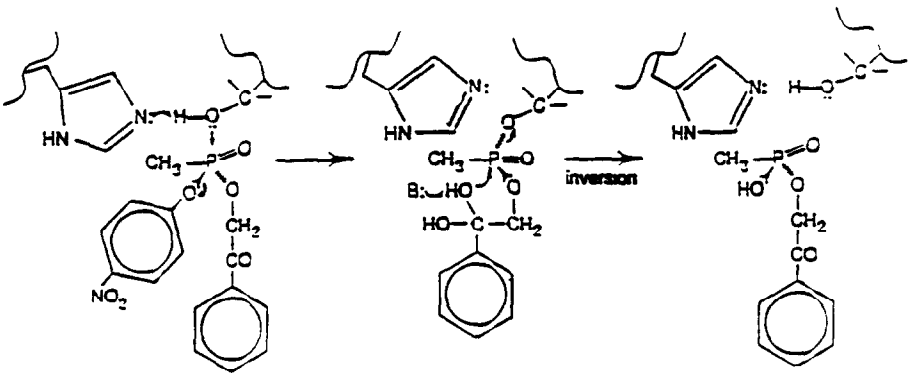
First order rate constants and solvent isotope effects (k_{obs}^{H}/k_{obs}^{D}) at pH 7.6 (pD 8.1) are shown in Table 1 for chymotrypsin recovery from its adduct with PMN at 34.4 ± 0.2 °C, for chymotrypsin recovery from its adduct with NPMN at 25.0 ± 0.1 °C and for trypsin, thrombin and plasmin recovery from their adducts with PMN at 25.0 ± 0.1 °C.

TABLE 1. First order rate constants (min^{-1}) for the reactivation of serine proteases from their phenacyl methylphosphonyl adducts in pH 7.6, 0.2 M phosphate buffer at 25.0 ± 0.1 °C

enzyme	inhibitor	$10^3\ k_{obs}$ (H_2O)	$10^3\ k_{obs}$ (D_2O)	k_{obs}^{H}/k_{obs}^{D}
chymotrypsin[a]	PMN	6.0 ± 0.2	5.0 ± 0.4	1.2 ± 0.1
chymotrypsin	NPMN	72 ± 5		
trypsin	PMN	8.2 ± 0.2	6.8 ± 0.4	1.2 ± 0.1
thrombin	PMN	18 ± 1		
plasmin	PMN	48 ± 17		

[a]34.4 °C

Substituted phenacyl 4-nitrophenyl methylphosphonate derivatives inactivated chymotrypsin, trypsin, thrombin and plasmin fully and very effectively. The intramolecular catalysis of enzyme reactivation also took place as expected and at relative rates as predicted (Scheme 1).



Scheme 1 Reversible inhibition of a serine protease by PMN

Chymotrypsin activity recovered rapidly from the adduct with the 4-nitro-substituted derivative and quite slowly with the unsubstituted derivative. Trypsin-like enzymes recovered faster from the phenacyl methylphosphonyl adduct than chymotrypsin, plasmin recovering the fastest. Thus, these enzymes participate in regeneration of their activity at a characteristic rate of their own, possibly by specific interactions at the active site. It appears that chymotrypsin better accommodates the phenacyl side chain in the specificity pocket than the trypsin-like enzymes and the tighter binding results in slower recovery of enzyme activity.

There was a slight dependence of the first order rate constans on phosphate dianion concentration. The second order rate constant for phosphate dianion catalyzed enzyme reactivation was calculated from the slope of the linear least squares line for the dependence of k_{obs} on $[HPO_4^{-2}]$ to be k_2 = (1.83 $\pm$ 0.44) x 10^{-2} M^{-1} min^{-1}. The dependence of chymotrypsin recovery from its adduct with PMN was studied for a wide range of pH values in 0.05 M phosphate buffers shown in Figure 1.

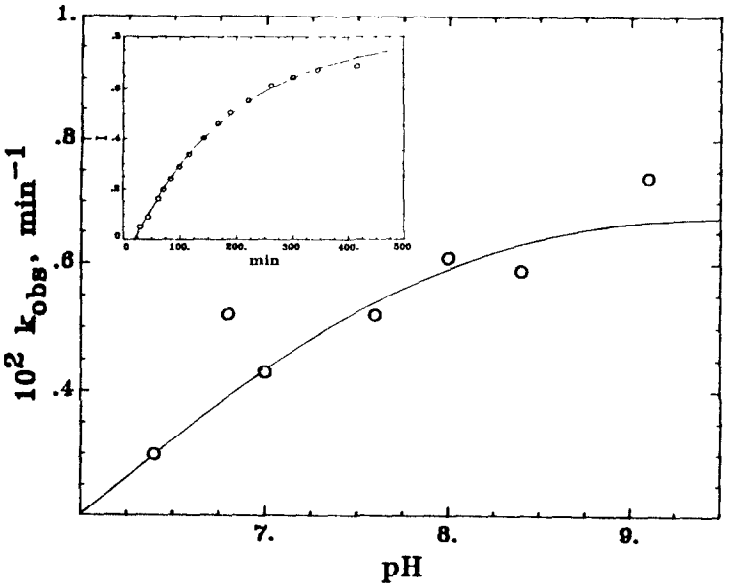


Figure 1. pH Dependence of the first order rate constants for the reactivation of chymotrypsin from the phenacyl methylphosphonyl adduct at 34.4 $\pm$ 0.2 °C. Insert: Time dependence of the recovery of chymotrypsin activity (indicated as fluorescence intensity, I, of the active site titrant) from the same adduct at pH 7.6 in 0.2 M phosphate buffer, at 34.4 0 $\pm$ 0.2 °C.

The data could be fitted to the following sigmoidal function:

$$k_{obs} = \frac{k^{lim}K_a}{K_a + [H^+]} + k_{HOH}$$

An upper limit of (6.5 ± 0.5) x 10^{-3} min^{-1} gives the maximal rate of recovery at pHs above 7.6. This recovery rate is slightly dependent on an ionizing group with an apparent pK_a of 7.2 ± 0.2. The identity of this group is uncertain, but could well be the active site His which has a pK similar to what is observed here. It may, however, be difficult for the His to get into the proximity of the hydrated keto group, which probably binds near the specificity pocket of chymotrypsin. Molecular modeling of the adduct could illuminate the question.[10] The lower limit to the reactivation rate, k = (3.0 ± 1.1) x 10^{-3} min^{-1} might represent the rate for the water-catalyzed reaction (k_{HOH}), ie., the reaction path in which water assists the proton removal from the carbonyl hydrate.

The buffer-independent pH-rate profiles of dephosphonylation also cover the physiological pH range for the adducts of trypsin, thrombin and plasmin with PMN. The pH dependence is small in all cases, which seems to suggest that, contrary to the hydrolysis of the parent compounds,[8] it is not hydroxide ion that is responsible for catalysis of the hydrolysis of the phosphonyl enzyme.

The small pH dependence and small solvent isotope effects, ~ 1.2, also support a mechanism in which proton transfer is not rate determining: Rapid general base-catalyzed hydration of the phenacyl group at the active site is followed by an intramolecular attack of the carbonyl hydrate anion at the central phosphorus atom and by removal of the enzymic Ser residue. Thus either P-O bond breaking to Ser or concerted displacement at P via cyclization determines the rate. The broad range of rates of serine protease reactivation is consistent with self-catalyzed enzyme reactivation with variant efficiency from the phenacyl methylphosphonyl adducts.

The very efficient recovery of the serine proteases from adducts formed with this group of phosphonate esters is unprecedented when compared to other known phosphonate ester inhibitors of these enzymes. Essentially no recovery of serine protease activity has been observed in all the other cases.[1,3] We attribute this reversible inhibition to the unique propensity of the phenacyl group to assist in an intramolecular displacement of the active site Ser from the covalent adduct as shown in Scheme 1.

Rational selection of the substituents in the 4-position of phenacyl will provide inhibitors that will regenerate enzyme activity on different time scales. Research is now in progress in this laboratory

to fully elucidate the mechanisms of enzyme inactivation, recovery and explore the range of inhibitor specificity. The potential for medical use of these compounds as temporary modifiers of enzyme activity, particularly in formulations, through controllable inactivation-reactivation cycles is fairly obvious.

Acknowledgement Generous sharing of experiences with the compounds is gratefully acknowledged to Mr. C. N. Lieske, US Army Medical Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010. This work was supported by the Wesley Foundation, Wichita KS through grant T8710003 at the University of Kansas and by the National Science Foundation through grant DMB 9009344 at the Catholic University of America.

References

1. (a) Kovach, I. M., Larson, M., & Schowen, R. L. *J. Am. Chem. Soc.* ***1986, 108, 5490-5494.*** (b) Kovach, I. M. *J. Enzyme Inhib.* ***1988, 2, 199.*** (c) Kovach, I. M., & Schowen, R. L. In *Peptides and Proteins: Recent Advances*, Schowen, R. L.; Barth, A. Eds.; Pergamon Press:, Oxford, 1987 pp. 205-212.

2. a) Kovach, I. M., Huber-Ashley H., J., & Schowen, R. L. *J. Am. Chem. Soc.* ***1988, 110, 590-593.*** b) Bennet, A. J., Kovach, I. M., & Schowen, R. L. *J. Am. Chem. Soc.* ***1988, 110, 7892.*** c) Bennet, A. J., Bibbs, J. A., & Kovach, I. M. *J. Am. Chem. Soc.* ***1988, 111, 6424.***

3. (a) Aldridge, W. N. & Reiner, E. *Enzyme Inhibitors as Substrates: Interactions of Esterases with Organophosphorus and Carbamic Acids*, American Elsevier: New York, 1972. (b) Main, A. R. *Pharmacol. Ther.* ***1979, 6, 579.***

4. Frank, D. S., & Usher, A. S. *J. Am. Chem. Soc.* ***1967, 89, 6360.***

5. Kluger, R., & Taylor, S. D. *J. Am. Chem. Soc.* ***1991, 113, 996.***

6. Steinberg, G. M., Lieske, C. N., Boldt, R., Goan, J. C., & Podall, H. E. *J. Med. Chem.* ***1970, 13, 435.***

7. Lieske, C. N., Miller Jr., E. G., Zeger, J. J., & Steinberg, G. M. *J. Am. Chem. Soc.* ***1966, 88, 188.***

8. Kovach, I. M., Zhao, Q., Keane, M., & Reyes, R. *J. Am. Chem. Soc.* to be submitted.

9. Leatherbarrow, R. J. ENZFITTER, Elsevier Science Publishers BV: New York; 1987.

10. (a) Kovach, I. M. *J. Molecular Structure (THEOCHEM 76)* ***1988, 170, 159.***
(b) Kovach, I. M., Huhta, D., & Baptist, S. *J. Molecular Structure (THEOCHEM 79)* ***1991, 226, 99.***