Mammalian Alkaline Phosphatases Are Allosteric Enzymes*

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Mammalian alkaline phosphatases (APs) are zinc-containing metalloenzymes encoded by a multigene family and functional as dimeric molecules. Using human placental AP (PLAP) as a paradigm, we have investigated whether the monomers in a given PLAP dimer are subject to cooperativity during catalysis following an allosteric model or act via a half-of-sites model, in which at any time only one single monomer is operative. Wild type and mutant PLAP homodimers and heterodimers were produced by stably transfecting Chinese hamster ovary cells with mutagenized PLAP cDNAs followed by enzyme extraction, purification, and characterization. [Gly429]PLAP manifested negative cooperativity when partially metalated as a consequence of the reduced affinity of the incompletely metalated AP monomers for the substrate. Upon full metalation with Zn2+, however, the negative cooperativity disappeared. To distinguish between an allosteric and a half-of-sites model, a [Gly429]PLAP-[Ser84]PLAP heterodimer was produced by combining monomers displaying high and low sensitivity to the uncompetitive inhibitor L-Leu as well as a [Gly429]PLAP-[Ala92]PLAP heterodimer combining a catalytically active and inactive monomer, respectively. The L-Leu inhibition profile of the [Gly429]PLAP-[Ser84]PLAP heterodimer was intermediate to that for each homodimer as predicted by the allosteric model. Likewise, the [Gly429]PLAP-[Ala92]PLAP heterodimer was catalytically active, confirming that AP monomers act independently of each other. Although heterodimers are structurally asymmetrical, they migrate in starch gels with a smaller than expected weighted electrophoretic mobility, are more stable to heat denaturation than expected, and are more sensitive to L-Leu inhibition than predicted by a strict noncooperative model. We conclude that fully metalated mammalian APs are noncooperative allosteric enzymes but that the stability and catalytic properties of each monomer are controlled by the conformation of the second AP subunit.

Alkaline phosphatases (AP) are ubiquitous enzymes found in most species from bacteria to man. Human APs are encoded by four genes (reviewed in Refs. 2 and 3), i.e. the placental (PLAP), germ cell (GCAP), intestinal, and tissue-nonspecific AP isozyme, respectively. APs are dimeric metalloenzymes that catalyze the hydrolytic transfer of phosphate to water or its transphosphorylation to amino alcohols (4), but when separated the monomeric subunits fail to display enzyme activity. Three metal ions (two Zn2+ and one Mg2+) in the active site (5) are essential for enzymatic activity. However, these metal ions also contribute substantially to the conformation of the AP monomer and indirectly regulate subunit-subunit interactions (6).

Fully metalated Escherichia coli AP dimers are symmetrical, both by crystallographic measurements and spectroscopic methods (7), but partially metalated dimers manifest structural asymmetry. As a result of such molecular asymmetry, APs have been claimed to be capable of accepting only one single-substrate molecule in a half-of-sites reactivity mechanism and to display negative cooperativity (8, 9). The existence of cooperativity for the interaction between AP subunits has been investigated intensively in the E. coli enzyme, and evidence for both the existence of positive and negative cooperativity has been presented (10, 11). During early studies, hybrid E. coli AP dimers were formed using mixtures of native and partially modified enzyme forms during a random reconstitution step following denaturation of the dimers with chaotropic agents (12, 13). More recently, heterodimers were generated upon controlled proteolysis of E. coli AP, not necessitating any AP denaturation (14). The latter study of the resulting hybrid APs clearly indicated asymmetry in these hybrids both in terms of structure and function, pointing to catalytically relevant subunit communication in the AP enzyme.

Mammalian APs have a unique surface loop not present in the E. coli enzyme that extends from amino acids 400–430 (15). This loop has been shown to play an important role in defining the conformation and stability of the AP molecule (16). The loop is also partially responsible for the interaction of APs with extracellular matrix proteins, such as collagen (17). We have also shown that this loop is responsible for the unique property of mammalian APs of being uncompetitively inhibited by a number of amino acids and small peptides (17, 18). We and others have found that a single amino acid substitution, E429G, was primarily responsible for the differential inhibition of PLAP and GCAP by L-Leu (19, 20). The degree of inhibition was further modulated by a second substitution, N84S, in GCAP, causing a conformational change in the molecule accompanied by a 50% drop in the degree of inhibition by L-Leu (16). Because the [Gly429]PLAP2 and the [Ser84]PLAP mutants dis-
play a 100-fold difference in $K_i$ for l-Leu, heterodimers of these mutants provided an optimal test system to study AP subunit interactions and negative cooperativity for mammalian APs. In this paper we report that AP dimers display negative cooperativity when the AP monomers are partially demetalated, but both AP monomers function essentially independently when both subunits are properly metalated. This behavior of mammalian APs fits the definition of an allosteric enzyme.

**EXPERIMENTAL PROCEDURES**

**PLAP Mutants**—The PLAP mutants [Ser<sup>429</sup>]PLAP ([S]PLAP) and [Gly<sup>429</sup>]PLAP ([G]PLAP) have been described elsewhere (19). Two charged substitutions, P209R and P479R, were introduced into the [G]PLAP cDNA to generate the [RGR]PLAP mutant. In addition, an S92A mutation was superimposed onto [RGR]PLAP to replace the active site S92 thus producing an inactive AP molecule ([ARGR]PLAP). Site-directed mutagenesis was performed as described (21).

The propagation of the mutagenesis primer pairs were as follows (underlined bases indicate changes): S92A, 5′-CTCTTCAGGGAGGAGCCACGCACCCGCCC-3′; and 5′-CTCTTCAGGGAGGAGCCACGCACCCGACCA-3′; P209R, 5′-CTCTTACTTTGTCGGAGACCC-3′; and 5′-CTCTTACTTTGTCGGAGACCC-3′; P479R, 5′-CTCTTCTGGCCGCCGCCGCCGCGCTGTTGGTG-3′. Finally, the sequence TESSEGSPE at positions 410–418 in [S]PLAP was replaced by SMDVYAHNN to produce the heat-labile mutant [S]PLAP-t (17). All mutant PLAP cDNAs were subcloned into the pSVT7 vector (22) and transfected into Chinese hamster ovary cells (23).

**Enzyme Catalysis of Mammalian Alkaline Phosphatases**

Interaction and cooperativity and to comply with the dimeric nature of the AP enzyme, AP catalysis can better be represented by the generalized model applicable for allosteric enzymes (28) (Scheme II). In this model, $K_1$ and $K_2$ represent the Michaelis constants for both subunits respectively, whereas $k_1$ and $k_2$ stand for the catalytic rate constants for each monomer. For a classical noncooperative allosteric dimeric enzyme, $K_1 = K_2 = K_M$, the kinetic analysis of these enzymes being described accurately by linear Lineweaver-Burk plots, with $k_{cat} = 2k_1$, $K_1 = K_2 = K_M$ (28).

However, because PLAP, respectively [S]PLAP, and GCAP, respectively [RGR]PLAP, have slightly different kinetic constants, in the case of [S]PLAP-[RGR]PLAP heterodimers Scheme II would be more accurately described by the following rate equation, at least for enzyme heterodimers in which both monomers act independently:

$$v = \frac{[E,E_0]^m}{1 + \frac{[S]}{K_1} + \frac{[S]}{K_2}}$$

(Eq. 1)

with $[E,E_0]^m$ being the total AP concentration and $k_1$, $K_1$ respectively $k_2$, $K_2$ being the rate constants and Michaelis constants for the individual AP subunits. Linear Lineweaver-Burk kinetics cannot be anticipated from Equation 1, based on previously determined kinetic constants for [S]PLAP and [RGR]PLAP, respectively, with $k_{cat} = k_1 + k_2$. The $K_m$ corresponds to the positive solution of the following second order function:

$$(k_1 + k_2)K_m^2 + (k_1 - k_2)K_m - K_1K_2 - k_1k_2K_m = 0$$

(Eq. 2)

This analysis assumes that in the heterodimers each monomer will catalyze phosphate substrates with similar catalytic efficiencies as in the parent homodimers.

Whereas no linear kinetics can arise when the SE,E,S intermediate would be the only active enzyme-substrate complex metabolized (i.e. $E_0E_0$ and $E_0E_0S$ are inactive), linear kinetics are also predicted when only one of both subunits (i.e., only $E_0E_0$ and not $E_0E_0S$) participates in the catalysis (i.e. $k_2 = 0$), because then Equation 1 reduces to a simple Michaelis-Menten form and $k_{cat} = k_1$, $K_m = K_1$. Such a situation would arise if as a result of structural cross-talk between both AP monomers; the second AP monomer is shut off for substrate positioning as a consequence of substrate binding to the first subunit, in agreement with a half-of-sites model. The reaction rate would then reduce to:

$$v = \frac{[E,E_0]^m}{1 + \frac{[S]}{K_1}}$$

(Eq. 3)
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$$V = \frac{(k_p/K_1 + k_i/K_d)[E_i,E_o]^*}{[K_p + 1/K_d + 1/[S]]}$$

(Eq. 3)

where

$$k_{cat} = \frac{(k_p/K_1 + k_i/K_d)}{[K_p + 1/K_d + 1/[S]]}$$

and describe a mechanism that results in linear double reciprocal plots of enzyme activity versus substrate over a wide range of substrate concentrations. Formally, under those conditions, enzyme kinetics for AP heterodimers match those for equal mixtures of both homodimers, with comparable degrees of saturation. In this model homodimeric APs are described kinetically by $k_{cat} = k_1$ and $K_m = K_1/2$. Formally this model also describes the kinetics of a heterodimer composed of one active and one inactive monomer, both in an allosteric and in a half-of-sites model.

When SE,E,S is only formed at much higher substrate concentrations than required to form either SE,E, or E,E,S, nonlinear double reciprocal plots of enzyme activity versus substrate concentration will be found even for homodimers, typical of negative cooperativity, as can be substantiated from Equation 1, because both AP monomers would be nonequivalent.

It is clear that the above equations do not enable an easy distinction between the allosteric and the half-of-sites model. The uncompetitive amino acid inhibitor L-Leu on the contrary enabled us to further distinguish between Equations 1 and 3. Uncompetitive inhibition of AP homo- and heterodimers was carried out at saturating concentrations of pNPP (10 mM) in the presence of increasing concentrations of L-Leu and the residual AP activity was measured. Because at high [pNPP] in the allosteric model (Equation 1) only the substrate intermediate SE,E,S is metabolized, schematically the inhibition of AP heterodimers can be represented as in Scheme III and be described by the following rate equation:

$$V = [E_i,E_o]^* \left( \frac{k_1}{1 + [1/K_{cat}] + k_2}{1 + [1/K_{cat}]} \right)$$

(Eq. 5)

On the contrary, according to the half-site model at saturating [pNPP], the inhibition is represented as in Scheme IV with the following rate equation:

$$V = \frac{[E_i,E_o]^* (k_p/k_1 + k_i/k_2)}{[K_p + 1/[S]]}$$

(Eq. 6)

Based on the known values of $k_1$, $k_2$, $K_1$, $K_2$, and $K_{cat}$ (18) and assuming no asymmetry-dependent cross-talk between both monomers in a nonsymmetrical heterodimer, it is possible to predict residual AP activities in the presence of L-Leu, according to both models (Equation 5, respectively 4) and to compare these predicted enzyme levels with actual data collected for heterodimers between [S]PLAP and [G]PLAP forms.

**RESULTS AND DISCUSSION**

*Active Site Zn$^{2+}$ Stability*—Double reciprocal plots of enzyme activity versus the concentration of pNPP were linear for various EDTA concentrations tested ($r$ ranging from 0.988 to 0.999) and intersected on the y axis (mean $\pm$ S.D. of the intersection equalled $A_{405\,nm} = 1.63 \pm 0.26$), compatible with competitive inhibition (Fig. 1A). Thus [S]PLAP activity was inhibited by EDTA with a $K_i$ equal to 0.51 $\pm$ 0.06 mM, indicative of the high stability of active site bound Zn$^{2+}$ ions; wt PLAP was likewise inhibited with a $K_i$ equal to 0.87 $\pm$ 0.3 mM. In agreement with these data, [S]PLAP could not be demetalated by Chelex, not even after prolonged incubations (not shown). On the contrary, [G]PLAP was also inhibited competitively by EDTA (Fig. 1B; $r$ of the regression lines ranging from 0.984 to 0.998; intersection at $A_{405\,nm} = 3.3 \pm 0.1$), but with a $K_i$ equal to 19 $\pm$ 1.4 $\mu$M. GCAP was inhibited to the same degree as [G]PLAP, with an inhibition constant equal to $K_i = 26 \pm 6 \mu$M. These data indicate that EDTA has a 30–40-fold higher affinity for Zn$^{2+}$ metal ions located in the GCAP (and [G]PLAP) active site than for those in the PLAP active site. In agreement with the different accessibility of L-Leu in the PLAP and [G]PLAP active sites, these large differences in the affinity of Zn$^{2+}$ can be ascribed to the substitution of one single amino acid (E429G) in PLAP.

When [G]PLAP, fully loaded with Zn$^{2+}$, is diluted in Chelex-treated buffered substrate solutions, a progressive loss of the AP activity is observed over time, independently of the substrate concentration, as shown by the decreasing slope of AP activity plots versus time (Fig. 2A). This behavior is in agreement with the rapid loss of [G]PLAP activity in the presence of EDTA and is indicative of the spontaneous dissociation of Zn$^{2+}$ from the active site. Replots of the slopes of these curves versus time were linear (Fig. 2B, $r = -0.986$ for the upper line and $-0.945$ for the lower line), i.e., compatible with a monophasic disappearance of enzyme activity with a half-life for the Zn$^{2+}$ dissociation in 1 mM DEA buffer, pH 9.8, estimated to be around 90 min. In agreement with the loosely bound Zn$^{2+}$ ions, the overnight treatment with Chelex inactivated [G]PLAP.
Because extrapolated V for the 1/\[S\] intercept [G]PLAP activity measured in the presence of 1 mM pNPP (●), 0.2 mM pNPP (○) and in the presence of 1 mM pNPP, combined with 37.5 μM EDTA (□). B, determination of the half-life of Zn^{2+} ion dissociation from semi-logarithmic plots of the residual AP activity versus time. Symbols are as in A.

**Active Site Zn^{2+} and Cooperativity**—Fully metalated [G]PLAP incubated with increasing substrate concentrations up to 100 mM (1000-fold above the Kₘ) in 1 mM DEA buffer, pH 9.8, shows no evidence of negative cooperativity. On the contrary, a mild inhibition is observed at substrate concentrations exceeding 10 mM (not shown). Similarly, measurements at pH 7.5, in 10 mM Tris-HCl buffer containing 20 μM ZnCl₂ and 0.5 mM of MgCl₂ but no transphosphorylating alcohol still shows no evidence of negative cooperativity for pNPP up to 100 mM, at which concentration a clear-cut substrate inhibition of about 50% is observed (Fig. 3A). Experiments in the presence of 0.5 mM guanidinium chloride, claimed to enhance AP activity (29), do not raise the AP activity measured at high pNPP concentrations, whereas in the presence of 1.0 mM guanidinium chloride, a cut-drop drop in enzyme activity is observed (Fig. 3A). These experiments indicate that fully metalated APs do not display negative cooperativity.

Whereas Chelex-treated [G]PLAP displays negligible enzyme activity, Zn^{2+} can restore the activity in a dose-dependent manner. Little or no activity is regained in the presence of 0.5 μM of ZnCl₂, partial reconstitution is observed with 2.5 μM ZnCl₂ and full reconstitution can be achieved in the presence of 20 μM ZnCl₂ (Fig. 3B), whereas the subsequent addition of MgCl₂ does not further increase the enzyme activity (r = 0.996 for the 1/v versus 1/[S] plot of the reconstituted enzyme with an intercept A_405 nm = 1.14 ± 0.04). Double reciprocal plots (Fig. 3B) of AP activity versus pNPP for the enzyme reconstituted with 2.5 μM ZnCl₂ are, however, linear only at low substrate concentrations (r = 0.999 for the linear part of the 1/v versus 1/[S] plot with a lower V_max as indicated by the higher intercept of A_405 nm = 3.16 ± 0.44) and display negative cooperativity. Because extrapolated V_max values are identical for the partially reconstituted and fully reconstituted enzyme, this implies that on substrate saturation both of the enzyme’s sites are occupied and that they function with identical catalytic rates, i.e. even though partial metalation increases k₅, it does not affect the rate of phosphorylation k₇ and dephosphorylation k₈ (Scheme 1). Together with the above findings, it follows that when fully metalated, AP dimers function noncooperatively.

**Kinetics Properties of Heterodimers**—To further study whether AP monomers acted independently, we made use of the differential L-Leu inhibition properties of PLAP and GCAP. Although the inhibition of wt GCAP (Kᵢ = 0.54 ± 0.01 mM) and wt PLAP (Kᵢ = 9.2 ± 1.2 mM) by L-Leu differ 17-fold, the inhibition of [S]PLAP (Kᵢ = 19 ± 1.5 mM) and [G]PLAP (Kᵢ = 0.2 ± 0.01 mM) differ 100-fold (18), and these mutants were therefore chosen for our experiments. To optimize the chromatographic separation of the AP heterodimers we also engineered two charge replacements, P209R and P479R in [G]PLAP ([RGR]PLAP). These substitutions were chosen, because they represent known allelic mutations in PLAP and GCAP and neither of them affect the catalytic properties of the enzyme (18, 25). Fig. 4 illustrates the predicted inhibition curves of heterodimers constructed between PLAP and GCAP (Fig. 4A) and between [S]PLAP and [G]PLAP (Fig. 4B) according to both test models. Whereas in the allosteric model an inhibition profile is expected to be intermediate between that of the AP homodimers, the half-of-sites model predicts an inhibition curve almost coinciding with that of the [G]PLAP mutant. A kinetic analysis of chromatographically purified homodimers revealed linear Lineweaver-Burk plots with Kᵢ values ranging from 0.3 to 0.4 mM for [S]PLAP and [G]PLAP-t (comparsible with the known Kᵢ of wt PLAP) to 0.1–0.2 mM for the [RGR]PLAP mutants, comparable with those reported for [G]PLAP and wt GCAP (18, 25), and confirming that homo- and heterodimers could adequately be separated via ion-exchange chromatography. The value Kᵢ = 0.54 ± 0.13 mM determined
for the [S]PLAP-t-[RGR]PLAP heterodimer was, however, higher than the expected $K_m$ values predicted by the models described by Equations 1 (expected $K_m = 0.2$ mM) and 3 (expected $K_m = 0.14$ mM), i.e., is higher than the weighted average of the parent molecules regardless of the model chosen. These data suggested structural asymmetry to influence AP subunit communication but made evident that a simple Michaelis-Menten analysis did not suffice to distinguish between models 1 and 2.

According to Equations 5 and 6, the uncompetitive inhibition by L-Leu of a heterodimeric AP molecule is fundamentally different for an allosteric enzyme or a half-of-sites enzyme. Fig. 5A shows the L-Leu inhibition profiles for [S]PLAP-t-[RGR]PLAP heterodimers, in comparison with the inhibition curves obtained for the corresponding chromatographically isolated [S]PLAP-t and [RGR]PLAP homodimers. Both homodimers respond to L-Leu according to inhibition curves in good agreement with those predicted by Fig. 4B and known to describe the inhibition by L-Leu of [S]PLAP and [G]PLAP, respectively. Experimentally measured [S]PLAP-t enzyme levels in the presence of L-Leu correlated well with predicted residual enzyme levels (Fig. 6A; $r = 0.974$, slope of the regression line equals 1.06 ± 0.08). Likewise, a good correlation existed between experimentally measured [RGR]PLAP enzyme levels in the presence of L-Leu and the predicted residual enzyme levels (Fig. 6A; $r = 0.98$, slope of the regression line equals 0.93 ± 0.07). Correlating via linear regression analysis the biphasic intermediate inhibition curve derived for the isolated [S]PLAP-t-[RGR]PLAP heterodimers with residual AP enzyme levels as predicted by the allosteric model (Fig. 6B; $r = 0.997$ and slope = 1.02) and by the half-of-sites model (Fig. 6B; $r = 0.86$ and slope = 0.75) revealed that this inhibition curve only matches the inhibition profile predicted by the allosteric model. Thus this analysis confirms that APs are allosteric enzymes and implies that covalently immobilizing a phosphate group by L-Leu in one active site (E-P intermediate) has no direct consequences for the catalytic efficiency of the adjacent subunit.

To confirm that AP enzymes are classical allosteric but non-
cooperative enzymes at least when fully metalated, we pro-
dered heterodimers in which only one of the monomers is active. By mutagenizing the active site Ser92 (S92A) in the noncooperative allosteric enzymes, but the stability and cata-
lytic properties of each monomer are controlled by the confor-
matic properties divergent from those of the parent homodimers. Hence, mammalian APs are
mammalian AP-GR heterodimers resemble much more that of [G]PLAP than that of [S]PLAP, further confirming that PLAP-[G]PLAP types of heterodimer structurally compare better with [G]PLAP than with the weighted PLAP-[G]PLAP average (Fig. 8). We reported that a surface loop, made up of amino acids 400–430 substantially contributed to the heat-stability of PLAP (17), because modifications in this loop dramatically reduced the resistance of the resulting PLAP mutant (PLAP-t) to denaturation by heat (PLAP-t homodimers were inactivated by 90% after 20 min at 56 °C; 17) without affecting the kinetic parameters of the PLAP-t mutant. To investigate in more de-
tail whether this loop participates in the structural cross-talk between both AP subunits, we have also analyzed the heat stability of the [S]PLAP-[G]PLAP heterodimers. It is evi-
dent that the heat inactivation pattern for the [S]PLAP-[G]PLAP heterodimers resembles that of [G]PLAP (Fig. 8), confirming that these heterodimers behave more as [G]PLAP, irrespective of the presence of the t-loop substitution in the [S]PLAP subunit. Thus, the amino acids 400–430 loop controls the active site stability in PLAP but is not involved in any stabilizing cross-talk between AP subunits, corroborating our findings that the E429G substitution in PLAP is associated with structural changes in this loop, facilitating the access for 

Structural Asymmetry in AP Heterodimers—Fig. 7 shows the well characterized electrophoretic migration on starch gel ele-
trohoresis of the common F and S homodimeric allozymes of PLAP as well as the pattern for the heterozygous FS variant.

Conclusions—Mammalian APs are allosteric enzymes in which both monomers act independently, at least when both AP subunits are completely metalated. It is, however, clear that for different AP isozymes, subtle amino acid substitutions in positions close to the active site may dramatically affect the affinity for Zn$^{2+}$ binding in the active site pocket. Therefore, in different tissues the mechanism of the actual AP catalysis will be determined by the local concentrations of available isozyme and zinc ions. It is also evident that heterodimers can form between structurally related mammalian APs. These heter-
dimers are not the weighted average of the parent homodimers; as a consequence of subunit interactions, AP enzy-
mes are formed that are structurally less asymmetrical than expected and that have catalytic properties divergent from those of the parent homodimers. Hence, mammalian APs are noncooperative allosteric enzymes, but the stability and cata-
lytic properties of each monomer are controlled by the confor-
mation of the second AP subunit.
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