Engineered Monomeric Human Histidine Triad Nucleotide-binding Protein 1 Hydrolyzes Fluorogenic Acyl-adenylate and Lysyl-tRNA Synthetase-generated Lysyl-adenylate*

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Hint1 is a homodimeric protein and member of the ubiquitous HIT superfamily. Hint1 catalyzes the hydrolysis of fluorogenic acyl-adenylate and lysyl-adenylate generated by lysyl-tRNA synthetase (LysRS). To determine the importance of homodimerization on the biological and catalytic activity of Hint1, the dimer interface of human Hint1 (hHint1) was destabilized by replacement of Val97 of hHint1 with Asp, Glu, or Arg. The mutants were shown to exist as monomers in solution by a combination of size exclusion chromatograph, static light scattering, and chemically induced dimerization studies. Circular dichroism studies revealed little difference between the stability of the V97D, V97E, and wild-type hHint1. Relative to wild-type and the V97E mutant, however, significant perturbation of the V97D mutant structure was observed. hHint1 was shown to prefer 3-indolepropionic acyl-adenylate (AIPA) over tryptamine adenosine phosphoramidate monoester (TpAd). Wild-type hHint1 was found to be 277- and 1000-fold more efficient (kcat/Km values) than the V97E and V97D mutants, respectively. Adenylation of wild-type, V97D, and V97E hHint1 by human LysRS was shown to correlate with the mutant kcat/Km values using 3-indolepropionic acyl-adenylate as a substrate, but not tryptamine adenosine phosphoramidate monoester. Significant perturbations of the active site residues were not detected by molecular dynamics simulations of the hHint1s. Taken together, these results demonstrate that for hHint1; 1) the efficiency (kcat/Km) of acylated AMP hydrolysis, but not maximal catalytic turnover (kcat), is dependent on homodimerization and 2) the hydrolysis of lysyl-AMP generated by LysRS is not dependent on homodimerization if the monomer structure is similar to the wild-type structure.

Histidine triad nucleotide-binding proteins (Hint)² are members of the histidine triad (HIT) protein superfamily of nucleotidyltransferases and hydrolyses (1). HIT proteins are named for the conserved nucleotide binding motif containing the sequence His-X-His-X-His-XX, in which X is a hydrophobic amino acid. The HIT proteins have been classified into three subfamilies according to their enzymatic function, sequence composition, and structural similarity: the Hint branch, the fragile histidine triad (Fhit) branch, and the galactose-1-phosphate uridylyltransferase (GaIT) branch (1).

The Hint branch is the most ancient and can be found in Archaea, Bacteria, and Eukaryotae. Hints are homodimeric proteins that have been found to be purine phosphorimidases (2–5). Recently, we have demonstrated that lysyl-adenylate (lysyl-AMP) generated by bacterial and human lysyl-tRNA synthetases (LysRS) are also substrates for the respective bacterial and human Hints (6). Escherichia coli hintT knock-out mutants exhibited salt-dependent cell growth, whereas HintT knock-out mice have an increased susceptibility to 7, 12-dimethylbenz[a]anthracene (DMBA)-induced ovarian and mammary tumors by the carcinogen DMBA and increased incidence of spontaneous tumors (2, 7, 8). In addition, Hint1 has been shown to have a potential role on cancer cells apoptosis and p53 expression (9). The mechanism(s) by which such a conserved protein as Hint effects such a variety of biological processes has not been fully elucidated. Eukaryotic Hint1 has been shown to associate with, and suppress the β-catenin Wnt signaling pathway transcriptional activity by direct interactions with Reptin and Pontin (10). Hint1 has also been associated with transcription factors such as, TFIIH (11), MITF (12, 13), and USF2 (14). In addition, the interactions between MITF and USF2 appear to be mediated by LysRS (13–15).

²The abbreviations used are: Hint, histidine triad nucleotide-binding protein; hHint1, human histidine triad nucleotide-binding protein 1; AIPA, adenosine 5′-indole-3-propionic adenylate; AMPPCP, adenosine 5′-([α,β-methylenediphosphate]; HIT, histidine triad; MTX, methotrexate; r.m.s. deviation, root mean square deviation; SEC, size exclusion chromatography; TpAd, tryptamine adenosine phosphorimidate monoester; ApA, diadenosine P1′,P3′-triphosphate; Fhit, fragile histidine triad; GaIT, galactose-1-phosphate uridylyltransferase; DHFR, dihydrofolate reductase.

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Monomeric Human Histidine Triad Nucleotide-binding Protein 1

The mechanism of the phosphoramidase and aminoacyl-AMP hydrolase activity catalyzed by Hints has been proposed to follow a reaction pathway similar to that for GalT and Fhit (1, 6). In the first step, the active site is nucleotidylated by nucleophilic attack at the substrate phosphorous by a conserved active site histidine (His151) for Fhit1. The nucleoside monophosphate product is subsequently released after water hydrolysis of the enzyme His-AMP intermediate. Evidence in support of formation of this intermediate with both the bacterial and human Hint has been obtained by radiolabeling experiments with LysRS and [α-32P]ATP (6). X-ray crystal structure and size exclusion chromatography analyses have revealed that HINT exists as a homodimer (2, 23, 24). Similar to other homodimeric enzymes, such as bacterial alkaline phosphatase, each monomer contains a well separated active site that does not participate in the dimer interface (25). Nevertheless, the C terminus of each monomer does form a range of contacts with the adjacent monomer in close proximity to the active site.

To the best of our knowledge no attempt has been made to characterize the role of protein dimerization on stability and catalysis of HINT, in general, and hHint1, in particular. Therefore, based on an analysis of the crystal structure of hHint1, we hypothesized that the replacement of an interface residue, Val97, with more sterically demanding and charged residues would allow the effect of destabilization of the dimer interface on stability and catalysis to be addressed. The Val97 to Asp, Glu, and Arg point site mutation were prepared characterized by CD spectroscopy, thermal stability, multianal static light scattering, size-exclusion chromatography (SEC), and molecular modeling. The effect of dimerization on the phosphoramidase hydrolase activity was assessed by steady-state kinetics, whereas the effect on acyl-AMP hydrolase activity was determined by steady-state kinetics with a new fluorogenic substrate. The effect of homodimerization on the hydrolysis of lysyl-AMP produced by LysRS was also determined.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—The DHFR fusion protein was constructed based on the pJLCF15DmH plasmid template previously constructed in our laboratory (2, 26). The pJLCF15DmH plasmid encodes a fusion protein containing a FLAG peptide at the N terminus of L54F-E. coli DHFR, linked by a 15- amino acid linker to hHint1. The following mutants were generated by using the QuikChange site-directed mutagenesis kit (Stratagene); the sequence of primers was listed in supplemental data (Table S1). The first mutagenesis was carried out to change the L54F back to wild-type DHFR with two primers (F54L-F and F54L-R). The FLAG peptide was deleted with primers (d-Flag-F and d-Flag-R) to generate pTFC15DH. The pTFC15DH was then truncated to a 7-amino acid linker with primers 7DH-F and 7DH-R to create wild-type pTFC7DH. The V97D-, V97E-, and V97R-pTFC7DH plasmids were generated with primers (V97D-F, V97D-R, V97E-F, V97E-R, V97R-F, and V97R-R) with wild-type plasmid pTFC7DH. V97D-pTFC7DH and V97E-pTFC7DH were generated with primers (V97D-F, V97D-R, V97E-F, and V97E-R) and with the pTFC15DH plasmid containing a thrombin-sensitive linker between DHFR and hHint1. Plasmids containing mutant DNA were sequenced by automated sequencing (Advanced Genetic Analysis Center, University of Minnesota).

Protein Expression and Purification—To avoid wild-type E. coli hinT contaminant, all expression plasmids were transformed into the E. coli hinT disrupted strain BB2 as previously described (2). Purification of wild-type fusion protein was published. Cell growth and cell lysate extractions were as previously described (26). The same purification procedure for each mutant was employed. The cell lysates containing overexpressed V97D-, V97E-, or V97R-hHint1-DHFR (7-amino acid linker) were loaded onto a methotrexate (MTX)-agarose column (Sigma) and the column was washed with buffer A (20 mM Tris, pH 7.0, 1 mM EDTA, 1 mM dithiothreitol, 100 ml) and 1 M NaCl in buffer A (800 ml), and the fusion protein was eluted with trimethoprim (150 μM in buffer A containing 1 M NaCl). Fractions (9 ml) were collected at a flow rate of 1 ml/min and an aliquot (10 μl) of each fraction was assayed for protein concentration with the Bradford dye reagent (Bio-Rad). Fractions containing more than 0.1 mg/ml of protein were analyzed by 4–12% SDS-PAGE (Invitrogen). Fractions containing more than 0.1 mg/ml of protein were analyzed by SDS-PAGE and assayed for phosphoramidase activity by fluorescence assay. The fractions containing the highest phosphoramidase specific activity were combined and concentrated with an Amicon stirred cell with a YM-10 membrane (Millipore). The protein solution was applied onto a DEAE column that had been equilibrated with buffer A. The column was eluted with 0–0.5 M NaCl gradient in buffer A. Fractions (9 ml) were collected at a flow rate of 1.5 ml/min. The absorbance at 280 and 260 nm of each fraction was determined. Aliquots (10 μl) of the selected fractions were analyzed by SDS-PAGE and assayed for phosphoramidase activity by fluorescence assay. The fractions containing the highest phosphoramidase specific activity were combined and concentrated with an Amicon stirred cell. To purify native V97D and V97E mutants, thrombin (10 units/mg) was used to cleave the corresponding DHFR-hHint1 fusion protein (15-amino acid linker), reaction mixture was applied onto a DEAE column. The column was eluted as described above. Glycerol was added to a final concentration of 10% and purified proteins were stored at −80 °C.

Molecular Mass from Static Light Scattering—Wild-type hHint1-DHFR (1 mg/ml, 31 μM, 500 μl) or V97D hHint1-DHFR (0.7 mg/ml, 22 μM, 500 μl) was subjected to size exclusion chromatography (Superdex G200, GE Healthcare) and eluted at a flow rate of 0.5 ml/min with P500 buffer (0.5 M NaCl, 50 mM potassium phosphate, 1 mM EDTA, pH 7.0, filtered through a 0.02-μm filter) and monitored by a in-line multianal light scattering detector with a power 690 nm argon laser light
source (DAWN EOS, Wyatt Technology), a refractive index detector (Altex), and a UV detector (Beckman Gold 166). Data were collected across a range of angles from 14.5 to 163.3 degrees: 14.5, 25.9, 34.8, 42.8, 51.5, 60, 69.3, 79.9, 100.3, 110.7, 121.2, 132.2, 142.5, 152.5, and 163.3 degrees. The instrument was calibrated with a molecular standard, bovine serum albumin sample (1 mg/ml, 500 μl). All samples were filtered with a 0.2-μm filter just prior to analysis. Molecular masses were calculated with the ASTRA software package (27).

Size Exclusion Chromatography—Protein samples were prepared in P500 buffer with 5% (v/v) glycerol. Samples were mixed in 200-μl vials (Chromtech). The molecular weight of recombinant purified fusion proteins was determined by analytical gel filtration chromatography on a Superdex G75 size exclusion column (GE Healthcare) and the proteins were eluted with P500 buffer. The retention time of proteins was monitored by absorbance at 280 nm with an in-line UV detector (Beckman Gold 166) and a PE1520 fluorescence detector (Jasco). The molecular standards used were blue dextran (200 kDa), albumin (66 kDa), DHFR(5-1DDG (36 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa), and aprotinin (6.5 kDa) (Sigma).

Circular Dichroism (CD) Spectroscopy—CD spectra of proteins were obtained at 10 °C with a J710 spectropolarimeter (Jasco) equipped with a temperature-controlled water bath. hHint1-DHFR fusion proteins at concentrations ranging from 3.2 to 5.4 μM in buffer A and native wild-type hHint1, V97D, and V97E proteins at 5 μM were analyzed in a quartz cuvette with path length of 1 mm, and spectra were accumulated and averaged over nine scans. The buffer background was subtracted from the protein spectrum with Excel. The CD signal at 207 or 222 nm was monitored as the temperature increased from 10 to 80 °C over 84 min to determine the thermal denaturation of wild-type and mutant of hHint1-DHFR and hHint1 proteins.

Synthesis of Adenosine 5′-Indole-3-propionic Adenylate (AIPA)—The title compound was synthesized according to the method by Berg (28), with a minor modification.3 Thus, to a cooled suspension (ice-bath) of indole-3-propionic acid (0.040 mM), DHFR2-1DDG (36 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa), and aprotinin (6.5 kDa) (Sigma).

Circular Dichroism (CD) Spectroscopy—CD spectra of proteins were obtained at 10 °C with a J710 spectropolarimeter (Jasco) equipped with a temperature-controlled water bath. hHint1-DHFR fusion proteins at concentrations ranging from 3.2 to 5.4 μM in buffer A and native wild-type hHint1, V97D, and V97E proteins at 5 μM were analyzed in a quartz cuvette with path length of 1 mm, and spectra were accumulated and averaged over nine scans. The buffer background was subtracted from the protein spectrum with Excel. The CD signal at 207 or 222 nm was monitored as the temperature increased from 10 to 80 °C over 84 min to determine the thermal denaturation of wild-type and mutant of hHint1-DHFR and hHint1 proteins.

Steady-state Kinetics—Steady-state kinetic studies were carried out with tryptamine 5′-adenosine phosphoramidate (TpAd) or AIPA as substrate and with the fluorescent activity assay as described (5). Excitation wavelength was set at 280 nm, fluorescence emission was measured at 360 nm, and excitation and emission slits were set at 10 nm for concentrations of substrates ranging from 0.05 to 1 μM for wild-type and 5 nm for concentrations ranging from 10 to 50 μM for V97D, V97E, and V97R mutants. Due to the inner filter effect, the highest substrate concentration within the linearity of fluorescence intensity is 50 μM. The fluorescence intensity was monitored for 2 min to obtain the baseline and allow the temperature to stabilize at 25 °C, and then enzyme (wild-type, 0.5 pmol; V97D mutant, 50 pmol; V97E mutant, 50 pmol; V97R mutant, 25 pmol) was added to initiate the reaction. The increase of fluorescence intensity was recorded for 2–10 min according to the rate of hydrolysis. The Michaelis-Menten constants, kcat (s⁻¹) and Km (μM), were determined by Jump IN nonlinear regression or double-reciprocal plots.

Assay of Ap3A and Ap4A Hydrolysis—A coupled assay was applied to determine the rate of hydrolysis of Ap3A and Ap4A as described for human Fhit (18). Initial rates were measured in a Cary 50 spectrophotometer at 25 °C. Ap3A or Ap4A (100 μM) was prepared in the assay solution and enzyme (500 pmol) was added to reaction mixture.

Molecular Modeling—A total of three molecular dynamics simulations were performed. The considered models were: wild-type monomer, V97E mutant monomer, and V97D mutant monomer. The initial coordinates were taken from the crystal structure of the hHint1 dimer, which was determined in complex with AMPPCP (Protein Data Bank code 1AV5) (24). All non-protein atoms of the model were deleted to study the unbound protein. The monomers were constructed by deleting the chain B of the original model. Mutations were performed using the biopolymer module of the Sybyl package (Sybyl 7.0). Simulations were carried out in the NPT ensemble, using the GROMACS package (29, 30) and the OPLS all-atom force field

3 A. Wolfson, personal communication.
developed for proteins (31). To remove bad contacts, the systems were first minimized in vacuum, using 1000 steps of the steepest descent algorithm. Next, each system was included in a rectangular box in which each edge of the box was placed at 0.9 nm away from the protein surface. The boxes were filled with pre-equilibrated SPC water molecules (32). The systems were neutralized with sodium counterions. From this point on, periodic boundary conditions and the Ewald summation method for long range electrostatics were applied (33). The LINCS algorithm was used to constrain the stretching modes involving hydrogen atoms (34). For each system, a simulation of the water molecules and counterions, at 300 K and 1 atm, was carried out to adjust them to the potential exerted by the protein, and to allow cavities on the surface of the protein to be filled. In this step, the protein atoms were kept restrained by using the conventional procedure of applying harmonic potentials. The equations of motion were integrated using the Verlet Leapfrog algorithm (35). Temperature and pressure were controlled using the Berendsen approach, in which external baths are weakly coupled (29). Each system was softly heated to 300 K using six blocks of simulation at constant pressure (1 atm) (36). Molecular dynamics simulations were started once the systems equilibrated at 300 K and 1 atm. Each simulation was performed using a time step of 0.002 ps and a total time of 10 ns.

RESULTS

Design of Monomeric Human Hint1—The homodimeric structure of hHint1 is shown in Fig. 1A with Val97 highlighted at the dimer interface. The interface encompasses 42% (4037 Å²) of the homodimer surface area and is composed of 12 residues residing on five β strands and two α helices (23, 24). Whereas the majority of the interfacial interactions are across a rather flat surface, the C-terminal loop of each protomer forms a series of bridging contacts with its partner in close proximity to the adjacent active site (Fig. 1B). Inspection of the interface revealed a set of core residues, Tyr94–Arg95–Met96–Val97–Val98–Asn99–Glu100 (Fig. 1C), that upon mutagenesis might lead to dimer destabilization. In particular, the hydrophobic interactions between the side chains of Val97 appeared to be strategically placed for the promotion of dimer destabilization when substituted with a larger, positively or negatively charged residue (Fig. 1D). The substitution of charged residues, such as Asp, Arg, and Glu into a protein dimer or oligomer interface has been shown to be a viable approach for preparing monomeric enzymes (25, 37–39).

Expression and Purification of Monomeric hHint1 Proteins—As previously described, we have developed a protein expression protocol for hHint1 as a fusion protein to E. coli DHFR (2). This approach facilitates the isolation of Hints that are unable to be purified by AMP affinity column by taking advantage of the binding of DHFR to a MTX affinity column. Wild-type hHint1-DHFR fusion protein was purified by an AMP affinity chromatography as described previously (2). However, the V97D, V97E, and V97R hHint1-DHFR mutants could only be isolated by MTX affinity chromatography, because initial attempts to use AMP affinity chromatography were unsuccessful. To remove small amounts of truncated fusion protein, the combined protein solution after MTX affinity chromatography...
Static light scattering and size exclusion experiments were carried out to evaluate the apparent molecular weight of fusion proteins followed by a DEAE column. Removal of the 15-amino acid linker was further purified with a DEAE anion exchange column. Even though SDS-PAGE indicated the homogeneity of the purified DHFR fusion proteins (data not shown) the analysis from SEC indicated the presence of a small amount of truncated V97E hHint1-DHFR (7%) and V97R hHint1-DHFR (22%) (data not shown). To obtain native V97D and V97E mutants, DHFR was removed by thrombin cleavage of the 15-amino acid linker fusion proteins followed by a DEAE column.

**Molecular Mass Determination by Static Light Scattering**—Static light scattering and size exclusion experiments were carried out to evaluate the apparent molecular weight of hHint1-DHFR fusion proteins. A Superdex G200 SEC column with in-line static light scattering detector was employed to assess the molecular weights of the wild-type and V97D hHint1-DHFR fusion proteins. As shown in Fig. 2A the wild-type protein is eluted as a single species with a molecular mass of 64.24 ± 0.07 kDa, consistent with the protein being a homodimer, whereas the V97D hHint1-DHFR mutant was found to be a monomer with a molecular mass of 40.2 ± 0.8 kDa (Fig. 2B).

**Size Exclusion Chromatography**—A Superdex G75 column with in-line UV and fluorescence detectors was employed to estimate the dissociation constant between two monomers. The wild-type hHint1-DHFR fusion protein eluted with a retention time of 18.5 min, and co-eluted with a molecular mass standard, bovine serum albumin (66 kDa, 18.7 min, data now shown) in good agreement with the theoretical mass of 64.2 kDa for the dimer. In contrast, the V97D, V97E, and V97R hHint1-DHFR mutants all eluted at 21.5 min, with our reference protein (DHFR$_{\text{e}}$-1DDG, mass 36 kDa, data not shown) in good agreement with the expected molecular mass for the monomeric protein (32.1 kDa). Typical SEC chromatograms of the wild-type, V97D, and V97E hHint1-DHFR proteins (5–6 μM) are shown in Fig. 3. Various lower concentrations (5–500 nM) of wild-type were examined (data not shown) and only dimer could be detected at a concentration as low as 5 nM (the detection limit); thus, the dissociation constant ($K_d$) of dimer formation for wild-type hHint1 is less than 5 nM. In contrast, higher concentrations of monomers were subjected to SEC analysis and only monomer could be detected at a concentration of 25 μM for V97E hHint1-DHFR and 20 μM for V97D hHint1-DHFR (data not shown); therefore, the $K_d$ of monomeric mutants is higher than 25 μM. Thus the $K_d$ for the wild-type and V97E hHint1-DHFR fusion proteins differs by at least a factor of 10$^4$. The native V97D and V97E hHint1 mutants were found to exist as monomers in solution (data not shown).

**Chemically Induced Dimerization**—Our laboratory has developed a system to study chemically induced protein dimerization of wild-type E. coli DHFR by bis-methotrexate ligand (Bis-MTX-C$_\text{a}$) (40, 41). To further confirm the existence of stable monomers in solution, the V97D hHint1-DHFR (5 μM) was mixed with various equivalents of chemical dimerizer (Bis-MTX-C$_\text{a}$, 0.1, 0.5, 1, and 2 eq), then subjected to Superdex G75 column chromatography as described previously (40, 41). The overlaid traces clearly demonstrated the amount of dimer formation increased with increasing amounts of added dimerizer (Fig. 4). The overlaid chromatograms of wild-type dimer, chemically induced dimer of V97D mutant, and monomorphic V97D mutant are shown in supplemental data Fig. 1A. Furthermore, the other monomorphic mutants V97E (5 μM) and V97R (2.5 μM) were mixed with 2 eq of Bis-MTX-C$_\text{a}$. The overlaid chromatograms of dimerized V97E and V97R mutants versus the corresponding monomers are displayed in supplemental data Fig. S1.

**Secondary Structure Analysis**—CD spectra of the wild-type and monomorphic mutants were determined in the far-UV region (190–260 nm). The result was expressed as the mean residue ellipticities. As can been seen in Fig. 5A, the CD spectra of the DHFR fusion protein for wild-type, V97D, V97E, and V97R enzymes are nearly superimposable and consistent with a nearly identical combination of α-helical and β-sheet secondary structural elements. The CD spectra of the recombinant wild-type, V97D, and V97E hHint1 were determined. The similar spectra observed for the wild-type and V97E mutant indicated that, whereas the α-helical structure of the mutant protein has likely been slightly decreased, overall the secondary structure of the mutant was not significantly perturbed relative to wild-type. However, the shift of the minimum point from 222 to 207 nm for V97D mutant indicates that the secondary structure has been significantly perturbed relative to wild-type, with the mutant protein likely containing a higher percentage of random coil content (Fig. 5B).

**Thermal Stability**—CD was also used to determine the thermal stability of the wild-type and mutant DHFR fusion proteins.
by monitoring the temperature-dependent change in ellipticity at 222 nm. The variable temperature CD spectra for the DHFR fusion protein of wild-type, V97D, V97E, and V97R enzymes are shown in Fig. 5 and for recombinant hHint1 proteins are shown in Fig. 5D. The transition midpoint temperatures are listed in supplemental data Table 2. The results indicated that each protein loses its structural integrity between 55 and 65 °C, clearly demonstrating that dimeric and monomeric hHint1-DHFR proteins have similar cooperative thermal transition. For the recombinant hHint1 proteins, wild-type and V97E mutant exhibited a similar Tm of 65 °C when the ellipticity at 222 nm was plotted against temperature. The V97D mutant displayed a similar Tm of 67.5 °C, when the ellipticity at 207 nm was plotted against temperature. However, the magnitude of the change was considerably larger, consistent with significant secondary structural differences between the V97D mutant and both wild-type and V97E mutant.

Steady-state Kinetic Characterization—The Michaelis-Menten constants, kcat and Km, were determined for hHint1-DHFR and recombinant hHint1 with the fluorescent phosphoramidate, TpAd (Table 1). Results indicated that DHFR did not appreciably affect the phosphoramidase activity of hHint1. Whereas the kcat values for wild-type and monomeric mutants were found to be similar, the Km values for V97D and V97E mutants were increased by 100- and 1000-fold, respectively. For V97E and V97R hHint1-DHFR fusion proteins, the Km values were found to be greater than 50 μM (the highest applicable substrate concentration due to fluorescence inner filter effect). Consequently, the kcat/Km values were calculated from the slope of the double reciprocal plots. The kcat/Km value for the V97E and V97R hHint1-DHFR mutants were found to be 104- and 105-fold less, respectively, than the value for wild-type (Table 1).

The Michaelis-Menten constants were also determined for recombinant wild-type, V97D, and V97E hHint1 proteins with the fluorescent acyl-adenylate, AIPA (Table 2). The kcat value for wild-type was found to be similar to that determined with TpAd. However, the Km value with AIPA was 3-fold lower than that observed for TpAd, resulting in a kcat/Km value of nearly 108 s⁻¹ M⁻¹ for AIPA. As observed for TpAd, the kcat for the V97D mutant was reduced by 5–6-fold. However, the kcat for the V97E mutant was only lowered 2-fold, relative to wild-type. In contrast, the Km values for AIPA and V97D and V97E mutants were increased by 110- and 128-fold, respectively, resulting in a 1000- and 277-fold decrease in kcat/Km. Consequently, although, similar efficiency of the V97D mutant catalysis was observed when the substrate was AIPA, wild-type and V97E mutant were found to prefer AIPA over TpAd by factors of 3.1 and 32, respectively.

Adenylation of Dimeric and Monomeric Hints by Human LysRS—Recently, we have demonstrated that hHint1 hydrolyzes lysyl-AMP generated by LysRS (6). Evidence for this reaction can be detected by observing the labeling of the hHint1-AMP intermediate formed during the reaction after treatment with LysRS and 32P-labeled ATP (6). As can be seen in Fig. 6, the hHint1 labeling experiments (lanes 2, 5, and 8) revealed that V97D and V97E mutant adenylation was at least 10- and 2-fold lower than wild-type, respectively. When product inhibition of lysyl-AMP formation by LysRS was relieved by the addition of pyrophosphatase, the adenylation of the V97E mutant was found to be comparable with that for wild-type, whereas the
labeling of V97D mutant was still ~2-fold lower than that observed for wild-type or V97E mutant. These results are consistent with the rank order of $k_{\text{cat}}$ values for the hHint1 proteins.

*Molecular Modeling*—Three molecular dynamics simulations were carried out on the wild type-hHint dimer, and the V97E and V97D monomers. To analyze the stability of the trajectories we calculated the root mean square (r.m.s.) deviation related to the initial structure (crystal structure of the hHint1 dimer, PDB code 1AV5) (24). The r.m.s. deviation was calculated using only $\alpha$-carbon atoms. The wild-type dimer showed low r.m.s. deviation values, indicating that no significant conformational changes have occurred during the simulation. For the V97E and V97D monomers, pronounced conformational changes were observed. For the monomers, the r.m.s. deviation stabilized after ~6 ns, with the r.m.s. deviation for V97E monomer reaching values greater than 0.3 nm. A large percentage of the effect on the r.m.s. deviation appeared to be due to the flexibility of the N and C termini and not core residues. When the r.m.s. deviation is re-calculated, excluding the first 10 and last 10

**FIGURE 4.** The chemically induced dimer of the monomeric hHint1-DHFR. SEC profiles for V97D hHint1-DHFR (5 $\mu$m) with 0, 0.1, 0.5, 1.0, and 2.0 eq added dimerizer, Bis-MTX, are overlaid. Data were collected in sequential runs, beginning with the 5 $\mu$m V97D hHint1-DHFR sample. The monomer peaks near 21.3 min decreased as the concentration of added dimerizer increased.

**FIGURE 5.** Circular dichroism spectroscopy. The spectra were collected from 190 to 260 nm for: A, DHFR fusion proteins of wild-type, V97D, V97E, and V97R hHint1; B, wild-type, V97D, and V97E hHint1; C, the spectra were collected at 222 nm with variable temperature ranging from 10 to 80 °C for DHFR fusion protein; and D, the spectra were collected at 222 nm with variable temperature for wild-type and V97E mutant and at 207 nm for V97D mutant.
residues, wild-type and V97E mutant were found to be most similar, with the greatest deviation being exhibited by V97D mutant.

Superimposition of the average structure of the wild-type dimer fitted to the crystal structure (PDB code 1AV5) revealed no significant structural changes (supplemental data Fig. S2). When the average structures of the mutant monomers and chain A of the wild-type dimer were superimposed, the β-sheet of the V97E mutant was shown to overlap with that of wild-type, whereas small differences between the V97D β-sheet and the wild-type were noticeable (Fig. 7A). The r.m.s. deviation relative to the wild-type for a superimposition considering residues 28 to 98 was 0.111 nm for the V97E mutant and 0.140 nm for the V97D mutant, demonstrating that for the more rigid structure, V97E mutant is modestly more similar to wild-type than the V97D mutant. Differences in the conformation of the loops surrounding the active site and the highly flexible N and C termini were observed. For both mutants, the induction of hydrogen bonding and ionic pairing between either the substi-

### TABLE 1
Comparison of steady-state kinetic parameters of hydrolysis of a fluorogenic phosphoramidate substrate, TpAd monoester, for wild-type and monomeric hHint1 and hHint1-DHFR fusion proteins.

Data were obtained at 25 °C in HEPE buffer (20 mM HEPES, pH 7.2, 1 mM MgCl2). Measurements were carried out in duplicate and variants are given as standard deviation.

* ND, not determined.

| Enzyme            | K_m (μM) | k_cat (s⁻¹) | k_cat/K_m (s⁻¹ M⁻¹) | Ratio of (k_cat/K_m) (Mutant/wild-type) |
|-------------------|----------|-------------|---------------------|---------------------------------------|
| Wild-type hHint1  | 0.13 ± 0.01 | 2.3 ± 0.1   | (1.7 ± 0.3) × 10⁷   | 1                                     |
| Wild-type hHint1-DHFR | 0.13 ± 0.01 | 3.15 ± 0.01 | (2.4 ± 0.1) × 10⁷   | 1.4                                   |
| V97D hHint1       | 10.7 ± 0.6  | 0.5 ± 0.01  | (4.7 ± 0.3) × 10⁴   | 2.7 × 10⁻³                             |
| V97D hHint1-DHFR  | 23 ± 2     | 0.59 ± 0.02 | (2.6 ± 0.3) × 10⁴   | 1.5 × 10⁻³                             |
| V97E hHint1       | 116 ± 31   | 0.71 ± 0.14 | (6.0 ± 3.0) × 10³   | 3.5 × 10⁻⁴                             |
| V97E hHint1-DHFR  | ND*       | ND*         | (3.5 ± 0.1) × 10³   | 2.0 × 10⁻⁴                             |
| V97R hHint1-DHFR  | ND*       | ND*         | (3.0 ± 0.5) × 10²   | 1.8 × 10⁻⁵                             |

### TABLE 2
Comparison of steady-state kinetic parameters of hydrolysis of a fluorogenic adenylate substrate, AIPA, for wild-type and monomeric hHint1.

Data were obtained at 25 °C in HEPE buffer. Measurements were carried out in duplicate and variants are given as standard deviation.

| Enzyme            | K_m (μM) | k_cat (s⁻¹) | k_cat/K_m (s⁻¹ M⁻¹) | Ratio of (k_cat/K_m) (Mutant/wild-type) |
|-------------------|----------|-------------|---------------------|---------------------------------------|
| Wild-type hHint1  | 0.04 ± 0.002 | 1.98 ± 0.02 | (5.3 ± 0.5) × 10⁷   | 1                                     |
| V97D hHint1       | 4.4 ± 0.5  | 0.23 ± 0.01 | (5.3 ± 0.1) × 10⁴   | 10⁻³                                  |
| V97E hHint1       | 5.1 ± 0.5  | 0.97 ± 0.03 | (1.9 ± 0.3) × 10⁵   | 3.6 × 10⁻³                             |
tuted Glu97 or Asp97 side chain and Arg95 was the major new interaction observed (data not shown). Nevertheless, relative to wild-type, V97E and V97D exhibited little difference in positioning of the catalytic residues, His51, His110, His112, and His114 (Fig. 7B). A slightly more perturbed orientation was observed for His114 of the V97D mutant. However, this appears likely to be due to slight differences in the position of the backbone and not to a direct interaction with the mutated side chain.

**DISCUSSION**

The physiological role of the Hint protein has not yet been determined, even though it is the most ancient and conserved branch of the HIT protein superfamily. To date, all of the members of the HIT superfamily have been shown to exist as homodimers (2, 22, 24, 42). Whereas the native function of Hints remains unknown, the, rabbit, human, yeast, and bacterial proteins have been shown to be purine nucleoside phosphoraminases (2, 3). Site-directed mutagenesis and x-ray crystallographic studies have revealed the hHint1 nucleotide-binding sites, which are positioned ~180° from each other and 25 Å apart (Fig. 1A) (23, 24). Consequently, the dimer interface does not contribute residues to the respective active sites, with one exception. The C terminus of each protomer forms a loop that protrudes across the interface and places Trp123 at the far end of the adjacent active site (Fig. 1, A and B).

After replacing the selected hot spot on the dimer interface with large and charged residues, Asp, Glu, or Arg, the overexpressed mutants lost their ability to bind to the AMP affinity column; thus, the MTX affinity column was in turn used to purify mutants. A combination of evidence from size exclusion chromatography and multiangle static light scattering, a well known method for measurement of absolute molecular weight of macromolecules in solution (27, 39), revealed that all three mutants were successfully destabilized to form monomers in solution (Figs. 2 and 3). These results were further supported by utilizing a Bis-MTX-C9 ligand to dimerize the existing monomers (Fig. 4, and supplemental data Fig. 1). The detailed design of this interesting chemically induced protein dimerization method was published previously (40) and first applied here to prove the existence of monomeric mutants.

To probe the effects of hHint1 monomerization on protein structure, the V97D and V97E mutants of hHint1-DHFR and hHint1 were characterized by CD spectroscopy (16). Little difference in the CD spectra or thermal stability between the wild-type hHint1-DHFR and the mutant fusion proteins was observed (Fig. 5, A and C). Because it is possible that changes in the secondary structure of hHint1 could be masked by DHFR, we carried out analyses of the recombinant versions of wild-type hHint1 and the monomeric mutants, V97D and V97E. CD spectra for both the wild-type and V97E mutant exhibited similar minima. A larger structural perturbation was observed for the V97D mutant, because the mean residue ellipticities minimum for the mutant shifted from 222 to 207 nm. This change is consistent with a possible increase in random coil content. In addition, although the thermal stability of the wild-type, V97D, and V97E mutants were similar, the degree of change for the
V97D mutant was considerably larger than for either wild-type or V97E mutant. Further indicating significant structural perturbations by the aspartic acid substitution.

Consistent with the structural studies, both for TpAd and AIPA, the $k_{cat}$ value for V97E mutant was closer in value to wild-type than the $k_{cat}$ value for V97D mutant (Table 2). However, the TpAd $K_m$ value for V97E and V97D mutants was found to be significantly higher than the corresponding AIPA $K_m$ values. Nevertheless, relative to wild-type, the AIPA $K_m$ values were found to have increased by greater than 100-fold. As a consequence, the monomeric hHint1 proteins are less efficacious enzymes than the wild-type hHint1 by factors ranging from 1000 (V97D mutant and AIPA) to 2833 (V97E mutant and TpAd) (Tables 1 and 2). Therefore, the catalytic efficiency of the mutants with model substrates was substantially reduced. Differences were observed for the selectivity of the mutants and the phosphoramidate or acyl-adenylate substrates. For TpAd, the order of efficiency was wild-type > V97D mutant > V97E mutant. In contrast, the order of efficiency for the more native-like substrate, AIPA, was wild-type > V97E mutant > V97D mutant.

Whereas a native phosphoramidate substrate has yet to be identified, we have shown, recently, that hHint1 is fully capable of catalyzing the hydrolysis of lysyl-adenylate produced by LysRS (6). We have demonstrated that this reaction is dependent on the adenylation of active site residue His$^{112}$. Formation of an exchangeable and hydrolytically unstable enzyme-AMP intermediate could be readily observed by treatment of hHint1 with [$\alpha^{-32}$P]ATP, lysine, and LysRS. Protein labeling was not observed when His$^{112}$ was replaced by either glycine or alanine. Consequently, we chose to examine the role of hHint1 dimerization on the hydrolysis of lysyl-AMP generated by LysRS (Fig. 6). Consistent with the AIPA kinetic results, the extent of hHint1-AMP formation by wild-type was 2-fold greater than that for the V97E mutant and 10-fold greater than for V97D mutant. When pyrophosphatase was added to reduce the level of PP$_i$ product inhibition, the discrepancy between the wild-type and V97E mutant vanished, whereas the amount of V97D mutant labeling was still found to be less than that for both the wild-type and V97E mutant. The degree to which the kinetic parameters for the model acyl-AMP substrate, AIPA, accurately reflect these single time point experiments will require further investigation. Nevertheless, as observed for model substrate hydrolysis, although hHint1 dimerization is not required for lysyl-AMP hydrolysis, the reaction is dependent on monomer structure.

To develop a rationale for the difference in the mutant, catalytic efficiency and structure, we carried out a series of molecular dynamic simulations of the V97D and V97E monomers and compared them to the simulations of both the wild-type dimer and monomer. Although, the overall structure of the V97E mutant more closely resembled wild-type, significant differences in the final structures of the two monomers were not observed, particularly with regard to the active site residues. Taken together, these results may indicate that the observed structural perturbations necessary for the decreased catalytic efficiency of the V97D mutant, relative to the V97E mutant, are quite subtle.

Whereas it appears that Hint dimerization is a pre-requisite for efficient phosphoramidate and acyl-adenylate hydrolysis, why hHint1, unlike Fhit, does not carry out Ap$_4$A and Ap$_3$A hydrolysis is not apparent. Upon close inspection of the structures of both hHint1 and Fhit, the C-terminal loop of hHint1 could be shown to occupy a potential binding site for the substrate for Fhit, Ap$_3$A. Therefore, we hypothesized that removal of the interaction with the C terminus of the other monomer might rescue the Ap$_3$A hydrolase activity of hHint1. Nevertheless, no activity (<0.0002 s$^{-1}$) for either wild-type or monomeric enzymes was observed. Consequently, although binding studies claim to have shown direct binding of Ap$_3$A to hHint1, the removal of the sterically bulky C-terminal loops does not appear to rescue the potential Ap$_3$A or Ap$_4$A hydrolysis activity (13).

To conclude, whereas the hHint1 dimer interface does not contribute to significant stabilization of each protomer, it is
probably necessary to maintain a tightly associated dimer to enhance catalytic efficiency through the contributions of the C-terminal loop of one monomer to the active site of the other. Ongoing steady-state and pre-steady state kinetic studies to delineate the kinetic and catalytic mechanism of hHint1 with model substrates and aminoacyl-tRNA synthetases will allow the contribution of the C termini and dimerization on substrate binding and active site adenylation and deacylation to be determined (Fig. 8). The identification of V97E as a catalytically functional and largely structurally unperturbed monomeric version of hHint1 should allow the contribution of dimerization and catalytic activity on the native function of hHint1 to be characterized. In principle, with the exception of the GaIT branch, a similar approach could be used to investigate the importance of homodimerization on other members of the HIT superfamily, such as Fhit, that do not rely on an active site composed of catalytic residues contributed from each monomer.

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