Structural Evidence for a Functional Role of Human Tissue Nonspecific Alkaline Phosphatase in Bone Mineralization*

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The human tissue nonspecific alkaline phosphatase (TNAP) is found in liver, kidney, and bone. Mutations in the TNAP gene can lead to Hypophosphatasia, a rare inborn disease that is characterized by defective bone mineralization. TNAP is 74% homologous to human placental alkaline phosphatase (PLAP) whose crystal structure has been recently determined at atomic resolution (Le Du, M. H., Stigbrand, T., Taussig, M. J., Ménez, A., and Stura, E. A. (2001) J. Biol. Chem., 276, 9158–9165). The degree of homology allowed us to build a reliable TNAP model to investigate the relationship between mutations associated with hypophosphatasia and their probable consequences on the activity or the structure of the enzyme. The mutations are clustered within five crucial regions, namely the active site and its vicinity, the active site valley, the homodimer interface, the crown domain, and the metal-binding site. The crown domain and the metal-binding domain are mammalian-specific and were observed for the first time in the PLAP structure. The crown domain contains a collagen binding loop. A synchrotron radiation x-ray fluorescence study confirms that the metal in the metal-binding site is a calcium ion. Several severe mutations in TNAP occur around this calcium site, suggesting that calcium may be of critical importance for the TNAP function. The presence of this extra metal-binding site gives new insights on the controversial role observed for calcium.

The alkaline phosphatases (EC 3.1.3.1) (APs) form a large family of dimeric enzymes common to all organisms. They catalyze the hydrolysis of phosphomoesters (1) with release of inorganic phosphate. Mammalian APs have low sequence identity with the Escherichia coli enzyme (25–30%), although the residues involved in the active site of the enzyme and the ligands coordinating the two zinc atoms and the magnesium ion are largely conserved. Therefore, the catalytic mechanism deduced from the structure of the E. coli AP is believed to be similar in eukaryotic APs (2). This mechanism involves the activation of the catalytic serine by a zinc atom, the formation of a covalent phosphoseryl intermediate, the hydrolysis of the phosphoseryl by a water molecule activated by a second zinc atom, and the release of the phosphate product or its transfer to a phosphate acceptor (3).

In humans, three out of four AP isozymes are tissue-specific; one is placental (PLAP), the second appears in germ cells (GCAP), and the third in the intestine (IAP). They are 90–98% homologous, and their genes are clustered on chromosome 2q37.1. The fourth, TNAP, 50% identical to the other three, is nonspecific and can be found in bone, liver, and kidney (4, 5, 6). Its gene is located on chromosome 1p34–36 (7), and mutations in the TNAP gene have been associated with hypophosphatasia, a rare inherited disorder, characterized by defective bone mineralization. The disease is highly variable in its clinical expression, due to the strong allelic heterogeneity in the TNAP gene. Such expression ranges from stillbirth without mineralized bone to pathological fractures developing only late in adulthood (8). Depending on the age of onset, five clinical forms are currently recognized: perinatal, infantile, childhood, adult, and odontohypophosphatasia. To date, 89 different mutations associated with this disease have been characterized (9–22). Correlation between genotype and phenotype are difficult to establish, because most patients are compound heterozygous for missense mutations, making difficult the determination of the respective roles of each mutation.

This difficulty arises mainly from the lack of data concerning the precise role that TNAP plays in bone mineralization. This may be partly solved by the use of site-directed mutagenesis of TNAP cDNA and cell transfection to assay residual activity of the mutant AP enzyme (16, 18, 20, 23–25). However, this only measures the ability of the enzyme to hydrolyze phosphomoesters. Transfection assays cannot distinguish structural mutations from functional ones, and mutations that exhibit residual activity in vitro may yield protein that in vivo is incompletely processed or incorrectly addressed to the cell membrane. To complement the study of TNAP missense mutations, a three-dimensional model was built from E. coli AP (ECAP) structure (18). However, ECAP and human TNAP are only 25% identical, and the resulting model allows the assignment of mutated residues mainly around the active site and at the homodimer interface. The model omits a large number of regions of deletions and insertions in TNAP relative to ECAP, in particular concerning the putative functional regions of TNAP. Such insertions are also present in human placental alkaline phosphatase (PLAP) which is 57% identical to TNAP in sequence and whose crystal structure has been recently solved to 1.8-Å resolution (26). PLAP contains fewer insertions and deletions and provides a better framework from which to interpret TNAP mutations. Compared with ECAP, PLAP has extra domains that have been acquired during evolution, probably to mediate functional specialization of the enzyme. Among these domains, we point out the metal-binding domain with its

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‡ The abbreviations used are: AP, alkaline phosphatase; PLAP, placental alkaline phosphatase; ECAP, E. coli alkaline phosphatase.
bound calcium. The homology between PLAP and TNAP at this location suggests that such a site is also present in TNAP. The presence of calcium at this site was suggested on the basis of the metal coordination but remained to verify (26).

Here we report the analysis of the metal composition of a dehydrated solution of PLAP by x-ray fluorescence using a synchrotron source. This study confirms the presence of calcium in PLAP and, by homology of the sequence, also in TNAP. We have built a three-dimensional model of TNAP on the basis of the PLAP structure, and we analyze the effect of the mutations that occur in TNAP and are associated with hypophosphatasia. Such a model allows us to map the distribution of the amino acid substitutions within the molecule. The correlation between the degree of severity of the mutations and their location within the specific domains of TNAP suggests a probable role for the enzyme in bone mineralization. In particular, an additional mutagenesis study at the calcium binding site suggests that this atom plays a fundamental role in TNAP function.

MATERIALS AND METHODS

Mutations Studies—Fifty-four of the 73 missense mutations studied here have been reported previously (9–22). The other mutations were found by our group and were not published or were directly submitted to the Human Gene Mutation Data base (Cardiff, United Kingdom).

Mutations associated with lethal or infantile hypophosphatasia were considered as severe, while mutations associated with less severe phenotypes (childhood, adult, and odontohypophosphatasia) were classified as either severe or moderate depending on their residual enzymatic activity, measured by transfection experiments, and on the nature of the second mutation found in the observed genotype (Table I).

Sequence Alignment of TNAP and PLAP—The TNAP sequence was first aligned to that of PLAP using the program BLAST as implemented at the internet site www.ncbi.nlm.nih.gov/blast2.html (27). The two molecules display 57% identity and 74% homology. TNAP has four insertions of one residue, one insertion of three residues, and one deletion of two residues relative to PLAP (Fig. 1). The hydrophobicity profiles of the two molecules were calculated with the program HCA (28) to check the conservation of hydrophobic patches associated to the secondary structure elements.

Synchrotron Radiation X-ray Fluorescence with Human Placental Alkaline Phosphatase—PLAP was purified as described previously (26). The protein was concentrated to 10 mg/ml in 10 mM Tris, 2 mM MgCl₂, 0.02% NaN₃, pH 7.0, using a Centricon Amicon 30. The experiment of synchrotron radiation x-ray fluorescence was performed at LURE on station D15. A 4-µl droplet of protein was deposited on a 1-µm mylar sheet and evaporated overnight. The elements (phosphorus, sulfur, chlorine, argon, potassium, calcium, titanium, vanadium, antimony, manganese, iron, nickel, copper, zinc) in the protein sample were assayed using highly sensitive synchrotron x-ray fluorescence. The sample on the mylar sheet and the mylar alone were illuminated with a 10.10 KeV source, the sample was positioned at a 45° angle, and the detector positioned at 45° and 3.00 cm from the sample and 90° from the source. The calcium/zinc concentration ratio was calculated after subtracting the mylar contribution. The area of a peak, corrected for the air absorption, for an element E, is given by the following formula,

\[ \text{S}_{E}^{\text{corrected}} = [E] \sigma_0(K,E) \frac{IK_{E1}(I_{K1} / I_{K2})}{I_{K2} / H_{9268}} \]

with [E] being the concentration in element E, \( \sigma_0(K,E) \) the fluorescence yield, \( (I_{K1} / I_{K2}) / (IK_{E1} / H_{9268}) \) the intensity ratio of Kα ray, \( \sigma_0(K,E) \) K shell photo-electric cross-section, \( \sigma_0(\text{tot},E) \) the total photoelectric cross-section and \( J_{E} \) the K edge jump ratio.

From the relation of proportionality (1), we cannot directly calculate the concentration in E. However, the ratio of the corrected areas of two peaks corresponding to elements E1 and E2 can be evaluated as the ratio of the concentrations of these elements (shown as follows).

\[ \frac{[E1]}{[E2]} = \left( \frac{SE_{E1}^{\text{corrected}}}{SE_{E2}^{\text{corrected}}} \right) \frac{\sigma_0(\text{tot},E1) \sigma_0(K,E1) I_{K1}}{\sigma_0(\text{tot},E2) \sigma_0(K,E2) I_{K2}} (I_{K1} / I_{K2}) / (IK_{E1} / H_{9268}) \]

We can therefore deduce the proportion present in the protein sample (Table I) (29, 30).

Site-directed Mutagenesis and Transfection Study of Mutation E218G—The cDNA with mutation E218G was obtained directly from the full atom model of TNAP was calculated with the program MODELLER (31). The program models three-dimensional protein structure by satisfying spatial restraints in the modeled molecule to accommodate the mutations made from the homologous structure (32). MODELLER produces three-dimensional structure by minimization of the variable target function using the conjugate gradient method and molecular dynamics with simulated annealing. The program PROCHECK (33) was used to verify the model geometry. The resulting model corresponds to a crystal structure in the 1.5–2.0-Å resolution range, suggesting that none of the mutations between the two enzymes results in major structural changes.

The range of action of the side chains of the mutated residues were visualized on a Silicon Graphic Octane station, with the program TURBO-FRODO (34).

Site-directed Mutagenesis and Transfection Study of Mutation E218G—The cDNA with mutation E218G was obtained directly from the normal cloned cDNA by using the QuikChange site-directed mutagenesis kit (Stratagene). Mutated and wild-type plasmids were transiently transfected in COS-1 cells. Transfections were performed with LipofectAMINE PLUS reagent (Life Technologies, Inc.) using the methodology recommended by the manufacturer. The plasmid pcDNA3.1/His/LacZ containing the β-galactosidase gene was used as a positive control of transfection and expression. The cells were treated as described previously (23) and AP activities determined using a VP Abbott dichromatic autoanalyzer with commercially available kits.

## Table I

| Location and severity of the hypophosphatasia mutations | Severe | Moderate |
|--------------------------------------------------------|--------|----------|
| Active site or active site vicinity:                   |        |          |
| G46V, T83M, A94T, A99T, G112R, N153D, H154Y, A159T,  |        |          |
| R167W, D277A, D277Y, G317D, A331T, A360V, D361V,    |        |          |
| H364R, R433C                                          |        |          |
| Homodimer interface:                                   |        |          |
| A23V, R54C, R54P, R54H, G58S, G103R, R374C, N400S,  |        |          |
| S428P, V442M, G456S, G459K, E459G, E459K, R433C,    |        |          |
| G46V, T83M, A94T, A99T, G112R, N153D, H154Y, A159T,  |        |          |
| R167W, D277A, D277Y, G317D, A331T, A360V, D361V,    |        |          |
| H364R, R433C                                          |        |          |
| Crown domain:                                          |        |          |
| R374C, N400S, V406A, G409C, S428P, R433C              |        |          |
| Calcium site or calcium site vicinity:                 |        |          |
| R206W, R207E, R218G, D277K, D277Y, E281K, D289V      |        |          |
| Others:                                                |        |          |
| A34V, R135H, G145V, A162T, C184Y, Q190P, N194D,      |        |          |
| R229S, G232V, F310C, F310L, C472S                     |        |          |

For mutations followed by “?” the severity remains unclear as these mutations were found in patients with moderate hypophosphatasa and compound heterozygotes for two missense mutations for which the respective effects were not determined by mutagenesis experiments. Few mutations appear more than once as they belong to overlapping domains.
RESULTS AND DISCUSSION

Sequence Alignment and Synchrotron Radiation X-ray Fluorescence—The sequence alignment of TNAP and PLAP shows 57% identity with four one residue insertions, one three residues insertion, and one two residues deletion in TNAP (Fig. 1). The hydrophobicity profiles calculated with the program HCA are similar between the two molecules, suggesting that the secondary structures are highly conserved. The extra domains observed in PLAP and not in ECAP are the N-terminal α-helix, the metal-binding domain, and the crown domain. They are respectively 35, 46, and 48% identical to the homologous regions of TNAP. In particular, in the metal-binding domain the metal ligands are strictly identical, in agreement with the presence of the same metal in both proteins.

By x-ray fluorescence, performed at LURE on station D15, the protein sample was found to contain chloride, zinc, sulfur, calcium, copper, and iron in order of peak height (Fig. 2). The chloride ion comes from the buffer solution, and the sulfur comes from the cysteines and methionines in the protein. In order of peak height, zinc is the second peak and calcium the fourth. The presence of iron and copper, in ionized form, are biological trace elements (Table II). Placenta, before purification is rich in mitochondria, which contains significant amounts of iron and copper ions, and the placenta is filled with fetal blood, containing trace amounts of iron and copper ions.

**TABLE II**

|                      | Calcium | Iron | Copper | Zinc |
|----------------------|---------|------|--------|------|
| \(E_{\text{corrected}}\) | 73,519  | 27,718 | 56,291 | 797,228 |
| \(\sigma_E\) | 89.9 | 165 | 209 | 226 |
| \(K_E/K_\alpha\) | 9.1 | 8.31 | 7.81 | 7.56 |
| \(K_E/K_{\beta}\) | 0.887 | 0.882 | 0.881 | 0.844 |
| \(\sigma_E\) | 0.163 | 0.340 | 0.440 | 0.474 |
| \([\text{Calcium}]_E\) | 1 | 10.2 | 8.31 | |

![Fig. 1. Sequence alignment of PLAP and TNAP as used for the modelization of TNAP.](http://www.jbc.org/)

Deletions are indicated with a dot, basic residues are written in blue, acidic in red, and the catalytic serine is in boldface. In the intermediate line, identical residues are written, homologous residues are indicated by +, divergent residues by *, the α-helices are highlighted in yellow, and the β-strands in green. The metal-binding residues are indicated with an arrow.
During the purification, heat denaturation of contaminating proteins could putatively cause the release of the bound metal ions. The coordination number observed in the metal-binding site of PLAP is 7, clearly different from that usually observed for either copper or iron, which ranges from 4 to 6 (35). The presence of such metals is therefore unlikely. After subtraction
PLAP, the charge distributions at the protein surfaces are clearly different (Fig. 3). In particular, the active site valley located on both sides of the active site, which contains a large hydrophobic area in PLAP, is punctuated by more polar residues in TNAP. Thus, the hydrophobic residues, Trp, Tyr, and Tyr are conserved, but in TNAP, they are surrounded by ionic residues. Close to the active site, the acidic residue Glu, which confers to PLAP the specificity toward the uncompetitive inhibitors L-Phe or L-Leu (40), is replaced by a basic one, Arg, in TNAP. The hydrophobic pocket that is deeper in the active site of PLAP is not conserved in TNAP. Residues Phe, Gln, and Phe are replaced in TNAP by Gln, Glu, and Arg, respectively. However, the tyrosine, which enters in the active site of the other monomer (Tyr in PLAP), is conserved in TNAP (Tyr). This reinforces the idea that Tyr may contribute to the allosteric properties shared by the two enzymes (41). All residues that are essential for the hydrolytic activity of the bacterial and the other mammalian phosphatases are preserved in TNAP, but those that confer substrate specificity are different.

The sequences alignment and the comparison of the hydrophobicity profiles shows that the structural features that are present in PLAP, but not in the E. coli AP, also occur in TNAP. These comprise the N-terminal α-helix involved in the dimer interface, the 76 residues of the calcium-binding-domain (residues 211–289), and the interfacial “crown-domain” formed by the insertion of a 60-residue segment (371–431) from each monomer. Within the crown domain, a site for collagen attachment has been localized in loop 405–435 (41). The TNAP model shows that this loop is highly accessible and located at the very tip of the crown domain. The functional relevance of these domains can be explained by an analysis of the mutations that lead to hypophosphatasia.

**Functional Regions of TNAP—**The location of the 73 missense mutations associated with hypophosphatasia in the three-dimensional model allowed us to define five crucial regions in the TNAP molecule.

The first region of TNAP is the active site where 20 substitutions are located within a 15-Å sphere centered around the phosphate group. Except for M45L, H154R, and R433H, all other mutations found in this sphere are classified as severe (Table I). Mutation M45L is structurally conservative; the patient with this mutation was affected with infantile hypophosphatasia, but with only odontologic symptoms. Apart from these more moderate mutations, most substitutions around the active site correspond to severe alleles as reported previously (18) (Fig. 4, upper panel).

The second region is located in the active site valley, which extends on both sides of the active site. Here, six mutations affecting four residues were observed: T117N, R119H, E174K, and R433H, all other mutations found in this sphere are classified as severe (Table I). This reinforces the idea that His may contribute to the allosteric properties shared by the two enzymes (41). All residues that are essential for the hydrolytic activity of the bacterial and the other mammalian phosphatases are preserved in TNAP, but those that confer substrate specificity are different.

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stabilization, the same function could be performed by other polar residues, suggesting that polarity and not charge is essential for substrate steering.

The third important region is the homodimer interface. Dimerization is particularly important for TNAP, not only for allosteric reasons (cannot exist without dimerization) but also because it appears that APs are active only in dimeric form (42). Since dimerization is such a fundamental aspect of APs, it is not surprising to find that of the 20 mutations in the homodimer interface, at least 15 of them are severe alleles. These residues may be directly involved in homodimer interactions or play a role in maintaining the correct fold to allow these interactions to form. The PLAP-based model allowed us to locate some mutated homodimer interface residues, G456S, E459K, and G474R, that were not identified from the *E. coli*-based model (18). These residues, associated with a severe form of the disease, belong to the C-terminal part of the molecule that have no equivalent in the *E. coli* structure.

The fourth region is the loop 405–435 within the crown domain, composed of a total of 65 residues. This region, which has been associated with the binding of collagen (40), corresponds to a long insertion loop with no counterpart in *E. coli*.

FIG. 4. Functional regions of TNAP. On the left panel TNAP is shown in ribbon representation with the residues involved in hypophosphatasia in red (active site and active site valley), purple (homodimer interface), blue (crown domain), or yellow (calcium binding site). On the right panel, the functional region are enlarged with the residues involved in hypophosphatasia in red if the mutation effect is severe or yellow if the mutation effect is moderate.
The 65 residues of the crown domain are well conserved (72% homologous) with PLAP with the exception of loop 405–435 where the two molecules share only 11 identical and 5 homologous residues. However, the hydrophobicity profile shows that the hydrophobic and hydrophilic residues adopt similar distribution. Among the 10 mutations associated with hypophosphatasia identified in this domain, V406A, G409C, Y419H, S428P, R433C, and R433H are located within this loop. The others, R374C, A382S, D389G, and N400S, in the portion of the crown domain involved in monomer–monomer interactions are disruptive of the interface, as is the case also for V406A. The substitution G409C rigidifies the loop backbone at the tip of the crown domain. Y419H belongs to an α-helix of the crown domain, and S428P occurs within a β-strand. As mentioned previously, Arg433 is found at the entrance of the active site pocket with a role in the substrate positioning. Its mutation to His433 is probably less severe than its mutation to Cys433, probably because it is more conservative. This is corroborated by the lethal effect associated to the homozygous genotype R433C and the moderate phenotype observed in patient R433H with childhood hypophosphatasia but just odontologic symptoms. Finally, we can observe that most of the mutations affecting this domain are severe, reinforcing the idea that collagen binding is crucial for the function of the enzyme.

The fifth region surrounds the calcium binding site (M4). Eleven distinct mutations were found in this region. The calcium atom is coordinated by the carboxylates of Glu218, Glu274, and Asp289, by the carboxyl of Phe273, and by a water molecule.

Conclusions—In this study, we have built a model of TNAp on the basis of the structure of PLAP. This model allowed us to position the substitutions responsible for hypophosphatasia. The distribution of the substitutions highlights five regions comprising 78% of the mutations, most of them being severe alleles. These regions are namely the active site and its vicinity, the active site valley, the homodimer interface, the crown domain, and the calcium-binding domain, clearly indicating that these regions are crucial for the enzyme function and bone mineralization. Two of these regions, the crown domain and the calcium-binding domain, are absent in the E. coli structure and were ignored in our previous model. This new model highlights two distinct mammalian-specific regions, one involved in collagen binding and the other involved in a novel function, which probably involves calcium.

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