Modulation of crystal formation by bone phosphoproteins: role of glutamic acid-rich sequences in the nucleation of hydroxyapatite by bone sialoprotein

Graeme K. HUNTER* and Harvey A. GOLDBERG

* Division of Oral Biology, Faculty of Dentistry, University of Western Ontario, London, Ontario N6A 5C1, Canada

Bone sialoprotein (BSP) is a bone-specific glycoprotein containing phosphoserine and sulphotyrosine residues and regions of contiguous glutamic acid residues. Recent studies in this laboratory have shown that BSP is capable of nucleating the bone mineral hydroxyapatite in a steady-state agarose gel system. We show here that chemical modification of carboxylate groups abolishes the nucleation activity of BSP, but enzymic dephosphorylation has no effect. Formation of hydroxyapatite is also induced by poly(L-glutamic acid) and poly(D-glutamic acid), but not by poly(L-aspartic acid) or poly(L-lysine). Calreticulin, a muscle protein with short sequences of contiguous glutamic acid residues, also lacks nucleation activity. These findings suggest that the nucleation of hydroxyapatite by BSP involves one or both of the glutamic acid-rich sequences. Based on these findings and others, we propose that polycarboxylate sequences represent a general site for growth-modulating interactions between proteins and biological crystals.

INTRODUCTION

The mineralization of bone occurs by the ordered deposition of hydroxyapatite (HA) crystals in an extracellular matrix consisting of type I collagen and various non-collagenous proteins. Because of the intimate and specific relationship between HA crystals and extracellular matrix structures, it has long been thought that matrix components are involved in the initiation of mineralization (for review, see Hunter, 1992). Specifically, it has been suggested by several workers that HA crystals are nucleated by a complex composed of type I collagen fibrils and an anionic protein, probably a phosphoprotein (Glimcher, 1984; Gorski, 1992; Veis, 1992).

The major phosphorylated proteins of bone are bone sialoprotein (BSP) (Fisher et al., 1983) and osteopontin (Franzen and Heinegard, 1985; Prince et al., 1987). Other bone proteins reported to contain bound phosphate include osteonectin (Termine et al., 1981), a collagen propeptide (Fisher et al., 1987) and BAG-75 (Gorski and Shimizu, 1988). Of these, BSP is most specific to bone (it is also found in trophoblasts and calcified cartilage) (Bianco et al., 1991), and is also associated temporally and spatially with initial calcification events (Chen et al., 1991; Kasugai et al., 1992; Bianco et al., 1993). For these reasons, BSP has been proposed to function in the initiation of HA formation (Chen et al., 1991). BSP has a molecular mass of approximately 57000 Da, of which almost half is carbohydrate (Fisher et al., 1983; Franzen and Heinegard, 1985). Other post-translational modifications include phosphorylation (Franzen and Heinegard, 1985) and tyrosine sulphation (Ecarot-Charrier et al., 1989). In all species studied, the primary sequence of BSP exhibits a high content of the acidic amino acids (particularly glutamic acid), an RGD cell attachment sequence and two regions containing contiguous glutamic acid residues (Oldberg et al., 1988; Fisher et al., 1990; Shapiro et al., 1993).

The effects of bone phosphoproteins on HA formation have been studied in several *in vitro* systems. Osteonectin has been shown to inhibit HA formation and growth in metastable calcium phosphate solutions (Menanteau et al., 1982; Romberg et al., 1986). In association with type I collagen fibrils, osteonectin has been reported both to promote (Termine et al., 1981) and to inhibit (Doi et al., 1989) HA formation. Osteopontin is a potent inhibitor of HA formation in metastable calcium phosphate solutions and in steady-state gelatin and agarose gels (Boskey et al., 1993; Hunter et al., 1994). The inhibitory activity of osteopontin appears to involve both phosphate groups and aspartic acid-rich sequences (Hunter et al., 1994). The effect of BSP on HA formation has recently been studied using a steady-state agarose gel system (Hunter and Goldberg, 1993). It was shown that BSP induces the formation of HA at $Ca \times PO_4$ concentration products below the threshold for spontaneous precipitation. Based on this finding, and consistent with its known biological properties, we have proposed that BSP is involved in the nucleation of HA crystals at the mineralization front of bone (Hunter and Goldberg, 1993). The present study was designed to identify the specific structural motifs in the BSP molecule that are responsible for the HA-nucleating effect.

EXPERIMENTAL

Materials

Bovine intestinal alkaline phosphatase bound to agarose beads (P-0762), 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide, poly(L-glutamic acid) [P-4761 (34000 Da) and P-4886 (51300 Da)], poly(D-glutamic acid) (P-4033; 41000 Da), poly(L-aspartic acid) (P-6762; 28800 Da) and poly(L-lysine) (P-7890; 25000 Da) were all obtained from Sigma Chemical Co. *N*-Hydroxysulphosuccinimide was obtained from Serva Biochemicals. Rabbit calreticulin preparations (Baksh and Michalak, 1991; Baksh et al., 1992) were kindly provided by Dr. Marek Michalak, University of Alberta, Edmonton, Alberta, Canada.

Steady-state system

Nucleation of HA was studied using a modification of the steady-state agarose gel previously described (Hunter and Goldberg, 1993). Agarose gels (1% agarose; volume approx.

Abbreviations used: HA, hydroxyapatite; BSP, bone sialoprotein; GEE, glycine ethyl ester.

* To whom correspondence should be addressed.

1 ml) containing 0.15 M NaCl, 10 mM Tris/HCl and 0.01 % NaN_3 were poured into the central cavities of modified equilibrium dialysis cells. Solutions containing 6.0 mM $CaCl_2$ or 6.0 mM sodium phosphate, pH 7.4, plus the above concentrations of NaCl, Tris/HCl and NaN_3 were pumped through chambers at either end of the agarose gels at a flow rate of 1 ml/h per gel. The entire apparatus was incubated at 37 °C for various times (usually 5 days). The gels were then ashed in concentrated nitric acid at 85 °C, diluted with deionized water and analysed for phosphate by the phosphomolybdate method (Chen et al., 1956) and for Ca by atomic adsorption spectrophotometry using a Varian SpectrAA 30/40.

Enzymic dephosphorylation of BSP

BSP was purified from adult porcine calvaria as previously described (Zhang et al., 1990). Dephosphorylation of BSP was performed by treatment of approx. 50 μg of protein with 0.5 unit of bovine intestinal alkaline phosphatase bound to agarose beads in 0.1 M Tris/HCl, pH 8.0 (total volume 1 ml). A control solution contained BSP and Tris, but no enzyme. These samples were incubated at 37 °C for 4 h on a tube rocker. The enzyme beads were then removed by brief centrifugation, and the supernatant dialysed against deionized water (three changes of 1000 volumes). Aliquots of alkaline phosphatase-treated and untreated BSP dialysates were used for organic phosphate determination by the method of Van Veldhoven and Mannaerts (1987).

Chemical modification of BSP

Carboxylate group modification of BSP was performed by a variation (Staros et al., 1986) of the method of Hoare and Koshland (1967). The reaction solution (volume 1 ml) contained approx. 100 μg of BSP, 10 mM glycine ethyl ester (GEE), pH 4.75, 50 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide and 5 mM *N*-hydroxysulphosuccinimide. A control solution contained BSP and GEE as above, but no catalyst. These solutions were incubated at 37 °C for 4 h without mixing, dialysed separately against deionized water and lyophilized. To determine the extent of modification, aliquots corresponding to approx. 10 μg of modified and unmodified protein were hydrolysed (6 M HCl at 115 °C for 18 h) and amino acid analysis was performed using a Beckman 119CL single-column analyser. From the difference in glycine content between the two preparations, the efficiency of derivatization was calculated.

RESULTS

Effect of dephosphorylated BSP on HA formation in steady-state gels

To determine whether phosphate groups are involved in the BSP-mediated nucleation of HA, porcine calvarial BSP was dephosphorylated by treatment with the enzyme alkaline phosphatase. This treatment removed approx. 85 % of the organic phosphate in BSP.

Alkaline phosphatase-treated and untreated BSP were incorporated at 5 μg/ml into steady-state agarose gels and incubated for 5 days prior to analysis of the gels for Ca and phosphate. As shown in Table 1, untreated BSP caused significantly higher levels of calcium phosphate accumulation than control gels (the $Ca+PO_4$ contents of control gels represent diffusion of reservoir solutions into the gels, not precipitation; Hunter and Goldberg, 1993). Alkaline phosphatase-treated BSP induced levels of calcium phosphate accumulation similar to those with untreated BSP, suggesting that phosphate groups are not required for the nucleation effect.

Effect of carboxylate-modified BSP on HA formation in steady-state gels

To determine whether carboxylate groups are required for HA nucleation, BSP was reacted with GEE in the presence of a water-soluble carbodi-imide. This reaction forms an amide bond between protein carboxylate groups and the amino group of GEE, resulting in loss of charge.

Amino acid analysis showed that modified BSP contained 84 glycine residues per mol more than the control protein. Porcine BSP contains 78 acidic amino acids per mol (22 Asp and 56 Glu) (Shapiro et al., 1993). Therefore GEE treatment results in essentially quantitative modification of carboxylate groups.

Incorporation of unmodified BSP at 5 μg/ml into steady-state agarose gels resulted in $Ca+PO_4$ contents significantly higher than those of control gels (Table 2). Incorporation of carboxylate-modified BSP, however, resulted in $Ca+PO_4$ contents similar to those of controls. Therefore modification of carboxylate groups completely abolishes the HA-nucleating activity of BSP.

Effects of synthetic polypeptides on HA formation in steady-state gels

To determine whether the polycarboxylate sequences of BSP are involved in the nucleation of HA, the effect of poly(L-glutamic acid) on HA formation was studied. Incorporation of poly(L-Glu) (5 μg/ml) into steady-state agarose gels resulted in an approximately linear accumulation of $Ca+PO_4$ during a 10-day incubation period, whereas poly(L-Asp) had no effect (Figure 1). At the same concentration of BSP, the rate of HA formation was approx. 1.5-fold higher than that with poly(L-Glu) (0.37 versus 0.24 mg of $Ca+PO_4$/gel per day; in both cases precipitation commenced after approx. 2 days of incubation. The mineral phase formed in the presence of poly(L-Glu) was identified as HA by powder X-ray diffraction (results not shown).

Incorporation of poly(L-Glu) at 2–20 μg/ml into steady-state gels resulted in $Ca+PO_4$ contents higher than the range with control gels at all concentrations (Figure 2). No strict proportionality between poly(L-Glu) concentration and the amount of precipitation was observed. Gels containing poly(L-Asp) at

Table 1 Effects of chemical and enzymic modification on the nucleation of HA by BSP

Alkaline phosphatase-treated, chemically modified and untreated BSP were incorporated into agarose gels at 5 μg/ml and incubated for 5 days. Calcium and phosphate contents were then determined as described in the Experimental section. Results shown are means ± S.D. of four determinations. Statistical significance was determined, compared with corresponding control values, using one-way analysis of variance and the Tukey multiple comparisons test; * $P < 0.001$. AP-BSP, alkaline phosphatase-treated BSP; GEE-BSP, carboxylate-modified BSP.

	$Ca+PO_4$ (mg/gel)
Control	0.436 ± 0.058
Unmodified BSP	1.480 ± 0.094*
GEE-BSP	0.444 ± 0.072
Control	0.353 ± 0.078
Untreated BSP	1.390 ± 0.109*
AP-BSP	1.270 ± 0.137*

Table 2 Effects of synthetic polypeptides on HA formation in steady-state agarose gels

Poly(L-Glu) (34000 Da), poly(D-Glu) (41000 Da), poly(L-Asp) (28000 Da) and poly(L-Lys) (25000 Da) were incorporated into agarose gels at 10 μg/ml and incubated for 5 days before analysis for Ca and phosphate (see the Experimental section). Results shown are means $\pm$ S.D. for numbers of determinations indicated in parentheses. Statistical significance compared with control value was determined as described in the legend to Table 1; * $P < 0.001$.

	$Ca + PO_4$ (mg/gel)
Control	0.445 ± 0.073 (6)
Poly(L-Glu)	1.080 ± 0.161 (6)*
Poly(D-Glu)	0.857 ± 0.193 (3)*
Poly(L-Asp)	0.388 ± 0.035 (6)
Poly(L-Lys)	0.425 ± 0.065 (3)

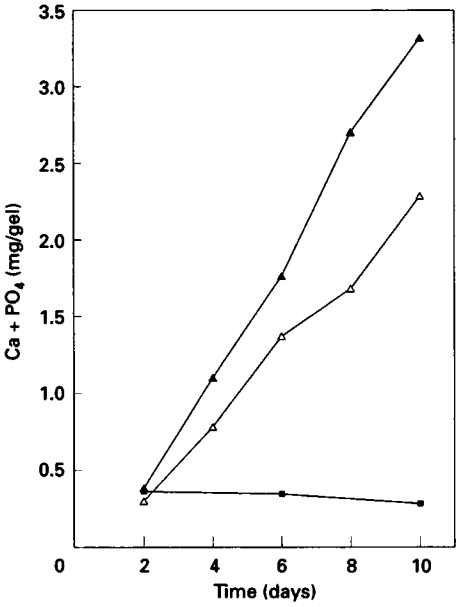

Figure 1 Kinetics of nucleation of HA by BSP and poly(L-Glu)

Agarose gels containing 5 μg/ml BSP (▲), poly(L-Asp) (28800 Da) (■) or poly(L-Glu) (51300 Da) (△) were harvested at various times during a 10-day incubation period, and analysed for Ca and phosphate as described in the Experimental section. The regression line for poly(L-Glu) (days 2–10) is described by the equation: $y = 0.24x - 0.18$ ($r = 0.996$). The regression line for BSP (days 2–10) is described by the equation: $y = 0.37x - 0.39$ ($r = 0.998$).

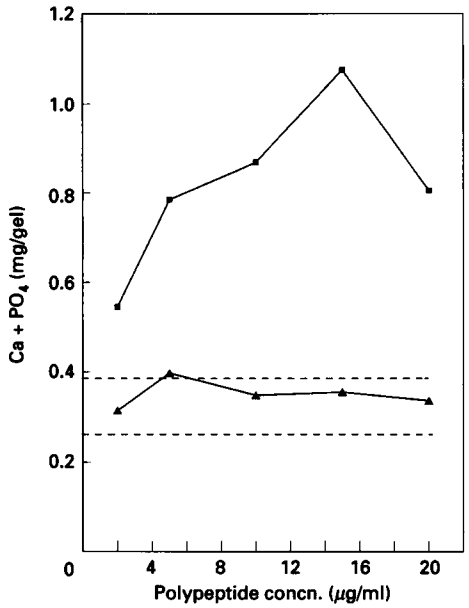

Figure 2 Effect of poly(L-Glu) and poly(L-Asp) concentration on HA formation in steady-state agarose gels

Poly(L-Glu) (34000 Da) (■) and poly(L-Asp) (28800 Da) (▲) were incorporated into agarose gels at various concentrations and incubated for 5 days prior to analysis for Ca and phosphate. The broken lines indicate the range of control values (mean $\pm$ 95% confidence intervals of three determinations).

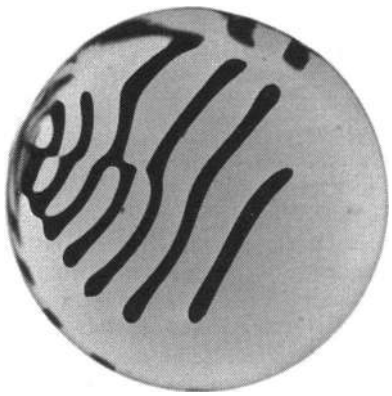

Figure 3 Morphology of precipitation induced by poly(L-Glu)

Poly(L-Glu) (34000 Da) was incorporated into an agarose gel at 10 μg/ml, and incubated for 5 days. A representative gel was photographed using backlighting on a Durst Chromatopro copy stand. The gel is viewed end-on from the calcium side of the apparatus. Magnification $\times$ 5.1.

2–20 μg/ml resulted in $Ca + PO_4$ contents within the range of those in control gels. Therefore poly(L-Glu) at concentrations as low as 2 μg/ml induced the formation of HA, whereas poly(L-Asp) at concentrations as high as 20 μg/ml had no effect.

To examine the stereospecificity of the poly(Glu)-mediated nucleation of HA, the effects of poly(L-Glu) and poly(D-Glu) on HA formation in steady-state gels were compared. At 10 μg/ml, both the D and L isomers of poly(Glu) induced the precipitation of calcium phosphate (Table 2). Therefore the nucleation activity of poly(Glu) is not stereospecific. Under similar conditions, poly(L-Asp) and poly(L-lysine) resulted in no formation of calcium phosphate (Table 2). Poly(L-lysine) likewise caused no formation of HA at 30 μg/ml (results not shown).

Incorporation of poly(Glu) into steady-state gels results in the formation of complex geometric patterns of precipitation, typically taking the form of partial or complete concentric rings, rather than the solid discs seen with BSP. This phenomenon is apparently responsible for the relatively high variability in the amount of precipitation induced by poly(Glu) (Table 2). A representative pattern of poly(L-Glu)-induced precipitation is shown in Figure 3.

Effect of calreticulin on HA formation in steady-state gels

Calreticulin is a calcium-binding protein from skeletal muscle that contains several sequences of contiguous glutamic acid residues in its C-terminal domain (Michalak et al., 1992). To determine whether these sequences have HA-nucleating activity, recombinant rabbit calreticulin was incorporated into steady-state agarose gels. At 20 μg/ml, neither calreticulin nor a fusion protein consisting of the calreticulin C-domain fused to glutathione S-transferase caused a significant increase in the $Ca + PO_4$ content of these gels (results not shown).

DISCUSSION

Previous studies performed in this laboratory have shown that BSP can induce the formation of HA in a steady-state agarose gel system under conditions of sub-threshold $Ca \times PO_4$ (Hunter and Goldberg, 1993). In the present study, chemical and enzymic modification and studies with synthetic polypeptides have been used to identify structural features of BSP that are required for this HA-nucleating activity.

To determine the role of phosphate groups in the nucleation of HA, BSP was treated with the enzyme alkaline phosphatase. This procedure removed approx. 85% of the organic phosphate. Alkaline phosphatase-treated BSP did not differ significantly from untreated protein in its ability to induce HA formation in steady-state agarose gels. Therefore either phosphate groups are not required for the nucleation effect, or else essential phosphate groups are resistant to the action of alkaline phosphatase. To determine the role of acidic amino acids in the BSP-mediated nucleation of HA, carboxylate groups in BSP were blocked by reaction with GEE. This procedure resulted in quantitative modification of carboxylate groups, suggesting that BSP has a relatively open structure, as previously indicated by rotary shadowing electron microscopy (Franzen and Heinegard, 1985). Chemical modification of BSP caused complete loss of nucleation activity, suggesting that carboxylate groups are necessary for the induction of HA formation.

Porcine BSP contains two regions that are enriched in glutamic acid, and in which several glutamic acid residues occur consecutively: EEEEEEEENSNEEENNEE (residues 61–78) and EEESDEDEEEEEENEE (residues 136–151) (Shapiro et al., 1993). Similar sequences are found in BSP from rat (Oldberg et al., 1988) and human (Fisher et al., 1990). In the light of the chemical modification studies described above, it was of interest to determine whether these sequences are involved in the nucleation of HA. Accordingly, we examined the effect of the synthetic polypeptide poly(L-Glu) on HA formation in the steady-state system. It was shown that poly(L-Glu), but not poly(L-Asp), induced the formation of HA in steady-state agarose gels under conditions similar to those used for BSP-mediated nucleation. This observation is consistent with a previous study by Garcia-Ramos and Carmona (1982), which showed that poly(L-Glu) increased the rates of formation of HA and brushite (dicalcium phosphate dihydrate) from metastable solutions. These workers suggested that acidic residues in proteins might promote calcification by scavenging protons released from phosphates during HA formation (Carmona and Rodriguez, 1987). However, the lack of nucleation observed with poly(L-Asp) in the present study does not support this mechanism.

Another protein that contains sequences of contiguous glutamic acid residues is calreticulin, a calcium-binding protein of skeletal muscle sarcoplasmic reticulum (Michalak et al., 1992). The C-terminal domain of calreticulin is highly enriched in glutamic acid and contains two EEEEE sequences. As described above, calreticulin has no HA-nucleating activity in steady-state agarose gels. Therefore nucleation appears to require either a longer sequence of glutamic acid residues or a conformation that is not adopted by the calreticulin C-domain.

Acidic amino acids have previously been implicated in the nucleation of calcite crystals in mollusc shell. The studies of Addadi and co-workers have shown that aspartic acid-rich proteins from the shell of the marine bivalve *Mytilus californianus* immobilized by binding to a solid-phase surface are capable of nucleating calcite crystals from calcium carbonate solutions (Addadi and Weiner, 1985; Addadi et al., 1987). The aspartic acid-rich proteins, which adopt a β-pleated sheet conformation in solution, bind to a specific face ([001]) of calcite and nucleate crystals from that face. A similar crystal face specificity may be involved in the interaction between bone proteins and HA (Moradian-Oldak et al., 1982). Osteonectin, osteocalcin and phosphophoryn have been reported to bind specifically to the [100] face of HA crystals (Fujisawa and Kuboki, 1991).

The studies on amino acid homopolymers described above were designed to generate information on the chemical specificity of the BSP-mediated nucleation of HA. Because the polypeptides used adopt different secondary structures, however, these studies can also provide insight into the conformational specificity of the nucleation activity. At low pH (< 5), spectroscopic analysis has shown that poly(L-Glu) exists in an right-handed α-helical conformation, which has 3.6 residues per turn and a rise of 0.15 nm per residue (McDiarmid and Doty, 1966). At higher pH, poly(L-Glu) adopts the conformation of a left-handed 'extended helix', with 2.4 residues per turn and 0.32 nm rise per residue (Tiffany and Krimm, 1969; Hiltner et al., 1972). Poly(D-Glu) exists as a left-handed α-helix at low pH (Brahms and Spach, 1963), and therefore presumably as a right-handed extended helix at neutral pH. Although less extensively studied than poly(L-Glu), poly(L-Asp) has been reported to lack ordered secondary structure (McDiarmid and Doty, 1966); however, a recent study indicates that poly(L-Asp) may adopt a β-pleated sheet conformation under some conditions (Addadi et al., 1991). Poly(L-Lys) has been reported to exist in an α-helical conformation at high pH (> 10), and an extended helix form at lower (neutral) pH (Tiffany and Krimm, 1969). At neutral pH, therefore, both poly(L-Glu) and poly(L-Lys) are in the left-handed extended helix conformation, poly(D-Glu) is in the right-handed extended helix conformation, and poly(L-Asp) is in a non-helical (possibly random coil) conformation.

Combining this structural information with the results of the present study allows some tentative conclusions to be reached about the conformational specificity of the nucleation effect. (i) Since poly(L-Glu) nucleates HA and poly(L-Asp) does not, nucleation activity appears to involve a helical conformation; specifically, the extended helix described above. (ii) Since poly(D-Glu) nucleates HA, the handedness of the helix does not appear to be important. (iii) Since poly(L-Lys) does not nucleate HA formation, the nucleation activity appears to be specific to extended helical polyanions. It is not yet clear whether these principles can be extrapolated directly to the nucleation activity of BSP. The predicted secondary structure of the porcine protein indicates that both sequences of contiguous glutamic acids are in α-helical regions (Shapiro et al., 1993). However, no experimental information on the conformation of BSP is available. To our knowledge, the extended helix has not been observed in any protein yet studied.

Another notable aspect of the poly(Glu)-mediated nucleation of HA is the formation of complex geometric patterns of precipitation. These typically consist of intact or partial concentric rings. In contrast, the HA precipitates formed in the presence of BSP, or by non-nucleated growth at higher $Ca \times PO_4$

concentrations (Hunter and Goldberg, 1993), consist of a solid disc of precipitate. The precipitation geometries observed with poly(Glu) are reminiscent of Liesegang rings, which are parallel bands of precipitation often seen when crystals are grown in gel-diffusion systems (Liesegang, 1896). However, Liesegang rings usually occur parallel to the direction of ionic diffusion, not perpendicular to the direction of diffusion as in the current case. Nor is it clear why these patterns should be seen with poly(Glu) but not with BSP.

In conclusion, the chemical and enzymic modification and model compound studies reported here indicate that nucleation of HA by BSP involves one or both of the glutamic acid-rich sequences. From the differing effects of various amino acid homopolymers, it appears that conformational factors are of great importance in the induction of HA formation. The findings described above, and those from the mollusc shell system, indicate that acidic amino acids represent a key structural determinant in the nucleation of biological crystals by extracellular matrix proteins.

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