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Review

# Molecular and cellular basis of hypophosphatasia

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ABSTRACT

*Background:* Hypophosphatasia (HPP) is an inherited disorder characterized by defective mineralization of the bone and teeth that is also associated with a deficiency of serum alkaline phosphatase (ALP). Patients with HPP exhibit a broad range of symptoms including stillbirth with an unmineralized skeleton, premature exfoliation and dental caries in childhood, and pseudo-fractures in adulthood. The broad clinical spectrum of HPP is attributed to various mutations in the *ALPL* gene, which encodes tissue-nonspecific alkaline phosphatase (TNSALP). Nevertheless, the molecular mechanisms underlying the genotypic and phenotypic relationship of HPP remain unclear.
*Highlight:* The expression of HPP-related TNSALP mutants in mammalian cells allows us to determine for the effects of mutations on the properties of TNSALP, which could contribute to a better understanding of the relationship between structure and function of TNSALP.
*Conclusion:* Molecular characterization of TNSALP mutants helps establish the etiology and onset of HPP.


**Contents**



*Abbreviations:* ALP, alkaline phosphatase; ENPP1, ectonucleotide pyrophosphatase/phosphodiesterase 1; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GPI, glycosylphosphatidylinositol; HPP, hypophosphatasia; MV, matrix vesicle; pNPP, *p*-nitrophenylphosphate; Pi, inorganic phosphate; PLP, pyridoxal-5′-phosphate; PPi, inorganic pyrophosphate; TNSALP, tissue-nonspecific alkaline phosphatase.

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## 1. Introduction

Hypophosphatasia (HPP) is an inherited condition that is caused due to an error in metabolism and is characterized by bone and dental hypomineralization (#OMIM 241510, 241500, and 146300). The biochemical hallmark of HPP is reduced alkaline phosphatase (ALP) activity in the serum, which may be caused by various loss-of-function mutations in the *ALPL* gene that encodes tissue-nonspecific ALP (TNSALP) [1–4]. HPP is characterized by various outcomes ranging from death *in utero* with poor skeletal mineralization to pseudo-fractures in adulthood. HPP is now classified into six categories: (1) perinatal severe, (2) perinatal benign, (3) infantile, (4) childhood, (5) adult, and (6) odonto HPP [1–4]. Odonto HPP is the least severe form of HPP and is characterized by the premature loss of primary teeth and/or severe dental caries without the skeletal mineralization abnormalities associated with other forms of HPP [2,3]. Severe forms are transmitted in an autosomal receptive pattern, whereas the milder forms have an autosomal recessive or autosomal dominant pattern of inheritance [2,3]. A total of 390 *ALPL* gene mutations have been reported worldwide as of Jun. 2019 (http://www.sesepuvsq/fr03_hypo_mutationsphp), most of which are missense mutations (70.4%); the others are small deletions (11.9%), splicing mutations (5.9%), nonsense mutations (4.4%), and small insertions (3.6%). The highly variable clinical phenotypes of HPP may be largely attributed to the high proportion of compound heterozygotes exhibiting various levels of residual ALP activity.

This review summarizes our attempts to understand the etiology of HPP based on the molecular characterization of TNSALP mutants. Standardized nomenclature where the N-terminal initiator methionine is designated as the first residue is used throughout this review except for Table I, in which both standardized and non-standardized nomenclatures have been used for comparison.

## 2. Alkaline phosphatases in humans

ALPs (EC 3.1.3.1) are present in bacteria and humans [1]. They catalyze the hydrolysis of phosphomonoesters with the release of inorganic phosphate (Pi). There are four ALP isozymes in humans: tissue-nonspecific (TNS)-, placental-, intestinal- and germ cell-like or placental-like ALP. The gene of TNSALP is located on chromosome 1, whereas the genes of the other three (tissue-specific) isozymes are on chromosome 2 and are 90–98% homologous to each other, indicating that they arise via gene duplication [1–4]. On the other hand, TNSALP and placental ALP show 57% identity and 74% homology [1]. No monogenic inherited disorder has been assigned to tissue-specific ALPs compared with TNSALP [4]. TNSALP is broadly expressed in the body, particularly in the liver, bone, and kidney. The physiological functions of TNSALP in the calcification of bone and teeth are well known. However, its role in extra-calcified tissues, including the brain, remains unclear [4]. *p*-Nitrophenylphosphate (pNPP) has been widely used as a substrate for the quick and convenient measurement of ALP activity. Inorganic pyrophosphate (PPi), phosphoethanolamine, and pyridoxal-5′-phosphate (PLP) have been proposed as physiological substrates of TNSALP [1–4]. These substances abnormally accumulate in the body of patients with HPP and TNSALP null mice [1–3].

## 3. TNSALP and matrix vesicle (MV)-mediated calcification

Osteoblasts are responsible for bone mineralization. They not only secrete collagen fibrils and non-collagenous proteins, such as osteocalcin and osteopontin, but are also the source of MVs, which bud from the cell surface [5]. MVs play roles in mineralization and are regulated by the concerted effort of several enzymes and transporters [6,7]. Increasing evidence shows that bone mineralization initiated inside the MVs [1–4,6]. Needle-shaped crystals then grow out of the MVs into the bone matrix and become mineralized nodules (also referred to as calcifying globules), which subsequently contribute to collagen fibril mineralization [1,7]. At least three hydrolases are involved in MV-mediated mineralization: orphan phosphatase 1, TNSALP, and ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) [6]. The former is localized inside MVs and appears to contribute to the nucleation of hydroxyapatite [6]. The latter two enzymes are located on the MV membrane and are involved in the growth of hydroxyapatite crystals by regulating local concentrations of PPi, a potent mineralization inhibitor, outside the MVs. ENPP1 generates PPi from extracellular ATP, whereas TNSALP enhances bone mineralization by hydrolyzing PPi and supplying Pi [4–7]. In HPP, various mutations in the *ALPL* gene can reduce ALP activity in osteoblasts and derived MVs. As a result, the accumulation of PPi and a shortage in the supply of Pi may lead to hypomineralization at the site of bone formation. This may also explain the mineralization of dentin and acellular cementum [6].

## 4. 3D structure of TNSALP

A computer-generated 3D structural model of TNSALP has been constructed based on the X-ray crystallography of PLAP [8]. TNSALP is a homodimer and consists of five principal functional domains [4,8]: 1) a catalytic site, 2) a calcium-binding site, 3) a crown domain 4) a homodimer interface, and 5) a N-terminal alpha helix (Fig. 1). The active center of the catalytic site is a serine residue at position 110 (Ser110) that covalently binds to phosphate during the catalytic reaction, which is surrounded by residues that function as ligands to three metal ions (2 $Zn^{2+}$ and 1 $Mg^{2+}$). Although *Escherichia coli* ALP and TNSALP are only 25% identical, the residues essential for catalytic function are well conserved between the two enzymes [1,8]. The calcium-binding site is located away from the catalytic site and may play a structural role. This domain is missing in bacterial ALPs; however, it is well conserved in vertebrate ALPs.

**Table 1**
A list of the TNSALP mutations described in this review. In the clinical form, P(S), I, C, A, and O denote perinatal-severe, infantile, childhood, adult, and odonto HPP, respectively. Database, The Tissue Non-specific Alkaline Phosphatase Gene Mutations Database (http://www.sesep.uvsq.fr/03_hypo_mutations.php). The residual activity of each mutant is expressed as a percentage of the wild-type enzyme in transiently transfected cells. In expression, wild-type and TNSALP mutants were analyzed in transiently expressed cells (T) and Tet-On CHO cells (Tet-On).

| | Amino acid change | | Genotype of the patient | Clinical form | Reference | Residual activity %WT/ Reference | Site of 3D structure (modification) | Expression |
|---|---|---|---|---|---|---|---|---|
| | Standardized nomenclature | Non standardized nomenclature | | | | | | |
| 1 | p.R71C | *R54C* | p.R71C/p.D294A | I | 17 | 1/15 | Homodimer | T |
| 2 | p..P108L | *P91L* | p.P108L/N | O | 22 | 0/24 | Catalytic site | T/Tet-On |
| 3 | p.A116T | *A99T* | p.A116T/N | A | 23 | 0/25 | Catalytic site | T/Tet-On |
| 4 | p.N170D | *N153D* | p.N170D/p.N170D | P(S) | 18 | 0/20 | Catalytic site | T |
| 5 | p.A179T | *A162T* | p.A179T/p.A179T | P(S) | 12 | 24/11 | Extra | T |
| 6 | p.C201Y | *C184Y* | c.-195C>T/p.C201Y | P(S) | 30 | <1/47 | Disulfide bridge | T/Tet-On |
| 7 | p.E235G | *E218G* | p.E235G/p.A399S | A | 29 | 0/11 | Calcium site | T |
| 8 | p.D294A | *D277A* | p.R71C/p.D294A | I | 17 | 35/15 | Catalytic site | T |
| 9 | p.D306V | *D289V* | p.D306V/p.D306V | I | 30 | 0/31 | Calcium site | T |
| 10 | p.G334D | *G317D* | p.G334D/p.G334D | P(S) | 19 | 0/21 | Catalytic site | T |
| 11 | p.N417S | *N400S* | p.N417S/c.648+1G>A | P(S) | 36 | 0/38 | Homodimer | T/Tet-On |
| 12 | p.G420A | *G403A* | p.G420A/p.E191K | P(S) | 37 | 2/39 | Homodimer | T |
| 13 | p.G420S | *G403S* | p.G420S/p.G420S | C | Data base | 5/39 | Homodimer | T/Tet-On |
| 14 | p.V423A | *V406A* | p.A116T/p.V423A | P(S) | 29 | 16/41 | Crown | T/Tet-On |
| 15 | p.G426D | *G409D* | p.G426D/p.E191K | C | 44 | <1/46 | Crown | T |
| 16 | p.G426C | *G409C* | p.K224A/p.G426C | I | 45 | 12/46 | Crown | T/Tet-On |
| 17 | p.G426S | *G409S* | p.G426S/p.G426S | I | Data base | 10/46 | Crown | T |
| 18 | p.N430S | *N413S* | p.N430S/p.E191K | I | 56 | 0/55 | N-glycan | T |
| 19 | p.R450C | *R433C* | p.R450C/p.R450C | I | 18 | 4/43 | Crown | T/Tet-On |
| 20 | p.R450H | *R433H* | p.D406G/p.R450H | O | 42 | >100/43 | Crown | T |
| 21 | p.C489S | *C472S* | p.C489S/c.997+2T>A | P(S) | 42 | <1/47 | Disulfide bridge | T/Tet-On |
| 22 | p.L520RfsX86 | *L503RfsX86* | p.E298K/c.1559delT | I | 52 | 3/53 | GPI-anchor | T |

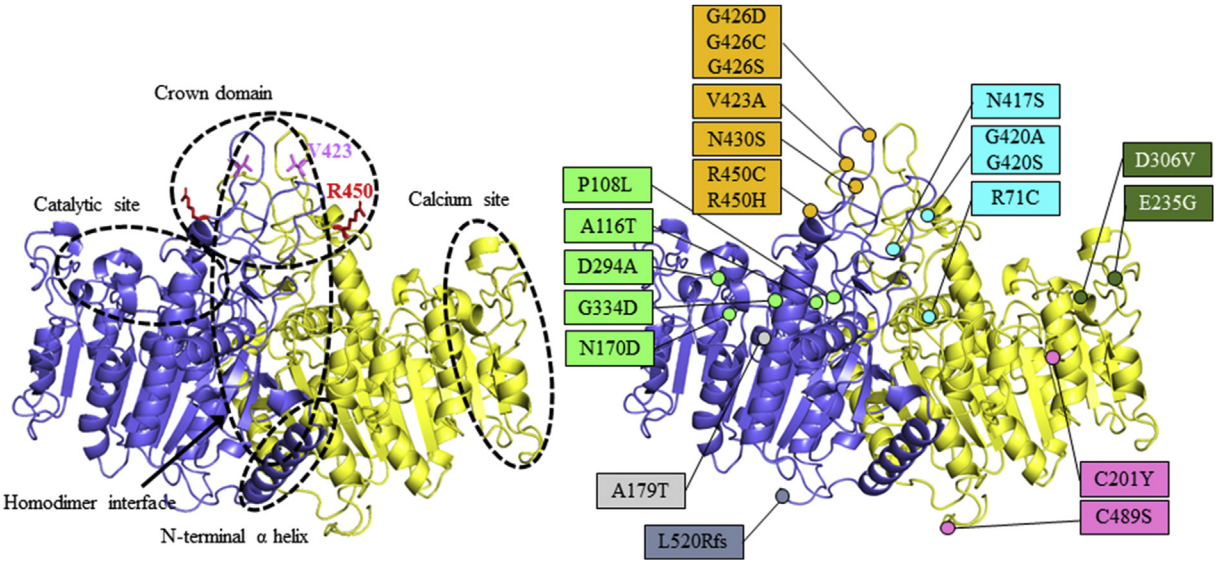


**Fig. 1.** A 3D model of human TNSALP based on the crystal structure of human placental alkaline phosphatase. The two monomers are shown in blue and yellow. Five functional domains are indicated. Valine at position 423 and arginine at position 450 in the crown domain are highlighted.

The crown domain is unique to mammalian ALPs and contributes to isozyme-specific properties, such as allosteric properties, heat stability, uncompetitive inhibition, and collagen binding [9]. At the homodimer interface, two homologous monomers bind to each other to become the dimeric enzyme. Part of the homodimer interface overlaps with the crown domain (the interfacial crown domain) and N-terminal alpha helix [4,9]. ALPs including TNSALP only exhibit catalytic activity in the dimeric form; however, TNSALP may exist as a tetrameric form on the cell membrane [10]. The N-terminal alpha helix is thought to stabilize monomer-monomer interactions [4].

## 5. Biosynthesis of TNSALP

TNSALP (W) is a membrane glycoprotein anchored to the cell membrane via glycosylphosphatidylinositol (GPI). During its biosynthesis process, as the nascent polypeptide of TNSALP elongates, it is thought to pass through the translocation channel in the endoplasmic reticulum (ER) with the guidance of the N-terminal 17-amino acid-long signal sequence. Once in the ER lumen, it starts to fold into the correct tertiary structure, which then binds to other tertiary structures and becomes the homodimeric functional enzyme. Concomitant with its translocation into the ER lumen,

TNSALP (W) is modified by N-glycans, disulfide bonding, GPI, and other modifications. These events may be crucial for the acquisition of the correct structure, which can exit the ER. TNSALP (W) is then delivered to the Golgi apparatus via vesicular transport, in which the 66 kDa immature form possessing high-mannose type N-glycans to become the ~80 kDa mature form bearing complex-type N-glycans [11]. This shift in the molecular size of TNSALP may be used to monitor its transport from the ER to the Golgi apparatus. Subsequently, TNSALP (W) is transported to the plasma membrane and localized on the cell surface via GPI. However, some TNSALP mutants, which fail to acquire transport competence due to misfolding, are retained in the ER or cis-Golgi network, resulting in their degradation, as described in section 6.7.

In theory, mutations associated with HPP may affect various stages of the protein folding and subunit assembly of TNSALP as well as its subsequent trafficking through the secretory pathway. Failures in these steps may lead to reduced and/or altered ALP activity in MVs, and ultimately result in hypomineralization.

## 6. Molecular characterization of TNSALP mutations associated with HPP

TNSALP was previously implicated in bone mineralization; however, its exact role was elusive. A missense mutation (p.A179T), in which the alanine (A) residue at position 179 is replaced with threonine (T), was the first HPP-associated mutation identified [12], demonstrating that TNSALP is essential for normal bone mineralization. Since then, 390 mutations have been reported as of Jun. 2019 (http://www.sesep. uvsq.fr/03_hypo_mutations.php). The assignment of various mutations to the five functional domains of the 3D TNSALP model has contributed to the current understanding of the genotypic and phenotypic relationship of HPP [8]. However, amino acid residues, which are transiently but significantly involved in the folding process of TNSALP, may be overlooked in the 3D structural model. A179, which does not belong to any functional domain of TNSALP [8], plays an important role in the folding and assembly of TNSALP, as described in section 6.1.

The expression of HPP-associated TNSALP mutants in mammalian cells has been characterized to determine the effects of each mutation using the 3D structural model of TNSALP. Table I shows a list of the TNSALP mutations examined by our group, including 1 frameshift mutation and 21 missense mutations. We started our analysis with the p.A179T mutant of TNSALP and made several interesting findings. In this review, these mutations have been divided into six groups based on their 3D structure and their effects on TNSALP [4,8]:

1) Extra-functional domain: p.A179T
2) Catalytic site: p.P108L, p.A116T, p.N170D, D294A, and p.G334D
3) Calcium-binding site: p E235 and D306V
4) Homodimer interface: p.R71C, p.N417S, p.G420S, and p.G420A
5) Crown domain: p.V423A, p.G426S, p.G426D, p.G426C, p.R450C, and p.R450H
6) Co- and post-translational modifications: p.C201Y, p. N430S, p.C489S, and p.Leu520ArgFsX86
7) Degradation

### 6.1. Extra-functional domain

p.A179 T was previously reported in a patient homozygous for this allele and diagnosed with perinatal severe (lethal) HPP [12] (Table I). A179 is conserved in *E. coli* ALP and human ALP isozymes [13]. In mammalian cells, a large fraction of newly synthesized TNSALP (p.A179T) is not expressed on the cell surface as it is trapped in the ER as disulfide-bonded high-molecular-weight aggregates that consist of immature 66 kDa molecular species, which result from misfolding and aberrant interchain cross-linking [11]. As a result, a reduced amount of the ~80 kDa functional enzyme reaches the cell surface (residual ALP activity = 24% of WT; Table I), indicating that the defective transport of TNSALP (p.A179T) to the cell surface is responsible for the onset of severe HPP.

An artificial *E. coli* ALP, with threonine in place of alanine at the position corresponding to A179 of TNSALP, was reported to have properties similar to those of the wild-type *E. coli* enzyme [14]. Furthermore, TNSALP (p.A179T) [15] and a secreted epitope-tagged form of TNSALP (p.A179T) [16] exhibit ALP activity similar to that of TNSALP (W) against pNPP. These findings suggest that threonine is acceptable at position 179 and does not disrupt the basic catalytic reaction of TNSALP. Therefore, it is possible that the substitution of alanine with threonine at position 179 of TNSALP reduces the probability of proper folding by altering the folding and assembly pathway, allowing only a small amount of TNSALP (p.A179T) to achieve transport competence and exert catalytic activity.

### 6.2. Catalytic site

p.P108L, p.A116T, p.N170D, p.D294A, and p.G334D are assigned to the catalytic site of the 3D model [8] (Table I); nevertheless, the latter three mutations are significantly different from the former two mutations in their effects on TNSALP. p.D294A was reported in a patient with infantile HPP [17], whereas p.N170D and p.G334D were detected in patients with perinatal severe HPP [18,19] (Table I). Similar to TNSALP (p.A179T), TNSALP (p.D294A) is characterized by aggregation with a small amount of the 80 kDa functional enzyme on the cell surface [15]. TNSALP (p.N170D) and TNSALP (p.G334D) exhibit almost no residual ALP activity because most of the TNSALP mutants form aggregates and accumulate in the ER [20,21], demonstrating the severity of the homoallelic forms of HPP (Table I). p.P108L and p.A116T were previously reported in patients with odonto HPP [22] and adult HPP [23], respectively (Table I). TNSALP (p.P108L) is mostly present in a monomeric form lacking residual activity in transfected cells and can suppress the activity of the wild-type enzyme when co-expressed with TNSALP (W) [24], indicating that the monomers of TNSALP (p.P108L) can interact with those of TNSALP (W). Interestingly, the monomeric form of TNSALP (p.P108L) can undergo N-glycan processing and become the ~80 kDa mature form, which localizes to the cell surface, suggesting that dimerization *per se* is not necessary for the cell surface localization of TNSALP. TNSALP (p.A116T) can also exert a dominant-negative effect on TNSALP (W) [25], which is consistent with its dominant inheritance [23]. However, in contrast to p.P108L, additional disulfide-bonded aggregates are present in transfected cells [25]. Both the monomers and aggregates were found to associate with TNSALP (W); nevertheless, the clinical relevance of these aggregates remains unclear. Therefore, similar to p.P108L, p.A116T may cause a subtle change in the homodimer interface away from the catalytic site, allowing for the interaction with TNSALP (W) (Fig. 1). Notably, the molecular phenotypes of p.P108L and p.A116T resemble those of missense mutations in the interfacial crown domain, as described in section 6.4. In another study, an A116T knock-in mouse model has been generated; this mouse has a primary dental phenotype that mimics odonto HPP [26].

Mutations in TNSALP not only reduce the overall catalytic activity to various degrees but also alter the catalytic properties of the enzyme, thereby increasing variability in clinical symptoms. Compared to the wild-type enzyme, TNSALP (p.A179T) and TNSALP (D294A) are almost incapable of hydrolyzing PLP [16]. This preferential substrate utilization may lead to epileptic seizures in patients with HPP carrying p.A179T or p.D294A due to a shortage of the

neurotransmitter gamma-aminobutyric acid in the inhibitory neurons of these patients [1–4]. Epileptic seizure is also a common phenotype in TNSALP-deficient mice [1–3]. In 1948, a study initially reported a patient with HPP who died of rickets and epilepsy [27]. More than 50 years later, this 3-week-old boy was found to have carried the genotype p.A114T/D294A [28].

### 6.3. Calcium-binding site

$Ca^{2+}$ is coordinated by four amino acid residues (E235, F290, E291, and D306) and a water molecule in TNSALP [8]. p. E235G and p.D306V were previously reported in patients with adult HPP [29] and perinatal severe HPP [30], respectively (Table I). The substitution of E235 and D306 with either glycine or valine could lead to the failure of TNSALP in capturing $Ca^{2+}$. The molecular phenotypes of TNSALP (p.E235G) and TNSALP (p.D306V) were found to resemble those of TNSALP (p.N170D) and TNSALP (p.G334D); widespread aggregates with a small amount of the 66 kDa monomeric form are present, both of which lack residual ALP activity [11,31]. These molecular properties suggest that the binding of $Ca^{2+}$ is essential for the proper folding of TNSALP and explain the null nature the p E235G and p.D306V alleles. TNSALP (p.A399S) may mask the severity of TNSALP (p.E235G) in a heterozygous patient diagnosed with adult HPP (Table I).

In addition, the cell surface expression of other TNSALP mutants could be affected to various degrees (p.T68M, p.R71S, p.R223W, p.L275P, p.F327L, p.T339N, p.Y388H, and p.R391H) [32–35], suggesting that defective transport is a major contributing factor to the onset of HPP.

### 6.4. Homodimer interface

Mornet et al. [8] reported a correlation between the mutations in the homodimer interface and the dominant-negative phenotype based on the 3D structure of TNSALP; This is largely consistent with our findings.

p.N417S, p.G420A, and p.G420S were reported in patients with perinatal severe HPP [36], childhood HPP [37], and perinatal severe HPP (http://www.sesep.uvsq.fr/03_hypo_mutations.php), respectively (Table I). A short stretch (LYN-417GPG-420Y) in the interfacial crown domain is identical among the four ALP isozymes, suggesting its importance in subunit assembly. The effects of the three mutations are similar; each TNSALP mutant exists as a monomer in transfected cells and can exert a dominant-negative effect on the wild-type enzyme [38,39], supporting that these alleles are inherited in a dominant pattern. Similar to TNSALP (p.P108L) and TNSALP (p.A116T) [24,25], the monomeric TNSALP (p.N417S), TNSALP (p.G420A), and TNSALP (p.G420S) have been observed on the cell surface, indicating that these mutations induce subtle conformational changes, but not large conformational alteration found in p.N170D and p.D306V. Therefore, the dominant negative properties of TNSALP mutants may be attributed to a mismatch between the monomers of TNSALP mutants and those of the wild-type enzyme. Nevertheless, another unique mechanism has been reported regarding to the dominant negative trait [40].

p.R71C was detected in a patient with infantile HPP [17] (Table I). Although R71C is located at the homodimer interface [8], the molecular phenotype of TNSALP (p.R71C) was found to resemble that of TNSALP (p.N170D) and TNSALP (p.D306V), which are characterized by impaired folding and aggregation [15]. This finding suggests that a new cysteine residue at position 71 abrogates the formation of two intrachain disulfide bridges, which are crucial for the proper folding of TNSALP, as described in section 6.6.1. TNSALP (p.R71C) could markedly suppress the residual ALP activity of TNSALP (p.D294A) when both mutants are co-expressed [15], indicating that interference between the two TNSALP mutants may occur in the cells of the patient with infantile HPP carrying p.R71C/p.D294A (Table I).

### 6.5. Crown domain

p.V423A, p.G426S, p.G426D, p.G426C, p. N430, p.R450C, and p.R450H are assigned to the crown domain [8]. The crown domain (Fig. 1) is formed by 65 residues from each monomer, and it partly overlaps with the homodimer interface [8]. The loop 422–452 is associated with enzymatic properties, such as allosteric properties, heat stability, and uncompetitive inhibition, and is involved in the collagen binding of TNSALP [9].

p.V423A was found in a patient with prenatal severe HPP [29] (Table I, Fig. 1). No significant differences have been observed in intracellular transport, dimer formation, or cell surface appearance between TNSALP (p.V423A) and TNSALP (W). Nevertheless, its residual ALP activity was found to be weaker than that of TNSALP (W) [41], indicating the importance of the crown domain for catalytic activity. This finding explains the severe phenotype of heterozygous patients carrying p.V423A and p.A116T, the latter of which results in no residual ALP activity, as described in section 6.2 (Table I). The conformational distortion may impair catalytic function, as demonstrated by the increased susceptibility of the mutant to protease digestion [41]. Initially, it was unclear how this conservative substitution between hydrophobic amino acids strongly affect catalytic activity. The substitution of valine with other hydrophobic amino acid residues has revealed the support of catalytic function by leucine and isoleucine but not phenylalanine [41], indicating that both the hydrophobicity of the side chain and its length are essential for catalytic function. This finding suggests that V423 on the monomer interacts with its counterpart on another monomer such that the dimeric enzyme adopts an optimal conformation for the efficient catalytic function of TNSALP (Fig. 1). Crystallographic analysis of human TNSALP is needed for a more definite conclusion. Placental and germ cell ALPs have leucine, whereas intestinal ALP has phenylalanine at the position corresponding to V423 of TNSALP [1,13].

p.R450C and p.R450H were previously reported in a homozygous patient with infantile HPP [18] and a compound heterozygous patient with odonto HPP [42], respectively (Table I). The amino acid residue at position 450 is not conserved even in human ALP isozymes (aspartate in placental and germ cell ALPs and serine in intestinal ALP) [1,13]. As shown in Fig. 1a, R450 hangs over the entrance to the catalytic pocket of TNSALP, suggesting its involvement in regulating the approach of the substrate and/or stabilization [8]. Unexpectedly, this mutant was found to be largely present as a 160 kDa form lacking catalytic activity in transfected cells [43]. It is possible that this 160 kDa form is a dimer cross-linked by an interchain disulfide bond. Enhanced susceptibility to protease digestion suggests that TNSALP (p.R450C) is distorted and warped such that it no longer contributes to an efficient catalytic reaction. As expected, a conservative replacement with histidine at position 450 was found to rather stimulate catalytic activity [43].

Three different missense mutations have been reported at position 426 of TNSALP. p.G426S, p.G426D, and p.G426C were reported in patients with infantile HPP [http://www.sesep.uvsq.fr/03_hypo_mutations.php], childhood HPP [44], and infantile HPP [45], respectively (Table I). The molecular properties of TNSALP (p.G426S) and TNSALP (p.G426D) are similar [46]. They predominantly exist as a monomeric form lacking ALP activity; however, a small amount of catalytically active dimers (p.G426S > p.G426D) may be present. In comparison with the hydroxyl group of serine, the bulky side chain and/or negative charge of aspartate may hinder dimerization more severely. p.E191K may mitigate the

clinical symptoms of a compound heterozygous patient with the genotype p.G426D/p.E191K (Table I). These mutants are not dominant-negative [46], indicating that both mutants are transmitted in a recessive manner. In contrast to TNSALP (p.G426D) and TNSALP (p.G426S), a 200 kDa disulfide-bonded form of TNSALP (p.G426C) lacking ALP activity is predominant [46]. This finding suggests that the additional cysteine residue in the crown domain induces interchain cross-linking between monomers such as those of TNSALP (p.R450C). However, both 200 kDa TNSALP (p.G426C) and 160 kDa TNSALP (p.R450C) were found to migrate more slowly than the dimeric form of TNSALP (W) in SDS-PAGE modified specifically for ALP [46]. Further studies are needed to construct their molecular models.

### 6.6. Co- and post-translation

#### 6.6.1. Disulfide bonding

There are five cysteine residues at positions 119, 139, 201, 489, and 497 per subunit of TNSALP [1,8]. Their positions are well conserved among four human ALP isozymes [1]. C139 and C489 are cross-linked to C201 and C497, respectively, to give rise to C-139-C-201 and C-489-C-497 disulfide bridges in the same subunit, whereas C119 is considered to be in a free state [1]. The replacement of C119 with serine does not affect the molecular properties of TNSALP (W) [43]. p.C201Y and p.C489S were previously reported in patients with perinatal severe HPP [30,42]. These mutants, lacking one of the two intrachain disulfide bonds, exhibit very low residual ALP activity [47], indicating the importance of disulfide bridges in the structure and function of TNSALP. On the other hand, TNSALP (p.C201Y) was found to be largely present as monomers in transfected cells, suggesting that the structure of TNSALP (p.C201Y) is completely changed such that the monomers are no longer capable of interacting with each other. The C-121-C-183 of placental ALP, which corresponds to the C-139-C-201 of TNSALP, plays a major structural role, whereas the carboxyl-terminal disulfide bond plays a minor role [48].

#### 6.6.2. GPI anchor

TNSALP is anchored to the outer leaflet of the phospholipid bilayer via the phosphatidylinositol moiety of GPI. As a result, when cells expressing TNSALP (W) are incubated with phosphatidylinositol-specific phospholipase C, which can cleave the linkage between phosphatidylinositol and an oligosaccharide chain in the GPI, TNSALP can be released into the culture medium as a soluble enzyme [11,49]. However, the amino acid residue of TNSALP to which GPI covalently binds remains unknown. In case of placental ALP, Asp484 is covalently bound to GPI, with the concomitant cleavage of the C-terminal 29-amino acid extension serving as a signal for GPI addition [50,51].

p.Leu520ArgFsX86, a frameshift mutation caused by the deletion of thymidine at cDNA number 1559 of TNSALP, was previously reported in a patient with infantile HPP [52] (Table I). This mutation has only been reported in Japanese patients with HPP having an allele frequency of approximately 40.9% [4]. The frameshift mutation may remove its original stop codon, giving rise to a larger TNSALP with a C-terminal extension, indicating that the C-terminal extension changes the amino acid sequence serving as a GPI anchor signal. This TNSALP mutant has reduced residual ALP activity and cannot be modified by GPI, and only a small amount of the mutant may be secreted as a soluble protein [53]. Consistent with this finding, a larger form of TNSALP has been detected *in vivo* in the serum of patients carrying this mutation [54]. However, the majority of this TNSALP mutant was found to accumulate within the cell as high-molecular-mass aggregates randomly crosslinked via interchain disulfide bonds [53]. Three cysteine residues in the C-terminal extension may be associated with the aggregation of this mutant.

#### 6.6.3. N-glycan

The cDNA of TNSALP (W) predicts five potential N-glycan consensus sites (N-X-S or T) [1,8], which serve as a signal for N-glycan addition. TNSALP (W) contains all five N-glycans at N140, N230, N271, N303, and N430 when expressed in mammalian cells [55]. p.N430S was previously reported in a patient with infantile HPP [56] (Table I). TNSALP (p.N430S) lacking one N-glycan is unable to form the dimeric enzyme [55]. However, the loss of N-glycan at position 430 does not cause the molecular defect. Other artificial mutants, such as TNSALP (p.N430D), TNSALP (p.S432A), and TNSALP (p.S432G), all of which are devoid of N-glycan at 430, exhibit properties similar to those of TNSALP (W). Therefore, serine at position 430 hinders the subunit assembly essential for the catalytic function of TNSALP. An analysis of another four single N-glycan deletion mutants of TNSALP (Q140, Q230, Q271, and Q303) has revealed that any single N-glycan does not play an important role in the structure and function of TNSALP. However, three N-glycans on N230, N271, and N303 may be a minimal requirement for the stability of TNSALP [55]. Consistent with these findings, the removal of the N-glycan of TNSALP by N-glycanase was reported to inactivate the enzyme [57]. In contrast to TNSALP, placental and germ cell ALPs have only two N-glycosylation sites, whereas the intestinal enzyme has three sites. The deletion of both N-glycan sites does not affect the molecular properties of germ cell-like ALP [58].

### 6.7. Degradation

Newly synthesized proteins are constantly monitored for their proper folding and correct assembly by a quality control system in the ER [59]. Instead of being transported to their destination, misfolded and incompletely assembled proteins accumulate and are eventually degraded by ER-associated degradation (ERAD) [59]. Mutants that could be degraded in this non-lysosomal pathway include TNSALP (p.N170D), TNSALP (p.C201Y), TNSALP (p.D306V), TNSALP (p.G334D), and TNSALP (p.Leu520ArgFsX86) [20,21,25,31,47,53]. These mutants, retained in the ER [21,25,47,53] or cis-Golgi network [20], are subjected to degradation in the proteasome. Furthermore, some of these mutants may be modified by polyubiquitination prior to degradation [31,47,53]. However, the effects of the accumulation of TNSALP mutants in cells on the overall cellular physiology (which may elicit various responses including an unfolding protein response to cope with the accumulation of defective products) and their involvement in the development of HPP remain unclear. Our initial attempts to establish conventional cell lines expressing TNSALP mutants have failed probably due to their cytotoxicity. We addressed this issue by establishing inducible cell lines (Tet-On cells), which can express TNSALP mutants in response to doxycycline.

## 7. Conclusions

We characterized the molecular phenotypes of TNSALP mutants associated with HPP. Although not mutually exclusive, these phenotypes may be largely classified into three groups:

1) Defective transport resulting from the improper folding and incorrect assembly of TNSALP was observed for severe alleles related to the catalytic site, calcium binding, and intrachain disulfide bridges as well as the first identified mutation p.A179T. A unique frameshift mutant found only in Japan also falls into this category. We found that defective products with severe folding

errors are polyubiquitinated prior to their destruction in the proteasome.
2) For dominant-negative traits, our findings are consistent with the predictions made by Mornet et al. [8]; the monomers of TNSALP mutants carrying mutations in the homodimer interface fail to assemble into the dimeric enzyme but negatively associate with the monomers of the wild-type enzyme.
3) Cell surface localization was observed for TNSALP mutants with amino acid substitutions in the crown domain; however, their catalytic activity could be attenuated or abrogated by conformational alterations, subunit disassembly, and unique intersubunit disulfide cross-bridges.

This diversity in the molecular phenotypes of TNSALP mutants may in part explain the variable expression of HPP.

## Ethical approval

Ethical approval was not required for this review.

## Conflict of interest

All authors state that they have no conflicts of interest.

## CRediT authorship contribution statement

**Keiichi Komaru:** Data curation, Formal analysis, Writing - original draft. **Yoko Ishida-Okumura:** Data curation, Formal analysis. **Natsuko Numa-Kinjoh:** Data curation, Formal analysis. **Tomoka Hasegawa:** Data curation, Formal analysis. **Kimimitsu Oda:** Data curation, Formal analysis.

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