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Effects of Zinc on Human Skeletal Alkaline Phosphatase Activity *In Vitro*

S. L. Hall, H. P. Dimai, J. R. Farley

Departments of Medicine and Biochemistry, Loma Linda University and Jerry L. Pettis Memorial Veterans Medical Center, 11201 Benton Street, Loma Linda California, 92357 USA

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Abstract. Inorganic phosphate (Pi) can regulate the level of skeletal alkaline phosphatase (ALP) activity in human osteoblast-like cells by stabilizing the enzyme (without affecting transcription, ALP release from the cell surface, or the amount of ALP protein). These observations suggest that Pi determines the level of ALP activity by modulating a process of irreversible inactivation. The current studies were intended to examine the hypothesis that this inactivation of ALP activity is caused by the dissociation of an active center Zn and that Pi inhibits that dissociation. Initial studies showed that Zn, like Pi, could increase ALP specific activity in human osteosarcoma SaOS-2 cells in a time- and dose-dependent manner (e.g., a 50% increase at 0.2 μmol/liter Zn, $P < 0.005$). This effect was specific for Zn (i.e., no similar effect was seen with Ca, Fe, Co, Mg, Mn, or Cu), but not for SaOS-2 cells. Zn also increased ALP specific activity in (human osteosarcoma) MG-63 cells and in cells derived from normal human vertebrae ($P < 0.001$ for each). The effect of Zn to increase ALP activity was not associated with parallel increases in total protein synthesis, collagen production, or tartrate-resistant acid phosphatase activity (no change in any of these indices), net IGF-2 synthesis (a Zn-dependent decrease, $P < 0.005$), or PTH-dependent synthesis of cAMP (a biphasic increase, $P < 0.02$). Kinetic studies of Pi and Zn as co-effectors of ALP activity showed that Zn was a mixed-type effector with respect to Pi, whereas Pi was competitive with respect to Zn. Mechanistic studies showed that (1) Zn reversed the effect of Pi withdrawal to decrease ALP activity, but not by reactivating inactive ALP protein (the process required protein synthesis, without increases in ALP mRNA or the level of ALP immunoreactive protein); (2) Zn increased the half-life of ALP activity in intact cells and after a partial purification; and (3) Pi inhibited the process of ALP inactivation by EDTA (which chelates active center Zn). All these findings are consistent with the general hypothesis that Pi increases the half-life of skeletal ALP by preventing the dissociation of active center Zn and with a mechanistic model of skeletal ALP activity in which active center Zn participates in Pi-ester binding and/or hydrolysis.

Key words: Inorganic phosphate — Alkaline phosphate — Zinc deficiency-SaOS-2 cells.

Correspondence to: J. R. Farley, c/o Research Service (151), J.L. Pettis Memorial Veterans Medical Center

Zinc (Zn) is an essential trace element and, as such, is required for a variety of metabolic functions including protein and DNA synthesis, wound healing, taste, and immune system activity, usually as an essential cofactor in metalloenzymes and proteins [1–3]. Although significant amounts of Zn are stored in bone and muscle, the available pools are too small to provide a metabolic buffer [4], so that serum Zn homeostasis is primarily dependent on the dietary intake. The nutritional requirement for Zn is 10.1–11.5 mg/day [4] and normal adult human serum contains 40–100 μg/liter Zn, most of which is protein bound [6]. Recent data indicate that mild Zn deficiency may be associated with age-related changes in diet and/or Zn absorption/excretion. The typical dietary intake of Zn decreases from 10 to 15 mg/day in young adults to an estimated average of 7–10 mg/day in elderly adults [7], and it has been suggested that as many as 39% of all women over 65 years of age may suffer from Zn deficiency [18]. Three observations have also suggested that this age-related Zn deficiency may be associated with the development of osteoporosis: (1) the skeletal content of Zn decreases with age, in proportion to the age-dependent decrease in bone strength [9]; (2) osteoporotic subjects have lower serum Zn levels than age-matched controls [10]; and (3) the occurrence of hyperzincuria is greater among osteoporotic subjects [8].

This circumstantial linkage between a chronic, age-related Zn deficiency and osteoporosis is all the more intriguing because Zn is required for normal bone growth and development [5, 6, 11–14]. Furthermore, animal studies have shown that Zn deficiency decreases the number of osteoblasts and chondrocytes in bone [15], and *in vitro* data indicate that supplemental Zn can increase the number of murine osteoblast-like cells (MC3T3E1 cells) in monolayer culture [16]. Zn has also been reported to increase collagen production in rat femora [17] and calvaria [18], and recent studies indicate that Zn may act to decrease bone resorption [19–21]. Thus, we can hypothesize that chronic Zn deficiency could result in decreased bone formation and/or increased bone resorption, but only if we assume that there are essential Zn-dependent biochemical processes in osteoblasts and/or osteoclasts.

One of the most likely candidates for a Zn-dependent activity in osteoblast-line cells is skeletal alkaline phosphatase (ALP) which is a Zn-metalloenzyme found on the surface of osteoblasts and in circulation [22–24]. Skeletal ALP contains 2 molecules of Zn/enzyme monomer, and this enzyme-bound Zn is required for ALP activity; removal of Zn by chelation results in an irreversible loss of skeletal ALP activity. Although we do not understand the precise bio-

chemical function of skeletal ALP, we know that the enzyme is somehow essential for normal bone formation and/or mineralization [25–27] and studies of subjects with hypophosphatasia and recent studies with ALP knock-out mice have suggested (but not proven) that pyridoxal-phosphate, phosphoethanolamine, and inorganic pyrophosphate may be the natural substrates for skeletal ALP [28–31]. With respect to the role of Zn in skeletal ALP activity, a comparison with bacterial ALP suggests that the active center Zn may participate in phosphate-ester binding and catalysis [32–34].

The following four observations have led to the hypothesis that the active center Zn in skeletal ALP may dissociate from the enzyme, causing an irreversible inactivation, and that phosphate (Pi) can modulate this process by preventing the dissociation: (1) the concentration of Pi (i.e., in serum-free cell culture) can regulate the level of skeletal ALP activity in human osteoblast-like cells [35]; (2) this effect of Pi is not mediated by changes in the level of ALP mRNA or changes in the rate at which skeletal ALP activity is released from the cell surface [35, 36]; (3) Pi increases the half-life of skeletal ALP activity in these intact cell cultures [35, 36]; (4) Pi withdrawal (i.e., a transfer to Pi-free medium) decreases the cellular level of skeletal ALP activity, but not the level of skeletal ALP immunoreactive protein [37]. Together, these findings suggest that Pi is affecting the level of skeletal ALP activity in human osteoblast-like cells by modulating a process of irreversible inactivation. Since the loss of active center Zn (e.g., by chelation) results in an irreversible loss of skeletal ALP activity without a change in immunoactivity, we postulate that Pi stabilizes ALP activity by preventing (or retarding) the dissociation of Zn from the active center of skeletal ALP in human osteoblast-like cells.

The current studies were intended to examine this hypothesis and, specifically, to accomplish the following three aims: (1) to determine the *in vitro* effects of Zn, alone and in combination with Pi, on the level of skeletal ALP activity in human osteoblast-like cells; (2) to determine whether any such effects of Zn on skeletal ALP activity might be associated with other effects on the cells (e.g., effects on PTH-dependent synthesis of cAMP, collagen production, net IGF-II synthesis); (3) to investigate the mechanism(s) by which Zn might regulate the level of skeletal ALP activity. We performed most of these studies using human osteosarcoma SaOS-2 cells because that cell line has characteristics of human osteoblasts [38, 39], including high steady-state levels of skeletal ALP activity [40, 41]. Furthermore, because the amount of skeletal ALP activity in subpopulations of SaOS-2 cells is proportional to the amounts of collagen production and PTH-dependent synthesis of cAMP [42], this cell line was well suited for testing the possible associations between Zn-dependent changes in ALP activity and Zn-dependent changes in these other aspects of osteoblast activity.

Materials and Methods

Materials

Fetal calf serum, p-nitrophenylphosphate (PNPP), 3-isobutyl-1-methyl-xanthine (IBMX), concanavalin-A agarose, cycloheximide and triton X-100 were from Sigma Chemical Co. (St. Louis, MO). Tissue culture dishes were from Corning (Corning, NY). Trypsin-EDTA and Dulbecco's minimum essential medium (DMEM), and Fitton-Jackson modified Biggers-Gwatkin-Jones medium (BGJ_b) were from GIBCO (Grand Island, NY). Bio-gel P10, Bio-spin disposable chromatography columns, and protein-dye-binding reagent were purchased from Bio-Rad Laboratories (Hercules, CA). Goat anti-mouse antibody and nonimmune mouse serum were from Scantibodies (San Diego, CA) and anti-IGF-II antibody was from Amano (Troy, VA). ^{32}P-labeled nucleotides and ^{125}I were from Dupont NEN Research Products (Boston, MA) and ^{3}H-proline was from ICN Biochemicals, Inc. (Irvine, CA). Bovine serum albumin (BSA) was purchased from Fluka (Ronkonkoma, NH). Materials for the murine serum osteocalcin assay (osteocalcin standards, I^{125}-labeled mouse osteocalcin, goat anti-mouse osteocalcin, nonimmune goat serum, and donkey anti-goat second antibody) were purchased from Biomedical Technologies, Inc. (Stoughton, MA). Enzymeimmunoassay (EIA) kits for cAMP were purchased from Amersham (Arlington Heights, IL). Ostase kits for the quantitation of skeletal ALP immunoreactive protein by immunoradiometric assay were purchased from Hybritech (San Diego, CA).

Maintenance of Cells in Culture

Human osteosarcoma SaOS-2 cells, MG-63 cells, and osteoblast-like cells derived from normal human bone were maintained in 100-mm diameter dishes in DMEM + 10% fetal calf serum (FCS) at 37°C in an atmosphere of 5% CO_2 and air in a humidified incubator. Subconfluent, replicate cultures ($n = 6–12$/group) were prepared in 24-place multiwell dishes (50,000–70,000 cells/well in 1 ml DMEM + 10% FCS) or in 6-place multiwell dishes (500,000–750,000 cells/well in 2 ml DMEM + 10% FCS). After 24–48 hours, the media were replaced with serum-free DMEM and, 2–5 hours after that, with serum-free BGJ_b containing the effector(s)—usually in volumes of 0.5 ml/24-place multiwell and 1.5 ml/6-place multiwell. Incubations were in BGJ_b medium with Pi and effectors as indicated. (Fitton-Jackson modified BGJ_b medium was prepared without Ca or Pi so those components could be added as desired. DMEM contains 1.2 mmol/liter Pi and 1.8 mmol/liter Ca, and BGJ_b normally contains 1.8 mmol/liter Pi, and 1.8 mmol/liter Ca.)

ALP Activity

After the indicated exposures to effectors, the cell-conditioned media were removed (and saved for analysis as desired) and the cell layers were rinsed with phosphate-buffered saline (PBS) and then scraped and homogenized in 0.3 or 1 ml of a solution of 0.01% triton X-100 in 25 mmol/liter $NaHCO_3$ buffer, pH 8, containing 0.005% azide (for 24- and 6-place multiwells, respectively). As in previous studies [35–37] ALP activity was assessed by the time-dependent formation of p-nitrophenolate (which absorbs light at 405 nm in alkaline solution) from PNPP. Typically, 25 μl of cell layer extract (diluted as required) was incubated in a total volume of 0.3 ml containing 10 mmol/liter PNPP, 1 mmol/$MgCl_2$, in 150 mmol/liter carbonate buffer, pH 10.3, in a 96-place microtiter plate (i.e., one sample in each microtiter well). Each sample was assessed in duplicate. The reactions were initiated by addition of substrate, and the time-dependent increases in absorbance at 405 nm were measured on a microtiter plate spectrophotometer (EAR400/340AT, Labinstruments, Vienna, Austria). ALP activities were calculated as U/mg protein or mU/mg protein, where 1 unit is defined as 1 μmole of product formed/minute.

TRAP Activity

Tartrate-resistant-acid phosphatase (TRAP) activity was also determined in aliquots of the triton X-100 cell layer extracts of replicate cell cultures ($n = 6$) that had been exposed to Zn (0, 1, 1.5, 2, 5, 10, and 20 μmol/liters) in serum-free, Pi-free BGJ_b medium, by the time-dependent increase in the concentration of p-nitrophenolate in 0.2 ml of a solution containing 10 μl of sample (i.e., cell layer extract), 10 mmol/liter PNPP, 80 mmol/liter tartrate, and 100 mmol/liter acetate buffer, at pH 5.5. The reaction

solutions were incubated in the individual wells of 96-well microtiter plates at 37°C for 60 minutes. The reactions were then stopped by the addition of 0.1 ml of 0.1 mol/liter NaOH to each sample. A control was included for each sample to correct for variation in basal absorbance due to variation in protein or other extract components. TRAP activity was calculated as mU/mg protein, where 1 unit is defined as 1 μmole of product formed per minute.

Protein Assays

Cell layer protein was measured by dye binding [43]. ^{3}H-Proline incorporation into macromolecular material was measured in replicate cultures of SaOS-2 cells (n = 6, in 24-place multiwells) during a 48-hour exposure to Zn (at 0, 1, 1.5, 2, 5, 10, and 20 μmol/liter, in Pi-free BGJ_b containing 0.1% of normal proline) as an index of effects on protein synthesis. Macromolecular ^{3}H-proline was assessed by trapping acid-insoluble material on cotton swabs. Effects of Zn on the synthesis of collagenase-digestible protein were determined by incubating replicate cultures of SaOS-2 cells (in 6-place multiwells) for 56 hours in 1.5 ml/well of proline-free, Pi-free BGJ_b containing ascorbic acid, β-aminoproprionitrile, and Zn (at 0, 1, 1.5, 2, 5, 10, and 20 μmol/liter) and measuring collagenase-digestible ^{3}H-labeled protein, as described in previous studies [42]. Skeletal ALP immunoreactive protein was measured in cell layer extracts by radioimmunoassay (RIA) using a commercial kit, according to the manufacturer's instructions, as in our previous studies [37, 41]. This assay is not affected by the Triton X-100 in our cell layer extracts and shows comparable activity toward tetrameric (insoluble, membrane-anchored) ALP and dimeric (soluble, anchorless) ALP [37].

IGF-II Measurements

IGF-II was measured in 48-hour, serum-free, cell-conditioned medium (BGJ_b + 0.1% BSA and Zn, at 0, 2, 5, 10, and 20 μmol/liter) collected from replicate cultures of SaOS-2 cells in 100 mm-diameter dishes (n = 5/group). The medium was concentrated (Speed-Vac centrifugation), resuspended in 1 mol/liter acetic acid, centrifuged on Bio-spin columns to separate free IGF-II from endogenous binding proteins, and quantitated by RIA [44]. IGF-II production was expressed as ng/mg cell layer protein.

PTH-Dependent Synthesis of cAMP

The effects of Zn on PTH-dependent synthesis of cAMP were determined in replicate cultures of SaOS-2 cells (in 6-place multiwell dishes, n = 6/group) after a 48-hour preincubation with or without Zn (i.e., at 0, 1, 1.5, 2, 5, 10, and 20 μmol/liter) in serum-free, Pi-free BGJ_b. The cultures were then incubated for 20 minutes in serum-free DMEM containing 1 mmol/liter IBMX, with or without 1 mmol/liter PTH. cAMP was measured in extracts of the cell layers [42] by EIA. Net cAMP synthesis per culture well was normalized to the amount of protein in the extract.

RNA Extraction and Northern Blot Analysis

Skeletal ALP mRNA was prepared from replicate cultures of SaOS-2 cells (three 100 mm-diameter dishes for each of three replicate extracts from each incubation condition) and measured by Northern blot analysis using ^{32}P-labeled cDNA [36]. The amounts of ALP mRNA in each of the replicate extracts were quantitated by densitometric analysis of the autoradiograms and normalized to both the 18S and 28S rRNA bands and β-actin, to correct for uneven loading. The data are presented as mean ± SEM of the three replicate mRNA preparations (with corresponding replicate Northern blot lanes) for each incubation condition, normalized for β-actin.

Partial Purification of Skeletal ALP

Skeletal ALP was prepared from a pool of Ca-free culture medium which had been conditioned by SaOS-2 cells (24 hours at 37° C, 3 ml/100 mm-diameter culture dish, 150 ml total volume containing 110 units of ALP activity at 1.1 U/mg protein). This pool of ALP was incubated with 1000 units of phospholipase C for 48 hours at 4° C (to convert insoluble, tetrameric, membrane-anchored ALP to the dimeric, anchor-free, soluble form), then filtered to remove insoluble material concentrated, and dialyzed against 25 mmol/liter $NaHCO_3$ buffer containing 0.005% azide and 10 μmol/liter Zn. (Zn was included to preserve enzyme activity during the purification.) The ALP activity was partially purified by separation on columns of concanavalin A-agarose (eluted with α-methyl mannoside) and sepharose CL-6B200. The overall purification was 13.4-fold with a recovery of 32%. The ALP was dialyzed against 25 mmol/liter $NaHCO_3$ buffer before our stability studies, to remove the free Zn from solution.

Statistical Analysis

ALP activities, TRAP activities, osteocalcin levels, and protein levels in bone extracts were all determined in duplicate for each sample. Data are presented as the averages of the replicate samples in each group (usually as mean ± SEM). Analytic methods included one and two-way analysis of variance, linear regression, and Pearson correlation, using Systat statistical software (Systat Inc., Evanston, IL). Correlations were determined using group mean values and individual values.

Results

Characterization of Effect(s) of Zn on ALP Activity

Preliminary studies showed that a 24-hour incubation in serum-free BGJ_b containing 10 μmol/liter Zn increased the cellular level of ALP activity in SaSO-2 cells, both in the absence of Pi (6.99 ± 0.18 U/mg cell layer protein, compared with 2.43 ± 0.16 U/mg in 0 Pi, 0 Zn controls, $P < 0.001$) and in the presence of 1.8 mmol/liter Pi (7.10 ± 0.23 U/mg protein, compared with 5.52 ± 0.27 U/mg in 0 Zn controls, $P < 0.005$). As shown in Figures 1 and 2, additional studies revealed that the effect of Zn to increase ALP activity in SaOS-2 cells was both time and dose dependent. Furthermore, these studies showed that ALP specific activity was increased at Zn concentrations of 2–50 μmol/liter, but cell layer protein was not. In fact, Zn doses of 100 and 200 μmol/liter decreased cell layer protein (i.e., to 26.3 ± 1.5 and 2.2 ± 0.4% of control, respectively, $P < 0.001$ for each), which we interpreted as evidence of a cytotoxic effect. The hyperbolic dose-response curve shown in Figure 2 indicates a K_A value for Zn of about 1 μmol/liter, in the absence of Pi. A similar K_A for Zn was also observed for cells cultured with supplemental BSA (i.e., at 10 mg/ml—data not shown), indicating that carrier protein did not alter the effect of Zn to increase ALP specific activity in SaOS-2 cells. Although this hyperbolic pattern was not observed in the presence of 1.8 mmol/liter Pi, ALP activity was nonetheless increased at Zn concentrations of 2 and 5μmol/liter (i.e., by 25–30%, $P < 0.005$ for each, compared with the Pi-only control, data not shown). Additional studies revealed that the effect of 10μmol/liter Zn to increase ALP specific activity was also independent of the initial cell density, at least within the range of 25,000–250,000 SaOS-2 cells/24-place culture well (i.e., 24 hours of Pi withdrawal decreased ALP specific activity by 59–68% and Zn in-

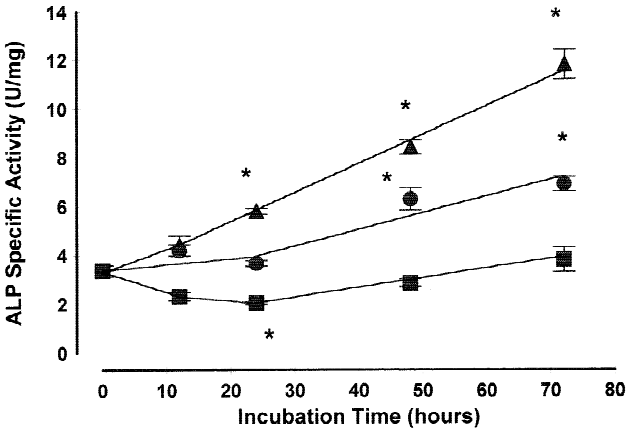

Fig. 1. Time-dependent effect of Zn on ALP specific activity in SaOS-2 cells. Replicate cultures of SaOS-2 cells (n = 6/group) were incubated for 12–72 hours in serum-free, Pi-free BGJ_b medium containing no additions (i.e., 0 Pi, 0 Zn—■); 1.8 mmol/liter Pi (●); or 10 μmol/liter Zn (▲). ALP specific activity was measured in cell layer extracts and expressed as U/mg protein (mean ± SEM). *A significant difference from the basal (0 hours) control, $P < 0.001$.

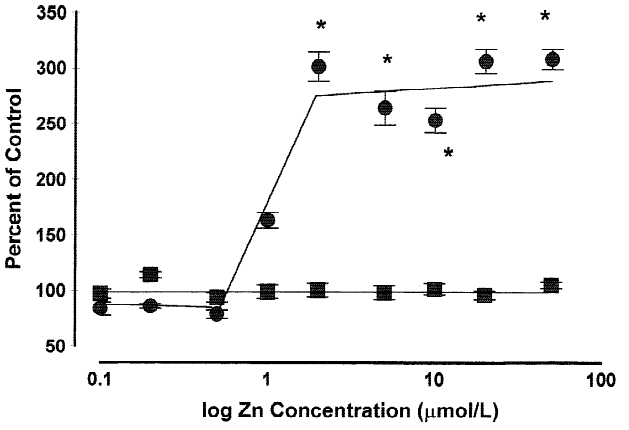

Fig. 2. Dose-dependent effects of Zn on ALP specific activity and cell layer protein in SaOS-2 cells. Note the logarithmic scale on the horizontal axis. Replicate cultures of SaOS-2 cells (n = 6/group) were incubated for 48 hours in serum-free, Pi-free BGJ_b medium containing either no additions (i.e., 0 Pi, 0 Zn) or the indicated dose of Zn. Cell layer protein (■) and ALP specific activity (●) were measured in cell layer extracts. Data are shown as percent of minus-Zn controls, mean ± SEM. Cell layer protein averaged 57.0 ± 1.8 μg/ml extract in the minus-Zn controls and ALP specific activity averaged 2.05 ± 0.12 U/mg protein. *A significant difference from the minus-Zn control, $P < 0.005$.

creased the level of ALP activity by 25–67%, compared with the initial values).

To investigate the nature of the interaction between Zn and Pi (i.e., as effectors of ALP specific activity), replicate cell cultures (n = 3/group) were incubated for 24 hours in serum-free medium containing one of four concentrations of Zn (0, 0.5, 2, or 10 μmol/liter) at each of 7 Pi concentrations (0.167, 0.2, 0.25, 0.33, 0.5, 1, and 2 mmol/liter). ALP specific activities were determined in cell extracts and the data were analyzed by double-reciprocal replots of 1/ALP specific activity versus 1/[Pi], as shown in Figure 3. The pattern

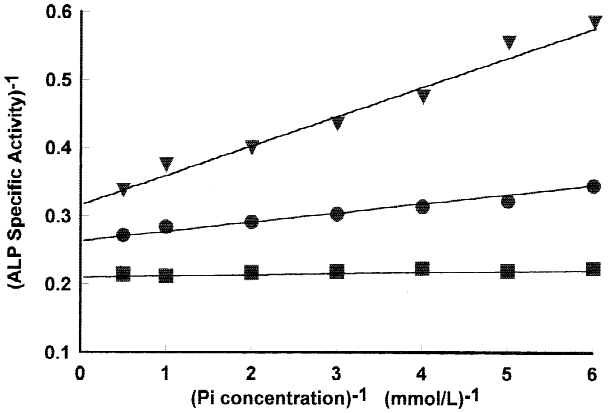

Fig. 3. Zn is a mixed-type activator of ALP specific activity, with respect to the dose of Pi. Replicate cultures of SaOS-2 cells (n = 3/group) were incubated for 24 hours in serum-free BGJ_b medium containing 0.167, 0.2, 0.25, 0.33, 0.5, 1, or 2 mmol/liter Pi and one of three Zn concentrations: 0 (▲), 0.5 (●), or 10 (■) μmol/liter. (Note: additional cultures were exposed to the same concentrations of Pi with 2 μmol/liter Zn—data not shown). ALP specific activities were measured in cell layer extracts, and average values were used to construct the double-reciprocal plot of 1/ALP specific activity versus 1/[Pi]. The almost horizontal line at 10 μmol/liter Zn implies that Pi has very little effect on ALP specific activity at a saturating dose of Zn.

of intersecting straight lines is typical of mixed-type interactions and the flat line (i.e., slope = 0) observed at 10 μmol/liter Zn implies that, at saturating Zn, ALP specific activity is essentially independent of the Pi concentration. The algebraic replots (i.e., of slope and vertical-axis intercept versus Zn concentration) were nonlinear, indicating a partial system in which the interactive effects of Zn and Pi as determinants of ALP activity approach a common maximum. $K_{apparent}$ for Pi (i.e., the kinetic constant for Pi as a determinant of ALP specific activity) was about 0.15 mmol/liter in the absence of Zn, and undefined at saturating Zn. A separate study revealed that the effect of Pi on ALP activity was competitive with respect to Zn (i.e., plots of 1/ALP specific activity versus 1/[Zn] produced a family of lines that intersected on the vertical axis—data not shown), further indicating that the effect of Pi on ALP activity is only evident at nonsaturating Zn. The value of $K_{apparent}$ for Zn apparent was 0.26 μmol/liter at 0 Pi and 0.11 μmol/liter at 2 mmol/liter Pi.

Specificity of Zn Effect on ALP Activity: Other Osteoblast-like Cells and Other Divalent Cations

In order to determine whether the observed effect(s) of Zn to increase ALP specific activity was unique to SaOS-2 cells, we assessed the effect of a 24-hour exposure to 10 μmol/liter Zn on ALP specific activity in SaOS-2 cells, human osteosarcoma MG-63 cells, and two normal human osteoblastic cell lines derived from explants of vertebral tissue (designated as HBV-146 and HBV-155). As shown in Table 1, we found that the effect of Zn to increase ALP specific activity was not unique to SaOS-2 cells or to osteoblast-like cells with high basal ALP levels. The effect of Zn was, however, cation specific, since neither Ca, Co, Cu, Fe, Mg, or Mn had similar effects to increase ALP specific activity. We found that a 24 hour exposure to 20 μmol/liter

Table 1. Zn increases ALP activity in human osteoblast-like cells

Cell type	ALP specific activity with the indicated effectors (percent of plus-Pi control)		
	Minus-Pi, minus-Zn	Minus-Pi, plus-Zn	Plus-Pi, plus-Zn
SaOS-2	50.5 ± 0.07[a]	127.5 ± 2.4[a]	123.0 ± 2.3[a]
MG-63	34.9 ± 2.5[a]	144.7 ± 2.6[a]	141.2 ± 2.1[a]
HBV-146	20.5 ± 1.1[a]	131.7 ± 1.1[a]	132.3 ± 2.1[a]
HBV-155	95.7 ± 2.7	138.7 ± 3.1[a]	146.0 ± 4.2[a]

Replicate cultures (n = 6/treatment group) of human osteosarcoma cell lines SaOS-2 and MG-63 and normal human bone cells prepared from vertebral tissue (designated HBV-146 and HBV-155) were incubated for 24 hours in serum-free, Pi-free BGJ_b medium containing no additions (minus-Pi); 1.8 mmol/liter Pi (the plus-Pi control); 10 μmol/liter Zn (minus-Pi, plus-Zn); or 1.8 mmol/liter Pi and 10 μmol/liter Zn (plus-Pi, plus-Zn). ALP specific activity (U/mg protein) was measured in cell layer extracts and expressed as a percentage (mean ± SEM) of the average value observed in the plus-Pi controls

[a] A significant difference compared with plus-Pi control, $P < 0.001$. ALP specific activity in the plus-Pi controls averaged 10.1, 0.04, 0.06, and 0.01 U/mg for the SaOS-2 cells, MG-63 cells, HBV-146 cells, and HBV-155 cells, respectively

Zn increased ALP specific activity (in Ca-free, Pi-free BGJ_b medium), compared with the Ca-free, 1.8 mmol/liter Pi control (i.e., 11.5 ± 0.2 versus 7.0 ± 0.4 U/mg cell layer protein, $P < 0.001$), whereas parallel exposures to 20 μmol/liter Ca, Co, Cu, Fe, Mg, and Mn (also in Ca-free, Pi-free BGJ_b medium) each decreased ALP specific activity (i.e., to 4.3 ± 0.1, 3.5 ± 0.2, 0.1 ± 0.01, 1.2 ± 0.03, 4.0 ± 0.1, and 4.6 ± 0.2 U/mg cell layer protein, respectively, $P < 0.001$ for each). Similar results were seen with 48-hour exposures to the same selection of cations at concentrations of 2, 10, and 20 μmol/liter.

Specificity of Zn Effect on ALP Activity: Other Indices of Osteoblast Function

To determine whether the observed effect of Zn to increase ALP activity in SaOS-2 cells might be associated with concomitant and/or parallel effects on other indices of osteoblast activity, we assessed the effects of a 48-hour exposure to Zn (at concentrations ranging from 0 to 20 μmol/liter) on total protein synthesis, collagen synthesis, net IGF-2 synthesis, PTH-dependent synthesis of cAMP, and TRAP specific activity (each with a parallel assessment of ALP specific activity as a positive control). The results of these studies showed that Zn had no effect on total protein synthesis, collagen production, or TRAP specific activity (data not shown). However, as summarized in Table 2, Zn caused a dose-dependent decrease in the net production of IGF-II. Net IGF-II synthesis was also negatively correlated with the amounts of ALP activity in the cell layer extracts ($r = -0.384$, $P < 0.05$) and in the cell-conditioned media ($r = -0.481$, $P < 0.01$). A 48-hour preincubation with Zn also increased PTH-dependent synthesis of cAMP in a biphasic (convex) manner, increasing cAMP at Zn concentrations of 1.5 and 2 μmol/liter (Table 2). Together these data indicate that, although the effects of Zn on SaOS-2 cells were not limited to the dose-dependent increase in ALP specific activity, Zn was not causing parallel changes in ALP activity and any other tested index of osteoblast activity.

Mechanistic Studies of the Effect of Zn to Increase ALP Specific Activity

Zn reversed the effect of Pi withdrawal to decrease the level of ALP activity in SaOS-2 cells (Table 3). In order to determine whether this effect of Zn required new protein synthesis or reflected a recovery of ALP activity that was lost during Pi withdrawal, SaOS-2 cells were preincubated for 48 hours in Pi-free, Zn-free BGJ_b and then transferred to fresh medium containing either Pi (1.8 mmol/liter), Zn (10 μmol/liter), the combination of Pi and Zn, or no additions (i.e., the minus Pi control), for an additional 24 hours. These studies were conducted in the presence of cycloheximide (3 μmol/liter) to inhibit protein synthesis. (Parallel studies measuring ^{3}H-proline incorporation into TCA-insoluble material confirmed that the cycloheximide decreased protein synthesis by more than 90%, in each treatment group.) We found that protein synthesis was required for the Zn-dependent increase in ALP activity (i.e., after Pi-withdrawal) and from this we concluded that Zn was not reactivating inactive ALP protein (Table 4). Although the effect of Zn to increase ALP activity required protein synthesis, it did not require an increase in ALP mRNA. Northern blot analysis of ALP mRNA in replicate extracts of SaOS-2 cells that had incubated for 48 hours in Pi-free BGJ_b medium containing no additions, 1.8 mmol/liter Pi, 10 μmol/liter Zn, or the combination of Pi and Zn (n = three extracts and three corresponding Northern blot samples/group) showed no difference in ALP mRNA (i.e., 0.81 ± 0.06, 1.00 ± 0.08, 0.93 ± 0.03, and 0.98 ± 0.07 densitometric units, normalized for β-actin, for no addition, + Pi, + Zn, and the + Pi + Zn combination, respectively).

To determine whether Zn was increasing the level of ALP activity in SaOS-2 cells by inhibiting release from the cell surface (i.e., into the culture medium), we measured the dose-dependent effect(s) of Zn on ALP specific activity in SaOS-2 cells and in the serum-free medium after a 28-hour exposure to various doses of Zn. As shown in Figure 4, Zn had an even greater effect to increase ALP activity in the cell-conditioned medium than it did in the cell layer. To test the assumption that Zn was increasing ALP activity by increasing the abundance of the enzyme, we also measured the cellular level of ALP immunoreactive protein in the same cell extracts. Surprisingly, those studies showed that Zn increased the level of ALP activity in SaOS-2 cells but not the level of skeletal ALP protein (Fig. 4).

Together, these data indicate that the *in vitro* effect of Zn to increase ALP specific activity in SaOS-2 cells did not reflect a decrease in the rate of ALP release from the cell

Table 2. Zn effects on indices of osteoblast activity

Characteristic	Index assessed in response to Zn		
	ALP specific activity	Net IGF-II synthesis	PTH-dependent cAMP
Pattern of response	Hyperbolic (positive)	Linear (negative)	Biphasic (convex)
Maximum effective dose	20 μmol/liter	20 μmol/liter	1.5 μmol/liter
Maximum response	+209%[b]	−18%	+68%[a]
Correlation coefficient	r = 0.802[b]	r = −0.510[b]	r = −0.048

Replicate cultures (n = 6/group) of SaOS-2 cells incubated for 48 hours at Zn doses of 0, 2, 5, 10, and 20 μmol/liter for IGF-2 synthesis and 0, 1, 1.5, 2, 5, 10, and 20 μmol/liter Zn for PTH-dependent synthesis of cAMP. Each dose range was also used for parallel assessments of Zn effects on ALP specific activity. ALP specific activity was measured in cell layer extracts, IGF-2 was measured in the cell-conditioned culture medium, and PTH-dependent synthesis of cAMP was measured during a 20-minute incubation at the end of the 48-hour exposure to Zn (see Methods for descriptions of each assay). The pattern of response refers to plots of the index versus Zn dose. The maximum observed response is shown as percent of untreated control at the maximum effective Zn dose (i.e., within the tested range). The correlation coefficient reflects the results of a linear correlation of index versus Zn dose
[a] A significant difference, compared with the minus-Zn control (by ANOVA) and/or a significant correlation (by linear regression), $P < 0.02$
[b] $P < 0.005$

Table 3. Zn reverses the effects of Pi withdrawal to decrease ALP specific activity

Effector(s)	ALP specific activity at indicated time (U/mg protein)	
	24 Hours	48 Hours
0 Pi, 0 zn	1.04 ± 0.07	1.25 ± 0.13[a]
1.8 mmol/liter Pi	1.74 ± 0.04[a]	2.32 ± 0.09[a]
10 μmol/liter Zn	2.39 ± 0.03[a]	3.42 ± 0.05[a]
1.8 mmol/liter Pi + 10 μmol/liter Zn	2.48 ± 0.05[a]	3.17 ± 0.06[a]

Replicate cultures of SaOS-2 cells (n = 6/group) were preincubated for 24 hours in serum-free, Pi-free BGJ_b medium (to decrease the cellular level of ALP activity), then transferred for an additional 24- or 48-hour incubation in serum-free, Pi-free medium containing no additions, 1.8 mmol/liter Pi, 10 μmol/liter Zn, or the combination of Pi and Zn. ALP specific activity was measured in cell layer extracts and expressed as mean ± SEM
[a] A difference from the specific activity observed at the end of the 24-hour Pi-free preincubation (i.e., 0.79 ± 0.04 U/mg)

surface, nor did it reflect an increase in the level of ALP protein. In other words, Zn was acting by increasing the ratio of ALP activity to ALP immunoreactive protein. Since our data also showed that Zn was not restoring activity to inactive ALP protein, we hypothesized that Zn might be increasing the half-life of ALP activity. We also hypothesized that if this was a direct effect of Zn it should not require any other component in the cell layer or in the cell-conditioned medium. As shown in Figure 5, we found that Zn was, indeed, increasing the level of ALP activity in SaOS-2 cells by stabilizing ALP activity. Consistent with previous data [33], Pi also increased the half-life of ALP activity in SaOS-2 cells, but not as effectively as Zn (i.e., $t_{1/2}$ for ALP activity was about 3 hours in Pi-free medium, 30–36 hours with 1.8 mmol/liter Pi, and >72 hours with 10 μmol/liter Zn).

To determine whether the effect of Zn to stabilize ALP activity was dependent on a medium- or cell-derived cofactor, we assessed the effect of Zn on the stability of ALP activity that had been partially purified from cell-conditioned medium (i.e., ALP activity that had been released from the surface of cells and, presumably, separated from any such cofactors during the purification). As shown in Table 5, we found that Zn prevented (or inhibited) the inactivation of partially purified skeletal ALP activity. Inorganic Pi also mimicked the effect of Zn in this cell-free stability study. In order to test the hypothesis that Pi was preserving skeletal ALP activity by preventing (or inhibiting) the irreversible dissociation of Zn from the enzyme active center, we examined the dose-dependent interaction between Pi, which increases ALP activity, and EDTA, which inhibits the enzyme by removing (chelating) active center Zn. Pi reduced the time-dependent effect of EDTA to inactivate ALP (Fig. 6). Kinetic studies further showed that EDTA was a noncompetitive, irreversible inhibitor of ALP activity with respect to the concentration of Pi [i.e., after 24 exposure to Pi concentrations of 0.167, 0.2, 0.25, 0.33, 0.5, 1, and 2 mmol/liter and EDTA doses of 0, 2.5, 5, and 10 μmol/liter (data not shown)]. In other words, these studies showed that EDTA decreased the maximal level of ALP activity without affecting the dose-dependence for Pi stabilization. The estimated value of K_A for Pi was 0.17 mmol/liter in the absence of EDTA and 0.16 mmol/liter at 10 μmol/liter EDTA. Replots of slope and intercept (i.e., of 1/ALP specific activity versus 1/[Pi]) versus EDTA concentration revealed K_I values for EDTA of 7 and 8 μmol/liter, respectively.

Discussion

The current studies were intended to accomplish three specific aims: (1) to define the *in vitro* effect of Zn alone and in combination with Pi, as a presumptive determinant of ALP specific activity in human osteoblast-like cells; (2) to determine whether any such effect of Zn on ALP specific activity might be associated with proportional and/or concomitant effects on other aspects of osteoblast cell function; (3) to assess the biochemical and/or kinetic mechanism(s) by which Zn and Pi might interact to regulate the level of ALP activity in human osteoblast-like cells. In these studies

Table 4. Cycloheximide inhibits the Zn-dependent increase in ALP specific activity

Effector(s)	ALP specific activity (U/mg protein)		Significance of difference ± cycloheximide
	Minus cycloheximide	Plus cycloheximide	
0 Pi, 0 Zn	0.58 ± 0.05	0.23 ± 0.02[a]	$P < 0.001$
1.8 mmol/liter Pi, 0 Zn	2.01 ± 0.05[a]	1.01 ± 0.09	$P < 0.001$
0 Pi, 10 μmol/liter Zn	3.60 ± 0.15[a]	1.73 ± 0.07[a]	$P < 0.001$
1.8 mmol/liter Pi, 10 μmol/liter Zn	3.52 ± 0.06[a]	1.70 ± 0.08[a]	$P < 0.001$

Replicate cultures of SaOS-2 cells (n = 6/treatment group) were preincubated for 48 hours in serum-free, Pi-free BGJ_b medium (to decrease the cellular level of ALP activity) and then transferred for an additional 24 hours incubation, with or without cycloheximide (at 3 μmol/liter to inhibit protein synthesis), in medium containing no additions (0 Pi, 0 Zn); 1.8 mmol/liter Pi; 10 μmol/liter Zn; or 1.8 mmol/liter Pi plus 10 μmol/liter Zn. ALP specific activity (U/mg protein) was measured in cell layer extracts and expressed as mean ± SEM
[a] A significance difference compared with the cellular ALP level at the end of the Pi-free preincubation (i.e., a specific activity of 0.97 ± 0.09 U/mg cell layer protein)

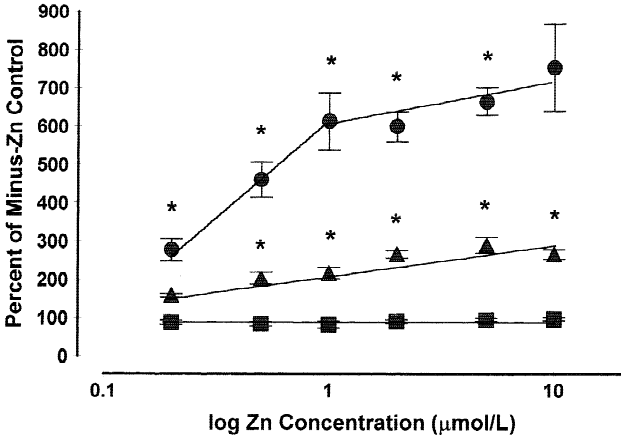

Fig. 4. Dose-dependent effects of Zn on ALP specific activity, ALP release, and ALP immunoreactive protein. (Note the logarithmic scale on the horizontal axis.) Replicate cultures of SaOS-2 cells (n = 6/group) were incubated for 28 hours in serum-free, Pi-free BGJ_b medium with Zn at the indicated concentration. ALP activity and ALP immunoreactive protein were measured in cell layer extracts, and ALP activity was also measured in the cell-conditioned medium. All ALP values were normalized for cell layer protein and expressed as percent of control (mean ± SEM). Control (i.e., minus Zn) values for ALP activity in the cell-conditioned medium (●), ALP activity in the cells (▲), and ALP immunoreactive protein in the cells (■) averaged 28 ± 4 mU/mg protein, 1.57 ± 0.04 U/mg protein, and 0.54 ± 0.03 ng/mg protein, respectively. *A significant difference from the minus-Zn controls, $P < 0.001$.

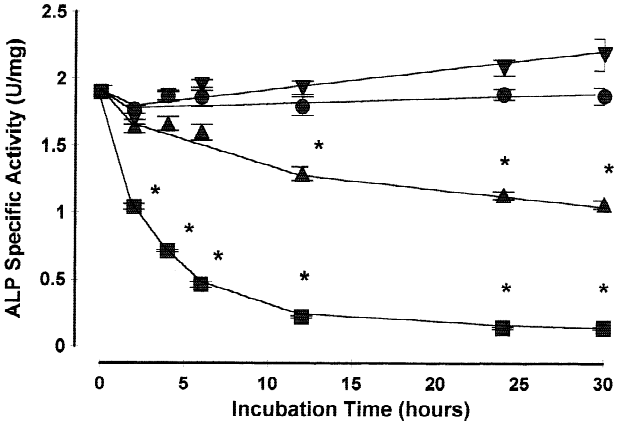

Fig. 5. Zn increases ALP specific activity by increasing the half-life of the enzyme. Replicate cultures of SaOS-2 cells were incubated for 0–30 hours in serum-free, Pi-free BGJ_b medium containing 3 μmol/liter cycloheximide (to inhibit protein synthesis) and no additions (i.e., 0 Pi, 0 Zn-■); 1.8 mmol/liter Pi (▲); 10 μmol/liter Zn (●); or the combination of 1.8 mmol/liter Pi and 10 μmol/liter Zn (▼). ALP specific activity was measured in cell layer extracts and shown as mean ± SEM. *A significant difference from the 0 hour controls, $P < 0.001$.

we assumed that ALP activity at pH 10.3, using PNPP as a substrate, was reflective of (and proportional to) ALP activity at physiological pH, with physiological substrates.

Our initial studies showed that Zn caused time- and dose-dependent increases in ALP specific activity in SaOS-2 cells, and prevented the effect of Pi withdrawal to decrease the cellular level of ALP activity. This Zn-dependent increase in ALP specific activity was apparently unique to Zn [i.e., no similar effect was seen with other divalent cations (Ca, Mn, Mg, Fe, Co, or Cu)], but not unique to SaOS-2 cells. Zn also increased the cellular level of ALP activity in human osteosarcoma MG-63 cells and in two cell lines established from outgrowths of normal human vertebral tissue. Our dose-dependence studies of the interaction(s) between Zn and Pi as determinants of ALP activity in SaOS-2 cells revealed that Zn was a mixed-type effector with respect to Pi whereas Pi was competitive with respect to Zn. This pattern of interaction implied that Zn could increase ALP specific activity at any given dose of Pi, but Pi could not increase ALP specific activity at a saturating (i.e., micromolar) dose of Zn. The effect of Zn to increase ALP specific activity in the absence of Pi was half-maximal at about 1 μmol/liter, even with albumin present, and our studies of Pi and Zn interactions indicated K_A values (i.e., from slope and intercept replots) for Zn of 0.11 and 0.26 μmol/liter. These *in vitro* values are consistent with the normal range of Zn in human serum (i.e., 0.6–1.5 μmol/liter) and are therefore consistent with the general hypothesis that Zn may determine the *in vivo* level of skeletal ALP activity in bone cells and/or serum. This idea is also sup-

Table 5. Effects of Zn and Pi on the stability of partially purified ALP activity.

Incubation (hours)	Time-dependent changes in partially-purified ALP activity (mU/mL) in the presence of the indicated effector		
	0 Pi, 0 Zn	1.8 mmol/L Pi	10 μmol/L Zn
0	28.3 ± 1.5	30.4 ± 0.2	28.7 ± 0.2
24	23.6 ± 0.6[a]	26.6 ± 0.2	29.3 ± 0.2
48	21.0 ± 0.4[a]	22.1 ± 0.4[a]	28.6 ± 0.8
72	18.4 ± 0.5[a]	21.6 ± 0.5[a]	27.4 ± 0.3

Replicate aliquots ($N = 4$/group) of partially purified skeletal ALP activity were incubated at room temperature (22°C) in serum-free, Pi-free BGJ_b containing: no additions (0 Pi, 0 Zn); 1.8 mmol/L Pi; or 10 μmol/L Zn. Aliquots of each solution were removed at the indicated times for determination of remaining ALP activity (data shown as mean ± SEM).
[a]Indicates a significant decrease from the 0 Pi, 0 Zn, time 0 control, $P < 0.001$.

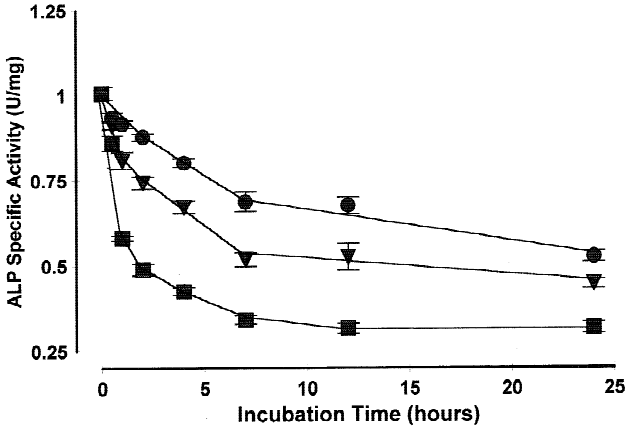

Fig. 6. Pi inhibits the effect of EDTA to inactivate ALP. Replicate cultures of SaOS-2 cells were incubated for 0–24 hours in serum-free BGJ_b medium containing 10 μmol/liter EDTA and 0 Pi (■), 1 mmol/liter Pi (▼), or 3 mmol/liter Pi (●). ALP specific activity was measured in cell layer extracts and expressed as mean ± SEM. ALP specific activity was significantly decreased ($P < 0.001$) at all times after 1 hour, under all incubation conditions. ALP specific activity was significantly greater in the presence of both 1 and 3 mmol/liter Pi, compared with 0 Pi, at all times after 0.5 hour, $P < 0.001$.

ported by previous animal studies which showed increases in skeletal ALP activity in the femoral diaphyses of rats that had been fed a Zn-rich diet [17, 18], a decrease in skeletal ALP activity in the bones of Zn-deficient rats [45], and a dose-dependent relationship between dietary Zn and skeletal ALP in the serum and tibia of adult female mice [46]. With regard to the effect of P_i, we interpret our data as evidence that P_i binding to the active center of skeletal ALP prevents or inhibits the irreversible dissociation of Zn. This is mechanistic information that may or may not be relevant to the physiological function of skeletal ALP. Although P_i is an inhibitor of ALP activity, it binds to the active center in a competitive manner with phosphoryl substrates. Our previous data suggested that phosphoryl substrates also increased the half-life of ALP activity [35, 37], presumably by preventing the irreversible dissociation of Zn.

Since the level of skeletal ALP activity in bone cells and in serum can provide a useful index of the rate of bone formation [47–49], and since we had previously shown [42] that subpopulations of SaOS-2 cells with different steady-state levels of ALP activity showed corresponding (proportional) differences in two other characteristics of osteoblast function (i.e., collagen production and PTH-dependent synthesis of cAMP), we wanted to determine whether the effect of Zn to increase the cellular level of ALP activity might be associated with proportional and/or concomitant increases in other aspects of osteoblast function. The results of those studies revealed no such Zn-dependent increases and very few effects. In our monolayer cultures of human osteoblast-like cells, Zn had no effect on total protein synthesis, collagen production, or TRAP specific activity. Zn did have a modest, biphasic effect to increase PTH-dependent synthesis of cAMP, but it was not proportional to the observed effect of Zn on ALP specific activity. The only dose-dependent effect of Zn that was associated with a concomitant Zn-dependent increase in ALP specific activity was the unexpected finding that Zn decreased the net synthesis of IGF-2 in a dose-dependent manner. This finding was in contrast to previous studies showing that Zn deficiency decreased serum IGF-1 levels in rats [50] and that supplemental Zn increased the production of IGF-1 by murine osteoblastic MC3T3-E1 cells [51], but consistent with the observation that human osteoblast-like cells with higher cellular levels of ALP activity had lower cellular levels of IGF-2 mRNA [52]. Resolution of these discordant results will require additional studies, but additional studies will not be required to answer our primary question as to whether the effect of Zn to increase ALP activity was associated with parallel, concomitant increases in other characteristics of osteoblast function. It was not. Although the *in vitro* action of Zn on human osteoblast-like cells was not strictly limited to the dose-dependent increase in ALP activity, no tested characteristic was altered to the same extent or in a proportional manner. Thus, we conclude that the effect of Zn to increase the specific activity of skeletal ALP was relatively specific for that enzyme in these cells.

But how does Zn have this effect? What can we deduce from out data regarding the biochemical mechanism of the Zn-dependent increase in skeletal ALP specific activity? Our data are consistent with the general hypothesis that Zn stabilizes existing skeletal ALP activity. The observed effect of Zn to increase ALP specific activity (and reverse the decrease in ALP specific activity that occurred with Pi withdrawal) was dependent on new protein synthesis and therefore did not reflect a reactivation of inactive ALP. However, this effect of Zn was not associated with an increase in ALP mRNA or an increase in the level of ALP-immunoreactive protein, suggesting that (1) the total level of skeletal ALP protein was constant; (2) some, but not all, of the ALP immunoreactive protein in these human osteoblast-like cells was enzymatically active; and (3) Zn was increasing the fraction of active ALP protein. Since our studies also showed that that Zn was increasing the half-life of skeletal ALP activity, both in monolayer cell cultures and in a solution of partially purified enzyme, we have favored the hypothesis of a direct effect of Zn on skeletal ALP protein and a direct interaction between Zn and Pi at nonsaturating Zn. (Further studies are required to assess the possibility of a Zn-dependent co-factor that copurifies with skeletal ALP activity, at least, through our partial purification.)

We had previously shown that Pi (and phosphoryl substrates) could increase ALP specific activity in human osteoblast-like cells [37], and hypothesized that this was due to an effect of Pi to bind to the enzyme (at or near the

catalytic center) and prevent the irreversible dissociation of active-center Zn. This hypothesis is supported by our current observations that Pi could offer a partial protection against inactivation by EDTA, and that the *in vitro* effect of Pi to stabilize skeletal ALP activity was obscured by the effect of Zn. But if Zn stabilizes the enzyme by inhibiting the irreversible inactivation that follows the dissociation of ALP-bound Zn, that dissociation must be, at least partly, reversible. Therefore, we now hypothesize that one or both of the two active center molecules of Zn in skeletal ALP is reversibly dissociable, and that the loss of Zn results in an irreversible inactivation of ALP by one of the following processes: (1) the irreversible dissociation of the second active center Zn; (2) an irreversible structural change that follows the dissociation of the first or second active center Zn (i.e., with a time constant that allows for reassociation); or (3) Zn binding to an allosteric site that prevents such dissociation from the enzyme active center.

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