Structure–Activity Relationship of Inhibitors of Hydroxyapatite Formation

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The relative abilities of some compounds to inhibit the transformation of amorphous calcium phosphate into hydroxyapatite were determined. Organic diphosphates, fructose 1,6-bisphosphate, pyrophosphate, imidodiphosphate and 3-phosphoglycerate all inhibited to various degrees. Strong inhibition was observed with phosphonoformate, and phosphocitrate proved to be the most powerful of the inhibitors examined. On the basis of these new findings an improved concept of the structure–activity relationship of inhibitors is proposed and mechanisms for inhibitor action are discussed.

The process of calcium phosphate deposition is fundamental to the formation of renal stones, mitochondrial granules and the hydroxyapatite of bone (Posner, 1973; Lehninger, 1977; Bygrave, 1978; Fleisch, 1978). Amorphous calcium phosphate is recognized as the precursor of hydroxyapatite both *in vitro* (Eanes & Meyer, 1977; Meyer & Eanes, 1978*a,b*) and *in vivo* (Dougherty, 1978; Posner & Betts, 1975). The ability of certain compounds to inhibit the conversion of amorphous calcium phosphate into hydroxyapatite has been extensively examined (Termine *et al.*, 1970; Sutor *et al.*, 1978). Pyrophosphate, for example, is a potent inhibitor of this conversion and appears to be important to bone mineralization (Fleisch & Russell, 1972). Other small ions such as Mg^{2+} and citrate are also inhibitors and, together with pyrophosphate, are considered to account for 77% of the ‘inhibitory power’ of urine (Bisaz *et al.*, 1978). ATP and Mg^{2+} have been shown to have a synergistic effect on the inhibition of the transition of amorphous calcium phosphate into hydroxyapatite and are believed to stabilize mitochondrial deposits of amorphous calcium phosphate (Blumenthal *et al.*, 1977).

Despite the range of compounds known to inhibit the transition and the mechanisms proposed to explain their activity, little definitive information has been published to enable a structure–activity relationship of inhibition to be deduced. It has been suggested that there is a link between an $H_2O_3P-X-PO_3H_2$ structure ($X = C, N$ or O) and inhibitor activity (Robertson & Fleisch, 1970), but the findings that fructose 1,6-bisphosphate and glycerol 2,3-bisphosphate can inhibit led Termine & Cohn (1976) to propose that ‘if inhibitor P–O–P bonds are not present, low molecular weight metabolites containing two ester phosphates (but not monophosphate or carboxylic acid groups) also inhibit’.

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Two compounds that do not fit into the structural criteria proposed, namely phosphocitrate and phosphonoformate, have recently become available. Phosphocitrate is of interest because it had been postulated to be a potent inhibitor of hydroxyapatite formation (Howard, 1976; Lehninger *et al.*, 1978), whereas the inhibitory power of phosphonoformate is unknown. In the present paper, we report on the ability of phosphocitrate and phosphonoformate to inhibit the transition of amorphous calcium phosphate into hydroxyapatite compared with that of other inhibitors. On the basis of the new data, a modified structure–activity relationship is proposed from which other compounds might be recognized as calcification inhibitors.

Materials and Methods

The transformation of amorphous calcium phosphate into hydroxyapatite was followed titrimetrically essentially as described by Meyer & Eanes (1978*a*). Portions (5.0 ml) of 0.50 M- $CaCl_2$ and 0.50 M- NaH_2PO_4 were added to 600 ml of stirred high-purity decarbonated water and equilibrated in a water bath. Sufficient 2 M- $NaOH$ was added over 90 s to adjust the pH to 7.40. The mixture was maintained at this pH by the addition of 0.05 M- $NaOH$ by using a pH-stat (Radiometer, Copenhagen, Denmark). Care was taken to exclude CO_2 from the system. The induction time (I_i) was measured as outlined by Meyer & Eanes (1978*a*). The difference in induction time with or without an inhibitor is designated ΔI_i and reflects the ability of a particular concentration of a compound to inhibit the transformation of amorphous calcium phosphate into hydroxyapatite. Inhibitory ability was measured at either 20 or 30°C. At 20°C any changes in the I_i were highlighted, whereas at 30°C I_i values were considerably shortened, but the same trends in inhibitor

strength were observed. Hence 30°C was used as a routine since it facilitated a more rapid determination of inhibitory power.

Adenosine phosphates, fructose phosphates, 3-phosphoglycerate and aminoethylphosphonic acid were products from Calbiochem, Sydney, N.S.W., Australia. Imidodiphosphate was prepared as outlined by Yount (1974), and phosphocitrate was synthesized from 2-cyanoethyl phosphate and triethyl citrate (G. Williams, unpublished work). Phosphonoformate was a generous gift from Astra Läkemedel A.B., Södertälje, Sweden. All chemicals were reagent grade.

Results and Discussion

As shown in Fig. 1, inhibitor activity can be readily assessed by a plot of ΔI_i against concentration of the test compound. Before the present investigation the potential of phosphocitrate to stabilize amorphous calcium phosphate was unknown, but, from Fig. 1, it is evident the phosphocitrate is a significantly more powerful inhibitor than imidodiphosphate, one of the most potent of the known inhibitors of hydroxyapatite formation (Fleisch, 1978). The finding of this remarkable inhibitory ability of phosphocitrate adds support to the suggestion that it may be the potent calcification inhibitor isolated from natural sources by others (Howard, 1976; Lehniger *et al.*, 1978).

Fig. 1. Effects of phosphocitrate (□) and imidodiphosphate (○) concentration on the induction time (I_i) of the conversion of amorphous calcium phosphate into hydroxyapatite. Induction times were determined at 20°C as described in the text.

Fig. 2 shows the results obtained with a wide range of compounds selected for structural features thought to influence inhibitor power. Of interest was the finding that phosphonoformate inhibited hydroxyapatite formation to a degree that was similar to that obtained with pyrophosphate, a compound accepted to be a strong inhibitor of calcification. Thus the fact that phosphonoformate (carboxylic monophosphate) and phosphocitrate (carboxylic phosphate ester) can both inhibit hydroxyapatite formation necessitates re-examination of the concept proposed by Termine & Cohn (1976) that at least two phosphate ester groups are required for activity. Citric acid was found to be a poor inhibitor over the concentration range tested (0.01–0.2 mM), whereas (not shown in Fig. 2) other carboxylic acids, including formic acid, succinic acid, malic acid and oxalic acid, failed to show any activity. Other compounds examined that did not inhibit were 1- and 2-aminoethylphosphonic acid, fructose 1-phosphate, *p*-nitrophenyl phosphate and EGTA.

These results suggest that, for strong inhibition, a molecule should possess at least one phosphate group and at some other position another acidic group, be it phosphate or a carboxylic acid group. Indeed, as predicted by our criteria, 3-phosphoglycerate was found to inhibit the formation, which is contrary to the experimental evidence obtained by Termine & Cohn (1976). We believed that there may be other

Fig. 2. Effects of inhibitor concentration on the induction time (I_i) of the conversion of amorphous calcium phosphate into hydroxyapatite.

Inhibitors tested were: ○, imidodiphosphate; ■, ATP; ◆, pyrophosphate; ◇, phosphonoformate; □, ADP; ▲, fructose 1,6-bisphosphate; ●, 3-phosphoglycerate; △, citrate. Inhibitory activity was determined at 30°C as described in the text.

natural phospho compounds as yet unrecognized as calcification inhibitors.

On consideration of the proximity of the two prime groups, it is evident that the closer they are together the more active is the molecule as an inhibitor. For example, pyrophosphate is more active than fructose 1,6-bisphosphate, and phosphonoformate inhibits to a greater degree than 3-phosphoglycerate. When the minimum requirement of a phosphate and an acidic group is fulfilled, it appears that the addition of further chelating groups to an inhibitor molecule confers increased inhibitory ability. Thus ATP is a more powerful inhibitor than ADP, and phosphocitrate inhibits to a greater degree than either phosphonoformate or 3-phosphoglycerate. In a study of the ability of multidentate organic phosphonates to inhibit hydroxyapatite formation, the number of chelating groups and their proximity to one another have been found to influence inhibitory power (Meyer & Nancollas, 1973). These findings conform to our proposals.

Despite the minimum group requirements, the stereochemical arrangement of these groups also must be important. For example, Meyer & Nancollas (1973) showed that 1-hydroxyethane-1,2-diphosphonate was a stronger inhibitor of hydroxyapatite formation than was nitrilotri(methylenephosphonic acid), even though the latter possessed three phosphate groups. Thus it was suggested that the tetrahedral carbon atom in 1-hydroxyethane-1,2-diphosphonate was superior to the trigonal nitrogen atom as a centre for radiating chelating groups. In this regard it is noteworthy that phosphocitrate possesses a tetrahedral carbon atom from which four chelating 'arms' radiate. The stereochemical factor may give the molecule extra affinity for hydroxyapatite defect sites.

Although our data do not allow positive identification of the modes of action of the inhibitors, it is useful to consider other factors that might affect inhibitor action and activity. All the inhibitors have the potential to chelate Ca^{2+} , but inhibition is not due to Ca^{2+} chelation as such because EGTA does not significantly alter I_i . Neither is activity due to attachment of a sterically bulky group to a phosphate moiety, since *p*-nitrophenyl phosphate (with a P-O-C linkage) and 1- and 2-aminoethylphosphonic acid (with a P-C linkage) failed to inhibit the transformation. Inhibition might have been expected if it were difficult to incorporate sterically bulky molecules into the hydroxyapatite lattice thus making hydroxyapatite energetically less favoured.

Obviously other inhibitory mechanisms are possible. From studies with ATP as an inhibitor Blumenthal *et al.* (1977) have suggested that the transition of amorphous calcium phosphate into hydroxyapatite occurs only after ATP is hydrolysed, thus removing this inhibitor of hydroxyapatite nucleation.

Another mechanism might be the adsorption of inhibitor molecules on particles of amorphous calcium phosphate, thus altering the lability and solubility of this material and affecting its stability relative to hydroxyapatite. Evidence for this concept comes from i.r. studies, which have shown that inhibitors have a strong affinity for amorphous calcium phosphate (Termine & Cohn, 1976). Inhibition of crystal growth is yet another possible mode of action, since the apparent solubility may then be greater than the thermodynamic solubility of the crystal material (Finlayson, 1978). 1-Hydroxyethane-1,2-diphosphonate is a crystal-growth inhibitor demonstrated to have this effect on brushite. Crystal growth is considered to proceed from defect sites, so molecules with affinity for crystal defects will, in our experimental system, destabilize hydroxyapatite. Of the compounds tested here, pyrophosphate and imidodiphosphate are both known inhibitors of hydroxyapatite crystal growth and delayed the transition of amorphous calcium phosphate into hydroxyapatite, as did phosphocitrate, which may have a stereochemistry especially suited to binding at defect sites.

Despite the range of inhibitory mechanisms possible, it is evident from our study that there is a certain minimum structural requirement to be fulfilled before a molecule can inhibit. We suggest that it should possess at least a phosphate group and an acidic group, the latter being either a phosphate group or a carboxylic acid group. These groups may confer to the molecule a special affinity for a calcium phosphate surface. Secondary stereochemical and structural factors will then determine what sites on amorphous calcium phosphate or hydroxyapatite are preferred. Once a molecule can bind sufficiently to the calcium phosphate surface then there exist many mechanisms, as discussed above, by which the transition of amorphous calcium phosphate into hydroxyapatite can be inhibited.

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References

- Bisaz, S., Felix, R., Neuman, W. F. & Fleisch, H. (1978) *Miner. Electrolyte Metab.* **1**, 74-83
- Blumenthal, N. C., Betts, F. & Posner, A. S. (1977) *Calcif. Tissue Res.* **23**, 245-250
- Bygrave, F. L. (1978) *Biol. Rev. Cambridge Philos. Soc.* **53**, 43-79
- Dougherty, W. J. (1978) *Metab. Bone Dis. Relat. Res.* **1**, 119-123
- Eanes, E. D. & Meyer, J. L. (1977) *Calcif. Tissue Res.* **23**, 259-269
- Finlayson, B. (1978) *Kidney Int.* **13**, 344-360
- Fleisch, H. (1978) *Kidney Int.* **13**, 361-371

- Fleisch, H. & Russell, R. G. G. (1972) *J. Dent. Res.* **51**, 324–332
- Howard, J. E. (1976) *Johns Hopkins Med. J.* **139**, 239–252
- Lehninger, A. L. (1977) *Horiz. Biochem. Biophys.* **4**, 1–30
- Lehninger, A. L., Reynafarge, B., Vercesi, A. & Tew, W. P. (1978) *Ann. N.Y. Acad. Sci.* **307**, 160–176
- Meyer, J. L. & Eanes, E. D. (1978a) *Calcif. Tissue Res.* **25**, 59–68
- Meyer, J. L. & Eanes, D. E. (1978b) *Calcif. Tissue Res.* **25**, 209–216
- Meyer, J. L. & Nancollas, G. H. (1973) *Calcif. Tissue Res.* **13**, 295–303
- Posner, A. S. (1973) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32**, 1933–1937
- Posner, A. S. & Betts, F. (1975) *Acc. Chem. Res.* **8**, 273–281
- Robertson, W. G. & Fleisch, H. (1970) *Biochim. Biophys. Acta* **222**, 677–680
- Sutor, D. J., Percival, J. M. & Doonar, S. (1978) *Clin. Chim. Acta* **89**, 273–278
- Termine, J. D. & Cohn, K. M. (1976) *Calcif. Tissue Res.* **22**, 149–157
- Termine, J. D., Peckauskas, R. A. & Posner, A. S. (1970) *Arch. Biochem. Biophys.* **140**, 318–325
- Yount, R. G. (1974) *Methods Enzymol.* **38C**, 420–427