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Phosphoryl group transfer: evolution of a catalytic scaffold

Karen N. Allen[1] and Debra Dunaway-Mariano[2]

[1]Department of Physiology and Biophysics, Boston University School of Medicine, 80 East Concord Street, Boston, MA 02118-2394, USA
[2]Department of Chemistry, University of New Mexico, Albuquerque, NM, 87131, USA

It is proposed that enzymic phosphoryl-transfer reactions occur by concerted, step-wise, associative (phosphorane-intermediate) or dissociative (metaphosphate-intermediate) mechanisms, as dictated by the catalytic scaffold and the reactants. During the evolution of a phosphotransferase family, the mechanism of the phosphoryl-transfer reaction is in constant flux, potentially changing with each adaptation of the catalytic scaffold to a new phosphoryl-donor–acceptor pair. Phosphotransferases of the recently discovered haloacid dehalogenase superfamily of enzymes, one of the largest and most ubiquitous of the phosphotransferase families characterized to date, are described in the context of the co-evolution of the catalytic scaffold and mechanism.

Cellular phosphoryl-transfer reactions, which add and remove phosphoryl groups to and from macromolecules and metabolites, form the centerpiece of biochemical processes [1]. The thermodynamics of these reactions vary greatly [2], but the kinetic barrier is consistently high [3]. Phosphotransferases, the enzymes that catalyze phosphoryl-transfer reactions [4–6], enhance the reaction rate by up to $\sim 10^{21}$ by lowering the energy barrier for the reaction via strong binding to the transition state ($K_d \sim 10^{-26}$ M) [3].

Solution phosphoryl-transfer reactions can proceed by three, mechanistically distinct, nucleophilic-substitution pathways (dissociative, concerted and associative) (Figure 1). These differ in the timing of the formation of the phosphorus–nucleophile bond and cleavage of the phosphorus leaving-group bond. The prevailing chemical pathway is determined by the nature of the nucleophile, electrophile and leaving group, and by the solvent [7–11]. What then, controls the chemical pathway of the enzyme-catalyzed phosphoryl-transfer reaction? There is evidence to support the view that all three types of chemical pathways are catalyzed by phosphotransferases [12–15], but the rules governing selection of a pathway for a given enzyme–reactant pair are not known.

The ensemble of electrostatic and steric interactions [12,14–20] that occur between enzyme and ligand along a reaction pathway are defined by the structure of the reactant(s) and by the catalytic scaffold of the enzyme. The catalytic scaffold of the enzyme is, in turn, determined by the backbone fold of the enzyme and, thus, the enzyme family [21]. Some catalytic-scaffold residues are conserved in all members of the enzyme family because they are used for catalysis of the core chemistry. Other positions on the scaffold are used to recruit residues that can expand either the substrate specificity or chemical range. The core chemistry of the phosphotransferases is 'phosphoryl-group transfer'. Numerous enzyme families have evolved phosphotransferases; the Protein Families database (http://www.sanger.ac.uk/Software/Pfam/) contains >50 families of phosphate-ester hydrolases (phosphatases). Each family brings a unique set of catalytic tools to the reaction, which constitutes a paradigm for an independently evolved 'successful strategy' for catalysis of phosphoryl transfer. During the evolution of a phosphotransferase family, the mechanism of the nucleophilic substitution pathway can be in constant flux and, potentially, changes with each adaptation of the catalytic scaffold to a new phosphoryl-donor–acceptor pair. This is because such adaptations can alter the nature of the nucleophile and electrophile as well as the ability of the enzyme to complement the steric and electrostatic properties of the transition state(s) encountered. The co-evolution of catalytic scaffold and mechanism is exemplified by the haloacid dehalogenase (HAD) superfamily of phosphotransferases [22], on which we center our discussion.

Despite the fact that it is named after a dehalogenase, the vast majority of the known catalytic activities in the HAD family are directed at phosphoryl transfer (reviewed in [23–25]). Moreover, based on the conservation of a metal-binding site in the 'unknown' or 'hypothetical' family members it is anticipated that these too, are phosphotransferases [26]. Presently, the HAD family contains >3000 sequenced proteins. Based on this dataset, it is clear that they are ubiquitous in prokaryotes and eukaryotes, and numerous within a given organism (29 members in *E. coli* and 58 in humans; J. Selengut, pers. commun.). ATPases and phosphatases are most prevalent, whereas diversification of catalysis to include phosphonate (P–C bond) hydrolysis (phosphonatase) and the transfer of phosphoryl groups between hexose hydroxyl substituents (phosphomutases) occur less frequently (known activities are listed in Table 1). Also striking is the range of phosphate monoesters that are

Corresponding author: Karen N. Allen (allen@med-xtal.bu.edu).
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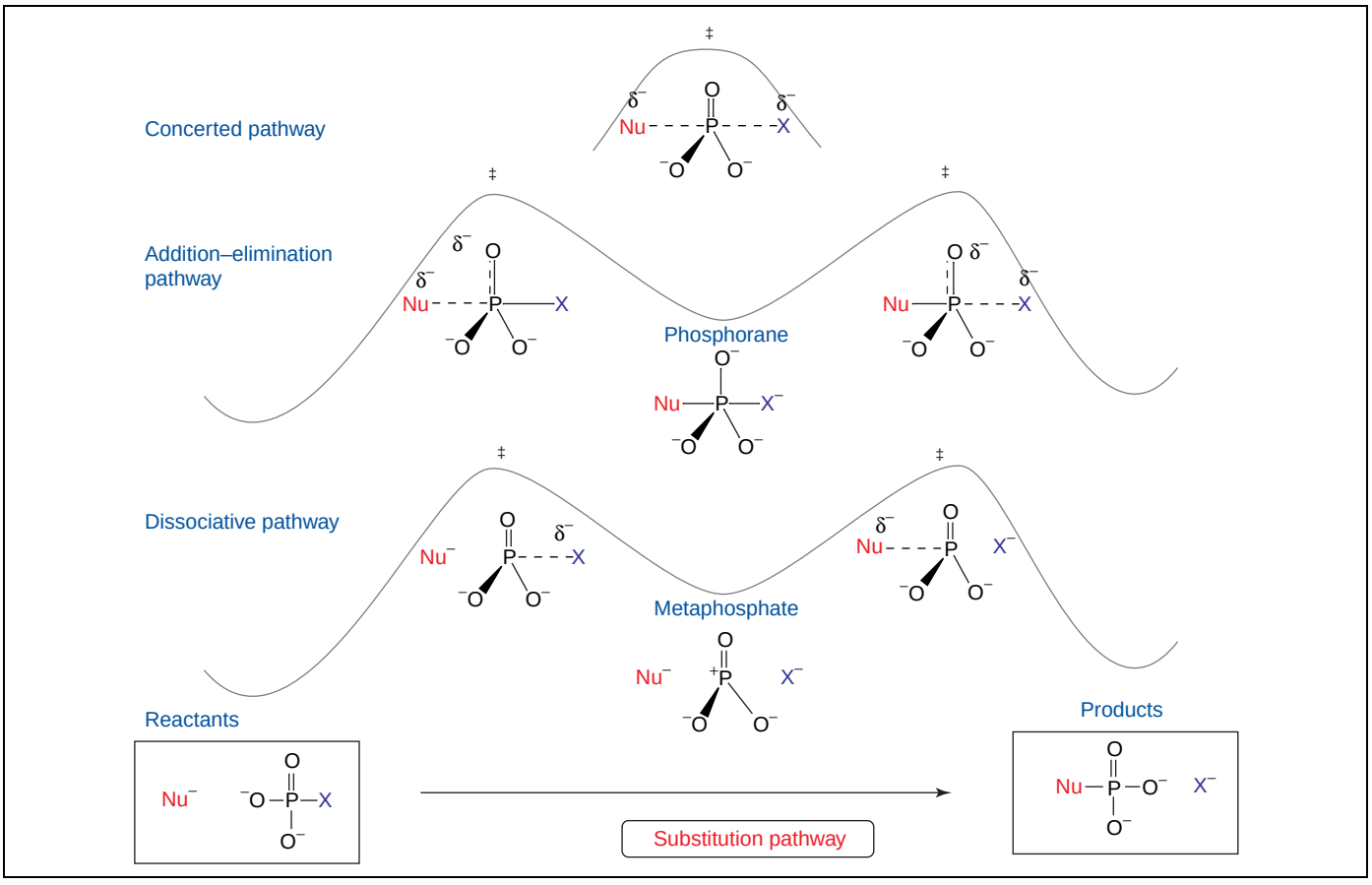

Figure 1. Three possible chemical mechanisms for phosphoryl transfer between two nucleophiles (Nu) differ in the timing of bond formation and cleavage (the substitution pathway). A hypothetical free energy diagram for each path is shown in the background. These highlight the fact that in the case of the addition–elimination (associative) and dissociative mechanisms an intermediate is formed, whereas there is no intermediate in the concerted reaction. In the addition–elimination mechanism, bond making to form a pentavalent phosphorane intermediate precedes bond breaking. In the dissociative mechanism, bond breaking to form a metaphosphate intermediate precedes bond making. In the concerted reaction, bonds are simultaneously made and broken in a single step, and the transition state resembles either metaphosphate or phosphorane intermediates.

accommodated by the HAD catalytic scaffold. These include small (e.g. phosphoserine, phosphoglycolate, hexose phosphates and histidinol phosphate), intermediate (e.g. nucleoside phosphates and 3-deoxy-D-manno-octulosonate-8-phosphate), and large substrates (tRNA and phosphoproteins). The HAD family has evolved catalysts to perform diverse, novel, physiological functions in primary and secondary metabolism, membrane transport, signal transduction and nucleic-acid repair. In the second theme of this review, we examine how this diversity of function has emerged through evolution of the HAD catalytic scaffold.

Table 1. Prominent members of the haloacid dehalogenase superfamily sorted by class and reaction type

Enzyme	Class	Substrate
C–Cl cleavage		
2-L-haloalcanoic acid dehalogenase	I	2-L-Haloalkanoic acid
C–P cleavage		
Phosphonoacetaldehyde hydrolase	I	Phosphonoacetaldehyde
CO–P cleavage		
Phosphoserine phosphatase	I	L-Phosphoserine
Mitochondrial 5′-(3′)deoxyribonucleotidase	I	dUTP
Sucrose-6^F-phosphate phosphatase	II	Sucrose-6^F-phosphate
Mg^{2+}-dependent phosphatase (MDP1)	III	Protein phosphotyrosine
8KDO phosphatase	III	3-Deoxy-D-manno-octulosonate 8-phosphate
CO–P cleavage/formation (mutase)		
β-phosphoglucomutase	I	β-Glucose 1-phosphate
Phosphomannomutase	II	α-Mannose 1-phosphate
PO–P cleavage		
Sarcoplasmic Ca^{2+}-ATPase	I	ATP
Cu^{2+}/H^{+}-ATPase	I	ATP

Utilization of an Asp residue in nucleophilic catalysis

Nucleophilic catalysis is a common tool used by phosphotransferases, especially phosphatases [27–29], which employ Cys (tyrosine-specific protein phosphatase family [30]), His (type 2 phosphatidic acid phosphatase superfamily [31]) and Ser-Thr (alkaline phosphatase containing metalloenzyme superfamily [32]) as the nucleophile. Using this catalytic strategy, a covalent bond is made to the nucleophile with the formation of a phosphoenzyme intermediate. In the HAD superfamily, the Asp nucleophile contributes to the adaptability of the catalytic scaffold. First, the high acidity of the carboxylate nucleophile obviates the need for activation by base catalysis and for electrostatically induced pK_a reduction, and extends the catalytic scaffold function to acidic pH, which is observed, for example, with the bacterial periplasmic class B acid phosphatase [33]. In all phosphotransferases of the HAD superfamily, there is an absolute catalytic requirement for the divalent metal ion Mg^{2+}. This cofactor is held by the metal-binding motif that is one of the consensus sequences that identifies HAD family members. The Mg^{2+} cofactor also binds to the nucleophilic Asp and phosphorylated substrate, providing orientation and charge shielding for nucleophilic attack [34]. The aspartylphosphate is intrinsically high in energy (the standard free energy for hydrolysis of acetylphosphate is $-44.7\ kJ\ mol^{-1}$ [35]), which can be used to drive phosphoryl transfer to water, the predominant phosphoryl-group acceptor. Because of its limited surface area for electrostatic interaction, a water nucleophile cannot provide much binding energy for transition-state stabilization.

The rate of hydrolysis of acetylphosphate is $\sim 10^{-4}\ s^{-1}$ under physiological conditions [36]. The rate of hydrolysis of the aspartylphosphate in HAD members varies according to function, but for most phosphohydrolases it is at least 10^5 faster than the solution rate [3]. In the phosphatases, the hydrolysis of the aspartylphosphate intermediate can be facilitated by a general base stationed at a specific location on the catalytic scaffold. In the type I ATPase, phosphoryl transfer to the Asp (at $191\ s^{-1}$) triggers a rate-limiting conformational change, which provides a physical pathway for ion transport across the cell membrane via a change in the interface between the cytoplasmic domains [37]. It is possible that the 'pause' needed for the conformational transition in the phosphorylated enzyme is provided by the relatively slow attack of water on aspartylphosphate (dephosphorylation rate is $5\ s^{-1}$ [38,39]). A reduced rate of hydrolysis is accomplished using a hydrogen-bond acceptor Thr (which contributes only 30-fold to the rate of dephosphorylation [38,39]) to interact with the water nucleophile in place of the general base, which, typically, contributes a rate enhancement of 10^2–10^4 [40]. The water, which is oriented but not deprotonated, attacks at a reduced rate. In the phosphotransfer reaction catalyzed by β-phosphoglucomutase (β-PGM) [12], the rate of hydrolysis of aspartylphosphate is reduced even further by a proposed 'substrate induced fit' mechanism, in which the sugar phosphate positions the base catalyst necessary for phosphoryl transfer, but a water molecule does not.

The conserved catalytic scaffold of the α/β-core domain

All members of the HAD superfamily possess an α,β-core domain consisting of a central parallel β-sheet flanked by α-helices on both sides. This supports four loops that comprise the catalytic scaffold (Figure 2). The Asp nucleophile is located on loop 1, whereas the carboxylate residues that form the Mg^{2+}-binding pocket are located on loop 4. Loops 2 and 3 station the Ser-Thr and Lys-Arg phosphoryl group-binding residues. In most HAD phosphotransferases, the Asp nucleophile in loop 1 is followed two positions downstream by a general acid-base that binds and, in many cases, protonates the substrate-leaving group of the first step and, possibly, binds and deprotonates the nucleophile of the second step (Figure 2b). As described above, this residue is Thr for the ATPase, the enolase-phosphatase subfamily employs Glu, many 'unknown' members use Ala, Val, Gly and Asn, and in the phosphomutases, it is Asp. The core domain lacks the ability to close off the active site from solvent and to recognize substrate. Different strategies to accomplish these tasks have evolved in the HAD superfamily.

Use of the cap domain for active-site desolvation and chemical diversification

The enzymes in the HAD superfamily are grouped into three subfamilies (I–III) based on the presence and the location of a second domain that functions as a cap over the active site of the core domain [41,42] (Figure 3). The small, α-helical-bundle cap domain of subfamily I is inserted between loops 1 and 2 of the core domain (Figure 3a), whereas the larger β-sandwich domain [43] of subfamily II is inserted between loops 2 and 3 (Figure 3b). Members of subfamily I include phosphonatase, phosphoserine phosphatase, 2,3-diketo-l-phospho-5-thiomethylpentane phosphatase, 2-deoxyglucose-6-phosphate phosphatase, glycerolphosphate phosphatase and β-PGM, and members of subfamily II include the phosphatase *TM0651* from *Thermotoga maritime,* trehalose-6-phosphate phosphatase and sucrose-6-phosphate phosphatase. Whereas the primary function of the cap domain might be active-site desolvation, it also brings diversity of catalytic function to the HAD phosphotransferases. In phosphonatase [41,42], β-PGM [44] and phosphoserine phosphatase [45–47], it has been shown that the cap domain – which is connected to the core domain by two solvated peptide linkers – moves as a rigid body by a hinge-like motion to open and close the catalytic site to solvent in response to substrate binding. The substrate is encapsulated and, thus, subfamilies I and II act predominantly on small substrates.

In subclass I, the catalytic scaffold is completed by the turn region of the conserved helix-turn-helix motif (loop 5) [48]. In typical phosphomonoesterases of subclass I, which are exemplified by phosphoserine phosphatase [45–47] and mitochondrial deoxyribonucleotidase (d-NT2) [49], residues from the cap domain bind the leaving-group portion of the substrate. In phosphoserine phosphatase, the phosphoserine substrate forms hydrogen bonds with both Glu20 (to the amino group of Ser) and Arg56 (to the carboxylate group of Ser) of the cap domain, which provide stereospecificity. In this instance, the strong hydrogen

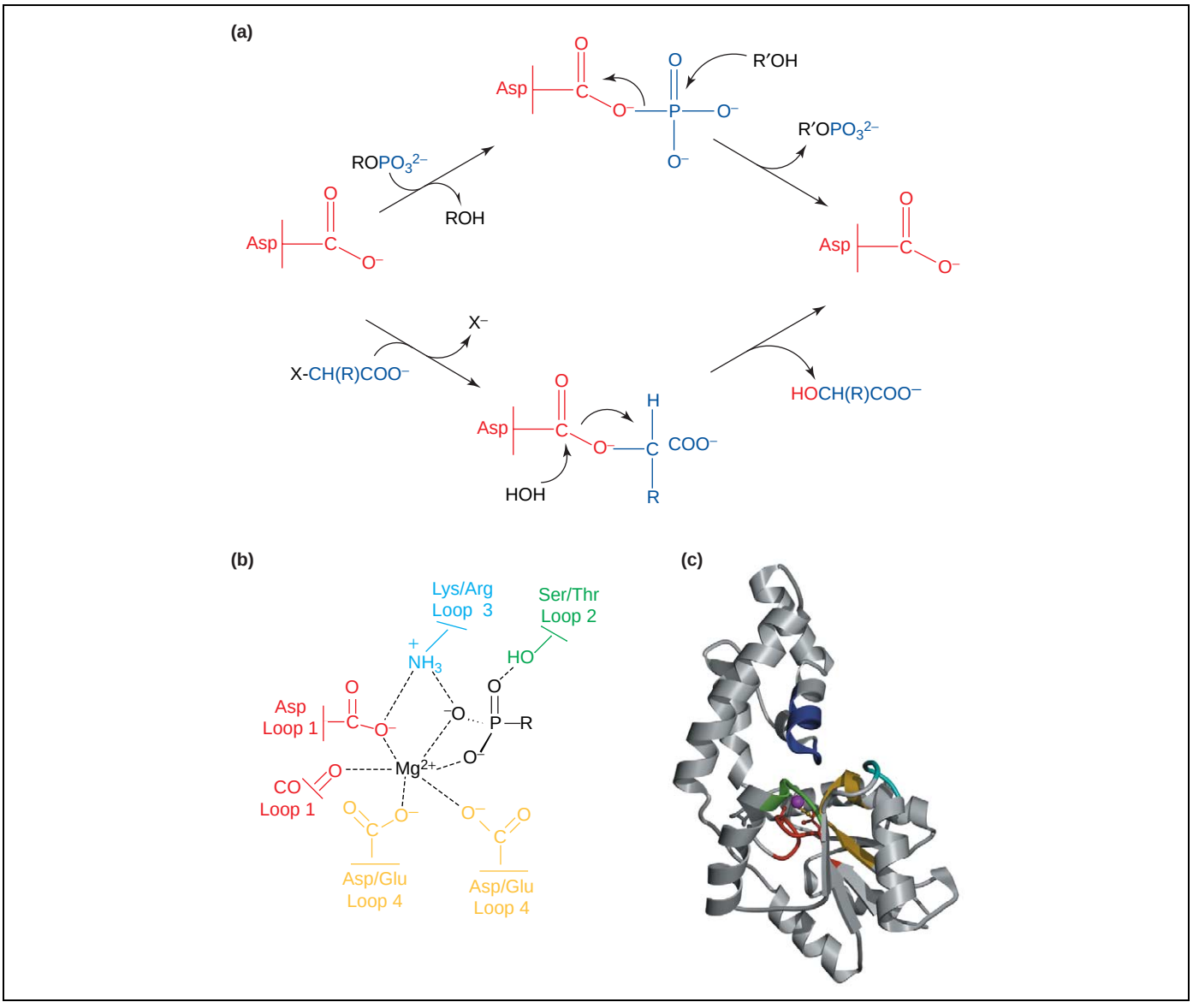

Figure 2. The catalytic scaffold in the haloacid dehalogenase (HAD) family of phosphotransferases. **(a)** In HAD enzymes, Asp [64–66] mediates carbon-group transfer to water (in the dehalogenases) and phosphoryl-group transfer to a variety of acceptors. Thus, the HAD superfamily is unique in catalyzing both phosphoryl-group transfer (top) and carbon-group transfer (bottom). **(b)** Schematic of the roles of the four loops that comprise the catalytic scaffold. The activity 'switch' is located on loop 4 of the catalytic scaffold (yellow) which positions one carboxylate residue to function as a general base for the dehalogenases and either two or three carboxylates to bind the Mg^{2+} cofactor essential for the phosphotransferases. CO represents the backbone carbonyl oxygen of the moiety that is two residues downstream from the loop 1 nucleophile (red). The side-chain at this position is also used as an acid-base catalyst by phosphatase and phosphomutase HAD members. Loop 2 (green) and loop 3 (cyan) serve to position the nucleophile and substrate phosphoryl moiety. **(c)** Ribbon diagram (core domain: loop 1, red; loop 2, cyan; loop 3, green; loop 4, yellow; cap domain: specificity loop, blue) of the fold supporting the catalytic scaffold of phosphonatase (1FES).

bond (2.5 Å) from Glu20 to the amino group orientates the NH_3 group to optimize an intrasubstrate hydrogen bond between NH_3 and Oγ of Ser, which provides substrate-mediated assistance to P–Oγ bond cleavage. Thus, the cap domain contributes residues that are tailored to the specific phosphoryl substrate being cleaved. The dNT-2 binds and orients the substrate pyrimidine ring via hydrogen bonds from the cap domain residues Glu50 (through water to the uracil NH) and Ser78 hydroxyl (to the uracil carbonyl). Hydrophobic contacts are also provided by cap residues Phe49 and Trp76. In both phosphoserine phosphatase and d-NT2, many of these residues, along with residues which provide a water-exclusive active-site environment, are contributed by the helix-turn-helix substrate-specificity loop (i.e. loop 5) of the cap domain.

The cap domain is also used to extend the range of chemistry in the HAD family beyond phosphate monoester hydrolysis. The cap domain of phosphonatase (Figure 2c) interacts with the substrate by covalent modification. Scission of the P–C bond in the phosphonoacetaldehyde substrate (Figure 4a) requires that an electron sink is created within the leaving group. This is accomplished by formation of a Schiff-base between the β-carbonyl and a Lys residue from loop 5 of the cap domain when the enzyme is in the closed conformation [50]. In an interesting example of catalytic group 'multitasking', the LysN-ethyl enamine adduct, which is formed on substrate cleavage, substitutes for the acid-base catalyst in loop 1 of the core domain. Serving as a base, it activates the water nucleophile of the ensuing apartylphosphate hydrolysis.

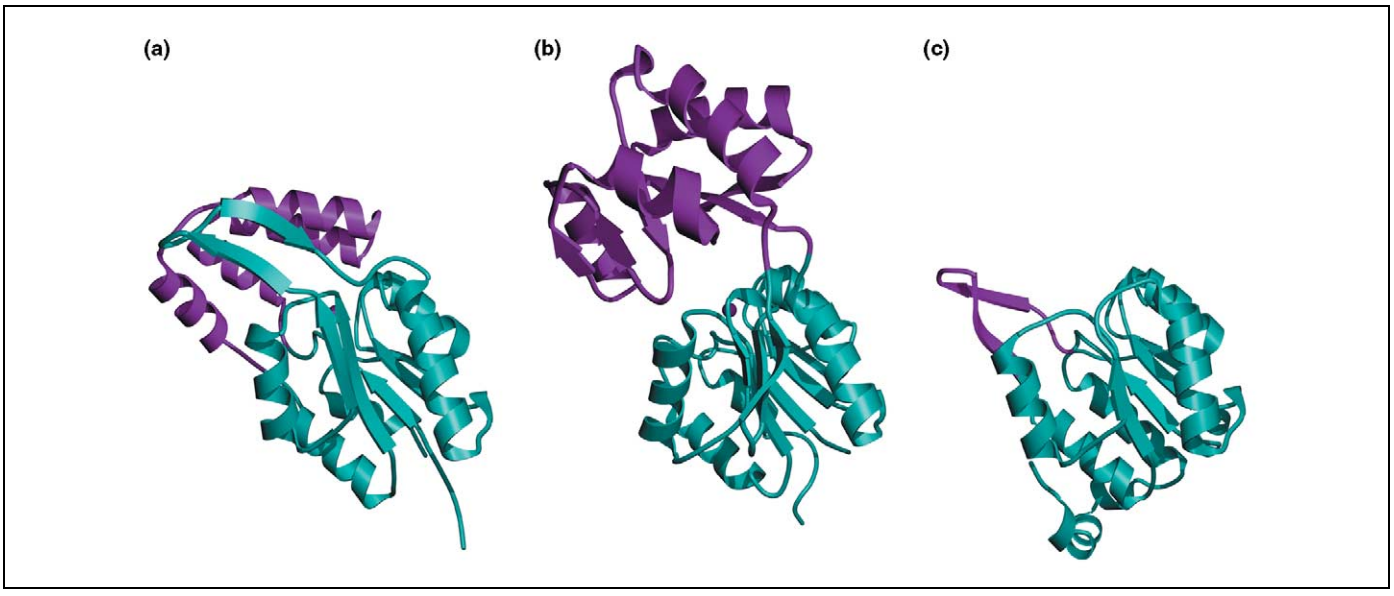

Figure 3. Ribbon diagrams highlighting the common core (cyan) and divergent cap domains (magenta) that distinguish the three subfamilies of the haloacid dehalogenase (HAD) superfamily. **(a)** Subfamily I, phosphoserine phosphatase (1F5S). **(b)** Subfamily II, phosphatase *TM0651* from *Thermotoga maritime* (1NF2). **(c)** Subfamily III, 8-KDO phosphatase (1J8D).

Protonation activates the LysN-ethyl enamine adduct for its hydrolysis. In phosphonatase, the core domain has lost sovereignty over hydrolysis of the phosphorylated enzyme.

In β-PGM, the cap contributes to catalysis in unique ways that switch the basic 'phospho-hydrolase' core-catalytic unit to a 'phospho-isomerase' mode (Figure 4b). For the Asp nucleophile to transfer phosphoryl groups to and from glucose phosphates, the aspartylphosphate must be protected from hydrolysis. This is a remarkable feat given that the core domain is tailored for hydrolysis. It is accomplished by coupling the closure of the cap domain over the active-site of the core domain with (i) movement of the general base (Asp10) in loop 1 of the core domain into position for catalysis, and (ii) binding between the loop 5 of the cap domain and the phosphoryl group of the bound substrate ligand. In the absence of substrate, cap closure is transient and activation of an opportunistic active-site water for hydrolysis of the aspartylphosphate is rare.

From comparison of the cap-open and cap-closed conformations of β-PGM we have inferred that the cap domain is an important determinant in the chemical pathway of phosphoryl transfer. For β-PGM, cap closure appears to compress the transferring phosphoryl group of the β-glucose 1,6-(bis)phosphate ligand against the Asp8 carboxylate group. The atomic resolution structure of β-PGM crystals grown from an equilibrium mixture of glucose-6-phosphate, glucose-1-phosphate, glucose 1,6-(bis) phosphate, and phosphorylated and unphosphorylated β-PGM provide a snap-shot of the fully loaded enzyme in the cap-closed conformation [12] (Figure 5). The electron-density map reveals that the β-glucose 1,6-(bis) phosphate ligand is joined covalently at the C(1)phosphoryl group to the Asp8 carboxylate (Figure 1). Apparently, the crystal packing forces do not allow the cap to open, hence the stabilized pentavalent phosphorane intermediate is trapped in a solid matrix. In our view, this is direct proof of the long-sought phosphorane intermediate of the associative phosphoryl-transfer pathway. We note however, that an alternative interpretation of this structural data is offered by Blackburn and coworkers [51] who observe that crystallization occurred in the presence of fluoride ions and proposed a hypothetical Asp8-MgF_3-OC(1)glucose 6-phosphate structure in place of the phosphorane intermediate.

HAD subfamily III does not use a cap domain

The HAD family members of subfamily III do not posses a cap domain (Figure 3c). Thus, unless an alternate structural unit substitutes for the cap domain, the active site of the core domain is open on one face to solvent. The four known structures in this subclass are the bifunctional T4 polynucleotide 5′kinase-3′phosphatase (which has N-terminal kinase and C-terminal phosphatase activity) [52], 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase (8-KDO) [53,54], the bacterial class B phosphatase [55] and the magnesium-dependent phosphatase 1 (MDP-1) [56]. The large size of the tRNA substrate explains why the two catalytic domains of T4 polynucleotide 5′kinase-3′phosphatase do not have cap domains and active site-gating loops. The entrance of the HAD active site of the core domain contains conserved residues that are likely to function in docking the tRNA, which can bind to both active sites simultaneously. The phosphatase MDP-1 is a monomer with several conserved polar and charged residues at its open active-site entrance. The substrate-activity profile and the shape of the catalytic pocket are compatible with a phosphotyrosine-like substrate (E. Peisach, unpublished results). Together, these findings indicate a phosphorylated protein as substrate, which, like the tRNA, docks over the core domain with the phosphorylated side chain projecting into the catalytic site.

In the case of 8-KDO (a tetramer), the entrance to the core domain is filled partially by an adjacent subunit. This creates a wormhole that is occluded by the carboxyhexose unit of the 3-deoxy-D-manno-octulosonate-8-phosphate substrate when it is docked at the protein surface. The

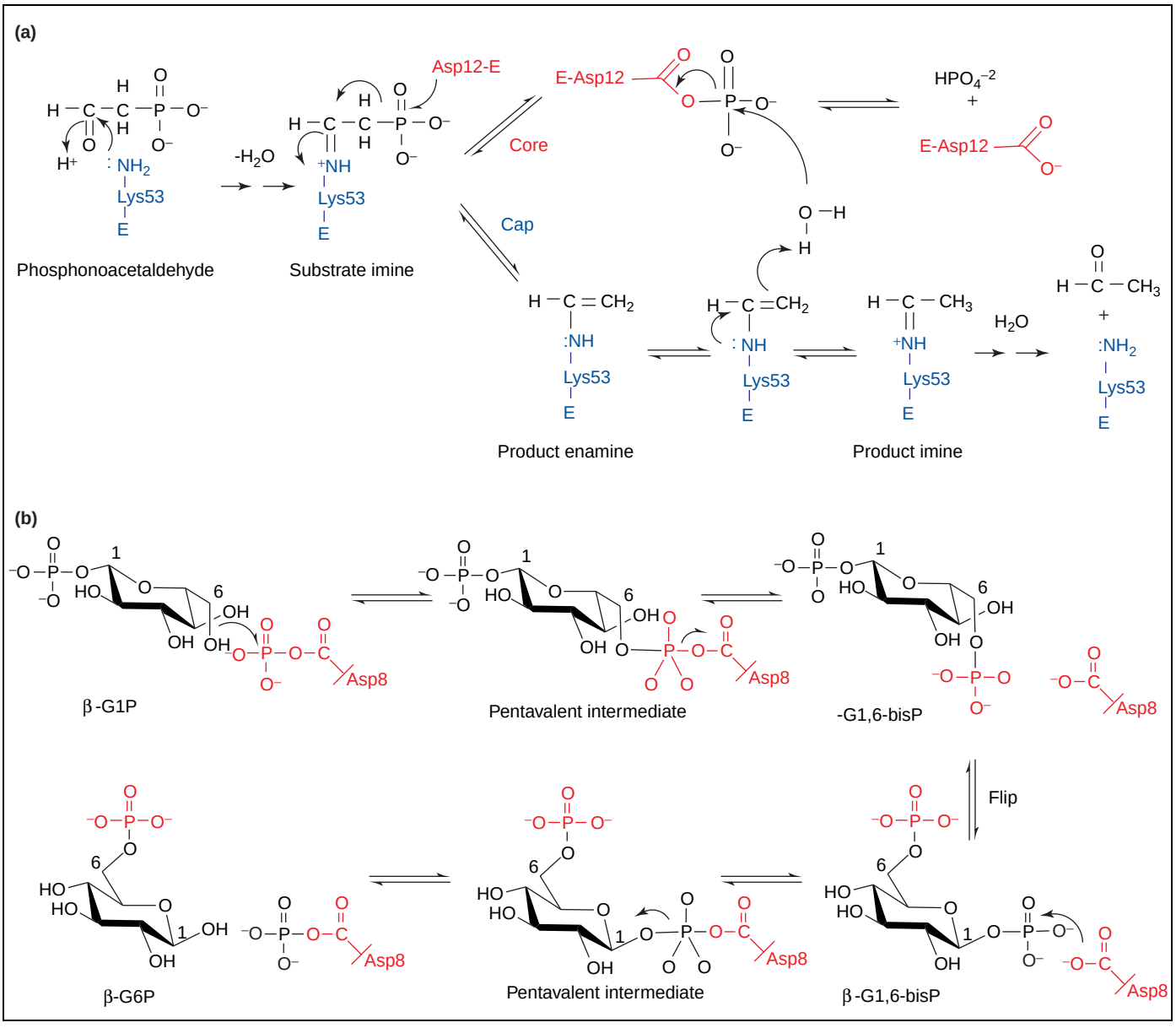

Figure 4. Reaction mechanisms and isolated intermediates of selected haloacid dehalogenase (HAD) family members. **(a)** In the P–C bond cleavage reaction catalyzed by phosphonoacetaldehyde hydrolase a catalytic lysine from the cap domain is utilized to form a Schiff-base (enamine) intermediate. This is followed by attack of the aspartate nucleophile from the core domain to form the aspartylphosphate intermediate. Hydrolysis of these intermediates from the cap and core follow. The utilization of the enamine intermediate as a general acid–base in the hydrolysis of the aspartylphosphate eliminates the need for the core scaffold to provide this catalyst. The modular nature of the reaction is underscored by the use of core residues (top) in the phosphoryl transfer chemistry and cap residues (bottom) in the Schiff-base chemistry. **(b)** The mutase reaction of β-glucose-1-phosphate (β-G1P) to β-glucose-6-phosphate (β-G6P) proceeds via two phosphoryl-transfer reactions with formation of a β-glucose-1,6-(bis)phosphate (β-G1,6-bisP) intermediate.

carboxylate of the substrate might bind to the conserved Arg at the mouth of the wormhole. The class B phosphatase is also tetramer but the subunits are arranged so as not to reduce the size of the entrance to the active site of the core domain. This enzyme is a nonspecific phosphomonoesterase that is believed to function in phosphate scavenging, and the absence of the cap domain is compatible with the lack of substrate specificity. The only contacts with substrate would be those made with the Mg^{2+} cofactor and the residues of the catalytic scaffold, which might recruit a third Asp residue from loop 1 to assist in catalysis. Because these interactions are restricted to the phosphoryl group and the wide 'mouth' of the active site is open to solvent, several phosphoryl group donors can be accommodated. The class B phosphatase functions at acidic pH, unlike alkaline phosphophatase, the metalloenzyme family phosphate scavenger, whose Zn^{2+}-activated Ser nucleophile functions optimally at basic pH [32].

Chemical pathway of phosphoryl transfer in the HAD family

The HAD family catalyzes two phosphoryl-transfer reactions for each catalytic cycle: in one, the loop 1 Asp is the nucleophile and in the other, the loop 1 Asp is the leaving group (Figure 2a). Each step proceeds via backside-in-line nucleophilic substitution with inversion of configuration at the phosphorus (net retention of configuration) [57]. In

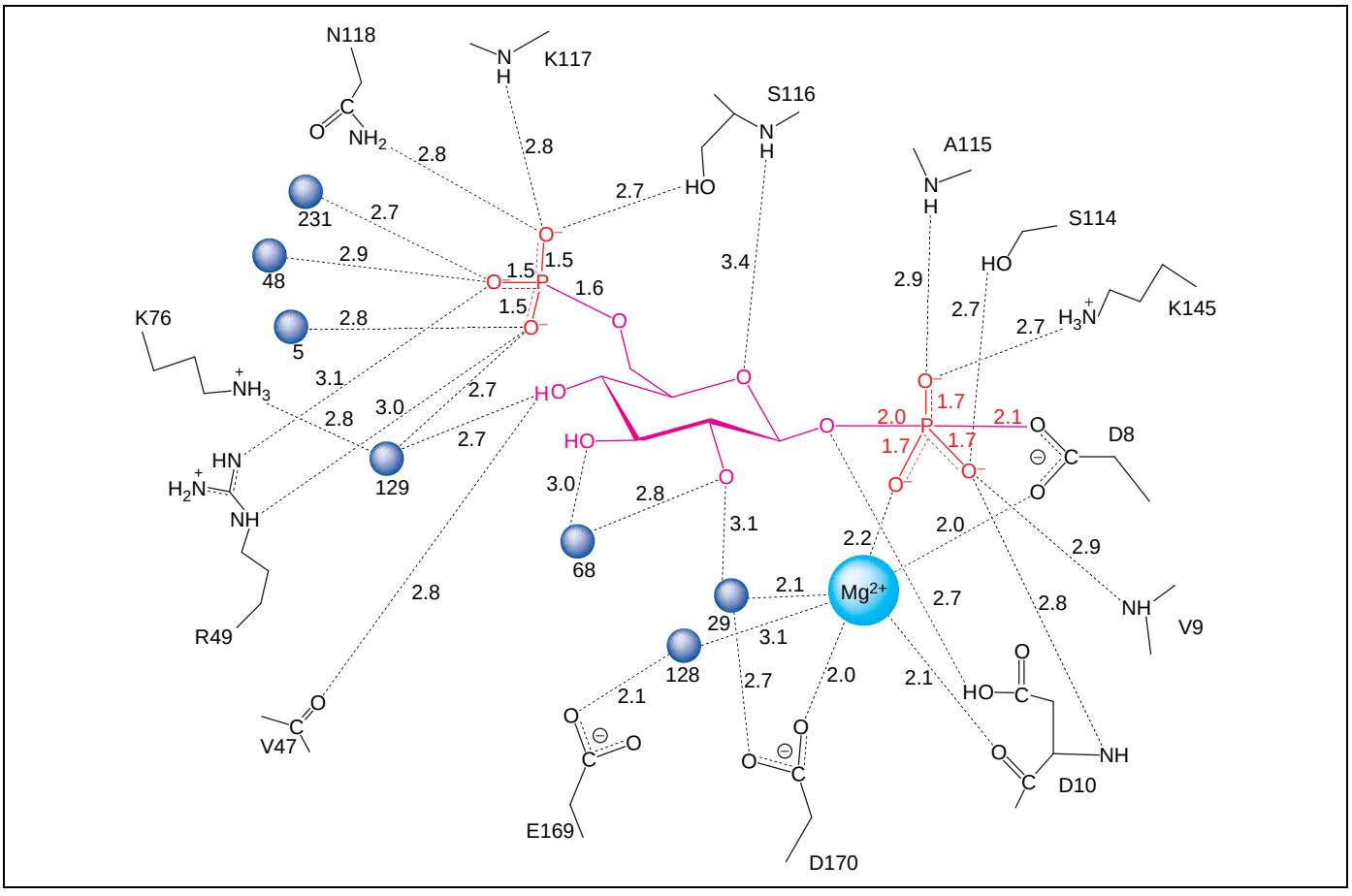

Figure 5. The structure of the pentavalent phosphorane intermediate determined by X-ray crystallography of crystals grown from an equilibrium mixture of glucose-6-phosphate, glucose-1-phosphate, glucose-1,6-(bis)phosphate, and phosphorylated and unphosphorylated β-phosphoglucomutase. The numerous hydrogen-bond interactions involved in binding and stabilization of the phosphorane intermediate and, thus, the ligand–enzyme interactions that dictate phosphoryl transfer via an associative pathway can be identified from the crystal structure. The hexose moiety is shown in red and the phosphoryl groups that undergo transfer are shown in orange. Crystallographically observed water molecules are shown as blue spheres. Reproduced, with permission, from Ref. [12] (www.sciencemag.org).

phosphonatase and β-PGM both steps occur in a desolvated active site with cap domain residues and core domain residues contributing to form the steric and electrostatic surface of the catalytic site. In the case of β-PGM, we know the mechanism of the phosphoryl transfer steps because of chance observation of the purported enzyme-phosphorane intermediate by X-ray crystallography [12]. The structure itself has raised several issues, the most controversial being the covalent bonding observed in the phosphorane [51,58]. Specifically, whereas the equatorial P–O bond lengths (1.7 Å) conform to those of chemical models, the axial P–O bond lengths (2.0 Å and 2.1 Å) are longer than anticipated. A second point of controversy is the apparent stability of the phosphorane. We have already given an explanation for the persistence of the intermediate in the crystalline matrix, but must now consider the free-energy profile for catalysis. For an intermediate to be observed experimentally, it must accumulate on the enzyme, which means that its energy is comparable to that of the enzyme-substrate complex. All previous tests of phosphotransferase mechanism (kinetic isotope effects and linear free-energy relationships) have been directed at the rate-limiting transition state rather than the actual intermediate. The intermediate could, in fact, correspond to only a slight dip in the energy barrier [59,60] and, therefore, be undetectable. However, we believe that this is not the case in β-PGM. The β-PGM phosphorane intermediate is stabilized by shortening of hydrogen bonds to the phosphorane center as the phosphorus transforms from tetrahedral to trigonal bipyramidal. Notable among these is loop 2 residue Ser114, and loop 3 residue Lys145, which contribute a rate factor of 10^3 and 10^5, respectively [61]. Increased coordination by Mg^{2+} is also important. Recent computational studies of bonding in phosphoranes indicate that elongation of the axial P–O bond (1.9–2.0 Å) occurs on intramolecular hydrogen-bond formation in small-molecule models [62]. The 2.0 Å and 2.1 Å axial P–O bonds observed in the β-PGM phosphorane intermediate are possible testament to the electrostatic interaction between the active site and the intermediate. This is illustrated in the active-site structure shown in Figure 5.

Concluding remarks

Although the phosphorane intermediate identifies the substitution pathway in β-PGM, the pathways of substitution in other members of the HAD family of enzymes are undetermined. Among the phosphatases, the variables in the first reaction step are the structure of the leaving group, the participation of cap-domain residues, the participation of residues that are recruited from the core domain catalytic scaffold and the exposure of the reaction

site to solvent. These variables can influence the mobility of the incipient leaving group, the accessibility and reactivity of the electrophile, and the orientation and reactivity of the Asp nucleophile as they are poised for reaction in the enzyme–substrate complex. Moreover, as the reaction proceeds, the landscape of the energy profile can be influenced by the steric and electrostatic interactions between ligand and catalyst as they progress together along the reaction coordinate.

The energy profile of the second step of the phosphoryl transfer process (i.e. hydrolysis of the phosphorylated enzyme) might be subject to less variation between HAD family members because the active site is solvated in this step, and catalysis is orchestrated solely by the core domain. The primary variables are activation of the water nucleophile by a general base and electron polarization in the aspartylphosphate moeity by core domain residues. Solution studies indicate a dissociative mechanism for the uncatalyzed hydrolysis of the acetylphosphate dianion [36], and dissociation to metaphosphate followed by water capture might be the preferred pathway for hydrolysis of the aspartylphosphate intermediate. By contrast, it is possible that interaction between the aspartylphosphate and the surrounding enzyme residues might retard metaphosphate formation, and that the utilization of a general base for water activation (see [63] for discussion of the effect of nucleophile activation on mechanism) might promote nucleophilic attack. An associative pathway for enzyme catalysis could, thus, replace the dissociative pathway of solution hydrolysis. Indeed, an associative pathway is consistent with the reported structure of the transition-state-analog complex of phosphoserine phosphatase-AlF_3 [45]. Although the structures of the rate-limiting transition states in the two phosphoryl-transfer reactions that are catalyzed by HAD phosphotransferases can be probed in future studies using kinetic isotope effect methodology, new methodology for transient spectroscopic detection is needed to test the presence of stabilized phosphorane and metaphosphate intermediates in these systems. Clearly, a rich area for future investigation remains.

Acknowledgements

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