Crystal Structure of Alkaline Phosphatase from Human Placenta at 1.8 Å Resolution

IMPLICATION FOR A SUBSTRATE SPECIFICITY*

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From the Département d’Ingénierie et d’Etudes des Protéines (DIEP), Commissariat à l’Energie Atomique, C. E. Saclay, 91191 Gif-sur-Yvette Cedex, France, Department of Immunology, Umeå University Sweden, and Laboratory of Molecular Recognition, The Babraham Institute, Babraham, Cambridge CB2 4AT, United Kingdom

Marie Hélène Le Du‡§, Torgny Stigbrand®, Michael J. Taussig, André Ménez‡, and Enrico A. Stura‡§

Human placental alkaline phosphatase (PLAP) is one of three tissue-specific human APs extensively studied because of its ectopic expression in tumors. The crystal structure, determined at 1.8-Å resolution, reveals that during evolution, only the overall features of the enzyme have been conserved with respect to Escherichia coli. The surface is deeply mutated with 8% residues in common, and in the active site, only residues strictly necessary to perform the catalysis have been preserved. Additional structural elements aid an understanding of the allosteric property that is specific for the mammalian enzyme (Hoylaerts, M. F., Manes, T., and Millán, J. L. (1997) J. Biol. Chem. 272, 22781–22787). Allostery is probably favored by the quality of the dimer interface, by a long N-terminal α-helix from one monomer that embraces the other one, and similarly by the exchange of a residue from one monomer in the active site of the other. In the neighborhood of the catalytic serine, the orientation of Glu-429, a residue unique to PLAP, and the presence of a hydrophobic pocket close to the phosphate product, account for the specific uncompetitive inhibition of PLAP by L-amino acids, consistent with the acquisition of substrate specificity. The location of the active site at the bottom of a large valley flanked by an interfacial crown-shaped domain and a domain containing an extra metal ion on the other side suggest that the substrate of PLAP could be a specific phosphorylated protein.

Alkaline phosphatases (E.C.3.1.3.1) (APs)1 form a large family of dimeric enzymes common to all organisms. In man, three of four AP isozymes are tissue-specific, one is placental (PLAP), the second is from germ cell (GCAP), and the third is intestinal. They are 90–98% homologous, and their genes are clustered on the same chromosome. The fourth (NSAP) is non-specific and can be found in bone, liver, and kidney. It is about 50% identical with the other three, and its gene is located on another chromosome (1–3). Like the other mammalian phosphatases, PLAP is post-translationally modified. This includes two N-glycosylation sites and a glycosylphosphatidylinositol anchor with which these APs are tethered to the membrane. The release of the enzyme may occur through the action of phosphatidylinositol-specific phospholipase C.

Irrespective of whether they are of mammalian or bacterial origin, APs catalyze the hydrolysis of phosphomonoesters (4) with release of inorganic phosphate and alcohol. PLAP has only low sequence identity with the Escherichia coli enzyme (30%), but residues involved in the active site of the enzyme (Ser-92, Asp-91, Arg-166) and the ligands coordinating the two zinc atoms and the magnesium ion are conserved. Therefore, the catalytic mechanism, which was deduced from the structure of the E. coli AP (5), was proposed to be similar in eukaryotic APs. In E. coli, the mechanism involves the activation of the catalytic serine by a zinc atom, the formation of a covalent phosphoseryl intermediate, the hydrolysis of the phosphoserine by a water molecule activated by a second zinc atom, and the release of the phosphate product or its transfer to a phosphoacceptor (6). However, as judged from various studies using mammalian APs, PLAP presents novel catalytic characteristics. It is an allosteric enzyme (7), and it is competitively inhibited by some L-amino acids, namely L-Phe, L-Trp, and L-Leu (8–10).

The tissue specificity of the human enzymes has been correlated to an additional function (3). In NSAP, this specificity involves binding to collagen (11, 12). In PLAP, evidence has accumulated over the past few years suggesting a role in cell division in both normal and transformed cells (13–16). This role probably occurs through its phosphatase activity against phosphorylated proteins (14, 17). In addition, it has been suggested that PLAP may be involved in the transfer of maternal IgG to the fetus (18–20).

PLAP is one of the first proteins found to be ectopically expressed by cancer cells, leading to the concept that dysregulation of embryonic genes plays a significant role in the cancer process (21). During the last 30 years, many clinical reports have been published concerning PLAP and its use as a tumor marker. However, the results are often controversial, mainly because the assays cannot distinguish between the closely related tumor markers GCAP and PLAP (3). The problem of cross-reactivity in the assays reveals that there is a need to elucidate the three-dimensional structure of at least one human AP.

PLAP is a good representative of all human APs because of
the high sequence homology between these enzymes. The knowledge of its three-dimensional structure is crucial to elucidating the distinctive properties of eukaryotic APs, which cannot be predicted with confidence by analyzing the bacterial AP structure. These properties include the uncompetitive mechanism of inhibition, the allosteric property related to catalytic activity, and the elements related to tissue specificity. In addition, the location of the residues specific to PLAP may help in the design of new diagnostic tools to eliminate the cross-reactivity observed in the current ones. We present here the crystal structure of PLAP at 1.8-Å resolution and a number of structural elements that may explain the specific properties of PLAP and other mammalian APs.

MATERIALS AND METHODS

Purification—PLAP was purified according to the method described by Holmgren et al. (22). The frozen placenta, SS homozygote phenotype, was slowly thawed and cleaned from blood and membranes and cut into pieces. Phosphate-buffered saline in the same amount as the weight of the pieces was added, and the mixture was homogenized. An equal volume of x-butanol was added, and PLAP was extracted by stirring for 30 min. After centrifugation at 7000 × g for 30 min, the upper butanol phase was removed, and the aqueous phase was heat-inactivated for 30 min at 56 °C. After a similar centrifugation and filtration, acetone was added (the same volume as the aqueous phase), and the mixture was washed with Tris-buffered saline. PLAP was then applied to a preparative isoelectric focusing column (Ampholytes pH 3–10, Amersham Pharmacia Biotech), eluted 48 h later and assayed for catalytic activity. The active fraction was dialyzed against 0.1 M Tris, 2 mM MgCl2, 0.02% NaN3, pH 7.0, using an Amicon Centricon 30. Crystallization screening and growth of crystals were carried out using sitting drop vapor diffusion plates (23). Crystals were obtained from 12% polyethylene glycol 4,000, 2 mM zinc acetate (essential), 200 mM sodium cacodylate, 2 mM zinc acetate, pH 8.5. The crystals grew as long needles with well defined pyramidal ends, 0.05 × 0.05 mm in cross-section, often as long as the whole length of the drop.

Cryogenic and Heavy Atom Soaking—Screening for cryo-protection conditions was carried out for crystals to be analyzed with synchrotron radiation. To make up the cryo-protectant, glycerol was added to the working solution with 0–15% xylitol. A few crystals were added to this solution. If the crystals dissolved, extra precipitant was added until the crystals were stable for a prolonged period of time (at least 20 min). Crystals were manipulated in the cryo-solution using loops. Cryoloops, mounting pins, and crystal caps were purchased from Hampton Research (Laguna Hills, Ca). Heavy atom soaking was carried out using 75–300 μl of soaking solutions in depression plates.

X-ray Data Collection—A first crystal was shock-cooled at 80 K and used to collect a native data set at 1.9-Å resolution. After a short soaking (20 min) in a solution containing 3 mM p-chlormercuri benzene sulphonate (PCMBS) in cryo-solution, a second crystal was shock-cooled at 80 K. One set of data was collected at 1.7-Å resolution. Both data sets were collected at Laboratoire pour l’Utilisation du Rayonnement Electromagnetique, station D41, on a Mar Research image-plate area detector. Data processing and internal scaling were carried out with HKL (24) (Table I). The space-group determination was performed using the autoindexation procedure as implemented in Denzo, and the enantiomer determination was performed by observation of the reflections extinction in the output file. The crystal belongs to the monoclinic space group C2221, with a = 88.8 Å, b = 114.5 Å, c = 106.9 Å. There is one monomer per asymmetric unit with a Vm of 2.74, corresponding to 50% solvent in the unit cell.

Structure Resolution—The structure was solved by molecular replacement, using the program AMoRe (25), and the coordinates of one monomer of the wild type E. coli alkaline phosphatase (ECAP) (entry code 1alk) (5) and the native data set. The best solution obtained was in the resolution shell of 8.0 to 3.5 Å, with a weak correlation coefficient (18.1) and a poor contrast between the first and second solution (Table I). However, when applying the rotation matrix and translation to the coordinates file, we observed that the dimer was recreated along a crystallographic 2-fold axis, which validated this solution as the correct one.

Model Building and Refinement—The re-building of the model was limited by the low phasing power of the ECAP model due to the low (30%) sequence identity between the two APs. Standard sequence alignment programs gave misleading results and uninterpretable maps. The method of hydrophobic cluster analysis as coded in the program HCA (26) was applied to both ECAP and PLAP sequences. The subsequent hydrophobic patch alignment resulted in a corrected sequence alignment between the two. The starting model contained most of the ECAP main chain without loops or insertions, leaving only the side chains for the conserved residues. The program WARP version 5.0 plus the routine Side Dock, kindly provided by A. Perrakis (27), was crucial in the rebuilding of the model as well as the data set collected at 1.7-Å resolution. The final PLAP model could then be re-built rapidly. In the active site, the peak height in the Fo − Fc map calculated from the
RESULTS AND DISCUSSION

Overall Structure—The overall structure of PLAP is a dimer; each monomer contains 479 residues, 4 metal atoms, 1 phosphate ion, and 603 water molecules. The two monomers are related by a 2-fold crystallographic axis. The surface of PLAP is poorly conserved with that of the *E. coli* enzyme, with only 8% residues in common, although the core is preserved. PLAP possesses additional secondary structure elements comprising an N-terminal α-helix (residues 9–25), forming an arm that embraces the other monomer; an α-helix and a β-strand in a highly divergent region (residues 208–280) and a different organization of the small β-sheet in domain 365–430. PLAP has two glycosylation sites at Asn-122 and Asn-249 that vary in their degree of glycosylation depending of the placental sample (Fig. 1). Two disulfide bridges are located at different positions from those in the *E. coli* enzyme. One is close to the first glycosylation site and may rigidify the loop that carries the carbohydrate chain. The other is located near to Asp-481, where the glycosylphosphatidylinositol anchor is attached, and may stabilize the orientation of PLAP relative to the membrane. In the active site, only the residues that are essential to the catalysis are preserved, i.e., the catalytic serine, the three metal ion sites (Zn1, Zn2, and Mg3) and their ligands, whereas most of the surrounding residues are different (Fig. 2). The upper part of the active site contains a large number of basic residues, and the lower part contains mainly aliphatic or hydrophobic residues. Among these hydrophobic residues, we observe Tyr-367, which belongs to the second monomer. The consequences of such a pattern of residues are typical for mammalian APs and will be discussed below.

Half of the enzyme surface corresponds to three clearly identifiable regions whose sequences largely vary among human APs and are lacking in nonmammalian enzymes (Fig. 3). The first is the long N-terminal α-helix described above. The second, an interfacial "crown domain," is formed by the insertion of a 60-residue segment (366–430) from each monomer. It consists of two small interacting β-sheets, each composed of three parallel strands and surrounded by six large and flexible loops containing a short α-helix. Third, a "metal binding domain," comprised of 76 residues (209–285) and folded into two β-strands flanked by two α-helices. It includes the glycosylation site at Asn-249, stabilized by a stacking interaction with Trp-248, and an additional metal ion. In the *F_o* − *F_c* map, calculated from the native data set, the peak for this metal corresponds to about 10 electrons. Its height and the crystallization conditions suggest the presence of a magnesium, but its coordination by carboxylates from Glu-216, Glu-270, and Asp-285 by Phe-269-CO and a water molecule is also in agreement with a calcium ion (30) (Fig. 1c). The crown domain and the metal binding domain correspond to novel folds, since a search for other structurally related domains using the program DALI (31) yielded no significant matches.

Allosteric Process—One important property that differentiates mammalian APs from their bacterial counterpart is the allosteric observed when the enzyme is fully metalized. Mutagenesis studies reveal that stability and catalytic properties of each monomer are controlled by the conformation of the second AP subunit (7). The principle of allostery implies that the binding of the substrate in one active site will affect the second binding site. The crystal form studied here requires the presence of the substrate p-nitrophenyl phosphate to grow. Therefore, the protein structure described corresponds to a conformation of the enzyme activated by substrate.

A second item to consider is the hydrophobic character of the dimer interface of PLAP. Less than 30% of the residues are involved in hydrogen-bonding interactions, which confer flexibility on the interface. The surface buried in the dimer interface is slightly larger in PLAP (4150 Å²) than in ECAP (3900 Å²). However, the residues involved in dimerization are mainly different, as is the nature of the interface. In ECAP, 36 out of 82 total residues at the dimer interface are involved in hydrogen bonds, whereas in PLAP only 24 out of 83 residues are involved in hydrogen bonding interactions, and only 9 of these are conserved between the two. It could be speculated that in PLAP many hydrogen bond interactions have been replaced by less specific van der Waals contacts, which are more likely to allow rearrangement of the two monomers.

Biochemical studies performed on PLAP showed that residues Asn-84, Tyr-367, and Glu-429 are involved in the allosteric (7). Our structure brings additional information concerning the precise role of these residues. First, Asn-84 is located at the dimer interface with its δ oxygen within hydrogen-bonding distance of Asp91-N (3.2 Å) and probably intervenes through this residue in the stabilization of the catalytic Ser-92. On the same loop, Val-85 and Asp-86 interact with Ala-1 and Ile-2 from the other monomer, and Lys-87 interacts with Leu-369 and Tyr-367, also from the other monomer. Therefore, Asn-84 may affect the enzyme activity through its interaction with Asp-91 and may intervene in the allosteric process because of its involvement in the dimerization (Fig. 4). Second, Tyr-367 is located at the entrance of the active site of the second monomer. Its hydroxyl is located 6.1 Å from the phosphate and 3.1 Å from His-432, which in turn chelates the zinc atom Zn1. The network consisting of Tyr-367 (monomer1)-His-432 (monomer2)-Zn1 (monomer2)-PO4 might also affect the step of phosphoseryl hydrolysis by modulating the reactivity of Zn1 (Fig. 4). The presence in the active site of a residue from the second monomer and its probable involvement in the enzyme reactivity is a new structural feature that could not have been postulated from the ECAP structure, as the loop containing residues 366 to 375 in PLAP is an insertion with respect to ECAP.

In their study, Hoylaerts et al. (7) report that the mutation E429G affects the affinity of Zn1 for PLAP (7). Glu-429 is solvent-accessible and located at the entrance of the cleft, which leads to the active site where the O1 oxygen interacts with the Nδ nitrogen of His-320 (distance = 3.4 Å). In turn, His-320-Nε interacts with Zn1 (distance = 2.4 Å). Therefore, the network consisting of Glu-429-His-320-Zn1-PO4 might affect the hydrolysis of the phosphoseryl moiety through the destabilization of Zn1 (Fig. 4). Therefore, Glu-429 may not be directly involved in the allosteric process but probably affects the activity and the metals in the enzyme.

Finally, the N-terminal α-helix described above is removed from the rest of its own monomer, but it interacts with the second monomer with a buried surface area of 555 Å², suggesting an involvement in enzyme dimerization. Residues of the N-terminal extremity interact with Arg-370 from loop 366–375 and with Asn-106 and Arg-117 from the second monomer. Therefore, the interaction of the N-terminal α-helix with the second monomer may help stabilize Tyr-367 and, therefore, affect the enzymatic activity (Fig. 4).
Specific Uncompetitive Inhibition—A second kinetic property of human APs, not shared by their bacterial ancestors, is that they are stereospecifically inhibited by L-amino acids through an uncompetitive mechanism. This type of rare inhibition implies that the inhibitor can bind to the enzyme-substrate complex but not to the enzyme alone. In the case of a two-step mechanism like that of PLAP, the complex can be the initial enzyme-substrate or any of the intermediate complexes. Previous studies on PLAP have shown that the molecular mechanism of this inhibition involves Arg-166 and Glu-429 and suggests the presence of a hydrophobic pocket in the active site. Glu-429 is of particular importance as it is the only active site residue specific to PLAP. The corresponding residue is a serine in intestinal AP, a glycine in GCAP, and a histidine in NSAP (8–10). The structure confirms the involvement of Arg-166 and Glu-429 and also allows precise description of the hydrophobic pocket, which probably stabilizes the hydrophobic moiety of the inhibitor. Located at the entrance of the cleft, which leads to the active site, Glu-429 borders a pocket that extends from the catalytic Ser-92 to the phosphate product. Arg-166 is found at the edge of a second pocket around the active site, also bordered by Phe-107, Gln-108, and Tyr-367 from the second monomer.
Fig. 2. Active site of PLAP. A Corey-Pauling-Koltun representation of the active site pocket of PLAP is shown. The residues conserved with ECAP are represented in white. The substituted residues are colored according to the residue type; Phe, Trp, and Tyr are violet; Asn, Cys, Glu, Met, Ser, and Thr are green; Arg, His, and Lys are blue; Asp and Glu are red; Ala, Gly, Thr, Leu, Pro, and Val are yellow. Residues from the second monomer are pink. This figure was made with TURBO.

Fig. 3. Overall structure. The overall structure of PLAP is shown in ribbon representation with the residue side chains of the three extra domains in ball and stick representation. The monomer I is shown in pale green, monomer II in blue, N-terminal α-helix in red, crown domain in orange, and metal binding domain in yellow. This figure was made with MOLMOL (35).

(Fig. 2). In Fig. 5, we have modeled the inhibition of PLAP by a L-Phe amino acid. This model shows that the geometry of Glu-429, Arg-166, and the hydrophobic pocket is ideal to fit an L-amino acid such as L-Phe. The hydrophobic moiety of the ligand would be stabilized in the hydrophobic pocket, its carboxyl group by Arg-166 and its amide group by Glu-429. This topology matches both the stereo-specificity and uncompetitive character of the inhibition.

The knowledge of the structural organization of these pockets offers a molecular basis for the design of more specific inhibitors, which may be useful in drug therapy when involvement of PLAP in tumor growth is confirmed. Our study of the complexes of PLAP and L-amino acids should further clarify the role of these two pockets and the mechanism of uncompetitive inhibition.

Elements Determining Enzyme Specificity—Although the target of each human AP remains unknown, the tissue specificity strongly suggests an adaptation for these enzymes toward specific proteins. For example, loop 400–430 of NSAP is directly involved in the binding of collagen (12). Although the case of PLAP is more controversial, our structural data allow us to speculate that the enzyme may have a specific target, whereas the bacterial enzyme has none. First of all, in the active site itself, there are two pockets that may accommodate...
residues from the target protein. Should such a protein bind at this position, Glu-429 would be ideally positioned to stabilize the ester moiety of the substrate in the initial step of the catalytic process. This residue is probably a key determinant for substrate selection. In addition, the hydrophobic pocket extends on one side of the active site into a large surface of about 1700 Å², which with the exception of Asp-173, comprises only neutral or hydrophobic residues from both monomers. The size and character of this region is similar to that typically observed in protein-protein interactions (32), and it would be an ideal location to stabilize a protein substrate. As we move away from the core of the active site, we notice a large valley with an accessible surface of 4000 Å², flanked on each side by the crown and metal binding domains (Fig. 3). The bottom of this valley is also lined by the loop containing residues 270–285 from the metal binding domain. This loop interacts with and may be stabilized by the putative calcium ion. It could therefore be responsible for the synergism observed between calcium and PLAP activity by intervening in substrate binding (15). In summary, the organization of the extended active site would be consistent with PLAP interacting with a large substrate. Such a substrate is likely to be a phosphorylated protein.

Comparison with the Other Human Alkaline Phosphatases—An important issue in the use of PLAP as a cancer marker is the assessment of the potential to differentiate it from the other human alkaline phosphatases present in serum. All mammalian APs have closely related sequences, conserving all the three specific elements: the four cysteines involved in the disulfide bridges, the glycosylation sites, and the residues that coordinate the fourth metal ion. Hence, it is likely that the
same ion and glycosylation sites are present in all APs. The core is thus largely preserved, and the divergence between the four proteins is concentrated in the elements of specificity. PLAP and GCAP are the closest 2, with only 10 substitutions between them (33). Eight of these positions have sufficient solvent exposure to differentiate between the surfaces of these two enzymes (Fig. 6). There are 58 substitutions between PLAP and intestinal AP, corresponding to about 12% of all residues (Fig. 6). Half of these are situated on the specific elements that encompass less than one-third of the protein residues. The greatest diversity exists between PLAP and NSAP, with 209 substitutions (Fig. 6). 56% of the 161 residues in the specific elements are substituted in NSAP, consistent with their involvement in the mediation of specificity and modulation of activity. Finally, three epitopic surfaces distinguish PLAP from the other APs. One corresponds to the active site pocket in proximity to Glu-429, the second corresponds to the metal-binding site, and the third corresponds to the region comprising Glu-15 and Ile-67 (Fig. 6).

Conclusion—The structure of human PLAP, the first structure of a eukaryotic example of this family of enzymes, gives some hints regarding the evolution of this enzyme. Compared with the bacterial homologues, the catalytic requirement has helped to preserve the overall core of the enzyme, the main secondary structure, and the residues necessary for the catalysis. However, the development of a placenta in mammals has been accompanied by the emergence of tissue specificity and the coadaptation of the enzyme to a specific protein target. The acquisition of allostery and the uncompetitive inhibition imply that this enzyme can be regulated in vivo. These new properties have led to extensive structural modification both in the active site and at the enzyme surface, in agreement with the hypothesis that the target of PLAP may be a phosphorylated protein.

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