Indian Journal of Chemistry
Vol. 23A, October 1984, pp. 834-838

Hydrolysis of Peroxodiphosphate by Acid Phosphatase

S Ch DHARMARAO & S N MAHAPATRO*†
Department of Chemistry
and
S N PADHY & B N MISRA
Department of Botany, Berhampur University, Berhampur 760 007

Received 27 *September* 1982; *revised* 27 *February* 1984; *accepted* 22 *May* 1984

The kinetics of hydrolysis of peroxodiphosphate to peroxomonophosphate by wheatgerm acid phosphatase has been studied in detail. The *p*H-rate profile shows a rate maximum at *p*H 5 in acetate buffer. A K_m value of 0.16 m*M* has been obtained from the Lineweaver-Burk plot. The dissociation constant (K_s) for the species $HP_2O_8^{3-}$ is significantly lower (0.0645 m*M*), indicating a strong preference for $HP_2O_8^{3-}$. The energy of activation of this reaction is 7.0 kcal/mol. Hydrolysis is competitively inhibited by orthophosphate and peroxomonophosphate.

Our current interest in the redox chemistry of peroxophosphates[1-3] and preliminary findings that the $-O-O-P$ moiety offers a potential site for the enzyme acid phosphatase[4] (crude enzyme in bleached white flour) prompted us to study the kinetics of cleavage of peroxodiphosphate by purified wheatgerm acid phosphatase in detail.

Materials and Methods

Triply distilled water was used for preparing all the solutions. Wheatgerm acid phosphatase (Sigma Chemicals) was used as such. All other chemicals were AR(BDH) reagents. *p*H in the range 4-7 was maintained by acetate buffer and measured on a Systronics digital *p*H meter 335. Potassium peroxodiphosphate ($K_4P_2O_8$) a gift sample from Food Machinery Chemicals Corporation, USA was 99% pure and used as such.

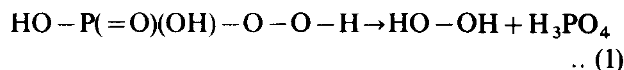
Kinetic measurements

All the kinetic measurements were carried out at 35° ±0.1°C. The differences in the reactivity of peroxodiphosphate and peroxomonophosphate towards iodide ions[5,6] were made use of to assay the enzymatic activity. At *p*H 4-5 the liberation of iodine by peroxomonophosphate is instantaneous; under the same conditions, the peroxodiphosphate-iodide reaction is negligibly slow.

In a typical kinetic experiment the enzyme solution (0.01% wt/vol, Sigma acid phosphatase, wheatgerm, 0.36 units/mg, one unit hydrolyses 1.0 mmol of *p*-nitrophenylphosphate per minute at *p*H 4.8 and 37°C) in appropriate buffer was equilibrated at 35°C for at least 20 min. Calculated volume of peroxodiphosphate solution in the same buffer, preequilibrated at the same temperature was added to initiate the hydrolysis in a total reaction volume of 100 ml. At regular time intervals, aliquots (10 ml) were pipetted out and the reaction quenched with 30 ml of chilled acetate buffer (*p*H 4-5). To this were added 10 ml of 5% KI solution and two drops of 1% ammonium molybdate solution. The liberated iodine was immediately titrated with standard thiosulphate to a starch end point. The problem of aerial oxidation of iodide did not occur at *p*H 4-5.

Velocity measurements for the variation of *p*H, temperature dependence and inhibition studies were obtained from initial rates, following the hydrolysis for 10 minutes only. Control experiments were performed with *p*-nitrophenyl phosphate under identical conditions. The enzyme activity was measured by quantitating *p*-nitrophenoxide at 420 nm.

The possibility of the second stage enzymatic cleavage of peroxomonophosphate to hydrogen peroxide, in accordance with Eq. (1) was checked.



$$HO-P(=O)(OH)-O-O-H \rightarrow HO-OH+H_3PO_4 \quad ..(1)$$

For this study peroxomonophosphate was prepared by the acid hydrolysis of peroxodiphosphate by a standard procedure[7]. The acid was neutralised by adding standard NaOH solution, the *p*H brought to near neutral and finally the desired *p*H adjusted by adding appropriate buffer. The difference in the two thiosulphate titres, i.e. one in the presence of ammonium molybdate[8] (peroxomonophosphate and H_2O_2 both estimated) and other in presence of titanyl sulphate[9] (peroxomonophosphate only estimated, H_2O_2 removed by complexation), provided the amount of H_2O_2 accumulated during the peroxomonophosphate hydrolysis.

† Presently at the Department of Chemistry, Hope College, Holland, Michigan, 49423 USA.

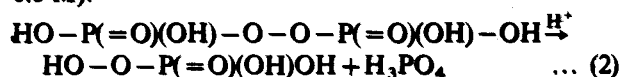
Inhibition studies

The inhibition caused by H_3PO_5 and H_3PO_4 was studied in the presence of added Na_2HPO_4 and peroxomonophosphate. In the presence of added peroxomonophosphate the amount of fresh peroxomonophosphate generated by enzymatic hydrolysis was calculated by substracting the pre-existing peroxomonophosphate level.

The inhibition of the hydrolytic activity was also studied in the presence of F^-, Cu^{2+} and Hg^{2+}. In the presence of Hg^{2+} and Cu^{2+}, the iodometric method was less satisfactory due to the catalytic effect of these ions on the peroxodiphosphate-iodide reaction[10,11] resulting in the frequent reappearance of the blue colour. Clear and reproducible titres were obtained by the addition of 2 ml of 1% EDTA before addition of KI.

Results and Discussion

The chemical hydrolysis of peroxodiphosphate requires drastic conditions of acidity[7] ($[H^+] < 0.1$-$0.5\ M$).



$$HO-P(=O)(OH)-O-O-P(=O)(OH)-OH \xrightarrow{H^+} HO-O-P(=O)(OH)OH + H_3PO_4 \quad \ldots (2)$$

The kinetics and mechanism of this hydrolysis (Eq. 2) has been studied in considerable detail[7,12,13] and a mechanism closely similar to the hydrolysis of pyrophosphate has been proposed[14]. The energy of activation (E_a) of this hydrolysis is acid-dependent and is in the range 18-28 k cal/mol[15]. In the *p*H range 4-9 peroxodiphosphate is very stable and hydrolysis is practically negligible.

The acid phosphatase catalyzed peroxodiphosphate cleavage follows first order kinetics. The *p*H-rate profile shows a maximum at *p*H 5 in acetate buffer (Fig. 1). The Lineweaver-Burk plot in a wide range of substrate concentrations is given in Fig. 2. The K_m value for peroxodiphosphate (0.16 m*M*) is lower than that of *p*-nitrophenyl phosphate (0.27 m*M*) under identical conditions.

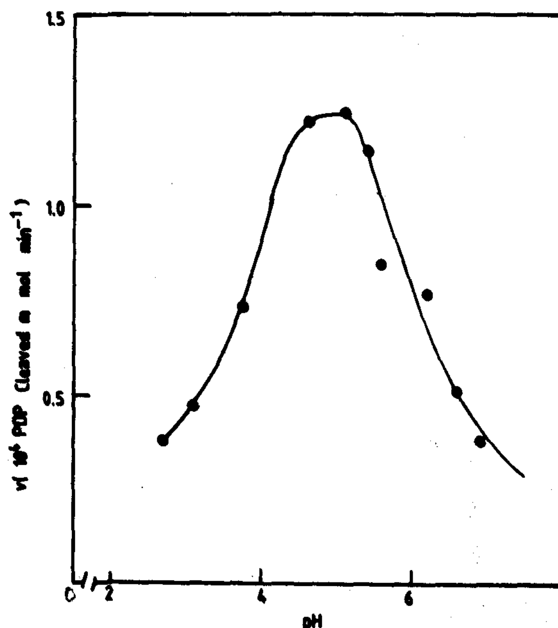


Fig. 1—Effect of *p*H on wheatgerm acid phosphatase catalyzed peroxodiphosphate cleavage [Acetate buffer, [PDP] = 0.577 m*M*, [enzyme] = 0.01% wt/vol, reaction mixture 10 ml, hydrolysis for 10 min, 35°C.

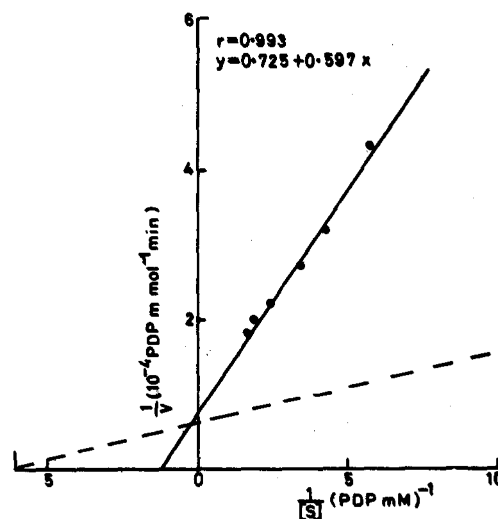


Fig. 2—Lineweaver-Burk plot for wheatgerm acid phosphatase catalyzed peroxodiphosphatase cleavage [*p*H 5.07 acetate buffer, [enzyme] = 0.01% wt/vol, reaction mixture 10 ml, hydrolysis for 10 min, 35°C]

Table 1—K_m and V_{max} Values for the Peroxodiphosphate Cleavage by Wheatgerm Acid Phosphatase*

*p*H	$10^4 K_m$ (mM^{-1})	V_{max} (10^4 PDP cleaved m*M* min^{-1})
4.0	5.26	2.8
4.48	1.92	2.0
5.0	1.6	1.6
5.34	2.35	2.2
5.58	2.86	2.2

*acetate buffer, 35°C. [Enzyme] = 0.01% wt/vol, 10 ml reaction mixture, hydrolysis for 10 min.

The K_m and V_{max} values of PDP at different *p*H values are given in Table 1. It is relevant to point out here that Hickey *et al* have reported the K_m values for the hydrolysis by wheatgerm acid phosphatase at *p*H 4.6 of a series of substrates like glucose-6-phosphate (3.8 m*M*) phenyl phosphate (0.9 m*M*), ATP (0.05 m*M*), D(−)-3 phosphoglycerate (0.5 m*M*) inorganic pyrophosphate (0.32 m*M*) and *p*-nitrophenyl phosphate (0.13 m*M*). The K_m value of PDP is well within the range of K_m values for other substrates, thus

Table 2—Michaelis-Menten Parameters in Hydrolysis of Peroxodiphosphate by Wheatgerm Acid Phosphatase[a]

[Inhibitor], mM	$Y=a+bx$	K_i (mM)	K_m (mM)	V_{max} 10^4PDP mM min^{-1}	Type of inhibition
None	$Y=0.62+0.99x$	—	0.16	1.61	—
Phosphate-(1)	$Y=0.53+0.135x$	1.7	0.254	1.88	Competitive (partial)
PMP + phosphate (1.87)	$Y=0.72+0.597x$	0.37	0.823	1.37	
Cu^{2+}(1.43)	$Y=1.63+0.136x$	0.88	0.083	0.614	Uncompetitive
Hg^{2+}(0.103)	$Y=2.5+0.165x$	0.034	0.067	0.405	Uncompetitive
F^-(2.1)	$Y=1.2+0.112x$	2.32	0.095	0.847	Uncompetitive

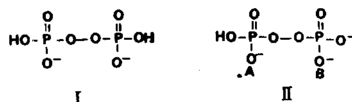
[a] pH 5 (acetate buffer), 35°C, [Enzyme] = 0.01% wt/vol., reaction mixture 10 ml hydrolysis for ten min , pNPP substrate $K_m=0.27$ mM at pH 5 (acetate buffer), 35°C.

demonstrating the nonspecific nature of the enzyme, cleaving a wide spectrum of phosphomonoester substrates.

The energy of activation of this reaction has been calculated from the Arrhenius plot (7.0 k cal/mol). The biocatalysis is clearly evident, as the acid catalyzed hydrolysis has an E_a value[15] in the range 18-28 kcal/mol. All the kinetic results are summed up in Table 2.

Determination of K_s

The rate maximum at pH 5, usual for acid phosphatase activity, on a closer scrutiny reveals certain interesting features. It is well established that, the binding of the substrate with acid phosphatase is preferred in its first ionisation stage[16,17] ($ROPO_3H^-$). Peroxodiphosphate is a tetrabasic acid and has the following dissociation constants[7]: $K_{a_1}=2.0$, $K_{a_2}=3.0\times10^{-1}$, $K_{a_3}=6.6\times10^{-6}$ and $K_{a_4}=2.1\times10^{-8}$. At pH 5, the relative abundance of $H_2P_2O_8^{2-}$(I) and $HP_2O_8^{3-}$(II) is about 60% and 40% respectively.



Formation of an N'-phosphorylhistidine intermediate in acid phosphatase reactions is now generally accepted[18-20]. $H_2P_2O_8^{2-}$ could form such a complex intermediate from either side because of the symmetrical nature of the dianion. In **II**, the binding site (A) is most probably preferred over site (B).

It was of interest to know the particular ionized species of peroxodiphosphate preferred by the enzyme. This was done by calculating the dissociation constant of the enzyme-substrate complex using Eq. (3).

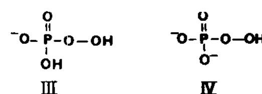
$$v=v_{max}\frac{[S]}{[S]+K_S(1+[H]/K_a)} \quad \ldots (3)$$

At pH 5, $K_a=K_{a_3}$. The K_s value was calculated to be 0.064 mM. If both (A) and (B) are equally preferred by the enzyme at this pH a situation may arise where $K_m=K_s$. The calculated K_s is less than the K_m indicating that all the ionized species are not involved in the reaction. As a matter of fact K_s is only 42% of the K_m value, which corresponds to the statistical abundance of $HP_2O_8^{3-}$ species in the reaction mixture at pH 5.

At pH 5, p-nitrophenylphosphate exists predominantly in its first ionization stage[16,17]. If the ionized species of the substrate is considered as the true substrate, K_s must be regarded as the true binding constant. In such a situation K_m is only an apparent binding constant. A comparison of the K_m value (0.27 mM) of p-nitrophenyl phosphate and the K_s value (0.06446 mM) of peroxodiphosphate indicates a substantial preference of the enzyme for PDP.

Reactions with peroxomonophosphate

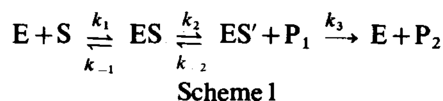
Peroxomonophosphate (PMP), the first product of enzymatic cleavage, is itself a potential substrate for the enzyme, as far as its structure is concerned. Independent experiments with PMP as a substrate, showed only a poor response and the could not compute the K_m value. The ionization constants[7] of PMP are $K_{a_1}=8.0\times10^{-2}$, $K_{a_2}=4.2\times10^{-6}$ and $K_{a_3}=1.6\times10^{-13}$. At pH 5, the population of $H_2PO_5^-$(III) and HPO_5^{2-}(IV) is approximately equal (50% each).



K_{a_3} refers to the ionization of the P–O–O–H hydrogen. A first ionization state is clearly available for binding with the enzyme. It is relevant to note here that the acid hydrolysis of PMP to H_2O_2 is two orders of magnitude slower than that of PDP to PMP and is appreciable[7,13] only at pH < 0.

The wide difference in the reactivities of PDP and PMP towards the enzyme may well be due to the

symmetrical nature of the peroxodiphosphate and electronic and steric factors, which we are unable to define precisely at this moment. In a recent study Lynn *et al.*[21] attempted a LFER correlation of log K_m versus σ (Hammett substituent constants) in the substituted phenyl phosphate cleavage by bovine milk phosphatase. A rho (ρ) value of -1.27 indicated that the transition state required electron accession and that k_3 in Scheme 1 was rate-limiting.



$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES'+P_1 \xrightarrow{k_3} E+P_2$$

Scheme 1

In the case of *p*-nitrophenyl phosphate cleavage, P_1 = *p*-nitrophenol, P_2 = phosphate. In PDP cleavage P_1 = peroxomonophosphate, P_2 = phosphate and in the PMP cleavage $P_1 = H_2O_2$ and P_2 = phosphate. The differences in the reactivities of several orders of magnitude in PDP and PMP could be explained on the basis of the above model, in which the release of H_2O_2 from the ES complex is not favoured thus making the k_2 step rate-limiting. The leaving group stabilities at *p*H ~ 5 (HPO_5^{2-} versus HO_2^-) may also be a contributing factor.

Product inhibition

The study of phosphate inhibition was carried out in the presence of Na_2HPO_4 at 1 m*M* concentration. The K_m' of the reaction was found to be 2.54×10^{-4} *M* while v'_{max} remained essentially same. This is a typical competitive inhibition. At subsaturated concentrations of PDP, Na_2HPO_4 (0.01 to 0.05 m*M*) accelerated the enzymatic hydrolysis up to 30%. We have frequently observed substrate inhibition of acid phosphatases and it is usual phenomenon with phosphatases[22-24]. Since orthophosphate is a competitive inhibitor it reverses the substrate inhibition at low concentrations. The data relevant to phosphate accelerated PDP cleavage are given in Table 3.

Table 3—Effect of Disodium Hydrogen Phosphate (Na_2HPO_4) on PDP Cleavage by Wheatgerm Acid Phosphatase[a]

10^2[Phosphate] (m*M*)	10^4[PDP] Cleaved (m*M* min^{-1})	10^2[Phosphate] (m*M*)	10^4[PDP] Cleaved (m*M* min^{-1})
0	1.08	15	1.2
2	1.11	20	1.21
5	1.19	30	1.28
7	1.16	50	1.24
10	1.2		

[a] *p*H 5, acetate buffer, 35°C.

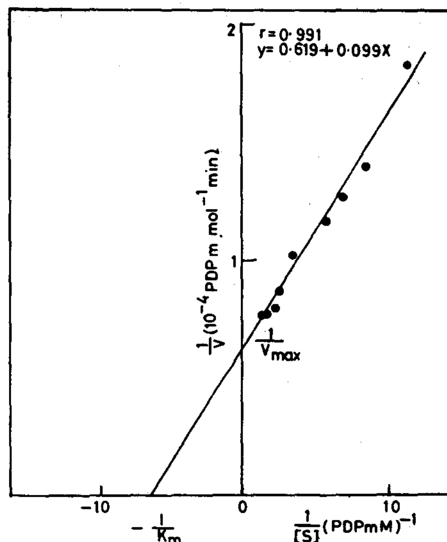


Fig. 3—Lineweaver-Burk plot for wheatgerm acid phosphatase catalyzed peroxodiphosphate cleavage showing mixed type of inhibition by PMPA + phosphate. [The dotted line represents the uninhibited reaction, *p*H 5.07, acetate buffer, [enzyme] = 0.01% wt/vol, reaction mixture 10 ml, hydrolysis for 10 min, 35°C]

PMP also inhibits the PDP hydrolysis significantly we could not use pure PMP free from orthophosphate, as PMP used was prepared by the acid hydrolysis of PDP, which yielded a equimolar concentration of PMP and phosphate (Eq. 2). The type of PMP inhibition was mixed (competitive and noncompetitive type, Fig. 3). PMP, though a substrate for the enzyme was poorly hydrolysed. The strong competitive inhibition by PMP in the presence of equimolar concentration of orthophosphate is most probably occurring by the same mechanism as that of phosphate alone. It is important to note that the K_i of PMP + phosphate inhibition (0.37 m*M*) is significantly lower than the K_i of phosphate (1.7 m*M*) (Table 2). The lowering of v_{max} also indicates a noncompetitive inhibition by PMP. In a typical noncompetitive inhibition, the inhibitor forms a complex with the enzyme as well as with the ES complex resulting in a situation of nonavailability of active sites. This and the substrate nature of PMP result in a mixed inhibition.

Inhibition by F^-, Cu^{2+} and Hg^{2+}

All the kinetic data relevant to inhibition studies are summed up in Table 2. Fluoride, a well known inhibitor of acid phosphatase[25] showed anomalous behaviour in peroxodiphosphate hydrolysis. Fluoride is reported to be competitive inhibitor for prostatic acid phosphatase, while it behaves as non-competitive inhibitor in the *trans*-phosphorylation reaction with glucose for the same enzyme. It also acts as a competitive inhibitor for plant acid phosphatases[26,27]. In the present study inhibition due to F^- turned out to

be uncompetitive. In a typical uncompetitive inhibition the ligand intervenes with the ES complex but not with the free enzyme. Studies with Cu^{2+} and Hg^{2+} showed an identical pattern. The biochemical implications of this cleavage are not known at this moment.

References

1 Mahapatro S N, Panda R & Panigrahi G P, *Indian J Chem*, **19A** (1980) 905.

2 Panda A K, Mahapatro S N & Panigrahi G P, *Bull chem Soc* (Japan), **54** (1981) 2507.

3 Dharma Rao SCh, Panda A K & Mahapatro S N, *J chem Soc, Perkin Trans II*, (1983) 769.

4 (a) Padhy S N, Misra B N, Pattnaik H & Mahapatro S N, *Indian J chem Educ*, **9** (1982) 21.
(b) *Food Nachinery Chemical Corporation USA*, Technical Report (7/70).

5 Indelli A & Bonore P L, *J Am chem Soc*, **88** (1966) 924.

6 Bharadwaj L M, Sharma D N & Gupta Y K, *J inorg nucl Chem*, **39** (1977) 1621.

7 Battaglia C J & Edwards J O, *Inorg Chem*, **4** (1965) 552.

8 Schneider J, Nagy L & Csanyi L J, *Acta chim Acad Sci Hung*, **89** (1976) 15; *Chem Abstr*, **86** (1977) 11533C.

9 Ledaal T & Bernatek E, *Anal chim Acta*, **28** (1963) 322.

10 Kapoor S, Sharma P D & Gupta Y K, *Talanta*, **22** (1975) 765.

11 Bharadwaj L M, Sharma D N & Gupta Y K, *Inorg Chem*, **17** (1978) 2469.

12 Crutchfield M M, *Peroxide reaction mechanisms*, edited by J O Edwards (Interscience, New York) 1962, 41.

13 Goh S H, Heslop R B & Lathbridge J W, *J chem Soc A*, (1966) 1302.

14 Van Wazer J R, *Phosphorus and its compounds* (Interscience, New York) 1958, 452.

15 Creasser I I & Edwards J O, *Topics in phosphorus chemistry*, Vol. 7, edited by E J Griffith and M Grayson (Wiley-Interscience, New York) 1972, 379.

16 Hickey M E, Waymack P P & Van Etten R L, *Arch Biochem Biophys*, **172** (1976), 439.

17 Alvarez E F, *Biochim Biophys Acta*, **59** (1902) 663.

18 Van Etten R L & McTique J J, *Biochim Biophys Acta*, **484** (1977) 386.

19 Van Etten R L & Hickey M E, *Arch Biochem Biophys*, **183** (1977) 250.

20 McTique J J & Van Etten R L, *Biochim Biophys Acta*, **523** (1978) 407.

21 Lynn K R, Clevette-Rodford N A & Chuaqui C A, *Bioorg Chem*, **10** (1981) 90.

22 Reid T D & Wilson B, *The enzymes*, edited by P D Boyer (Academic Press, New York) Vol. IV (1971), 373.

23 Fernley H N, *The enzymes*, edited by P D Boyer (Academic Press, New York) Vol. IV (1971) 449.

24 Hollander V P, *The enzymes*, edited by P D Boyer (Academic Press, New York) Vol. IV (1971), 449.

25 Vescia A & Chance E K, *Biochim Biophys Acta*, (1958), 446.

26 Newmark M Z & Wanger B S, *Arch Biochem Biophys*, **89** (1960) 110.

27 Shaw J G, *Arch Biochem Biophys*, **117** (1966) 1.