INOSITOL PHOSPHATE PHOSPHATASES OF MICROBIOLOGICAL ORIGIN. OBSERVATIONS ON THE NATURE OF THE ACTIVE CENTRE OF A BACTERIAL (*PSEUDOMONAS* SP.) PHYTASE

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Abstract

The pentaphosphates produced by a bacterial phytase acting on *myo*- and L-*chiro*-inositol hexaphosphates as substrates were identified by ion-exchange chromatography. On the basis of the structures of these pentaphosphates and the previously published properties of the bacterial phytase, a simple model for the active centre of the enzyme is proposed which is capable of explaining the intermediates, as far as the diphosphate stage, isolated by Cosgrove from the hydrolysates of *myo*-, D-*chiro*-, and *scyllo*-inositol hexaphosphates due to the action of this enzyme. With slight modification this model for the active centre is also capable of explaining, as far as the diphosphate stage, the intermediates isolated by Tomlinson and Ballou from the hydrolysate of *myo*-inositol hexaphosphate by wheat-bran phytase.

I. INTRODUCTION

Very little has been published about the nature of the active centres of acid phosphomonoesterases. London, McHugh, and Hudson (1958), on the basis of a study of the competitive inhibition of human prostatic acid phosphomonoesterase by anions, proposed for the active centre of that enzyme a model having a pair of electropositive sites and a pair of hydrogen-bonding sites. In order to be bound by the active centre of the enzyme, an anion required a negative charge separated by 2·5–3·0 Å units from a single pair of electrons.

The bacterial (*Pseudomonas* sp.) phytase described in a preceding paper is an acid phosphomonoesterase which is fairly specific for inositol hexaphosphates and appears to hydrolyse all the inositol hexaphosphate isomers studied at a single type of active centre (Irving and Cosgrove 1971). On the basis of the structures of the inositol pentaphosphates isolated from the *Pseudomonas* phytase hydrolysates of *myo*-, D-*chiro*-, and L-*chiro*-inositol hexaphosphates, this paper outlines the minimum stereochemical requirement in the substrate for the enzyme to bind it in an active complex. This stereochemical requirement is used to define a model of an active centre, similar in concept to that proposed for human prostatic acid phosphomonoesterase by London, McHugh, and Hudson (1958). The proposed model is capable of explaining the pathways, outlined by Cosgrove (1970), for the *Pseudomonas* phytase degradation of *myo*-, D-*chiro*-, and *scyllo*-inositol hexaphosphates, as far as the diphosphate stage.

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II. Materials and Methods

(a) *Enzymic Hydrolysis of Inositol Hexaphosphates*

(i) myo-*Inositol Hexaphosphate*

Neutral sodium *myo*-inositol hexaphosphate (0·347 μmoles) was incubated in 30·0 ml of incubation mixture (Irving and Cosgrove 1971). Sufficient *Pseudomonas* phytase was added to bring about the hydrolysis of *c.* 50% of the total ester phosphate present in the substrate during incubation for 7 min at 40°C. The reaction was stopped by the addition of 1 drop of 10N sodium hydroxide solution, yielding a solution suitable for immediate ion-exchange chromatography.

(ii) L-chiro-*Inositol Hexaphosphate*

Neutral sodium L-*chiro*-inositol hexaphosphate (*c.* 68 μmoles), prepared from 100 mg of the barium salt (Cosgrove 1970), was incubated in 50 ml of incubation mixture (Irving and Cosgrove 1971). Sufficient *Pseudomonas* phytase was added to bring about the hydrolysis of *c.* 0·2% of the total ester phosphate present in the substrate during incubation for 30 min at 40°C. The reaction was stopped by the addition of a quantity of Dowex-50W H^+ form ion-exchange resin, yielding a solution suitable for immediate ion-exchange chromatography.

(b) *Ion-exchange Chromatography*

The pentaphosphate components from both the above preparations were isolated as their barium salts (Cosgrove 1970, method A). In each case, the first pentaphosphate fraction eluted from the ion-exchange column was further examined (Cosgrove 1970, method B).

(c) *Phosphate Analysis*

Inorganic orthophosphate was determined by a method described elsewhere (Irving and Cosgrove 1970).

(d) *Nomenclature*

The I.U.P.A.C.-I.U.B. Tentative Cyclitol Nomenclature Rules (1968) are used.

III. Results and Discussion

The results of the ion-exchange chromatography of the pentaphosphate components of the *Pseudomonas* phytase hydrolysates of *myo*- and L-*chiro*-inositol hexaphosphates are summarized in the following tabulation:

Inositol Pentaphosphate	Substituent Nos.	Structure No. (see Fig. 1)	Percentage Detected
D-*myo*	1,2,4,5,6	VI	92
L-*myo*	1,2,3,4,5*	VII	8
L-*chiro*	1,2,3,5,6	IX	89
L-*chiro*	1,2,3,4,6	X	4
L-*chiro*	1,2,3,4,5	XI	7

* And/or its enantiomer.

No other pentaphosphate isomers were detected in the hydrolysates of these two inositol hexaphosphates.

In this paper it is assumed that the structures and most favoured chair conformations of the inositol hexaphosphates are analogous to those of the corresponding free inositols. The assumed structure for *myo*-inositol hexaphosphate is therefore that proposed by Anderson (1914) and not that proposed by Neuberg (1908). Johnson and Tate (1969) recently published evidence which favoured the Anderson structure

for *myo*-inositol hexaphosphate and their argument assumed that the conformation of the molecule was the same as that for the most favoured chair conformation of *myo*-inositol. The assumed structures of the inositol hexa- and pentaphosphates are illustrated in Figure 1.

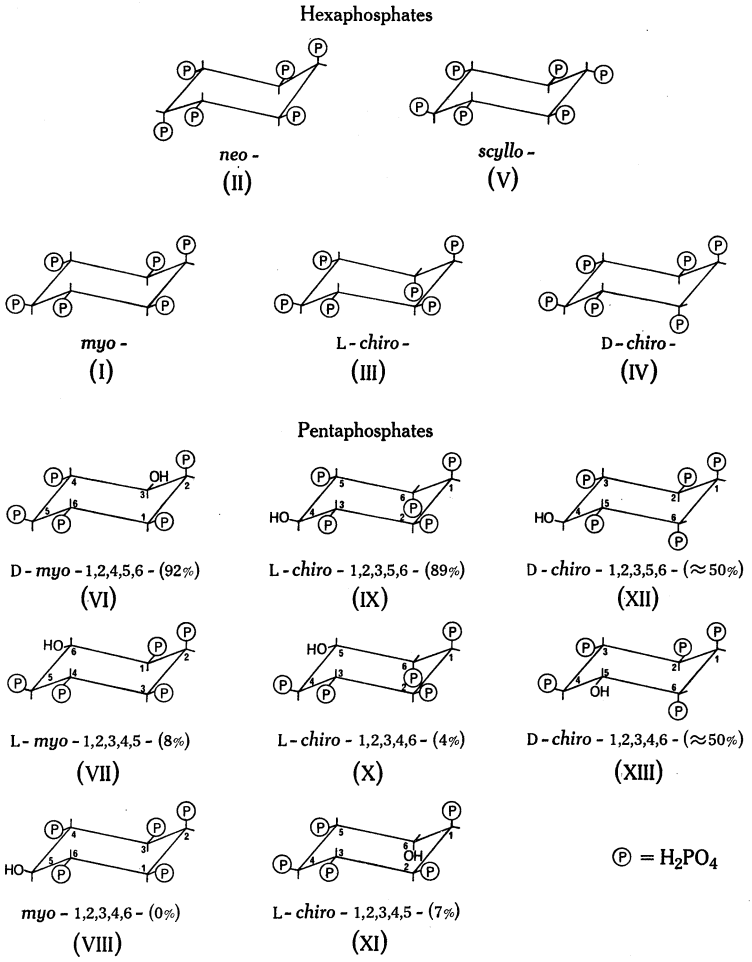

Fig. 1.—Assumed structures of the inositol hexaphosphates and pentaphosphates.

The action of *Pseudomonas* phytase on *myo*-inositol hexaphosphate (I) yields predominantly D-*myo*-inositol-1,2,4,5,6-pentaphosphate (VI) with a small amount of what is either L-*myo*-inositol 1,2,3,4,5-pentaphosphate (VII), its enantiomer, or a mixture of both. Under similar conditions L-*chiro*-inositol hexaphosphate (III) yields predominantly L-*chiro*-inositol-1,2,3,5,6-pentaphosphate (IX) with traces of the other two possible pentaphosphates. *Pseudomonas* phytase hydrolysis of D-*chiro*-inositol hexaphosphate (IV) yields a roughly equimolar mixture of D-*chiro*-inositol-1,2,3,5,6-pentaphosphate (XII) and D-*chiro*-inositol-1,2,3,4,6-pentaphosphate (XIII) (Cosgrove 1970).

The above facts show that the phosphate group which is hydrolysed to yield the pentaphosphate is usually a member of a pair of vicinal *trans*-equatorial phosphate groups whose configuration is illustrated in Figure 2. Such pairs of phosphate groups can exist in one of two mirror-image configurations and therefore if *Pseudomonas*

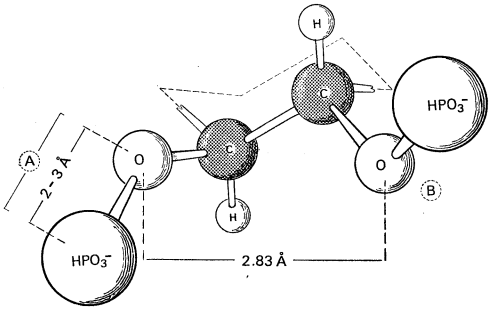

Fig. 2.—Configuration of a pair of vicinal *trans*-equatorial phosphate groups which is required for the formation of an active complex with the enzyme.

phytase is to distinguish the configuration in Figure 2 the enzyme would need to interact with it at a minimum of three points (Ogston 1948). These considerations, taken in conjunction with the model of London, McHugh and Hudson (1958), have led us to propose a model for the active centre of *Pseudomonas* phytase (Fig. 3) which is capable of explaining some of the properties of the enzyme.

The figure illustrates how the enzyme could bind the C3 and C4 phosphate groups of *myo*-inositol hexaphosphate (I). The C3 phosphate group, bound at site A, would be hydrolysed yielding D-*myo*-inositol-1,2,4,5,6-pentaphosphate (VI). By binding the C3 phosphate group oxygen atoms specified (Fig. 3), *Pseudomonas* phytase could catalyse the hydrolysis of that group by stabilizing the trigonal–bipyramidal intermediate of an S_N2 type hydrolysis in which the ester-bond oxygen atom occupies an apical position. Westheimer (1968) has shown that the chemical hydrolysis of a phosphate ester proceeds most readily when the ester group is in an apical position.

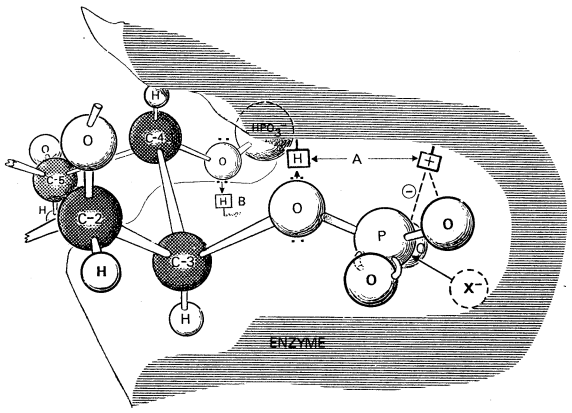

Fig. 3.—Diagrammatic representation of the active centre showing the possible spatial arrangement of sites A and B and how they could bond to a pair of vicinal phosphate groups having the necessary stereochemistry. The displacing group X may be attached to the enzyme or be in solution.

To be bound in an active complex by the active centre illustrated in Figure 3 a substrate would require a nucleophilic group separated by about 2·83 Å units from the ester-bond oxygen atom. This is the interatomic distance between a pair of vicinal oxygen atoms attached to an inositol ring (Posternak 1965).

myo-Inositol hexaphosphate (I) possesses two pairs of vicinal *trans*-equatorial phosphate groups with the required stereochemistry. These are the C3–C4 pair and the C5–C6 pair. Binding of the C3–C4 pair, as illustrated in Figure 3, would yield D-*myo*-inositol-1,2,4,5,6-pentaphosphate (VI). The trace of the second pentaphosphate detected in the hydrolysate could arise in two ways. If the structure of the enzyme in the region of the active centre is such as to permit rotation of the plane of the inositol ring through 180°, then the enzyme could also bind the C3–C4 pair with the C4 phosphate group at site A, yielding L-*myo*-inositol-1,2,3,4,5-pentaphosphate (VII) on hydrolysis. Similar binding of the C5–C6 pair of phosphate groups would yield *myo*-inositol-1,2,3,4,6-pentaphosphate (VIII) or the enantiomer of (VII). If the proportions of the pentaphosphates detected in the *Pseudomonas* phytase hydrolysate of *myo*-inositol hexaphosphate (I) can be taken as reflecting their rates of formation rather than their rates of hydrolysis, then the absence of *myo*-inositol-1,2,3,4,6-pentaphosphate (VIII) and the predominance of D-*myo*-inositol-1,2,4,5,6-pentaphosphate (VI) must be taken as implying either the preferential binding of the C3–C4 pair of phosphate groups or a greater ease of hydrolysis of the C3 phosphate group.

The C3 phosphate group of *myo*-inositol hexaphosphate (I) is a member of a *cis*-1,2,3 trio and this grouping appears to be important in determining the rates of hydrolysis of inositol hexaphosphates by *Pseudomonas* phytase (Irving and Cosgrove 1971). Both the *myo*- and *neo*- isomers, which have this grouping (Fig. 1), are hydrolysed at the same rate while those isomers which are epimers of *myo*-inositol hexaphosphate (I) at C1, C2, or C3 are hydrolysed at very much lower rates (Irving and Cosgrove 1971). The *cis*-1,2,3 grouping could accelerate the hydrolysis of the C3 phosphate group of *myo*-inositol hexaphosphate (I) by steric assistance, due to the inevitable crowding resulting from such an arrangement of phosphate groups. A second possibility is that the "space" created beneath the plane of the inositol ring by the axial orientation of the C2 phosphate group is important for the functioning of the enzyme.

neo-Inositol hexaphosphate is hydrolysed at the same rate as the *myo*- isomer (Irving and Cosgrove 1971). It has a single pair of phosphate groups with the required stereochemistry (Fig. 2). However, both members of this pair are equivalent to the C3 phosphate group of the *myo*- isomer with respect to the stereochemistry of nearest neighbours and therefore it could be expected that the *neo*- isomer should be hydrolysed at a greater rate than the *myo*- isomer. The equality of the rates of hydrolysis suggests that the rate-limiting step is the breakdown of the enzyme substrate complex and not its formation.

D-*chiro*-Inositol hexaphosphate (IV) has two equivalent pairs of suitable phosphate groups. These are the C2–C3 pair and the C4–C5 pair. It is easily seen how binding of either of these pairs would yield D-*chiro*-inositol-1,2,3,5,6-pentaphosphate (XII) and D-*chiro*-inositol-1,2,3,4,6-pentaphosphate (XIII).

A critical test for the proposed model is provided by the results of hydrolysis of L-*chiro*-inositol hexaphosphate (III). Since it has only a single pair of suitable phosphate groups, *Pseudomonas* phytase hydrolysis of this substrate would be expected to yield predominantly a single pentaphosphate, L-*chiro*-inositol-1,2,3,5,6-pentaphosphate (IX). This was found to be the case. The traces of the two other

possible pentaphosphates detected show that with this substrate the enzyme is not completely specific for the suggested structure (Fig. 2).

scyllo-Inositol hexaphosphate (V) possesses three pairs of suitable phosphate groups. However, binding and hydrolysis of any one of these pairs would yield the same pentaphosphate.

Cosgrove (1970) in his study of the hydrolysis of *myo*-, D-*chiro*-, and *scyllo*-inositol hexaphosphates by *Pseudomonas* phytase found evidence that the hydrolysis of pentaphosphate and lower phosphate esters of inositols involves the preferential hydrolysis of a phosphate group which is vicinal to a hydroxyl group. Tomlinson and Ballou (1962) had previously made a similar observation in connection with the pathway of degradation of *myo*-inositol hexaphosphate (I) by wheat-bran phytase. In terms of the proposed model this preferential hydrolysis, by *Pseudomonas* phytase, of phosphate groups which are vicinal to a hydroxyl group can be explained by the fact that a hydroxyl oxygen atom would be a stronger nucleophile than a phosphate ester-bond oxygen and so would compete more effectively for site B (Fig. 3).

D-*myo*-Inositol-1,2,4,5,6-pentaphosphate (VI) would therefore preferentially combine with the active centre so as to place the C3 hydroxyl oxygen atom at site B and the C4 phosphate group at site A, yielding on hydrolysis D-*myo*-inositol-1,2,5,6-tetraphosphate. This combination of substrate and enzyme assumes that the plane of the inositol ring may be rotated through 180° and that the enzyme will still actively bind the substrate in this new orientation.

Application of the concept outlined above to the results of Cosgrove (1970) can explain the most probable pathways of hydrolysis of *myo*-, D-*chiro*-, and *scyllo*-inositol hexaphosphates by *Pseudomonas* phytase as far as the diphosphate stage. In addition, if sites A and B in Figure 3 are transposed, a model is created which is capable of explaining the intermediates, as far as the diphosphate stage, detected by Tomlinson and Ballou (1962) in the hydrolysate of *myo*-inositol hexaphosphate (I) by wheat-bran phytase.

IV. Acknowledgment

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