Inhibition of iron-catalysed hydroxyl radical formation by inositol polyphosphates: a possible physiological function for myo-inositol hexakisphosphate

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1. The ability of myo-inositol polyphosphates to inhibit iron-catalysed hydroxyl radical formation was studied in a hypoxanthine/xanthine oxidase system [Graf, Empson and Eaton (1987) J. Biol. Chem. 262, 11647–11650]. Fe2+ present in the assay reagents supported some radical formation, and a standard assay, with 5 μM Fe2+ added, was used to investigate the specificity of compounds which could inhibit radical generation. 2. InsP4 (phytic acid) was able to inhibit radical formation in this assay completely. In this respect it was similar to the effects of the high affinity Fe2+ chelator Desferral, and dissimilar to the effects of EDTA which, even at high concentrations, still allowed detectable radical formation to take place. 3. The six isomers of InsP6 were purified from an alkaline hydrolysate of InsP6 (four of them as two enantiomeric mixtures), and they were compared with InsP6 in this assay. Ins(1,2,3,4,6)P6 and D/L-Ins(1,2,3,4,5)P6 were similar to InsP6 in that they caused a complete inhibition of iron-catalysed radical formation at > 30 μM. Ins(1,3,4,5,6)P6 and D/L-Ins(1,2,4,5,6)P6, however, were markedly less potent than InsP6, and did not inhibit radical formation completely; even when Ins(1,3,4,5,6)P6 was added up to 600 μM, significant radical formation was still detected. Thus InsP6 lacking 2 or 1/3 phosphates are in this respect qualitatively different from InsP6 and the other InsPs. 4. scyllo-Inositol hexakisphosphate was also tested, and although it caused a greater inhibition than Ins(1,3,4,5,6)P6, it too still allowed detectable free radical formation even at 600 μM. 5. We conclude that the 1,2,3 (equatorial–axial–equatorial) phosphate grouping in InsP6 has a conformation that uniquely provides a specific interaction with iron to inhibit totally its ability to catalyse hydroxyl radical formation; we suggest that a physiological function of InsP6 might be to act as a 'safe' binding site for iron during its transport through the cytosol or cellular organelles.

INTRODUCTION

myo-Inositol hexakisphosphate (phytic acid, InsP6) is a major component of plant storage tissues (Cosgrove, 1980), where it is generally believed to be a phosphate store, and Ins(1,3,4,5,6)P6 is found in large quantities in erythrocytes of avian, reptilian and amphibian species (Johnson and Tate, 1969), where it modulates haemoglobin oxygen binding (see Bartlett, 1982). Until the fairly recent past, these two higher inositol polyphosphates, with these specific functions, were believed to be confined to these particular tissues. However, investigations of inositol phosphates in animal cells revealed that InsP6 is found probably in all animal cells [see Stephens et al. (1991) for references], and presently there is no known exception to suggest other than that InsP6 is universal in eukaryotic tissues. InsP6 are also found in various extents in animal tissues, the predominant isomer being Ins(1,3,4,5,6)P6 (Stephens et al., 1991).

The levels of Ins(1,3,4,5,6)P6 can be modulated after cell stimulation, probably because there are complex routes of synthesis to it from receptor-generated Ins(1,4,5)P3 [e.g. Stephens and Downes (1991) and see Shears (1989) for a review]. However, at least in the slime mould Dictyostelium discoideum, presently the only organism in which the route of synthesis of InsP6 has been fully elucidated, InsP6 can be synthesized [with Ins(1,3,4,5,6)P6 as an obligate intermediate] by sequential phosphorylations of inositol and with no lipid intermediate (Stephens and Irvine, 1990). The breakdown route of InsP6 is not documented yet, but in slime moulds it is predominantly dephosphorylated in the 3 and 5 positions (Stephens and Irvine, 1990; Stephens et al., 1991), and in animals there is a 3-phosphatase with a high affinity for InsP6 (Nogimori et al., 1991).

The reasons for the apparently universal occurrence of InsP6 in eukaryotic cells are not known. It is found at estimated levels up to 600 μM (Martin et al., 1987), and clearly represents a considerable investment in cell energy. Various functions have been suggested, and these have been summarized elsewhere (Stephens and Irvine, 1990; Carpenter et al., 1989). In 1984, Graf et al. showed that InsP6 was a particularly effective inhibitor of iron-catalysed hydroxyl radical (OH·) formation, and suggested that it might make a useful food additive (Graf et al., 1984, 1987; Graf and Eaton, 1990). They also suggested that, given its high level in dry storage tissues of higher plants, evolution may already have put it to that use, i.e. that it is there as a natural antioxidant (Graf et al., 1987; Berridge and Irvine, 1989). A natural extension of this line of reasoning is that animal cells may also use InsP6 as an endogenous iron chelator. It is clear that animal cells possess iron in a low-molecular-mass pool which may be important for the transport of iron between transferrin (the major route of iron entry into cells), ferritin (the major iron depot in cells), and various cell destinations (e.g. haem-containing enzymes); this pool may also be important in various disease states (e.g. haemochromatosis: Jacobs, 1977; Grohlich et al., 1979; Crichton, 1979; Morgan, 1977; Fontecave and Pierre, 1991; Britton et al., 1990; Nielsen et al., 1993). The nature of the low-molecular-mass iron pool is presently unclear, but it has in the past been postulated to represent iron chelated with citrate (Morley and Bezhorovaiyi, 1983), phosphate (Pollack et al., 1985), amino acids (Bakkeren et al., 1985; Deighton and Hider, * Present address: Department of Pharmaceutical Sciences, Aston University, Birmingham B4 7ET, U.K.
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1989) and ATP (Weaver and Pollack, 1989). However, given the apparently high affinity and kinetic lability of iron-InsP₄ complexes (e.g. Cosgrove, 1980; Poyner et al., 1993) and the documented ability of phosphates to exchange iron between iron-binding proteins (e.g. Cowart et al., 1986; Morgan, 1977), it seemed an attractive possibility that InsP₄ represents a component of this low-molecular-mass iron-pool.

Direct evidence for such a function for InsP₄ will be difficult to obtain (it may have to come from eliminating InsP₄ from a cell by genetic means), but we have here sought some indirect evidence by investigating the specificity of the interaction of inositol polyphosphates with iron. We find that although all the inositol polyphosphates tested will interact with iron and inhibit OH⁻ formation (generated from superoxide anion and H₂O₂), only those with a 1,2,3 (equatorial-axial-equatorial) phosphate grouping cause a complete inhibition. This precise and specific effect lends some credibility to the possibility that this is one reason why eukaryotic cells maintain fairly high levels of cytosolic InsP₄.

**MATERIALS AND METHODS**

In all experiments involving ferric iron, the desired concentrations were obtained by diluting (at least 20-fold) concentrated stock solutions immediately before use; this was done to avoid the formation of insoluble iron hydroxide precipitates.

**InsP₄ and InsP₅ isomers**

These were prepared from InsP₄ (purchased from Sigma) by alkaline hydrolysis and ion-exchange chromatography as described in Stephens (1990) and Stephens et al. (1991). They were checked for purity by chromatography on polyethyleneimine-cellulose (Spencer et al., 1990), and, as shown in Figure 1, each InsP₄ fraction is distinct and apparently largely free of contamination with other inositol phosphates. The InsP₄ was also purified by h.p.l.c., and in some experiments, InsP₄ from mung beans (*Phaseolus aureus*) (a gift from L. Stephens) was used. *scyllo*-Inositol hexakisphosphate was generously given by Dr. M. F. Tate (Waite Institute, Adelaide, South Australia, Australia).

**Catechol-decolouring assay**

Solutions were made containing 0.25 mM catechol and the appropriate concentration of chelator, and then made up to 0.25 mM Fe³⁺ with FeCl₃. The pH was adjusted to 7.0 with dilute HCl or NaOH and after 15 min the absorbance was read at 575 nm (at 25 °C). The chelator-catechol complexes had no significant absorbance at this wavelength in the absence of added iron.

**Hydroxyl radical generation assay**

This was essentially performed as described by Graf et al. (1984, 1987) with some modifications. The principle of the assay is the iron-catalysed formation of OH⁻ from superoxide anion radical (O₂⁻⁻) (generated by xanthine oxidase/hypoxanthine) and H₂O₂ (the reactions involved are thought to be:

Fe³⁺ + O₂⁻⁻ → Fe²⁺ + O₂
Fe²⁺ + H₂O₂ → Fe³⁺ + OH⁻ + OH⁻)

This assay is based on the original work of Beauchamp and Fridovich (1970). The formation of OH⁻ is monitored by reaction with dimethyl sulphoxide and measurement of resulting formaldehyde by the Hantz reaction (Nash, 1953). We tried to make

![Figure 1](image-url)  
(a) The six isomers of InsP₄ (four of them as two enantiomeric mixtures) were prepared as described in the Materials and methods section, and this Figure illustrates their apparent purity when analysed by polyethyleneimine-cellulose l.c. (see the Materials and methods section for details). Lanes are as follows: 1, Ins(1,2,3,4,6)P₅; 2, c/-Ins(1,2,4,5,6)P₅; 3, c/-Ins(1,2,3,4,5,6)P₅; 4, Ins(1,3,4,5,6)P₅; 5, Ins(2). (B) We illustrate, in its predominant chair conformation (Emsley and Niazi, 1981), InsP₄ (left) with its carbon atoms numbered. Note that the 2-phosphate is axial, whereas the others are all equatorial; thus the 1,2,3 phosphates form a unique equatorial-axial-equatorial grouping. Below, for comparison, is *scyllo*-inositol hexakisphosphate, which has all six of its phosphates equatorial.
this assay linear with respect to enzyme, substrates, time and added Fe³⁺. Under the conditions originally described by Graf et al. (1984), the reaction saturated with enzyme concentration and over time, even under conditions where the original substrate (hypoxanthine) was consumed to < 10% as calculated by formaldehyde formation and the enzyme was still active (as judged by further substrate addition). Eventually, however, we arrived at assay conditions essentially linear with everything but added Fe³⁺ concentration (see the Results section); these conditions included the addition of a small quantity of H₂O₂ to encourage a ‘pseudo-first-order’ generation of OH⁻ radicals with respect to added iron. The standard assay (e.g. as used for Figures 5 and 6 below) mixture contained, in a final volume of 1 ml, 20 mM Trizma (Sigma) buffered to pH 7.5 with HCl, 50 mM dimethyl sulphoxide, 0.3 mM hypoxanthine, 5 μM FeCl₃ (added from 50 μM stock freshly prepared), 0.5 μM H₂O₂, 18 m-units of xanthine oxidase (Grade III; Sigma; from buttermilk). After 15 min (at 25 °C) the fractions were quenched by 2 min immersion in a boiling-water bath, and 250 μl formaldehyde reagent was added. This consists of 15 g of ammonium acetate, 0.3 ml of acetic acid and 0.2 ml of acetylacetone in a final volume of 100 ml of water. The tubes were incubated at 60 °C for 15 min to develop the colour, and the absorbance was measured at 410 nm.

RESULTS

Interaction of Fe³⁺ and higher inositol polyphosphates

Although this investigation is concerned primarily with the relative efficacy of some naturally occurring inositol polyphosphates to inhibit iron-catalysed OH⁻ formation, we began by investigating the overall ability of InsP₄, and the InsP₆ isomers used here, to bind Fe³⁺. This was done indirectly, and in two ways. First, increasing concentrations of InsP₄ were mixed with 1 mM Fe₃(SO₄)₂ (unbuffered, pH 6.5, or buffered to pH 7.0) and their ability to prevent precipitation of Fe(OH)₃ was assessed by the formation of stable clear solutions. InsP₄ at 100 μM had a significant inhibitory effect on precipitation, and at 1 mM inhibited precipitation completely (results not shown). Given the extremely low solubility of Fe(OH)₃ these data imply a very high affinity of Fe⁴⁺ for InsP₄ (i.e. an overall affinity constant for Fe⁺⁴ binding to InsP₄ > 10⁻¹¹; Biederman and Schindler, 1957). Because of the semi-quantitative nature of this assay we did not explore extensively the potency of the four InsP₄ isomers (see below), but, when equimolar with Fe⁴⁺, all four also completely inhibited precipitation, implying that they too have a very high affinity for Fe⁺⁴. The important point is that in the experiments on iron-catalysed OH⁻ formation described below, when any InsP₆ or InsP₄ is added in excess of Fe⁺⁴, the Fe⁺⁴ will be very largely bound to the inositol phosphate.

Some idea of the relative affinity of InsP₆ for Fe⁺⁴ was deduced by competition experiments measuring the decolorization of FeCl₃/catechol complexes (see the Materials and methods section). Any compound that is able to compete with catechol for Fe⁺⁴ in the same concentration range as the Fe⁺⁴–catechol complex (0.25 mM in this case) must have an affinity for Fe⁺⁴ that is of a similar order to, or greater than, that of catechol (the Kᵣ for which is approx. 10⁻⁹; Martell and Smith, 1982). The data (Figure 2) show that InsP₆, EDTA and Desferral all fall into this category; the greater potency of InsP₆ compared with the other two chelators is presumably because InsP₆ has multiple phosphates which are capable of chelating Fe⁺⁴ with high affinity (i.e. more than one Fe⁺⁴ can be bound per InsP₆; Graf et al., 1987). The discontinuity in the InsP₆ inhibition curve (Figure 2) may imply differing affinities between the phosphates, or interactions between the phosphates as they progressively bind Fe⁺⁴, but we have not explored this further. Another point that emerges from Figure 2 is that InsP₆ is considerably more potent in this assay than ATP. We do not know exactly the intracellular concentrations of InsP₆ (see the Introduction), but the data imply that, even in the presence of physiological levels of ATP, InsP₆ is likely to compete very well with ATP for this cation. Finally, we investigated the relative affinities of other cations by using the radical formation assays described below and exploring the ability of the cations to reverse the inhibitory effect of InsP₆ on radical formation catalysed by 5 μM Fe⁺⁴. Of these, only Al⁺³ was effective at releasing Fe⁺⁴ from InsP₆ (as judged indirectly by radical formation), whereas Zn⁺², Ni⁺², Mn⁺², Pb⁺² and Ca⁺² were without discernible effect (results not shown). Taken all together, these data suggest a very high affinity interaction between inositol polyphosphates and Fe⁺⁴, and our aim was then to investigate to what extent this interaction inhibits the ability of Fe⁺⁴ to generate OH⁻ radicals.

Linearity of radical forming assay

As we were interested in quantitative differences between the ability of inositol polyphosphates to interact with iron, we have tried to make the hypoxanthine/xanthine oxidase system linear with respect to added Fe⁺⁴. This has provided some problems (see below and the Materials and methods section). The formation of the OH⁻ radical is a multi-stage process involving more than one oxidation state of iron [see the Materials and methods section and Graf et al. (1984, 1987)] and the reaction between OH⁻ and dimethyl sulphoxide causes a variety of products to be formed (see Halliwell and Gutteridge, 1985), only one of which (formaldehyde) is being measured in the assays, and these factors may explain some of the complexities that we have encountered. OH⁻ formation proceeded in the absence of added Fe⁺⁴, and this was inhibited by low concentrations of the Fe⁺⁴ chelator Desferral (Halliwell and Gutteridge, 1985; Graf et al., 1987). From this we deduced that it is due to Fe⁺⁴ in the reagents, and
we traced the Tris buffer as the principal source; the reaction was to some degree proportional to Tris concentration, and changing to a purer form of Tris (Trizma, Sigma) decreased the reaction rate. Nevertheless, we could not eliminate this entirely, and titration with Desferral (Figure 3) suggested that less than 1 μM Fe³⁺ was present.

Addition of more Fe³⁺ (Figure 4a) gives an erratic but highly reproducible curve, with a high level of activity at 1 μM added Fe³⁺ which then decreased and levelled off. We did not see this erratic behaviour with 40 μM EDTA present (Figure 4b), and so we believe this is to do with the inherent difficulties of (a) multiple reactions and products, of which we are only measuring one [see above and Hallwell and Gutteridge (1985)] and (b) the inevitable difficulty of working with very low levels of unbuffered multivalent cations.

As a final compromise we added 5 μM Fe³⁺, and we have had to accept that quantitative conclusions must be limited. For blanks we used heat-killed enzyme, though there was a very slight colour development (< 5% of that with enzyme) in its absence. Extensive exploration of blanks has convinced us that a high concentration of Ins⁶ or Desferral (in excess of Fe³⁺) does truly reduce OH⁻ generation to zero within the limits of detection of this method.

Inhibition of radical formation by myo-inositol polyphosphates

The effect of all the Ins⁶ isomers [four of them as two enantiomeric pairs; see Stephens et al. (1991)] on OH⁻ generation in our standard assay is shown in Figure 5. If 50 μM Fe³⁺ was used instead of 5 μM, similar results were obtained for Ins⁶ and Ins(1,3,4,5,6)⁶P₆ (the only two InsPs of which we had sufficient for high concentrations), but, as is to be expected, more of each inositol phosphate was needed to achieve the same percentage inhibition (results not shown). It is clear that the compounds fall into two distinct groups (Figure 5). Ins⁶ at about 10–30 μM causes a complete inhibition (see above), and we found that Ins⁶ preparations from several sources (prepared from mung beans; h.p.l.c.-purified from Sigma Ins⁶) were similar in their potency and ability to inhibit completely the radical generation (results not shown). Ins(1,2,3,4,6)⁶P₆ and D/L-Ins(1,2,3,4,5,6)⁶P₆ are similar to Ins⁶. They appear slightly less potent than Ins⁶, but it is clear that they reach the same end point, i.e. complete inhibition under our assay conditions. Ins(1,3,4,5,6)⁶P₆ and D/L-Ins(1,2,4,5,6)⁶P₆ are profoundly different. They are less potent and, most importantly, they do not cause a complete inhibition under our assay conditions. We did not have sufficient D/L-Ins(1,2,4,5,6)⁶P₆ to increase it to a very high concentration, but we increased Ins(1,3,4,5,6)⁶P₆ to 600 μM, and still it had not inhibited radical formation completely (Figure 5). Neither Ins(1,4,5)⁶P₆ nor Ins(1,3,4,5)⁶P₆ caused any inhibitory effect at all up to 500 μM, and both in fact caused a slight (approx. 20%) stimulation, and ATP at 1 mM caused only a small inhibition (results not shown).

The implication from these data overall is that the 1,2,3 phosphate grouping in Ins⁶ is essential for the complete inhibition of free-radical formation. In this context, it is noteworthy...
that, notwithstanding the semi-quantitative assay, we can see that a considerable excess of InsP6 over Fe3+ is required (Figure 5), which contrasts with the data of Figure 2, from which we deduced that multiple phosphate moieties on InsP6 are binding Fe3+ tightly; this apparent contradiction could be because to cause complete inhibition of radical formation the 1,2,3 grouping must bind all the iron (and moreover it must do so in competition with the other phosphate moieties on the InsP6). We should note, however, that Graf et al. (1984) found complete inhibition of radical formation at iron/InsP6 ratios of less than 1, and we have no explanation for this other than differences in the assay (see above).

**Inhibition by other potential chelators**

In agreement with Graf et al. (1984, 1987) and Graf and Eaton (1990) we found that whereas Desferral caused a complete inhibition (Figure 3), EDTA did not (see also Beauchamp and Fridovich, 1970). EDTA was more potent than InsP6 at low concentrations, but even at millimolar levels there was still some reaction occurring, whereas, in parallel incubations, InsP6 at 30 μM caused complete inhibition (results not shown).

The 2-phosphate is the only axial one in InsP6 in the predominant chair conformation (Emsley and Niazi, 1981; Figure 1b), and it is therefore an intriguing possibility that an equatorial-axial-equatorial conformation has the precise geometry for this complete inhibition of radical generation. We were fortunate to gather some more direct information on this by the gift of some synthetic *scylo*-inositol hexakisphosphate from Dr. M. F. Tate. *scylo*-inositol hexakisphosphate is identical with InsP6, except that all its phosphate groups are equatorial (Figure 1b). When we tested this in the assay we found that it is clearly more effective at reducing OH· formation than Ins(1,3,4,5,6)P6, especially at high concentrations, but it is also clearly and reproducibly unable to block this completely (Figure 6). This goes some way to confirming the implication above, that it is the equatorial-axial-equatorial grouping of InsP6 (*myo*-inositol hexakisphosphate) which is uniquely (in the present inositol phosphate context) able to prevent iron entirely from catalysing OH· formation.

**Conclusions and physiological significance**

The data presented above enable us to group the ion chelators studied into two divisions, based on whether, given sufficient concentrations, inhibition of the iron-catalysed OH· radical formation is complete. Graf et al. (1984) drew a similar line: only Desferral and InsP6 in their experiments would stop this completely, whereas other chelators allowed some activity to proceed. The reasons for this are not clear, though Graf et al. (1984, 1987) suggested that the exclusion of water from the co-ordination complex or iron and an inhibition of Fe3+ reduction (which forms Fe2+, an essential catalyst in the generation of OH· radicals by the mechanisms studied here; see the Materials and methods section) might both contribute to this phenomenon. The precise mechanisms are not our concern here. What is striking is that our results suggest that a unique conformation of the phosphate grouping in InsP6 is probably responsible. The binding of iron to InsP6 is likely to be very complex, with a number of different iron/InsP6 stoichiometries possible, each possessing individual and possibly quite different stability constants [compare, for example, the large polynuclear complexes described containing Fe3+ and ATP (Mansour et al., 1985)], and thus detailed physical studies are likely to be required before this postulated interaction can be interpreted at the molecular level. It is interesting, however, that the distinctive behavior of the InsP6 isomers in the radical generation assay (i.e. the qualitative difference between D/L-Ins(1,2,4,5,6)P6/Ins(1,3,4,5,6)P6 and D/L-Ins(1,2,3,5,6)P6/Ins(1,3,4,5,6)P6) is closely mirrored by their relative abilities to inhibit InsP6 binding to cerebellar membranes (Poyner et al., 1993) and their grouping by chromatography in the presence of HCl on polyethyleneimine-cellulose (Figure 1) and Dowex-1 resin (Cosgrove, 1980), indicative perhaps of some common physical basis for these observations.

As discussed in the Introduction, our current knowledge of InsP6 metabolism in animal cells implies that the 2-phosphate may be the last to be added, and the 3-phosphate the first to be removed; also, it is apparent that Ins(1,3,4,5,6)P6 is probably the
only InsP₆, which is found at cellular concentrations close to (sometimes greater than) InsP₇ (Stephens et al., 1991; McConnell et al., 1991). So, one could argue that InsP₆ is unique among inositol phosphates in its high cellular levels, and its ability to bind iron entirely ‘safely’, i.e., in a way that completely prevents iron-catalysed formation of OH radicals. Since iron-catalysed OH radical formation is an event potentially lethal to living cells (Halliwell and Gutteridge, 1985), this property further serves to suggest that InsP₆ may be a physiologically relevant ligand for iron in cells (see the Introduction).

In conclusion, as Graf et al. (1987) have emphasized, the ability of InsP₆ to prevent OH radical formation entirely is unusual among chemical compounds, and on present knowledge it is unique amongst endogenous chemicals. Our observations here point to this property being due to the 1,2,3 (equatorial–axial–equatorial) phosphate grouping, a conformation probably only found in InsP₆ out of the inositol phosphates which exist in cells at high levels. We believe that it is therefore a reasonable suggestion that this may be one reason for InsP₆’s universal occurrence in cells, and that it provides a possible physiological function of this molecule.

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