We have probed the structural/functional relationship of key residues in human placental alkaline phosphatase (PLAP) and compared their properties with those of the corresponding residues in Escherichia coli alkaline phosphatase (ECAP). Mutations were introduced in wild-type PLAP, i.e. [E429]PLAP, and in some instances also in [G429]PLAP, which displays properties characteristic of the human germ cell alkaline phosphatase isozyme. All active site metal ligands, as well as residues in their vicinity, were substituted to alanines or to the homologous residues present in ECAP. We found that mutations at Zn2 or Mg sites had similar effects in PLAP and ECAP but that the environment of the Zn1 ion in PLAP is less affected by substitutions than that in ECAP. Substitutions of the Mg and Zn1 neighboring residues His-517 and His-153 increased $K_{cat}$ and increased $K_m$ when compared with wild-type PLAP, contrary to what was predicted by the reciprocal substitutions in ECAP. All mammalian alkaline phosphatases (APs) have five cysteine residues (Cys-101, Cys-121, Cys-183, Cys-467, and Cys-474) per subunit, not homologous to any of the four cysteines in ECAP. By substituting each PLAP Cys by Ser, we found that disrupting the disulfide bond between Cys-121 and Cys-183 completely prevents the formation of the active enzyme, whereas the carboxy-terminally located Cys-467-Cys-474 bond plays a lesser structural role. The substitution of the free Cys-101 did not significantly affect the properties of the enzyme. A distinguishing feature found in all mammalian APs, but not in ECAP, is the Tyr-367 residue involved in subunit contact and located close to the active site of the opposite subunit. We studied the A367 and F367 mutants of PLAP, as well as the corresponding double mutants containing G429. The mutations led to a 2-fold decrease in $k_{cat}$, a significant decrease in heat stability, and a significant disruption of inhibition by the uncompetitive inhibitors L-Phe and L-Leu. Our mutagenesis data, computer modeling, and docking predictions indicate that this residue contributes to the formation of the hydrophobic pocket that accommodates and stabilizes the side chain of the inhibitor during uncompetitive inhibition of mammalian APs.

Alkaline phosphatases (APs; EC 3.1.3.1) are a family of dimeric metalloenzymes catalyzing the hydrolysis of monoesters of phosphoric acid (1). APs also catalyze a transphosphorylation reaction in the presence of large concentrations of phosphate acceptors. APs occur widely in nature and are found in many organisms from Escherichia coli to man. APs are homodimeric enzymes, and each catalytic site contains three metal ions (two Zn ions and one Mg ion) that are necessary for enzymatic activity (2). Whereas the main features of the catalytic mechanism are conserved between mammalian APs and the E. coli enzyme, there are important differences. Mammalian APs (a) have higher specific activity and $K_m$ values, (b) have a more alkaline pH optimum, (c) are inhibited by L-amino acids and peptides through an uncompetitive mechanism, and (d) display lower heat stability. These properties, however, differ noticeably within the group of mammalian AP isozymes. Many of the isozyme-specific properties have been attributed to a flexible loop region or “crown” domain of the molecule (3, 4). Some mammalian APs, such as the human intestinal isozyme, are activated by magnesium ions, whereas the human placental AP is more similar to the E. coli enzyme in that its activity is not enhanced by the addition of magnesium (1).

For many years, E. coli AP (ECAP) was the only source of structural information on APs (2), but now the three-dimensional structure of the first mammalian AP, i.e. human placental alkaline phosphatase (PLAP), has been solved (4). As had been predicted from sequence comparisons (5), the central core of PLAP, consisting of an extended $\beta$-sheet and flanking $\alpha$-helices, is very similar to that of ECAP. The same is true in the immediate vicinity of the three catalytic ions. However, a number of distinctive features, including a different positioning of the amino-terminal segment of the molecule and the expanded top loop or “crown” domain, are now apparent (4). An additional noncatalytic metal-binding site not present in ECAP was uncovered, which appears to be occupied by calcium (4, 6). ECAP has four cysteine residues that are all involved in disulfide bond formation (7), whereas PLAP has five nonhomologous residues. Furthermore, whereas ECAP is located in the periplasmic space of the bacterium, PLAP is an ectoenzyme bound to the plasma membrane via a glycosyl-phosphatidylinositol anchor (8, 9).

In the present paper, we embarked on a wide-range mutagenesis study on structure-function relationships in mammalian APs, using the PLAP structure as a paradigm. The aim was to pinpoint the features of PLAP responsible for the spe-
cific properties of this AP isozyme, as well as the properties of mammalian APs in general. The active site metal ligand residues were mutated to alanines or to the analogous residues in the structure of ECAP, and cysteines were mutated to serines. We have also defined the location of the hydrophobic pocket that participates in stabilizing the side chains of competitive inhibitors in the immediate vicinity of the active site of mammalian alkaline phosphatases.

**EXPERIMENTAL PROCEDURES**

**PLAP Mutagenesis**

Site-directed mutagenesis was performed by PCR with mutated oligonucleotide primers. The strategy involves the introduction of restriction sites for enzymes that cut at a distance from their recognition sites (BsaI, BspMI, Alw261, and EarI) or the end of endogenous restriction enzyme sites. Either the PLAP-FLAG/pCDNA3 or PLAP/pSVT7 plasmid was used as a template in PCR reactions. PCR products were subcloned into a pCR2.1 or PCRII-TOPO cloning vector (Invitrogen), and the mutations were confirmed by sequencing. The restriction fragments were then cut and ligated with the fragments of PLAP-FLAG/pCDNA3 and pCDNA3 plasmid (Invitrogen). All final constructs were verified by restriction enzyme analysis and sequencing. Plasmid DNA was prepared by the alkaline lysis procedure. Sequencing was performed using Sequenase according to the manufacturer’s protocol (Amersham Biosciences).

**Metal Ligand Mutations**—The sequences of the oligonucleotide primers used for amplifying the site-directed mutagenized fragments in the case of the active site metal ligands are described in this section. The name of the primer (all shown 5’ to 3’) is given first, followed by the sequence (positions that denote the mutations are underlined):

- D42A, GCC-GTC-TCC-TGG-GGG-CTG-GT; 42 →
- ATG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153A, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153D, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153E, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153F, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153G, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153H, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153I, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153J, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153K, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153L, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153M, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153N, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153O, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153P, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153Q, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153R, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153S, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153T, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153U, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153V, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153W, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153X, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153Y, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG; H153Z, CCG-GTC-TGG-CCC-AGG-AGG-ATG-AG.

To measure relative specific activities, microtiter plates were coated with 2 µg/mL M2 anti-FLAG monoclonal antibody (Sigma). PLAP-FLAG proteins were purified by affinity chromatography with anti-FLAG M2 antibody gel (Sigma) according to the manufacturer’s instructions.

**Characterization of Recombinant APs**

To measure relative specific activities, microwell plates were coated with 2 µg/mL M2 anti-FLAG monoclonal antibody (Sigma). After the addition of recombinant APs, the activity of the bound enzymes was measured as the change in absorbance at 405 nm over time at 37 °C.
upon the addition of 20 mM p-nitrophenylphosphate as substrate in 1.0 M diethanolamine buffer (pH 9.8), 1 mM MgCl₂, and 20 μM ZnCl₂. PLAP-FLAG served as a reference for each microtiter plate. The p-nitrophenol concentration formed was calculated using an extinction coefficient of 10,080 liter cm⁻¹ mol⁻¹. To calculate Kₘ, substrate concentration was varied between 0.2 and 1.6 mM p-nitrophenylphosphate, and the initial reaction rate was measured at 37 °C over a time interval of 5 min. Results were fit by nonlinear regression to the Michaelis-Menten equation using GraphPad Prism version 3.02 (GraphPad Software, San Diego, CA).

AP pH/activity profiles were done using 20 mM p-nitrophenylphosphate as substrate in 50 mM buffer with 1 mM MgCl₂ and 20 μM ZnCl₂. The phosphate ion present in the enzyme structure was included in the energy-minimized form, with all hydrogens added and formal charges calculated using the flexible ligand docking program FlexX (14). The manual “base fragment” selection (14) option was used, and either the carboxylic, amino, or hydrophobic groups of the ligand were chosen as the base fragment.

RESULTS AND DISCUSSION

To pinpoint the residues important for catalysis and stability of human PLAP, we constructed and characterized 23 individual site-directed mutants of PLAP (Fig. 1) as well as 7 double mutations. Residues serving as ligands to catalytically important metal ions, i.e. Asp-42, His-153, Ser-155, Glu-311, Asp-316, His-320, Asp-357, His-358, His-360, and His-432, were mutagenized into Ala. Two residues in the vicinity of the Mg-binding site in PLAP that are not conserved in ECAP, i.e. His-153 and His-317, were mutagenized into Ala or to Asp and Lys, respectively. Some active site mutations were studied in the context of both wild-type PLAP, i.e. [H153A, E429G], and [G429]PLAP because this substitution has been shown to have profound influences on the behavior of the enzyme by conferring upon it germ cell alkaline phosphatase characteristics (11), i.e. [H153A, E429G], [H317A, E429G], and [H319A, E429G]. Furthermore, the mammalian APs have 5 cysteine residues/monomer in positions that are not homologous to the 4 cysteines of ECAP (7), and we mutagenized each of these 5 cysteines of PLAP to Ser and also studied the [G429]PLAP because this substitution has been shown to have profound influences on the behavior of the enzyme by conferring upon it germ cell alkaline phosphatase characteristics (11), i.e. [H153A, E429G], [H317A, E429G], and [H319A, E429G].

To simplify the recovery and purification of the recombinant PLAP variants, the glycosylphosphatidylinositol anchoring sequence of PLAP was replaced by the FLAG octapeptide, and all mutants were expressed as secreted, epitope-tagged, enzymes. We had previously determined that the addition of the FLAG sequence does not interfere with the kinetic properties of the molecule (15). This strategy facilitated the production of large amounts of recombinant protein and enabled the fast and efficient isolation of each mutant AP using anti-FLAG affinity purification. Two substitutions resulted in mutant enzymes that did not have any AP activity over background levels in the culture media, i.e. C121S and C183S. We could not detect any FLAG-tagged protein in the media for these mutants by Western blot analysis, indicating that these mutations had severe effects on AP activity.
adverse consequences on protein structure, proper folding, and/or secretion.

**Mutagenesis of Metal Ligands in PLAP**—Fig. 2 shows a detailed comparison of the structure of the active site region of PLAP and ECAP including all the metal ligands that were mutagenized in this study. When substituting the Zn1 ligands in PLAP, i.e. Asp-316, His-320, and His-432, two of the three mutants, i.e. [A316]PLAP and [A432]PLAP, retained significant activity (Table I). The \( k_{\text{cat}} \) and \( K_m \) of [A316]PLAP showed a 2.8- and 2.25-fold decrease relative to wt PLAP. Thus, the catalytic efficiency (\( k_{\text{cat}}/K_m \)) of the [A316]PLAP mutant remains comparable to that of wt PLAP. The \( k_{\text{cat}} \) of [A432]PLAP was also reduced 2.7-fold, but its \( K_m \) increased 3.7-fold for a resulting 5.8-fold reduction in catalytic efficiency. In contrast, the introduction of the H320A mutation reduced the specific activity of PLAP by >200-fold. Saturation of each of the mutants with Zn2+ concentrations of up to 10 mM did not result in any increase in activity. It should be noted that analogous mutations in ECAP were reported to have very different consequences. Notably, the D327A substitution in ECAP (analogous to Asp-316 in PLAP) resulted in a 3000-fold decrease in \( k_{\text{cat}} \) and a 2000-fold increase in \( K_m \) for a 106-fold decrease in catalytic efficiency that was not reversible by the addition of Zn2+ (16). In contrast, the activity of the H412A mutant in ECAP (analogous to H432A in PLAP) was responsive to 0.2 mM Zn2+, reaching \( k_{\text{cat}} \) and \( K_m \) values only 2-fold lower that those of wt ECAP (17). The H311A mutation (analogous to H320A in PLAP) has not been studied in ECAP. These results indicate that there are significant differences in the environment of Zn1 in the PLAP structure compared with the ECAP structure and that substitutions of the Zn1 ligands are better tolerated in PLAP than in ECAP. This may reflect the fact that the top flexible loop, or crown domain, that harbors E429 in PLAP appears to provide additional stabilization to the active site environment, so that Zn2+ cannot easily diffuse in or out of the PLAP molecule, as we have previously observed (18). Thus, even though the state of coordination of Zn1 is affected by the mutations, the Zn2+ ion remains in place and is able to function in catalysis.

![Comparison of the residues coordinating to the active site metals in PLAP and ECAP.](Image)

**TABLE I**

| PLAP mutants | \( k_{\text{cat}} \) (s\(^{-1}\)) | \( K_m \) (mM) | \( k_{\text{cat}}/K_m \) (s\(^{-1}\)mM\(^{-1}\)) |
|--------------|----------------|-------------|------------------|
| wt PLAP      | 460 ± 11       | 0.36 ± 0.03 | 1288             |
| [G429]PLAP   | 344 ± 14       | 0.10 ± 0.005| 3400             |
| Conserved active site residues | | | |
| [A42]PLAP    | 6.4 ± 0.8      | 0.68 ± 0.04 | 9                |
| [A155]PLAP   | 5.1 ± 0.5      | 0.38 ± 0.02 | 14               |
| [T357]PLAP   | 529 ± 37       | 0.18 ± 0.01 | 2831             |
| [A311]PLAP   | 2.8 ± 0.4      | 1.26 ± 0.08 | 2                |
| [A316]PLAP   | 193 ± 6        | 0.16 ± 0.01 | 1073             |
| [A320]PLAP   | 1.8 ± 0.9      | ND           | ND               |
| [A357]PLAP   | 15 ± 3.7       | 0.21 ± 0.02 | 78               |
| [A358]PLAP   | <1.0           | ND           | ND               |
| [A432]PLAP   | 170 ± 6        | 1.36 ± 0.18 | 221              |
| Nonconserved active site residues | | | |
| [D152]PLAP   | 313 ± 14       | 0.71 ± 0.03 | 442              |
| [A153]PLAP   | 989 ± 53       | 1.22 ± 0.08 | 825              |
| [A153, G429]PLAP | 546 ± 35     | 0.31 ± 0.03 | 1761             |
| [K317]PLAP   | 906 ± 64       | 0.80 ± 0.03 | 1097             |
| [A317]PLAP   | 999 ± 31       | 1.13 ± 0.19 | 884              |
| [A317, G432]PLAP | 797 ± 38     | 0.25 ± 0.02 | 3188             |
| [D153, K317]PLAP | 400 ± 18     | 1.2 ± 0.1   | 340              |

**Active site neighboring residues**

| [A319]PLAP | 6.5 ± 1.5 | ND       | ND |
| [A319, G429]PLAP | 12.8 ± 2.2 | ND       | ND |
| [A360]PLAP | 552 ± 23  | 1.4 ± 0.1 | 608 |
| [A367]PLAP | 195 ± 11  | 0.35 ± 0.02 | 557 |
| [F367]PLAP | 178 ± 14  | 0.27 ± 0.02 | 659 |
| [A367, G432]PLAP | 292 ± 6   | 0.22 ± 0.01 | 918 |
| [F367, G432]PLAP | 290 ± 11  | 0.17 ± 0.01 | 1176 |

**Cysteine residues**

| [S101]PLAP | 489 ± 13 | 0.41 ± 0.05 | 1193 |
| [S467]PLAP | 206 ± 15 | 0.36 ± 0.05 | 572 |
| [S474]PLAP | 232 ± 11 | 0.33 ± 0.03 | 670 |
| [S467, S474]PLAP | 244 ± 11   | 0.40 ± 0.04 | 610 |
| [S121]PLAP & [S183]PLAP | ND       | ND       | ND |

\( a \) ND, not determined.

Alanine substitutions of the Zn2 ligands in PLAP, i.e. Asp-42, Asp-357, and His-358, resulted in significant decreases in specific activity, ranging from >25-fold (D357A) to undetectable levels (I358A). None of these values changed in response to the addition of Zn2+; whereas the \( K_m \) of [A42]PLAP nearly doubled, the \( K_m \) of [A357]PLAP decreased slightly. Thus, the catalytic efficiencies of [A42]PLAP and [A357]PLAP were reduced by 130- and 16-fold, respectively. Whereas no studies have been performed in ECAP on residues analogous to Asp-357 and His-358, the [A51]ECAP mutant, analogous to [A42]PLAP, was shown to be >800-fold less active than the wt ECAP (19).

Ala-42 is a bidentate ligand, coordinating not only to Zn2 but also to Mg. Alanine substitution of the other two Mg ligands, i.e. Ser-155 and Glu-311, reduced the specific activity of PLAP ~100- and 200-fold, respectively. The \( K_m \) for [A155]PLAP did not change, but it increased about 4-fold for [A311]PLAP. A similar pattern was seen for the corresponding E322A mutation in ECAP (20). Interestingly, the S155T substitution hardly affects the activity of the resulting mutant and even doubles its catalytic efficiency (Table I).

**Mutagenesis of Nonconserved Active Site Residues in PLAP**—Whereas most of the AP active site residues are perfectly conserved throughout evolution, some important differences exist in the neighborhood of the Mg ion (Fig. 2). His-153 and His-317 in PLAP are homologous to Asp-153 and Lys-328, respectively, in ECAP. The substitution of D153H and K328H in ECAP produced enzymes with kinetic properties similar to...
those of mammalian APs. For example, the D153H/K328H double ECAP mutant displayed a 5.6-fold higher $k_{\text{cat}}$ and a 30-fold higher $K_m$, a decrease in heat stability, and a shift in pH optimum to alkaline pH values (21). We constructed the reciprocal mutations, i.e. H153D and H317K, in PLAP as well as the double mutation (H153D/H317K). We also introduced a H153A and H317A mutation in both PLAP and [G429]PLAP. The expectation was that by reverting to the original PLAP residues found in ECAP, one would confer ECAP-like properties to PLAP. Surprisingly, however, no decrease in $K_m$ was observed in any of the mutants. Instead, the $K_m$ values consistently increased for all the mutants. The effects on $k_{\text{cat}}$ were variable. There were no significant changes in the pH dependence or heat stability of the mutants compared with the wt PLAP (data not shown).

In the ECAP active site, the environment of the Mg ion is one of octahedral coordination, including three amino acids and three water molecules (2). These water molecules are further coordinated and stabilized by other amino acid residues including Asp-153. The D153H mutation in ECAP was shown to destabilize the octahedral Mg coordination in favor of a tetrahedral one and resulted in an enzyme that had reduced Mg$^{2+}$ affinity and increased Zn$^{2+}$ affinity and was significantly activated by Mg$^{2+}$ (21). This is strongly reminiscent of the behavior of the IAP isozyme (1) but not of human PLAP, which binds Mg$^{2+}$ tightly and in which the further addition of Mg$^{2+}$ does not increase activity. A possible explanation for our results comes from the analysis of the structure of PLAP around the Mg ion (see Fig. 2). In PLAP, His-153 and His-317 are positioned so that they can serve the same purpose as the corresponding Asp and Lys in ECAP, i.e., they are direct ligands to active site water molecules and indirect ligands to the Mg ion and the noncovalently bound phosphate group. We propose that His-153 and His-317 in PLAP are already well positioned to stabilize these water-mediated interactions and that introducing different residues at these positions would result in a decrease in affinity for phosphate, thus increasing $K_m$ and $k_{\text{cat}}$ rather than decreasing these parameters. Because the IAP isozyme is more dependent on Mg$^{2+}$ activation, one can speculate that the structure of IAP in the immediate environment of the Mg ion is more similar to and can be better modeled by the structure of ECAP, rather than the structure of PLAP. Upon mutagenizing H153A or H317A in PLAP, both mutant enzymes displayed about a 2-fold increase in $k_{\text{cat}}$ and about a 3-fold increase in $K_m$ compared with wt PLAP. This can be explained by the disruption of their water-mediated interactions with the phosphate group (see Fig. 2) via the same water molecule. Disruption may lead to an enzyme with smaller affinity for both substrate and product, thus having higher $k_{\text{cat}}$ and $K_m$ values. Interestingly, by combining the H153A and H317A mutations with the G429 mutation, we were able to engineer mutant enzymes with increased catalytic efficiency when compared with wt PLAP but not when compared with [G429]PLAP. The [A153, G429]PLAP and [A317, E429G]PLAP mutants had $K_m$ values that were restored to those of wt PLAP while largely preserving the increase in $k_{\text{cat}}$. This is especially true of [A317, G429]PLAP, in which both specific activity and catalytic efficiency ($k_{\text{cat}}/K_m$ values) were increased 2-fold compared with wt PLAP and 5-fold when compared with [G429]PLAP.

**Other Conserved Residues in the Active Site Area**—Two other active site residues, His-360 and His-319, that are perfectly conserved in mammalian APs were also investigated. In ECAP, His-372 is 3.8 Å away from Zn1 in the active site, and correspondingly, His-360 is located within 4.4 Å from the Zn1 ion in PLAP. The side chain of His-360 forms a hydrogen bond to the side chain of Asp-316, which is a direct ligand to Zn1. The [A372]ECAP showed changes in catalytic behavior, such as a 20% reduction in $k_{\text{cat}}$ and a 4-fold reduction in $K_m$ in the presence of a phosphate acceptor (22). In contrast, [A360]PLAP displayed an increase in specific activity 20% over that of wt PLAP and a 3.8-fold increase in $K_m$.

Judging from its three-dimensional positioning, residue His-319 should mainly have a structural role, being involved in hydrogen bonds with Thr-48 and Tyr-393. The H319A and H319A/E429G mutations, however, displayed a 70-fold and 37-fold decrease in specific activity, respectively, indicating that the interactions formed by His-319 are important for maintaining an optimal conformation in the active site.

**Hierarchical Significance of the Five PLAP Cysteines**—PLAP, as well as all mammalian APs, has 5 cysteine residues/subunit in positions 101, 121, 183, 467, and 474 (Fig. 3A). They form two disulfide bonds, Cys-121-Cys-183 and Cys-467-Cys-474, whereas the Cys-101 residue remains in free form. We found that the cysteine recombinant PLAP mutants C101S, C467S, C474S, and C467S/C474S were appropriately expressed by transfected COS-1 cells and retained residual activity, whereas the C121S and C183S mutants were degraded...
inside the cell. The PLAP structure reveals that the sequence contained within the Cys-121-Cys-183 disulfide bond (Fig. 3) harbors three important elements of secondary structure: (a) a stretch of the central β-strand; (b) the Arg-166 residue known to be crucial for catalysis (13); and (c) the ligands stabilizing the fourth Ca ion. Thus, interfering with Cys-121-Cys-183 disulfide bond formation appears to be incompatible with proper enzyme folding.

The $k_{cat}$ and $k_m$ values for the active mutants are summarized in Table I. No significant changes were observed in the substrate affinity of these mutant enzymes as compared with wt PLAP. In the case of [S101]PLAP, even the catalytic efficiency was similar to that of wt PLAP. We examined the possibility that Cys-101 would be available for covalent modification (Fig. 4A). This confirms that Cys-101 is not likely to play a role in regulating activity or stability of the enzyme. In contrast, [S467]PLAP, [S474]PLAP, and [S467, S474]PLAP enzymes all displayed $k_{cat}$ values that were 2-fold lower than the wt PLAP value of 460 s$^{-1}$. The double [S467, S474]PLAP mutant was marginally more active than the [S467]PLAP or [S474]PLAP enzymes. The stability of these PLAP mutations toward heat inactivation was tested at 68 °C in the same buffer used for the activity measurements (Fig. 4B). All four cysteine mutants displayed stability similar to that of wt PLAP, with [S467]PLAP and [S474]PLAP being slightly less stable than the other enzymes. When compared with other PLAP mutations known to affect the heat stability, such as the E429G mutation, one can conclude that no significant stability changes occurred with any of these cysteine mutations.

**Significance of the Conserved Y367**—An interesting structural feature of PLAP, with no counterpart in the ECAP structure, is Tyr-367. This residue is part of the subunit interface in the PLAP dimer, where it protrudes from one subunit and is positioned within 5.6 Å of the catalytic Zn1 ion in the active site of the other subunit (Fig. 5A). The HO• group of Tyr-367 also forms a hydrogen bond to the peptide group nitrogen of His-432, a direct ligand to the Zn1 ion (4). The location of Tyr-367 in the structure and the fact that this residue is perfectly conserved in mammalian APs (Fig. 1) implicate this residue in an important structural/functional role.

One of the specific properties of APs of higher organisms is their ability to be inhibited stereospecifically by L-amino acids and peptides through an uncompetitive mechanism (23). Our previous studies have revealed that the nature of residue 429 has a profound effect on the inhibition. The E429G, E429S, and E429H substitutions, all naturally occurring substitutions (Fig. 1), had the same effect of facilitating the access of the ligands stabilizing the fourth Ca ion (4). The location of Tyr-367 in the structure and the fact that this residue is perfectly conserved in mammalian APs (Fig. 1) implicate this residue in an important structural/functional role.

We mutated Tyr-367 to Phe as the closest possible structural analogue of Tyr and to Ala. Both mutations were done in the context of wt PLAP and [G429]PLAP. Residue 429 is situated close to Tyr-367, so it was of interest to check the effect of the double substitutions. The effects of the substitutions at Tyr-367 on the kinetic properties of PLAP were very similar for all four recombinant mutants studied, i.e. [A367]PLAP, [F367]PLAP, [F367, G429]PLAP, and [A367, G429]PLAP (Table I). The $k_{cat}$ values were in the range of 39–45% of the wt PLAP value. The $K_m$ values were not significantly changed from the value of wt PLAP (0.35 mM). However, the Y367A and Y367F substitutions significantly compromised the heat stability of the mutant PLAP enzymes (Fig. 6). In addition, whereas [F367]PLAP, like wt PLAP, displayed a monophasic inactivation curve, [A367]PLAP instead displayed a biphasic inactivation mechanism. Interestingly, the [A367]PLAP mutant was more stable than the [F367]PLAP enzyme after a prolonged incubation, despite a higher initial rate of inactivation.

We then examined the inhibition properties of the mutants toward L-Phe, L-Leu, and L-(2-phenyl)-glycine (Fig. 7). Because all these enzyme variants had comparable kinetic parameters, any observed changes in inhibition properties should mainly reflect the changes in true binding constants for the inhibitors studied (13). The Y367A mutation in PLAP was found to have a very profound destabilizing effect on the inhibition by L-Phe and L-Leu increasing their $K_i$ values by 17.5- and 12.7-fold,
respectively (Table II). The Y367F substitution had similar but less pronounced consequences, increasing $K_i$ values by 3.2- and 4-fold, respectively. These results show that the side chain of Tyr-367 is crucial for the binding of inhibitors to PLAP, most likely by providing a local binding area for the hydrophobic side chains of Leu and Phe. In the double mutants, [A367, G429]PLAP and [F367, G429]PLAP, the pattern of inhibition by L-Leu and L-Phe was intermediate. The effect of the Y367A substitution overweighed that of E429G, whereas the effect of Y367F was completely rescued by the E429G mutation (Fig. 7).

Inhibition of [F367]PLAP and [A367]PLAP by D-Phe was even less pronounced than the inhibition by L-Phe and was so low that accurate determination of $K_i$ was difficult ($K_i > 90$ mM) (data not shown). Whereas the effect of the Y367A mutation was the most significant, the H317A substitution also had a mild influence on inhibition by both l-Phe and l-Leu, decreasing $K_i$ by 2.6- and 2.5-fold, respectively. The [A317, G429]PLAP double mutant displayed a similar decrease in $K_i$ compared with [G429]PLAP. We also studied the inhibition of PLAP mutants by L-(2-phenyl)-glycine, which, from a structural viewpoint, can be considered a “truncated” form of l-Phe. The results (Table II) show that l-(2-phenyl)-glycine is as potent an inhibitor of PLAP and PLAP mutants as L-Phe and L-Leu. However, L-(2-phenyl)-glycine does not discriminate between the Y367A and Y367F mutations, suggesting that its shorter hydrophobic side chain makes less contact with Tyr-367. Our results indicate that substitutions at positions 367 and 429 can act independently in determining inhibition properties of PLAP. It is clear that removal of Glu-429 facilitates access of the inhibitor to the active site, whereas removal of Tyr-367 eliminates stabilization of the inhibitor’s positioning at the active site.

To better explain the inhibition results, we performed a docking simulation of the binding of l-Phe and l-Leu to wt PLAP. For that purpose, we used the program FlexX (14), which performs flexible ligand docking by an incremental construction mechanism. Ligands with nonprotonated amino groups were used, in agreement with the conclusions from previous experimental studies on the pH dependence of the inhibition (13). The enzyme active site was defined as a spherical area 9 Å around the Zn1 atom. In agreement with the discussion in Ref. 14, we found the results of docking simulation to be sensitive to the selection of the “base fragment” of the ligand, i.e. the first group for which the program tries to find the putative positions in the active site. Automatic selection of the base fragment led to a cluster of solutions located far from the active site atoms, which was not relevant for the inhibition modeling. Hence we used manual selection of the base fragment and tried all three main fragments of ligand: carboxylic, amino, and hydrophobic group (the phenyl ring for phenylalanine and a methyl group for leucine). After manual selection of the carboxylic group as the base fragment, results were similar to the automatic mode. When the amino group or the hydrophobic moiety was chosen as the base fragment, the ligands docked close to the active site Zn1, and a similar orientation of the hydrophobic chain was obtained.
L-Phe and L-Leu are presented in Fig. 5, for both inhibitors. Furthermore, using the hydrophobic group as L-(2-phenyl)-glycine.

...Y367F, [A317]PLAP (H317A), [A367, G429]PLAP (Y367A), [F367, G429]PLAP (E429G), [A367, G429]PLAP (E429G), [A317, G429]PLAP (H317A), [A367, G429]PLAP (Y367A/E429G), [F367, G429]PLAP (Y367F/E429G), and [A317, G429]PLAP (H317A/E429G).

Fig. 7. Inhibition curves of PLAP mutants by L-Phe, L-Leu, and L-(2-phenyl)-glycine. The graphs plot residual activity versus inhibitor concentrations. The mutants tested were wt PLAP, [G429]PLAP, [F367, G429]PLAP, [A367, G429]PLAP (Y367A/E429G), and [A317, G429]PLAP (H317A/E429G).

TABLE II

| PLAP mutants | Inhibition constant $K_i \pm S.D.$ | $nm$ |
|--------------|-----------------------------------|------|
| wt PLAP      | 0.80 ± 0.03                       | 4.5 ± 0.2 |
| [G429]PLAP   | 0.36 ± 0.05                       | 0.46 ± 0.003 |
| [A367]PLAP   | 2.08 ± 0.05                       | 4.3 ± 1.0 |
| [F367, G429]PLAP | 2.52 ± 0.175                   | 7.0 ± 0.5 |
| [A367, G429]PLAP | 3.42 ± 0.113                   | 6.0 ± 0.5 |
| [F367, G429]PLAP | 0.52 ± 0.03                       | 0.48 ± 0.04 |
| [A317, G429]PLAP | 0.04 ± 0.01                       | 0.43 ± 0.03 |

Whereas the hydrophobic phenyl ring of Tyr-367 is important for the formation of the hydrophobic pocket in PLAP, the HO group may additionally be involved in a hydrogen bond with the polar groups of the ligand, as was found in some of the docking solutions.

In conclusion, our analysis of the structure-function relationship of residues conserved throughout evolution from the E. coli to the mammalian enzymes has revealed largely conserved functions for those residues that stabilize the active site Zn and Mg metal ions. The nonhomologous disulfide bonds differ in their structural significance, and the free cysteine in mammalian APs can be substituted without major consequences to enzyme function. Significantly, we found that nonconserved residues that contribute active site stabilization and cross-talk between enzyme monomers also determine the heat stability and uncompetitive inhibition properties of mammalian alkaline phosphatases.

REFERENCES

1. McComb, R. B., Bowers, G. N., and Posen, S. (1979) Alkaline Phosphatases, Plenum Press, New York
2. Kim, E. E., and Wyckoff, H. W. (1991) J. Mol. Biol. 218, 449–464
3. Bossi, M., Hoylaerts, M. F., and Millan, J. L. (1993) J. Biol. Chem. 268, 25049–25056
4. Le Du, M. H., Stigbrand, T., Tausig, M. J., Menesz, A., and Stura, E. A. (2001) J. Biol. Chem. 276, 19158–19165
5. Kim, E. E., and Wyckoff, H. W. (1996) Clin. Chim. Acta 186, 175–187
6. Mornet, E., Stura, E., Lia-Baldini, A. S., Stigbrand, T., Menesz, A., and Le Du, M. H. (2001) J. Mol. Biol. 311, 1171–1178
7. Sone, M., Kishigami, S., Yoshihisa, T., and Ito, K. (1997) J. Biol. Chem. 272, 6174–6178
8. Miconovic, R., Brink, B. L., Gerber, L., Pan, Y.-C., Hulmes, J. D., and Udenfriend, S. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 1398–1402
9. Ogata, S., Hayashi, Y., Takami, N., and Ikebara, Y. (1988) J. Biol. Chem. 263, 10489–10494
10. Hoylaerts, M. F., and Millán, J. L. (1997) Eur. J. Biochem. 252, 605–616
11. Hummer, C., and Millán, J. L. (1991) Biochem. J. 274, 91–95
12. Watanabe, T., Wada, N., Kim, E. E., Wyckoff, H. W., and Chou, J. Y. (1991) J. Biol. Chem. 266, 21174–21178
13. Hoylaerts, M. F., Manes, T., and Millán, J. L. (1992) Biochem. J. 266, 23–30
14. Rayer, M., Kramer, B., Lengauer, T., and Klebe, G. (1996) J. Mol. Biol. 261, 479–489
15. Di Mauro, S., Manes, T., Hesse, H., Kuzlenkov, A., Piaumo, J. M., Hoylaerts, M. F., and Millán, J. L. (2002) J. Bone Miner. Res., in press
16. Xu, X., and Kantrowitz, E. R. (1992) J. Biol. Chem. 267, 16244–16251
17. Ma, L., and Kantrowitz, E. R. (1994) J. Biol. Chem. 269, 31614–31619
18. Hoylaerts, M. F., Manes, T., and Millán, J. L. (1997) J. Biol. Chem. 272, 22781–22787
19. Tibbits, T. T., Murphy, J. E., and Kantrowitz, E. R. (1996) J. Mol. Biol. 257, 700–715
20. Xu, X., and Kantrowitz, E. R. (1993) Biochemistry 32, 10683–10691
21. Murphy, J. E., Tibbits, T. T., and Kantrowitz, E. R. (1998) J. Mol. Biol. 253, 604–617
22. Xu, X., Qin, X. Q., and Kantrowitz, E. R. (1994) Biochemistry 33, 2279–2284
23. Fishman, W. H., and Sie, H. G. (1971) Enzymologia 41, 140–167
24. Ramasamy, L., and Butterworth, P. J. (1975) Biochem. Biophys. Acta 384, 146–158
25. Humphrey, W., Dalke, A., and Schulten, K. (1996) J. Mol. Graphics 14, 33–38
