

Current Topics

Hint, Fhit, and GalT: Function, Structure, Evolution, and Mechanism of Three Branches of the Histidine Triad Superfamily of Nucleotide Hydrolases and Transferases[†]

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Received April 10, 2002; Revised Manuscript Received May 19, 2002

ABSTRACT: HIT (histidine triad) proteins, named for a motif related to the sequence H ϕ H ϕ H ϕ ϕ (ϕ , a hydrophobic amino acid), are a superfamily of nucleotide hydrolases and transferases, which act on the α -phosphate of ribonucleotides, and contain a \sim 30 kDa domain that is typically either a homodimer of \sim 15 kDa polypeptides with two active-sites or an internally, imperfectly repeated polypeptide that retains a single HIT active site. On the basis of sequence, substrate specificity, structure, evolution, and mechanism, HIT proteins can be classified into the Hint branch, which consists of adenosine 5'-monophosphoramidate hydrolases, the Fhit branch, which consists of diadenosine polyphosphate hydrolases, and the GalT branch, which consists of specific nucleoside monophosphate transferases, including galactose-1-phosphate uridylyltransferase, diadenosine tetraphosphate phosphorylase, and adenylyl sulfate:phosphate adenylyltransferase. At least one human representative of each branch is lost in human diseases. Aprataxin, a Hint branch hydrolase, is mutated in ataxia-oculomotor apraxia syndrome. Fhit is lost early in the development of many epithelially derived tumors. GalT is deficient in galactosemia. Additionally, ASW is an avian Hint family member that has evolved to have unusual gene expression properties and the complete loss of its nucleotide binding site. The potential roles of ASW and Hint in avian sexual development are discussed elsewhere. Here we review what is known about biological activities of HIT proteins, the structural and biochemical bases for their functions, and propose a new enzyme mechanism for Hint and Fhit that may account for the differences between HIT hydrolases and transferases.

Mammalian Hint and GalT Structurally Define the HIT Superfamily¹

Galactose-1-phosphate uridylyltransferase (GalT), the second enzyme in the Leloir pathway of galactose utilization (1), was crystallized and its structure determined to under-

stand the mechanistic basis for transferring UMP between glucose-1-phosphate and galactose-1-phosphate (2–4). Histidine triad nucleotide-binding protein (Hint), purified as a purine nucleoside/nucleotide-binding protein from rabbit heart cytosol (5) and shown to have a widely conserved sequence (6), was crystallized and its structure determined to establish whether the conserved sequence formed the basis for a new mode of nucleotide-binding (7). GalT (2) and Hint are both dimers (8), but the GalT monomer is more than twice the size of the Hint dimer. Though GalT (9) and Hint (6) homologues could be cloned by similarity and the crystal

[†] This work was supported by grants from the NIH (CA75954 and CA77738).

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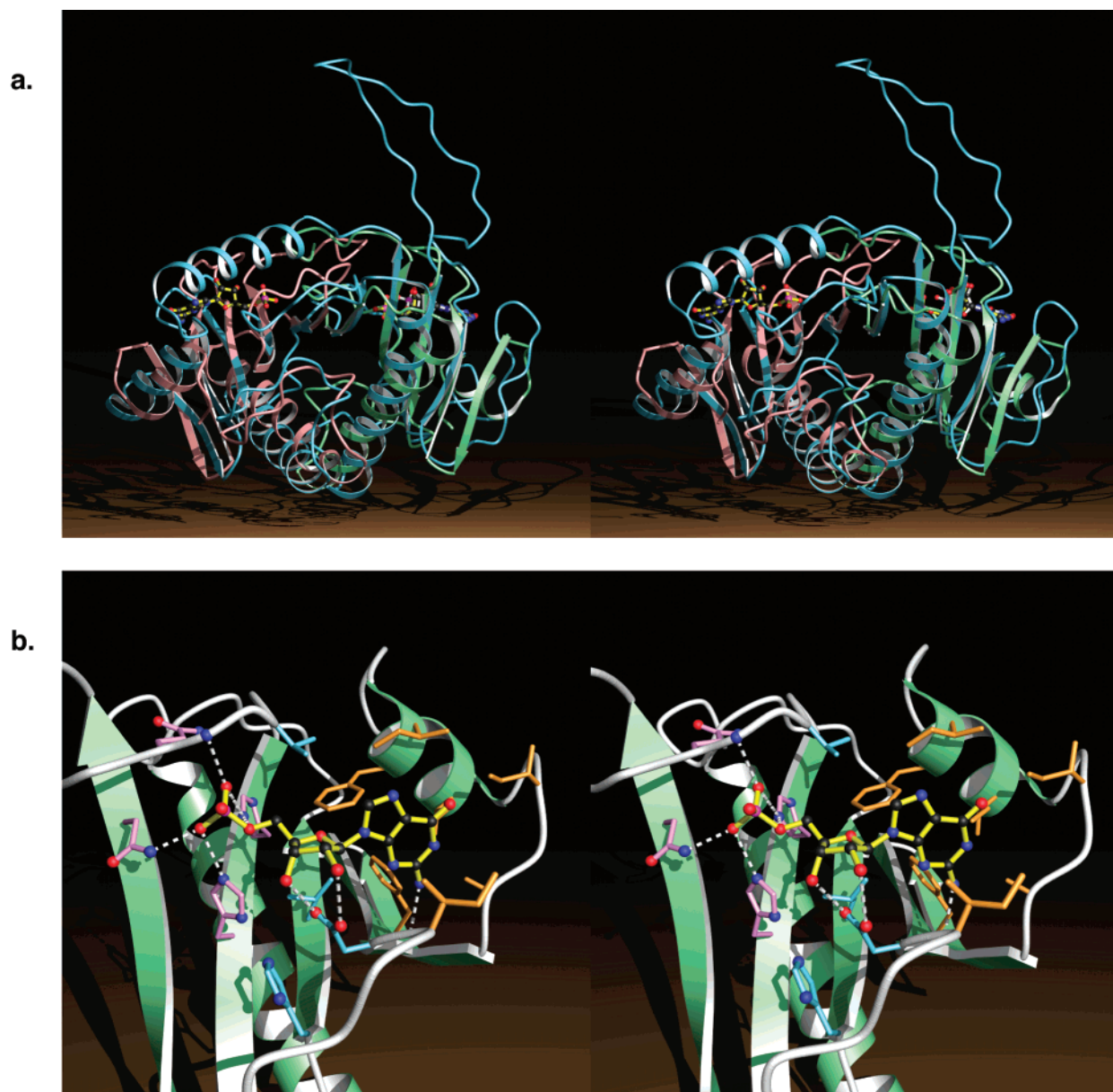


FIGURE 1: Structural definition of HIT proteins. (A) Stereoview of the GalT half-barrel (blue) superimposed on the Hint dimer (green and pink). The half-barrel consists of nine (GalT) or 10 (Hint) antiparallel β strands that enclose a pair of antiparallel α helices. GalT possesses a distinct loop structure and a helix that connects the N-terminal portion (right) of the half-barrel with the C-terminal portion (left) of the half-barrel. The GalT monomer has a single Hint-homologous active site in its N-terminal portion below the large loop where UMP is bound. UMP bound to GalT superimposes with GMP bound in the right-hand Hint monomer. (B) Highly conserved residues in Hint define a new mode of nucleotide-binding on top of the β -sheet. One of the two identical active sites is shown bound to GMP (yellow). The figure is based on reported X-ray structures (2, 7) and analysis (7).

structures (2, 8) coexisted in protein databases, mutual similarity was not noted until inspection of the rabbit Hint crystal structure (7) and computational analysis (10) indicated that a GalT monomer and a Hint dimer can be superimposed and that their nucleotide-binding sites retain some of the same

residues for binding a nucleoside monophosphate (7) (Figure 1). The level of sequence similarity of Hint and GalT is difficult to distinguish from noise in a standard BLAST search but can be picked up by PSI-BLAST (11). Nonetheless, the structures of Hint and GalT and modes of nucleotide binding are convincingly similar, and the mechanisms are related as discussed below.

Hint and Hnt1 are Enzymes with Adenosine 5'-Monophosphoramidase Activity and Hnt1 Enzyme Activity Positively Regulates Yeast Kin28

Because Hint are found in virtually every sequenced genome and appear to be the common link to fragile histidine triad (Fhit) branch and GalT branch sequences, it is considered that Hint is the ancestral HIT protein (7) and Hint homologues constitute Branch I of the HIT superfamily.

¹ Abbreviations: HIT, histidine triad; ϕ , hydrophobic amino acid; GalT galactose-1-phosphate uridylyltransferase; Hint, histidine triad nucleotide-binding protein; Fhit, fragile histidine triad; APTX, Aprataxin gene; ASW, avian sex-specific W-linked gene; EST, expressed sequence tag; Ap_nA, diadenosine polyphosphate; CAK, Cdc2 activating kinase; TFIIF, general transcription factor TFIIF; TFIIF, mammalian Cdk7-cyclin H-MAT1 or yeast Kin28-Ccl1-Tfb3 trimer; AOA, ataxia-oculomotor apraxia syndrome; PNK, polynucleotide kinase; PANT, polynucleotide kinase and Aprataxin N-terminus; FHA, forkhead associated; NMBA, N-nitrosomethylbenzylamine; AppppA, diadenosine tetraphosphate; ApppA, diadenosine triphosphate; HNPCC, hereditary nonpolyposis colon cancer; NSP2, rotavirus nonstructural protein two.

Table 1: A Genomic View of HIT Proteins: Three Branches in Three Genomes^a

branch	<i>H. sapiens</i>				<i>G. gallus</i>			<i>S. cerevisiae</i>		
	gene	a.a.	map	locus ID	gene	a.a.	map	gene	a.a.	map
(I) Hint	<i>HINT1</i>	(126)	5q31.2	3094	<i>HINT</i>	(126)	Z	<i>HNT1</i>	(158)	YDL125C
	<i>HINT2</i>	(163)	9p11.2	84681	?	?	?	—	—	—
	<i>HINT3</i>	(182)	6q22.33	135114	?	?	?	—	—	—
	<i>HINTP1</i>	(126)	7q35	168334	?	?	?	—	—	—
	<i>APTX</i>	(342)	9p13.3	54840	<i>APTX</i>	(?)	?	<i>HNT3</i>	(217)	YOR258W
---				<i>ASW</i>	(130)	W	—	—	—	
(II) Fhit	<i>FHIT</i>	(147)	3p14.2	2272	<i>FHIT</i>	(147)	?	<i>HNT2</i>	(217)	YDR305C
(III) GalT	<i>GALT</i>	(379)	9p13	2592	<i>GALT</i>	(?)	?	<i>GAL7</i>	(366)	YBR018C
	?				?			<i>APA1</i>	(321)	YCL050C
	?				?			<i>APA2</i>	(325)	YDR530C

^a Gene names are official except chicken *APTX*, *FHIT*, and *GALT*, which have been observed as mRNAs but not mapped. Human map positions and locusIDs are from LocusLink (<http://www.ncbi.nlm.nih.gov:80/LocusLink/index.html>) (14). Chicken and *Saccharomyces* genomic databases are at <http://www.thearkdb.org/browser?species=chicken> (15) and <http://genome-www.stanford.edu/Saccharomyces> (16), respectively. Sizes of primary translation products are for monomers of the largest splice forms. The size of chicken Fhit is inferred from an expressed sequence tag (EST).

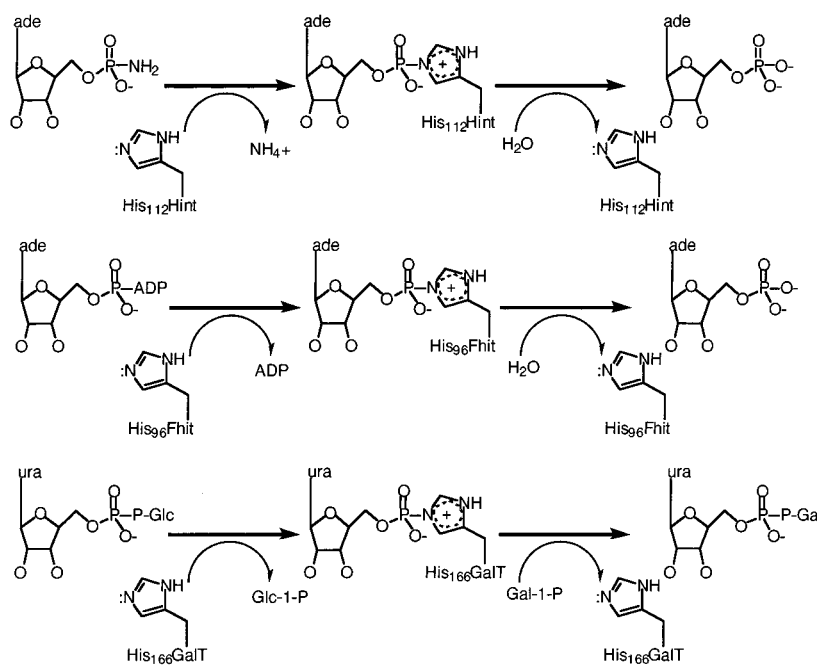


FIGURE 2: Substrates and forward reaction schemes for prototypical members of the three branches of the HIT superfamily. Note that Hint and Fhit hydrolyze nucleotidylated enzyme intermediates while the GalT nucleotidylated intermediate, stable to water, transfers to a second substrate, namely, a phosphorylated sugar.

Branch I includes at least four human, three chicken, and two yeast proteins (Table 1). Unfortunately, certain sequence annotations of HIT proteins persistently refer to the HIT motif as a zinc binding site or to Hint homologues as protein kinase C interactors or inhibitors. These annotations are based on data that have been publicly withdrawn in the case of protein kinase C (12) and on a paper whose data prove that zinc does not bind (8), as we have reviewed earlier (13). Crystallographic analysis demonstrated that 14 of the most highly conserved residues in the HIT protein superfamily mediate interactions with the purine base, the ribose, and the 5' monophosphate (7). However, while this analysis indicated that Hint homologues are conserved as nucleotide-binding proteins, the enzymatic and physiological functions of Hint homologues were not immediately clear.

Specific substrates for rabbit Hint and its yeast ortholog, Hnt1, emerged alongside physiological assays for the yeast *HNT1* gene (17). While deletion of *hnt1* was tolerated by haploid yeast cells on glucose media at all temperatures and

deletion of *hnt1* did not lead to a galactose- phenotype at 30 °C, *hnt1* deletion produced cells that are galactose- at 38–39 °C. Rabbit Hint and yeast Hnt1 were both purified from *Escherichia coli* and treated with a panel of nucleotides. As Fhit and GalT both cleave substrates that can be described as NMP-X (Figure 2), a large panel of dinucleoside polyphosphates (Ap_nA), nucleoside di- and triphosphates, nucleotide sugars, and other compounds were tested as rabbit Hint and yeast Hnt1 substrates. The only compounds hydrolyzed at greater than $\sim 1 \text{ nmol min}^{-1} \mu\text{g}^{-1}$ were the adenosine 5'-monophosphoramidate-linked compounds AMP morpholidate, AMP-*N*-alanine methyl ester, AMP-*N*- ϵ -(*N*- α -acetyl lysine methyl ester), and AMP-NH₂. AMP-NH₂ was hydrolyzed at greater than $10^6 \text{ s}^{-1} \text{ M}^{-1}$ by both rabbit and yeast enzymes. This activity was at least 200,000-fold dependent upon the His116 nucleophile of Hnt1, which corresponds to His112 of mammalian Hint. Just as yeast Hnt1 enzyme activity was destroyed by the H116A mutation, so the *hnt1-H116A* allele was without function in supporting growth of yeast cells on

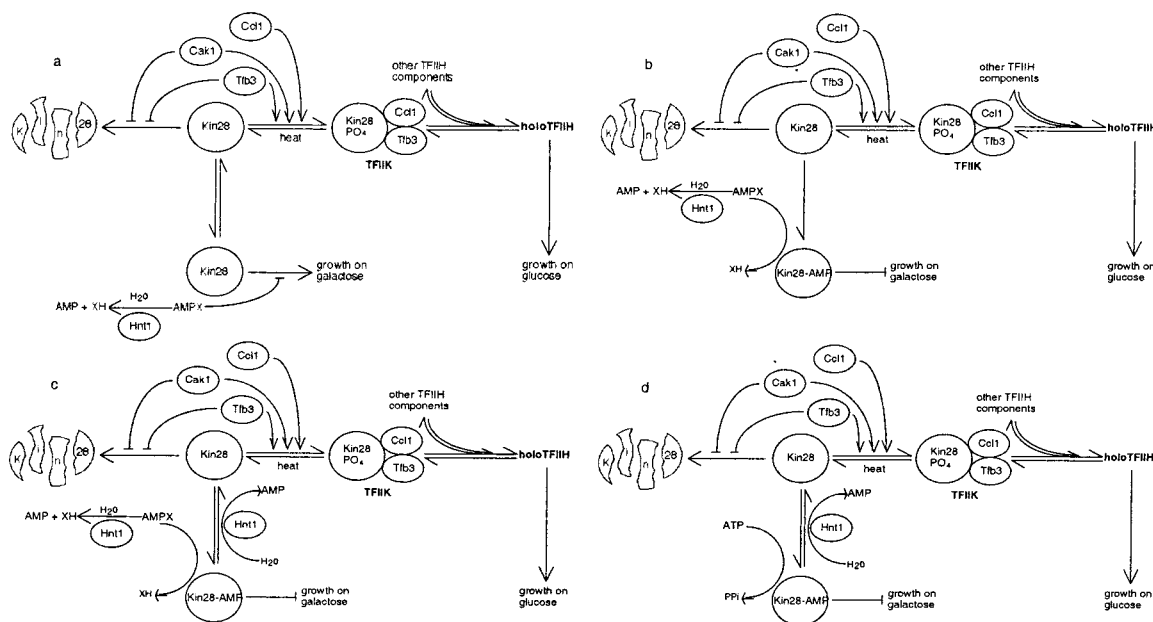


FIGURE 3: Models for regulation of Kin28 function by Hnt1 enzyme activity. See text for details.

galactose at 39 °C. Because the Hnt1-H116A protein is not unstable, it was concluded that hydrolysis of an Hnt1 substrate is required for growth of yeast cells at 39 °C (17).

A major clue to Hint function was provided by the identification of a two-hybrid interaction between human Cdk7 and human Hint1 (18). Cdk7 is the kinase catalytic subunit of CAK, the Cdc2 activating kinase complex, consisting of Cdk7, cyclin H, and MAT-1 (19–21). Additionally, this heterotrimer is part of a larger complex termed general transcription factor TFIIF that contains the major RNA polymerase II C-terminal domain kinase activity (22, 23). Adding to the significance of the findings, the yeast Cdk7 homolog, Kin28, had a weak two-hybrid interaction with yeast Hnt1, and it was demonstrated that the growth of a temperature-sensitive mutant in *kin28* was impaired by *hnt1* deletion on galactose media (18). Though the two-hybrid isolation of Hint as a Cdk7 interactor initially suggested a physical basis for regulation (18), expression of *hnt1-H116A* in *kin28-ts* backgrounds was as deleterious as the *hnt1* knockout, indicating that Hnt1 enzyme activity is a positive regulator of Kin28 function on galactose media (17).

Though fungal and plant orthologs of mammalian enzymes are typically 50% identical at the amino acid sequence level, the *Saccharomyces cerevisiae* Hint1 ortholog Hnt1 is only 22% identical, maintaining little more than the residues that form the nucleotide-binding site and the dimer interface (7). To address the possibility that Hnt1 enzymatic activity is not only necessary but sufficient for Kin28 regulation, we expressed rabbit Hint in place of yeast Hnt1 and showed full suppression of every *hnt1Δ* phenotype (17).

One obvious mechanism by which loss of Hnt1 enzyme activity might result in inhibition of a protein kinase was that the as yet unidentified *in vivo* substrate of Hint, provisionally termed AMP-X, which might be AMP-NH₂ or a chemically related molecule, could be an ATP-competitive protein kinase inhibitor. Further, because Cdk7 is both CAK and the kinase component of TFIIF, it was considered that Hnt1 enzyme activity might be a positive regulator of either Kin28 or Cak1. Consistent with the

possibility that Cak1 might be the target of Hnt1 regulation, *cak1-ts* mutants are synthetically less viable with *hnt1* mutations. However, when *hnt1* mutations were put into a strain that contains *CDC28* alterations that bypass the essential function of Cak1, poor growth was observed, indicating that Hnt1 does not act as a positive regulator of Cak1 (17). In fact, in the *cak1Δ hnt1Δ* background, poor growth was observed with glucose and galactose as carbon sources. This experiment excluded Cak1 as the target of Hnt1 regulation and also excluded the possibility that the galactose-dependent nature of *hnt1* phenotypes might have been due to AMP-X being produced solely as a byproduct of the Leloir pathway of galactose utilization.

With genetic experiments having shown that Hnt1 functions downstream of Cak1 and that *hnt1* mutations synergize with nonphosphorylatable Kin28, overexpressed Kin28, and temperature-sensitive mutations in *kin28*, *ccl1*, and *tfb3* (17), it remains to be determined precisely what the nature of Hnt1 regulation is and why the defect is easier to discern on galactose media than on glucose. Four models that account for all available data are shown in Figure 3. In each model, the glucose-essential function of Kin28 is depicted as residing in the TFIIF complex. Kin28 is also shown as existing in a CAK-like trimer consisting of Kin28, Ccl1, and Tfb3, which has recently been shown to exist *in vivo* (24), that we refer to as TFIIFK. We further illustrate the TFIIFK trimer as reversibly dissociable to its constituent polypeptides, with the equilibrium favoring monomers at higher temperature and as a function of temperature-sensitive alleles of Kin28, Ccl1, and Tfb3. It is known that the combination of temperature-sensitive Tfb3 and nonphosphorylatable Kin28 leads to Kin28 proteolysis (25). On the contrary, *hnt1* deletion with nonphosphorylatable Kin28 is synthetically lethal on galactose media, but *hnt1* deletion does not destabilize the Kin28 polypeptide (17). For these reasons, each model hypothesizes that the Kin28 monomer, released from TFIIFK at elevated temperature and with mutations in Cak1, Kin28, Ccl1, or Tfb3, is susceptible to inhibition in the absence of Hnt1 enzyme activity and that the *hnt1*-

inhibited form of Kin28 is disadvantaged in complex formation. Additionally, each model suggests that the *hnt1*-inhibited form of Kin28 is stable and inhibits growth on galactose. Further, each model hypothesizes that with mutations in Cak1, Kin28, Ccl1, or Tfb3 combined with the loss of Hnt1, sufficient Kin28 is drawn down to the poorly complexing form to deplete Kin28 from TFIIF and thus reduce viability on glucose.

The models consider alternate mechanisms by which Hnt1 enzyme activity may regulate Kin28 function. In model A, the small-molecule Hnt1 substrate provisionally termed AMP-X is a noncovalent inhibitor of Kin28, and the function of Hnt1 is to reduce the concentration of this molecule in the nucleocytoplasm. In model B, AMP-X is an Hnt1 substrate that covalently modifies Kin28. In model C, AMP-X is an Hnt1 substrate that adenylylates Kin28 as in model B, and additionally, Kin28-AMP is an Hnt1 substrate. Finally, in model D, Kin28 becomes adenylylated by virtue of reaction with ATP, and Kin28-AMP is the only Hnt1 substrate. In models A–C, small molecules are proposed to be important Hnt1 substrates. In models C and D, adenylylated Kin28 protein is proposed to be an important Hnt1 substrate. Experiments are in progress to test these models.

Aprataxin: A Zn Finger-Containing Hint Family Member That is Mutated in Ataxia-Oculomotor Apraxia

Ataxia-oculomotor apraxia syndrome (AOA) is an early onset, progressive neurological disease characterized by balance and facial motor problems, cerebellar atrophy, and hypoalbuminemia, whose neurological symptoms are difficult to distinguish from ataxia-telangiectasia (26). At the halfway point of a survey intended to catalog every case of hereditary ataxia in Portugal, AOA is second in occurrence only to Friedreich's among the autosomal recessive ataxias (27). Despite literature that is sparse outside of Portugal (27) and Japan (28), AOA has been found in many ethnic groups and does not appear to be a Portuguese or Japanese disease but rather a disease that is rare but ubiquitous (26). Two groups succeeded in positional cloning of an AOA disease locus to the *APTX* gene encoding Aprataxin on 9p13 (29, 30).

Human Aprataxin is expressed in two splice forms (30). The minor splice form encodes a predicted polypeptide of 168 amino acids consisting of a Hint domain and an apparent C(2)H(2) zinc finger C-terminal to the Hint domain (29, 30). The Hint domain of Aprataxin shows 31% amino acid identity with rabbit and human Hint in an 86 amino acid stretch that begins with the first β strand and ends with the HIT motif in the fifth and last strand (7). Though this segment does not include helix 1 and conserved sequences C-terminal to strand 5, it appears to be a minimum Hint domain we predict to dimerize and possess adenosine 5'-monophosphoramidase activity. C-terminal to the Hint domain, Aprataxin has the sequence CX(2)CX(12)HX(3)H that may be a nucleic-acid-binding motif consisting of a single zinc finger (31, 32).

The major splice form of Aprataxin is extended 174 amino acids at the N-terminus with respect to the minor splice form. Residues 6–102 of the N-terminus of Aprataxin are 41% identical with the N-terminus of human polynucleotide kinase (PNK), an enzyme with 5'-DNA kinase, 3'-phosphatase activity that is located C-terminal to this domain (33, 34).

The observation that PNK and Aprataxin share an N-terminal domain suggested that Aprataxin may have a role in single-strand break repair (30). Indeed, the PNK and Aprataxin N-terminal domain, termed PANT (30), was recognized as a forkhead-associated (FHA) domain by Keith Caldecott (personal communication). FHA domains are phosphothreonine-binding protein association domains that have been found in a variety of proteins involved in responses to DNA damage (35–38).

Many eukaryotes appear to have multiple Hint family members, one or more of which are Hint homologues, and one which is an Aprataxin homologue (Table 1). These sequences can be distinguished by degree of similarity to Hint and Aprataxin. Though Aprataxin homologues only align with Hint sequences inclusive of the β sheet, they are typically larger than Hint homologues because they are extended at the N- and C-termini and sometimes by insertions between helix 2 and strand 4. To date, we have only observed PANT/FHA domains in Aprataxin-homologous proteins from vertebrates. Invertebrate Aprataxin homologues, such as *S. cerevisiae* Hnt3 (17), possess a domain structure similar to the minor splice form of human Aprataxin (30). The molecular targets and pathways involving Aprataxin and its homologues remain to be discovered. With identification of such targets, it is hoped that new light can be shed on brain development and motor coordination.

Evolutionary of HIT Proteins: Bacteria to Birds

The observation that Hint homologues are nearly ubiquitous and that they are encoded in the smallest observed bacterial genomes suggested that Hint was present at the cellular root of the tree of life (7). Given the ability of Hint homologues to hydrolyze AMP–NH₂ and to hydrolyze AMP linked to a lysine side chain (17), we speculate that Hint may have evolved as an enzyme to cleanse spontaneously adenylylated proteins, which are potentially a consequence of life at high temperature with high levels of ATP. During evolution, certain proteins may have developed the ability to react in a suicide manner with adenosine nucleotides, leading to specific sites of high-occupancy adenylylation. In addition, specific proteins may have become sensitive to noncovalent effects of accumulated Hint substrates. Developments such as these could be responsible for specificity in the consequences of loss of Hint homologues, such as has been observed in yeast. Specificity in function of Hint homologues clearly exists because *hnt1* phenotypes are easy to observe in cells that are *HNT3+* (17) and because the genetics of AOA indicate that affected individuals have loss of function *APTX* mutations (29, 30) but should contain normal *HINT1-3* genes.

As will be discussed below, it appears that early to midway through eukaryotic evolution, a HIT protein underwent mutation and selection to become a Fhit-homologous Branch II enzyme. As illustrated in Figure 2, Fhit substrate specificity differs from that of Hint, with Fhit homologues preferring to hydrolyze substrates such as AppppA (39), ApppA (40), γ -(*m*-Nitrobenzyl) adenosine 5'-O-triphosphate (41), and ApppBODIPY (42). This amounts to an altered specificity for the adenylylation step of the reaction because adenylylation of each of these substrates produces an identical Fhit-AMP intermediate that, like Hint-AMP, is enzymatically

hydrolyzed. Thus, if the original enzyme was a Branch I enzyme, the new enzyme was altered to change its adenylation specificity from attack of AMP–NXH to attack of ApppX.

We have previously observed that a HIT protein may have undergone a tandem duplication with addition of a connecting helix and loss of one active site to produce a GalT-like nucleotidyl transferase (7). As shown in Figure 2, GalT and other Branch III enzymes have substrates that conform to the structure NMP-X and become nucleotidylated with release of the leaving group. Unlike Hint and Fhit intermediates, nucleotidylated GalT intermediates are stable in 55 M H₂O (3, 43). Not being hydrolases, Branch III enzymes enforce dependence of the intermediate reacting with a second substrate, typically phosphate or a phosphorylated hexose, to produce the product. A possible basis for the differences between HIT hydrolases and transferases is provided in the mechanistic section below.

Apart from Aprataxin (29, 30), which is a divergent member of the Hint branch, and the branch II and branch III enzymes Fhit (44) and GalT (45), the human genome appears to encode at least two additional Hint homologues (46). As shown in Table 1, *HINT2*, *HINT3*, and the putative pseudogene *HINTP1* appear to be encoded at 9p11.2, 6q22.33, and 7q35, respectively. Expressed sequence tags (ESTs) and/or cDNAs corresponding to *HINT2* and *HINT3* have been deposited, indicating more restricted expression patterns than that of the prototypical *HINT* (now *HINT1*) gene at 5q31.2 (47). *HINT2*, expressed in liver, is predicted to encode a Hint homologue with a 35 amino acid N-terminal extension with respect to the canonical 126 amino acid rabbit and human Hint. *HINT3* is predicted to encode a primary translation product of 182 amino acids, extended 31 and 25 amino acids at the N and C-termini, respectively. An ortholog of Hint3 including the C-terminal extension can be found in several other metazoans, and these genes are clearly expressed at the mRNA level. The *HINTP1* locus is apparently intronless, and its expression is supported by no publicly available EST. Conceptual translation of *HINTP1* produces a primary translation product of 126 amino acids with the peculiar substitution of two conserved histidine residues, H51R and H110Q. In the absence of evidence for expression of *HINTP1*, this locus has been annotated as a pseudogene. The physiological basis for the complexity of Hint homologues remains to be determined.

Perhaps the most remarkable sequence related to Hint occurs in birds on the W chromosome. ASW, which has every chemically important residue and nearly all nucleoside-binding residues mutated with respect to Hint, is an apparently catalytically inactive Hint family member encoded in an array of approximately 40 tandem repeats on avian W chromosomes (48, 49) whose mRNA may be stabilized by a unique mechanism (50). A canonical Hint homologue is encoded on avian Z chromosomes (49). Female birds are the heterogametic (WZ) sex while males are the homogametic (ZZ) sex. As the genetic basis for sex determination is not known in birds (51) and ASW is expressed in a temporal and spatial manner that could relate to feminization (48, 49), it will be important to investigate the function of ASW and avian Hint genetically, biochemically, and structurally. Heterodimerization as a mode of ASW inhibition of Hint was suggested in the absence of knowledge that Hint

function depends on enzyme activity (48–50). However, because Hint is a dimeric enzyme (7) that shows no cooperativity with respect to substrate (17), it is not obvious how heterodimerization of one inactive subunit with one active subunit would reduce specific activity by more than 50%. Elsewhere, we suggest three mechanisms by which ASW may plausibly function as a dominant negative form of Hint to promote feminization in avian development. The most interesting single feature in the ASW sequence that could make this molecule not only negative but dominant is Gln127, which is substituted for Trp123 in Hint, a residue that is buried across the Hint dimer interface in the interior of the other monomer (7). Molecular model of the proposed Hint–ASW heterodimer suggests that ASW Gln127 may pull Hint His114 out of its active conformation, accounting for dominant negativity.

Fhit Homologues: Tumor Suppressors and Ap₄A Hydrolases

Branch II of the HIT superfamily consists of orthologs of human Fhit. The first member of this branch to be characterized biochemically (52), cloned (39), and characterized physiologically (53) was aph1, a diadenosine tetraphosphate (AppppA) hydrolase from *Schizosaccharomyces pombe*. As aph1 possesses the canonical H ϕ H ϕ H ϕ ϕ motif, this enzyme was recognized as a member of the HIT superfamily (39), then populated by Branch I sequences, which were not yet known to encode nucleotide-binding proteins or enzymes. In addition, careful sequence inspection allowed these investigators to recognize that aph1 is distantly related to the HXHXQ ϕ ϕ -containing AppppA phosphorylases (39), which we now recognized as members of Branch III of the HIT superfamily. Thus, despite absence of statistically convincing sequence similarity and presence of mechanistic difference between hydrolases and phosphorylases, prescient observations made upon cloning aph1 (39) and later expanded in the review literature (54) form the basis for our current classification of three branches of HIT proteins.

Study of aph1-related molecules took on a sense of urgency with the discovery that the gene at the human chromosome 3 fragile site, which is disrupted in a familial chromosomal translocation associated with early-onset renal carcinoma and is lost in many cancers of the digestive tract, is the human aph1 homologue (44). Spanning the chromosome 3 fragile site FRA3B, which is the most fragile site in the human genome, and possessing a HIT motif, the gene was named *FHIT* for fragile histidine triad gene (44). It is now clear that *FHIT* deletions are among the earliest and most frequent genetic alterations in development of tumors in environmentally exposed tissues such as the lung (55, 56). Because the chromosomal location of *FHIT* is fragile and susceptible to deletions, the null hypothesis stated that losses at the *FHIT* locus are not contributing causes of cancer but rather consequences of the genome instability of p53- tumors (57). Though the kinetics of *FHIT* inactivation in the lung are inconsistent with this hypothesis (loss of Fhit protein occurs earlier and more frequently than *TP53* mutation, ref 55), it was critically important to determine whether re-expression of the *FHIT* gene in *fhit*- cancer cell lines suppressed tumorigenicity. This has been demonstrated convincingly in several experimental systems (58–65) and has been recently reviewed (66).

The murine *fhit* gene is encoded on a syntenic portion of chromosome 14 and also spans a fragile site (67, 68). Murine *fhit* is not an essential gene and *fhit*^{-/-} mice survive and develop a wide range of tumors including lymphomas and sebaceous tumors at higher incidence than wild-type mice (69). Taking advantage of the *fhit* gene's susceptibility to mutational inactivation, it was discovered that treatment of *fhit*[±] mice with low doses of the carcinogen *N*-nitrosomethylbenzylamine (NMBA) lead rapidly to development of Fhit- stomach and sebaceous tumors (70). Interestingly, stomach and sebaceous tumors are observed in a subset of hereditary nonpolyposis colon cancer (HNPCC) termed Muir-Torre syndrome that is thought to be due to loss of mismatch repair genes, potentially indicating that loss of *FHIT* is an important target in HNPCC (70).

Because loss of *FHIT* in development of lung cancer occurs in preneoplastic lesions (55) and *FHIT* re-expression in *fhit*^{-/-} cells suppresses tumor formation by induction of apoptosis (59, 60, 62, 63, 65), introduction of *FHIT*-expressing viruses has been explored as a preventative strategy in the NMBA-tumor induction model. Remarkably, administration of *FHIT* via viral vectors shortly after NMBA administration prevents tumor development (64), presumably by killing the cells that suffer their second hit at *fhit*.

Though *FHIT* re-expression in *fhit*- human or mouse cells induces death by apoptosis (59, 60, 62, 63, 65), relatively little can be said about the cellular pathway through which Fhit acts. There are three clues, however. First, the tumor-suppressing function of Fhit does not depend on hydrolysis of Ap_nA (58, 61) but may depend on forming a complex with Ap_nA (71). Second, there is a Rosetta Stone relationship (72, 73) between Fhit and Nit, a novel member of the nitrilase superfamily (74, 75). Third, Fhit homologues are found through a sufficient swath of eukaryotic evolution to predate many players in developmentally regulated apoptotic pathways and are likely to have a more fundamental cellular function, whose alteration triggers apoptotic consequences in animals.

Fhit homologues from different organisms vary in their substrate specificity for AppppA and diadenosine triphosphate (ApppA), but the enzymes that have been characterized kinetically bind both nucleotides very specifically (42, 75). Whereas aph1 from *S. pombe* prefers AppppA (52), human Fhit (40) and *S. cerevisiae* Hnt2 (76, 77) prefer ApppA. Fhit homologues from human and *Caenorhabditis elegans* do not discriminate significantly between ApppA and AppppA in terms of K_m , both enzymes being saturated by both substrates in the low micromolar range (42, 75), and thus Fhit homologous enzymes are thought to discriminate between substrates in the k_{cat} term (42, 75). Moreover, as the deadenylylation step for ApppA and AppppA is not different, these enzymes must discriminate on the basis of the rate of adenylylation of substrates that are bound with nearly identical K_m values (42).

Ap_nA are made in side reactions of certain tRNA synthetases (78) and are the subject of a comprehensive recent review (79). Because the functional consequences of elevated Ap_nA are not known, we considered three possible connections between the tumor-suppressing function of Fhit and Ap_nA (71). First, elevated Ap_nA may have a proliferative or antiapoptotic function that is potentiated by loss of Fhit. Second, Ap_nA may signal through Fhit in an antiproliferative

or proapoptotic manner that is lost with loss of Fhit. Third, the tumor-suppressing function may be Ap_nA-independent (71). Because the H96N mutant of Fhit had been shown to be extremely deficient in enzymatic activity (40) and yet functional in tumor suppression (58), it was important to characterize the nature of the biochemical defect. The mutant turned out to be more than 4 million-fold reduced in k_{cat} and less than 4-fold increased in the K_m term with respect to the wild-type enzyme in hydrolysis of ApppA (71). This was clearly inconsistent with the idea that Fhit must reduce the concentration of Ap_nA to function in tumor suppression. Because the H96N mutant retained a low micromolar K_m for ApppA and was found crystallographically to bind nonhydrolyzable ApppA in a manner nearly identical to that of wild-type Fhit, it was argued that the tumor-suppressing function of Fhit may depend on forming an Ap_nA complex (71). Furthermore, the crystallographically defined Fhit dimer surface that becomes filled with two Ap_nA molecules was proposed as a possible site of effector interactions (71). The critical test of this model depends either on discovery of an effector that binds Fhit-Ap_nA, potentially in a manner that retards hydrolysis, or showing that Fhit mutants that are defective in substrate-binding are poor tumor suppressors (71). These are both active research areas.

In the course of cloning *FHIT*-homologous genes from invertebrates, it was discovered that flies and worms encode Fhit in a fusion protein containing a novel ~300 a.a. polypeptide at its N-terminus that is a member of the nitrilase superfamily (74). Analysis of domain fusion events in sequenced genomes suggested that such fusions might be "Rosetta Stones" that decode a previously hidden cellular or biochemical interaction between proteins unrelated by sequence (72, 73). It was argued that fusions between domains that share a common phylogenetic profile (both genes are either represented in or both genes are absent from a genome) and which are coordinately expressed in organisms in which they are not fused are most likely to be indicative of a common cellular pathway (80).

Despite the fact that the "Nit" domain of fly and worm NitFhit had not been previously observed in any mammal, *NIT* genes were discovered in human and mouse as separate genes from *FHIT*, and murine *nit1* and murine *fhit* were shown to have nearly identical patterns of tissue-specific mRNA accumulation (74). Furthermore, discovery of homologues of the Nit domain of NitFhit in frogs, fission yeast and budding yeast supported the co-evolution of these polypeptides in the organisms that have Fhit homologues (75). The crystal structure of worm NitFhit showed that Nit is a tetramer that binds two Fhit dimers and that the C-terminus of Nit is conserved to interact with Fhit (75). The Nit-interacting surface of Fhit is not the Fhit-Ap_nA surface (75), but the architecture of the 200 kDa NitFhit complex is such that it could potentially bind two Fhit-Ap_nA interacting molecules at opposite poles of the 140 Å tetramer.

The active site of all members of the nitrilase superfamily was inferred from the NitFhit structure to be a Glu-Lys-Cys catalytic triad (75). The nitrilase superfamily consists of at least 13 branches of enzymes, members of only one of which have been shown to possess nitrilase activity (81). Eight branches of the nitrilase superfamily have either demonstrated or predicted amide hydrolase or amide con-

denation activities for a variety of substrates and domain fusion events have been common in the nitrilase superfamily (81). Biochemical studies have yet to identify a substrate for the Nit active site of NitFhit or a Nit ortholog.

In addition to fungi (52, 76, 77), mammals (44, 67, 68), and invertebrates (74), Fhit homologues can be found in the genomes of microsporidia such as *Encephalitozoon cuniculi* (82), mycetozoa such as *Dictyostelium discooidium* (unpublished), and green plants such as *Arabidopsis thaliana* (83). According to the tree of eukaryotic life constructed from protein data (84), unless Fhit is found in plants due to a lateral transfer, the earliest rift that would have preceded appearance of Fhit was the separation of plants from animals, fungi, microsporidia, and mycetozoa. It will therefore be important to look for Fhit homologues in organisms such as *Tetrahymena*, *Plasmodium*, *Trypanosomes*, and *Euglena*, as they are thought to be on the plant side of the plant/animal rift (84) that potentially preceded the appearance of Fhit. Because several apoptotic players including p53 and Bcl-2 family proteins are in animal-specific pathways, it is hard to reckon the primary function of Fhit being in direct management of apoptosis even though the biological effect of *FHIT* re-expression in *fhit*- cells has been apoptotic (59, 60, 62, 63, 65). A role more fundamental to eukaryotic cell biology, such as regulation of protein localization, which could have consequences that result in apoptosis in animal cells, seems more likely.

GalT-Related Enzymes are Specific Nucleoside Monophosphate Transferases that Contain a HXHXQ $\phi\phi$ Motif

Branch III of the HIT superfamily consists of GalT homologues and other related nucleotide transferases. The second step in the Leloir pathway for galactose utilization requires galactose-1-phosphate to be transferred to UMP to form UDP-galactose (1). As shown in Figure 2, the reaction is catalyzed by GalT, which consumes UDP-glucose to form a covalent UMP intermediate with His166 (43, 85) releasing glucose-1-phosphate, and then reacts with galactose-1-phosphate to form UDP-galactose. Humans with mutations in the *GALT* gene are the largest class of galactosemics and are initially diagnosed with failure to thrive in the first days of life. Despite being put on a galactose-free diet, the long-term outcomes for *GALT* galactosemics include problems with cataracts and intelligence (86).

S. cerevisiae (87) and *E. coli* (88) GalT sequences were similar enough to guide cloning of human GalT (9). GalT sequences coexisted with Hint sequences (6, 89) in the databases for several years without notice. In fact, the first Hint structure (8) and the first GalT structure (2) were initially considered to be unrelated to any other solved structures before Hint-nucleotide structures were solved (7). Independently, computational approaches based on structural coordinates (10) and on sequence alone (11) were able to detect the similarity.

While the Leloir pathway and thus GalT homologues may be extant in all organisms that utilize galactose as a carbon source (1), at least two other enzymes exist in subsets of organisms that are recognizable by sequence and mechanism to be members of Branch III of the HIT superfamily. *S. cerevisiae* and some other fungi and bacteria possess

AppppA phosphorylases (90–92) that produce ATP and ADP (not AMP) with overall retention of configuration (93). Like GalT, the reaction proceeds through a covalent nucleotidylated intermediate (94) with the release of ATP. This enzyme uses inorganic phosphate rather than water to phosphorylate the adenylylated intermediate to produce the ADP product. The three *S. cerevisiae* Branch III enzymes, Gal7, Apa1, and Apa2, are included in Table 1. Finally, *Thiobacillus denitrificans* possesses a GalT-related enzyme that is now termed adenylyl sulfate:phosphate adenylyltransferase, whose NMP-X substrate is AMP-SO₄ (95). After producing a covalent adenylylated intermediate that was detected by mass spectroscopy, this enzyme reacts with inorganic phosphate to produce ADP (95). Branch III enzymes are recognizable by related sequences that include a HXHXQ $\phi\phi$ motif and mechanisms that depend on transferring the covalently bound nucleoside monophosphate to phosphate or a phosphorylated second substrate, rather than hydrolyzing it with water (Figure 2).

Rotaviruses Utilize an Enzyme Resembling Hint That May Have Arisen via Convergent Evolution

Rotaviruses, which are major causes of diarrhea in humans and other animals, consist of six structural and six nonstructural proteins encoded by 11 segments of double-stranded RNA. Nonstructural protein two (NSP2) is a conserved basic protein that has been shown genetically to be required for RNA packaging (96, 97), which possesses intrinsic single stranded RNA-binding activity and magnesium-dependent nucleoside triphosphatase activity for GTP, ATP, UTP, and CTP to the corresponding nucleoside diphosphates (98). The crystal structure of the NSP2 octomer revealed that an ~80-residue segment within the C-terminal domain of the 317 amino acid NSP2 monomer adopts a Hint-like three-dimensional fold that is superimposable with β strands 1–3, helix 2, and strands 4 and 5 of Hint (99). Though NSP2 is an octomer, NSP2 has no dimer interface that aligns with Hint's dimer interface at helix 2 and strand 4; nor is there sequence conservation with Hint that would have indicated similarity by descent (99). While His221 of NSP2 can be aligned with His112 of Hint, no other residues important for nucleotide-binding or hydrolysis in Hint are found at corresponding positions in NSP2. The fact that NSP2 hydrolyzes any nucleoside triphosphate in a magnesium-dependent manner at the γ -phosphate while HIT proteins are specific for adenylylation or uridylylation in a cation-independent manner at the α phosphate suggests that NSP2 is not a HIT protein. However, we will wish to revisit our tentative exclusion of NSP2 from the HIT superfamily in light of a nucleotide-bound NSP2 crystal structure. Should NSP2 have a mode of nucleotide recognition that is nearly isosteric with that of Hint, one might be able to consider NSP2 a distant relative that has substituted a metal and new sequences for conserved residues, lost the characteristic dimer, and altered the site of nucleotide chemistry. For now, we agree that the Hint-NSP2 resemblance is striking but convergent (99), and not mechanistically related.

Mechanistic Insights in the HIT Superfamily: Hydrolases Versus Transferases and a Potential Role for Acid–Base Catalysis

Truly related sequences not only fold similarly but function similarly, so the extensive mechanistic dissection of GalT serves as a model for the entire HIT superfamily. In this regard, it is clear that both the nucleotidylation and the nucleotidyl transferase steps depicted in Figure 2 proceed with inversion of configuration at the α phosphate, leading to overall retention of configuration (100, 101). The GalT mechanism is thus extremely symmetrical: a UDP–hexose brings UMP in for covalent nucleotidylation of His168, producing a hexose-1-phosphate leaving group, and a hexose-1-phosphate retraces those steps to reclaim the nucleotide as part of a UDP-hexose product. The residues involved in recognizing the hexose-1-phosphate leaving/attacking groups have been defined crystallographically and, indeed, glucose and galactose are bound at the same site (4). The key difference between the HIT hydrolases in the Hint and Fhit branches and the transferases in the GalT branch is that hydrolases do not await a second substrate to accept the histidine-bound nucleotide but instead are competent to transfer the nucleotide to water. Might the nearly isosteric nature of the GalT leaving and attacking groups be the basis for GalT–UMP intermediates being resistant to hydrolysis? The existence of AppppA phosphorylases and adenylyl sulfate:phosphate adenylyltransferase makes us argue against this idea. Both of these enzymes are GalT-related transferases (95) that go through covalent intermediates (94, 95) with known stereochemistry (overall retention) in the case of AppppA phosphorylase (93). Because the AppppA leaving group, ATP, is not isosteric with the second substrate, phosphate, it is difficult to argue that substrate-binding sites remote from the nucleotidylated histidine enforce transfer rather than hydrolysis. We argue instead that Branch III enzymes lack the final histidine residue in the HIT motif, which is necessary to activate water for hydrolysis.

Kinetic studies with $O^{18} H_2O$ and with stereospecific α -thio-labeled γ -(*m*-Nitrobenzyl) adenosine 5'-*O*-triphosphate demonstrated that Fhit hydrolyzes substrates with overall retention of configuration and addition of water to the AMP product (41). Given physical evidence (102) for a covalently adenylylated intermediate and the stereochemical evidence (41) for retention of configuration, it is reasonable to assume that the hydrolytic water comes "in-line" from the position formerly occupied by the α - β bridging oxygen of a Fhit substrate. The fact that Branch I and II enzymes and the non-GalT Branch III enzymes are less symmetrical than GalT (i.e., water or inorganic phosphate as the nucleotide acceptor as opposed to another hexose-1-phosphate) does not make us think that the geometry of hydrolysis or phosphorylysis is any different than the geometry of nucleotide transfer in GalT (4).

Taken this way, some of the catalytic efficiency of Fhit may derive from the enzyme providing the same entry route to a polarized water as it provided an exit route for ADP. In the case of Hint, whose substrates include phosphoramides such as AMP–NH₂ and AMP linked to amino acids (17), the hydrolytic water's final approach to attack the enzyme adenylylate should retrace the initial escape path of the amine leaving group. In both cases, the enzyme has a role in getting

the leaving group to leave the confines of the active site, which is the area in which a leaving group can reattack. Altering the ionization state or solvating the leaving group is thus important because by jettisoning the leaving group into bulk solvent, what was microscopically reversible becomes effectively irreversible.

We make these points to disagree with one aspect of the Fhit mechanism proposed as a result of stereochemical analysis (41). According to that proposed mechanism, His98, the final His of the HIT motif, which aligns with Gln168 of GalT, makes a hydrogen bond with an α phosphate oxygen of an ApppA substrate. In our structures of wild-type and mutant Fhit bound to ApppA analogue, IB2, which has a phosphorothio substitution on one α phosphate and substitution of a methylene group for the other α - β bridging oxygen (103), we observed His98 to be positioned to interact with the α - β bridging oxygen (71) and not an α phosphate oxygen like Gln168 of GalT (3). The distance between the His98 ϵN and the α - β bridging oxygen in the cocrystal structure of Fhit H96N with nonhydrolyzable ApppA was 2.5 Å (71), which is in the range of low barrier hydrogen bonds that are frequently found at enzyme active sites (104). As shown in Figure 4, we propose that the final His in HIT hydrolases is positioned to interact with the amine or ADP leaving group and the attacking water as a general acid–base catalyst. The other invariant His residue in HIT hydrolases, Hint His51/Fhit His35, is positioned to stabilize the proposed positive charge of Hint His114/Fhit His98 in the ground state. As we interpret our X-ray structures (7, 71), the exit path for the leaving group and the entry path for the hydrolytic water is between Hint His114/Fhit His98 and Hint Asn99/Fhit Gln83. Referring back to Figure 1B, which is a product complex between Hint and GMP (7), the α phosphate oxygen interpreted to result from water addition is the lower left oxygen shown between His114 and Asn99. In the case of Fhit, this mechanism also accounts for the catalytic roles for His35 and His98 in addition to the role of the nucleophilic His96, as defined by mutagenesis (40). Further, in an accompanying paper (52), we describe how alteration of the position of the proposed general acid–base catalyst, His114 in chicken Hint, in the proposed Hint-ASW heterodimer (48–50) may reduce the activity of the heterodimer substantially more than 2-fold.

Though we have structural but not kinetic evidence for Hint His114/Fhit His98 as a general acid–base, this mechanism is compelling for Hint because the amine product must be protonated to leave, and the incoming water would gain nucleophilicity while restoring His114 to an acid. In the case of Fhit, we hesitated to suggest that His98 protonates an ADP or ATP leaving group because the terminal phosphate has two negative charges in bulk solvent at pH 7. We now consider, however, that donation of a proton from His98 would render the terminal oxygen a poor nucleophile to reattack the α phosphate. In fact, a low-barrier hydrogen bond may be so favorable to form that the rate-limiting step in adenylylation may be getting the leaving group to take the proton and go. We have previously observed that substrates with fundamentally unaltered chemical lability such as ApppA, AppppA, ApppBODIPY, and GpppBODIPY are cleaved with increasingly poor rates, suggesting that slow substrates may prefer to reattack than to exit (42). Ultimately, the hydrogen bond network between His35 and His98 is

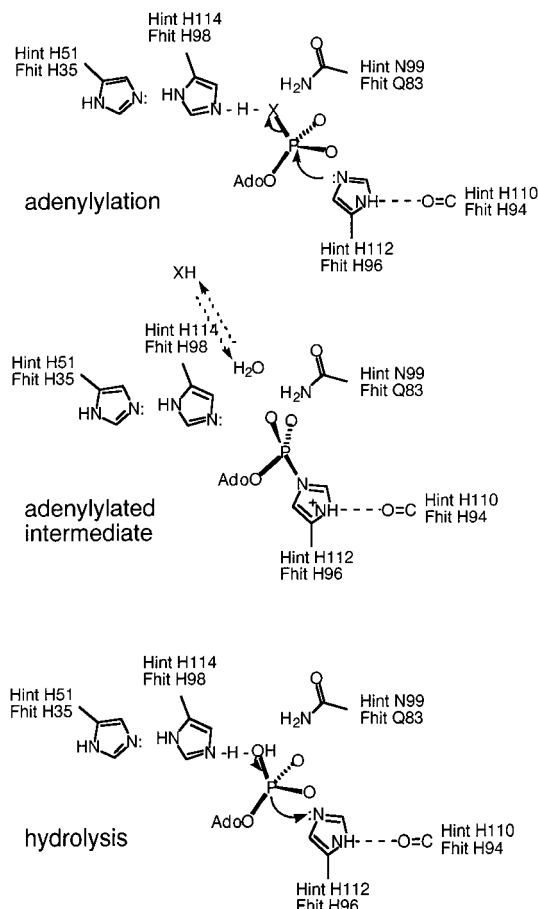


FIGURE 4: Proposed catalytic mechanism for HIT hydrolases. Hint and Fhit substrates are depicted as AMP-X and their leaving groups as XH. Upon adenylation of the middle His of the HIT motif, the atom at position X (a phosphoramidate N for Hint substrates and the α - β bridging O for Fhit substrates) receives a low barrier hydrogen bond from the C-terminal His of the HIT motif. Transfer of this proton is postulated to favor leaving over readenylation. The C-terminal His is then proposed to accept a low barrier hydrogen bond from the incoming water molecule that hydrolyzes the adenylylated intermediate.

postulated to promote transfer of the His98 N ϵ proton to the leaving group, rendering the adenylation reaction not immediately reversible and His98 basic to activate a hydrolytic water.

The most common galactosemic mutation in human *GALT*, Q188R, corresponds to the *E. coli* mutant Q168R that has been characterized and found to be reduced 1 million-fold in rates of uridylylation and deuridylylation (105). It will be interesting to characterize a site-directed Q168H mutant *in vitro* to see whether alteration of the HXHXQ $\phi\phi$ motif to a hydrolase-like HXHXH $\phi\phi$ motif is sufficient to produce some hydrolytic activity. Though deletions and mutations in *GALT* are not usually lethal once galactose is eliminated from the diet (86), it is possible that mutations in *GALT* that produce UDP-hexose hydrolase activity have not been observed because of the potentially lethal consequences of depleting UDP-glucose.

ACKNOWLEDGMENT

In preparing this review, it would be difficult to overstate the value of the collected works of Dr. Perry A. Frey and co-workers on GalT structure and function and the contribu-

tions of Drs. Preston Garrison and Larry D. Barnes on identifying enzymatic activities and the sequence and structural basis for function in the HIT superfamily. Additionally, I thank Drs. Dagmar Ringe, Charles P. Scott, Kay Huebner, Helen C. Pace, Keith W. Caldecott, and G. Michael Blackburn for helpful discussions.

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BI025942Q