Essential Roles of Zinc Ligation and Enzyme Dimerization for Catalysis in the Aminoacylase-1/M20 Family*

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Holger A. Lindner‡, Vladimir V. Lunin, Alain Alary, Regina Hecker, Mirosław Cygler, and Robert Ménard

From the Biotechnology Research Institute, National Research Council of Canada, Montréal, Québec H4P 2R2, Canada

Members of the aminoacylase-1 (Acy1)/M20 family of aminoacylases and exopeptidases exist as either monomers or homodimers. They contain a zinc-binding domain and a second domain mediating dimerization in the latter case. The roles that both domains play in catalysis have been investigated for human Acy1 (hAcy1) by x-ray crystallography and by site-directed mutagenesis. Structure comparison of the dinuclear zinc center in a mutant of hAcy1 reported here with dizinc centers in related enzymes points to a difference in zinc ligation in the Acy1/M20 family. Mutational analysis supports catalytic roles of zinc ions, a vicinal glutamate, and a histidine from the dimerization domain. By complementing different active site mutants of hAcy1, we show that catalysis occurs at the dimer interface. Reinterpretation of the structure of a monomeric homolog, peptidase V, reveals that a domain insertion mimics dimerization. We conclude that monomeric and dimeric Acy1/M20 family members share a unique active site architecture involving both enzyme domains. The study may provide means to improve homologous carboxypeptidase G2 toward application in antibody-directed enzyme prodrug therapy.

Zinc peptidases play roles in metabolic and signaling pathways throughout all kingdoms of life. A growing number of these enzymes have been found to contain two zinc ions at their active sites. Some are regarded as potential pharmaceutical targets (1). Recently, Wouters and Husain (2) pointed out that members of the MH and MF clans1 of dizinc peptidases, together with the MC clan of monozinc peptidases display three different catalytic zinc centers that have evolved in a similar structural scaffold, which is exemplified by carboxypeptidase A of clan MC. Although they all appear to employ the same general base-like catalytic mechanism, neither all catalytic residues nor the substrate-binding sites are conserved among the three clans. A glutamate residue representing a putative catalytic base, for instance, resides in different regions of the polypeptide chain in the MH and MC clans. In the MF clan enzyme leucine aminopeptidase, a bicarbonate ion replaces the glutamate residue. Moreover, whereas the monozinc center in the MC clan is structurally equivalent to one of the two zinc-binding sites in the dinuclear zinc center in the MH clan, it does not share any homology with the dinuclear zinc center in the MF clan anymore. As a consequence, members of families from each clan must be examined individually in order to gain a detailed understanding of their catalytic machineries.

Aminoacylase-1 (Acy1; EC 3.5.1.14) was discovered in 1881 by virtue of its ability to hydrolyze hippuric acid in crude kidney homogenates (4) and is now classified in the M20 family of clan MH, also referred to as the Acy1 family (5). Acy1 plays a general role in the cytosolic breakdown of N\(^{\text{acetyl}}\)-acylated amino acids (6) generated during protein degradation (7). Other functional aminoacylase enzymes from the Acy1/M20 family (Table I) are implicated in the bacterial biosynthesis pathways of arginine (N\(^{\text{acetylnorlthione}}\) deacetylase), lysine, and the cell wall (succinyldiaminopimelate desuccinylase) (8). Several enzymes of the Acy1/M20 family are known as exopeptidases (Table I). They include CPG2 (9–11), Saccharomyces cerevisiae carboxypeptidase Y (12), PepT and the dipeptidase enzyme PepV from various bacterial sources (13–15), Escherichia coli X-His dipeptidase (16), and human nonspecific dipeptidase and a brain-specific carboxinase that possibly plays a role in aging and neurodegenerative or psychiatric diseases (17). The enzymatic function of a related drought-induced polypeptide-1 from wild watermelon (Citrullus lanatus) remains unknown (18).

Enzymes of the Acy1/M20 family have shown potential for different applications. In biocatalysis, the high stereoselectivity of Acy1 allows the preparation of L-amino acids from racemic mixtures of N-acyl-L-amino acids (19). Succinylidiaminopimelate desuccinylase is considered as a potential anti-bacterial target (1), and CPG2 is considered a therapeutic agent in ADEPT for cancer treatment (18). However, compared with the well characterized active sites of AAP (21) and SGAP (22) from the M28 family of clan MH (Table I), relatively little is known about the Acy1/M20 family. The crystal structures of two Acy1 homologs, CPG2 (23) and PepT from Salmonella typhimurium (24), are each folded into a metal-binding domain and a smaller dimerization domain, which is inserted in the middle of the sequence of the metal-binding domain. In the structures of both enzymes, the two domains display an open conformation. Porcine Acy1 was shown by limited proteolysis to have a closely similar domain structure (25, 26). The metal-binding domains in CPG2 and PepT exhibit high structural similarity to the two single-domain proteins AAP and SGAP.

1 This report follows the MEROPS classification of peptidases (available on the World Wide Web at merops.sanger.ac.uk) (3). This system groups peptidases with significant sequence similarity into a family and assigns families of common evolutionary origin to a clan.

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1 The abbreviations used are: Acy1, aminoacylase 1; CPG2, Pseudomonas sp. carboxypeptidase G2; PepT, amino tripeptidase T; PepP, peptidase V; ADEPT, antibody-directed prodrug therapy; AAP, Aeromonas proteolytica aminopeptidase; SGAP, Streptomyces griseus aminopeptidase; hAcy1, human Acy1; r.m.s., root mean square.
Ants were expressed in a baculovirus expression vector system and versions of the mutagenic primers are listed in Table II. The sequences including Acy1, appear to exist as homodimers (8, 17, 26 and are thought to be responsible for catalysis. Besides CPG2, AspPepV was determined in a complex with an inhibitor, main and is referred to as the lid domain. The structure of additional domain in PepV is inserted in the zinc-binding do-

S. cerevisiae
delbrueckii
catalysis occurs at the dimer interface.

EXPERIMENTAL PROCEDURES
Site-directed Mutagenesis—Site-directed mutagenesis was performed on the baculovirus transfer vector pVL1393-hAcI (30) using the QuikChange™ XL site-directed mutagenesis kit (Stratagene). Forward versions of the mutagenic primers are listed in Table II. The sequences of the mutant hAcy1 genes in the resulting transfer vectors were confirmed by DNA sequencing.

Enzyme Expression and Purification—Wild-type hAcy1 and its vari-

Representative enzymes of the M20 and M28 families

| Enzyme | MEROPS identifier |
|--------|-------------------|
| N-Acylamino acid amidohydrolases | |
| Human aminoacylase-1 (N-Acylamino acid amidohydrolase) | M20.973 |
| Bacillus N-acetylornithine deacetylase | M20 nonpeptidase homologue |
| Bacillus succinylidaminopimelate desuccinylase | M20 nonpeptidase homologue |
| Carboxypeptidases | |
| Pseudomonas sp. carboxypeptidase G2 | M20.001 |
| S. cerevisiae carboxypeptidase Y | M20.002 |
| Human glutamate carboxypeptidase II | M28.010 |
| Amino tripeptidases and dipeptidases | |
| Bacterial amino tripeptidase T | M20.003 |
| Bacterial peptidase V | M20.004 |
| E. coli X-His dipeptidase | M20.007 |
| Human nonspecific dipeptidase | M20.005 |
| Human carnosinase | M20.006 |
| A. proteolytica aminopeptidase | M28.002 |
| S. griseus aminopeptidase | M28.003 |
| Specificity unknown | |
| Citrullus lanatus drought-induced polypeptidase | M20 nonpeptidase homologue |

The first part of the MEROPS identifier indicates the family assign-

Assays of Enzyme Activity and Stability—Acy1 activity was deter-

as described before (30). Kinetic data were evaluated by nonlinear regression anal-

As a measure of stability, the “melting temperature,” Tm, and the denaturation energy, ΔGH2O, of each enzyme variant were deter-

Structure-based Alignment and Structure Comparison—Structure-

The atomic coordinates for the crystal structure of this protein are available in the Protein Data Bank (http://www.rcsb.org/pdb) under PDB number 1Q7L (40).
of peptidases from the MH clan: CPG2, PepT, PepV, AAP, and SGAP. The models of the compared proteins were fitted, and r.m.s. calculations for Cα atoms were carried out using the Swiss-PDBViewer (41). For some secondary structure elements, additional local fitting was performed manually.

Figs. 1, 2, and 3B were made with Raster3D (available on the World Wide Web at www.bmsc.washington.edu/raster3d) (42).

RESULTS AND DISCUSSION

Structure of the Zinc-binding Domain in hAcy1—During crystallization screening of different mutants of hAcy1, which are under investigation in our laboratory, only the T347G mutant gave crystals. Deposition of an intervening sequence, from residue 199 to 320, corresponding to the dimerization domain was observed, and only the large zinc-binding domain remained. The deposition occurred only after the protein was concentrated to 5–8 mg/ml for the crystallization trials. The intact T347G mutant only showed a 6.5-fold increase in [S]₀.₅ and a reduction of Tm by 7 °C (Table V), indicating that the mutation did not affect the catalytic machinery.

The crystal structure shows two molecules in the asymmetric unit with residues 7–198 and 321–408 well defined in molecule A (Fig. 1) and residues 8–197 and 321–405 in molecule B. The remaining domain adopts a typical carboxypeptidase A fold (43). It consists of an eight-stranded mixed β-sheet, flanked by three short α-helices on one side and five long α-helices on the other side. The model also contains 665 water molecules in the asymmetric unit and two zinc ions per molecule. The model shows good overall quality (see “Experimental Procedures”).

The two molecules in the asymmetric unit are virtually identical and superimpose with a r.m.s. deviation of 0.33 Å for 273 Cα atoms. In the following, we only refer to molecule A.

His80, Asp113, Glu148, Glu175, and His273 define a dinuclear zinc-binding site per molecule (Fig. 2). Each zinc ion is bound in pentadentate coordination to the side chains of a histidine, a glutamate, and a bridging aspartate residue. Furthermore, an additional small ligand molecule bridges the two zinc ions. Since the crystallization drop contained 10 mM 1-norleucine, we interpreted the electron density of the ligand as a norleucine with a disordered side chain and modeled it as a glycine (Fig. 1).

Zinc Centers in the Acy1/M20 and M28 Families—The overall structure of the zinc-binding domain in the T347G mutant of hAcy1 (Fig. 1) is similar to the structures of the corresponding domains in the related enzymes CPG2, S. typhimurium PepT, and L. delbrueckii PepV as well as of the two single-domain enzymes AAP and SGAP from the M28 family. There are between 130 and 190 residues (of 280 residues) in our models, compared with 170 in the other proteins.

| Mutant | Predicted role in hAcy1 | Oligonucleotide sequence* |
|--------|-------------------------|--------------------------|
| E148A  | Zinc binding            | 5′-GGCGTAGGGGCGTGTGACCC-3′ |
| H373A  | Zinc binding            | 5′-CCGATCCGGGACGCGGAGCT-3′ |
| H580A  | Zinc binding            | 5′-CTCAACCACGCGGCATGAGG-3′ |
| E175A  | Zinc binding            | 5′-GGCTTTGGCCGGCTGAGGACAC-3′ |
| D113A  | Zinc binding            | 5′-GGGATCCGGGACGCGGAGCT-3′ |
| E147A  | General base            | 5′-CCGATCCGGGACGCGGAGCT-3′ |
| E147Q  | Catalytic               | 5′-GGGATCCGGGACGCGGAGCT-3′ |
| E147D  | Catalytic               | 5′-GGGATCCGGGACGCGGAGCT-3′ |
| H206N  | Catalytic               | 5′-GGGATCCGGGACGCGGAGCT-3′ |
| T347G  | Catalytic               | 5′-GGGATCCGGGACGCGGAGCT-3′ |

* Altered nucleotides are underlined.

** Table III

X-ray diffraction data collection statistics

| Data set | Hg-Peak | Native |
|----------|---------|--------|
| Wavelength | 1.0057 | 0.979700 |
| Resolution range (Å) | 50–1.5 | 50–1.4 |
| Last shell (Å) | 1.55–1.50 | 1.45–1.40 |
| Rmerge (%) | 0.074 | 0.056 |
| Last shell | 0.344 | 0.503 |
| Completeness | 95.8 | 98.4 |
| Last shell | 81.9 | 85.9 |
| No. of reflections | 529,534 | 618,690 |
| Unique reflections | 158,345 | 103,337 |

** Table IV

Refinement and overall crystal structure statistics

| Used data set | Native |
|---------------|--------|
| Resolution range (Å) | 50.9–1.4 |
| R-factor (Rmerge) (%) | 13.3 (17.2) |
| No. of non-hydrogen protein atoms | 4443 |
| No. of water molecules | 665 |
| Average B-factor for chain A (B) (Å²) | 11.4 (12.8) |
| Side chain atoms | 14.3 (15.8) |
| Water molecules | 25.5 |
| Metal ions | 10.7 |
| Substrate molecules | 16.1 |
| r.m.s. deviation bond length (Å) | 0.023 |
| r.m.s. deviation bond angle (degrees) | 1.890 |
| Ramachandran plot |
| Residues in most favorable regions (%) | 89.5 |
| Residues in additional regions (%) | 10.1 |
| Residues in disallowed regions (%) | 0.0 |

** Table II

Forward versions of mutagenic primers

| Oligonucleotide sequence* |
|--------------------------|
| 5′-GGCGTAGGGGCGTGTGACCC-3′ |
| 5′-CCGATCCGGGACGCGGAGCT-3′ |
| 5′-CTCAACCACGCGGCATGAGG-3′ |
| 5′-GGCTTTGGCCGGCTGAGGACAC-3′ |
| 5′-GGGATCCGGGACGCGGAGCT-3′ |

* Altered nucleotides are underlined.
Nevertheless, the zinc-zinc and most of the zinc-ligand distances in the dinuclear centers are similar among the compared structures (Table VI). The zinc 1-ligand distances in PepT, however, are 0.3–0.8 Å longer than in the other enzymes. This suggests that zinc 1 in PepT may show higher Lewis acidity.

An analysis of the Acy1/M20 sequence family revealed that all homologs with proven aminopeptidase or dipeptidase specificity, including PepT, PepV, X-His dipeptidase, and mammalian nonspecific dipeptidase and carnosinase (15, 17, 44) (Table I), contain an aspartic acid in this zinc ligand position. On the other hand, Acy1/M20 family members that exhibit either aminopeptidase AAP and SGAP and also in human glutamate carboxypeptidase II, also known as prostate-specific membrane antigen (46).

Characterization of hAcy1 Mutants—All mutants of hAcy1 generated during this work (see Table II) showed the same expression and purification behavior as reported for the wild-type enzyme (30). They also revealed the same electrophoretic mobility as the wild-type enzyme in SDS and native polyacrylamide gels (not shown). Effects of the mutations on protein stability and catalytic activity were investigated. The stabilization energy, \( \Delta G(H_2O) \), was determined by recording the loss of cumulative tryptophan fluorescence after equilibrium denaturation with guanidine hydrochloride and therefore represents the overall protein stability (Table V). The “melting temperature,” \( T_m \), was determined by monitoring enzymatic activity and therefore reflects loss of active site integrity (Table V). The transition curves obtained during the stability measurements indicate a folded state for all but one of the mutant enzymes. The E147D mutant became increasingly unstable at low denaturant concentrations so that \( \Delta G(H_2O) \) could not be determined quantitatively.

Kinetic measurements were performed using \( N^\beta\text{-actyl-}L\text{-methionine as enzyme substrate (Table V). The same relative values for kinetic parameters were obtained with } N^\beta\text{-formyl-}L\text{-norleucine as the substrate (not shown). All mutations discussed below resulted in a reduction of the catalytic constant, } k_{cat}, \text{ by 3–4 orders of magnitude. No activity could be detected for the E147D mutant. The substrate concentration at half-saturation, which throughout is given by the } [S]_{0.5} \text{ or } K_m \text{ values}

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### Table V

| hAcy1 variant | \( k_{cat} \) | \( [S]_{0.5} \) | Hill coefficient (n) | \( \Delta G(H_2O) \) | \( T_m \) |
|---------------|--------------|----------------|----------------------|----------------|---------|
| Wild type     | 38.3 ± 0.8   | 0.43 ± 0.03    | 1.16 ± 0.07          | 18.7 ± 0.10    | 64      |
| T347G         | 46.0 ± 0.77  | 2.78 ± 0.11    | 1.28 ± 0.04          | ND             | 57      |
| E147A         | (10 ± 0.3)\times 10^{-3} | 1.06 ± 0.11 | 18.0 ± 0.50 | 61 |
| E147G         | (4.43 ± 0.1)\times 10^{-3} | 0.71 ± 0.06 | 13.2 ± 0.10 | 64 |
| E147D         | ND           | NM             | 14.8 ± 0.24          | 45             |
| H80D (Zn2)‡  | (15.89 ± 0.75)\times 10^{-3} | 2.02 ± 0.30 | 1                  | 16.0 ± 0.20    | 50      |
| D113A (Zn1)‡ | (2.19 ± 0.01)\times 10^{-3} | 0.57 ± 0.10 | 1                  | 11.5 ± 0.10    | 46      |
| E148A (Zn1)‡ | (52.5 ± 3.9)\times 10^{-3} | 2.96 ± 0.05 | 1                  | 11.5 ± 0.10    | 46      |
| E175A (Zn2)‡ | (0.84 ± 0.02)\times 10^{-3} | 0.71 ± 0.05 | 1.16 ± 0.08         | 14.9 ± 0.10    | 47      |
| H373A (Zn1)‡ | (1.35 ± 1)\times 10^{-3} | 2.82 ± 0.30 | 1                  | 16.0 ± 0.20    | 50      |
| H206N‡        | (15.2 ± 0.4)\times 10^{-3} | 2.02 ± 0.15 | 1                  | 16.8 ± 0.20    | 54      |
| H206N/E147Q   | 10.26 ± 0.35 | 0.74 ± 0.06 | 1.13 ± 0.05         | ND             | 58      |
| H206N/E147D   | 2.18 ± 0.01 | 0.72 ± 0.04 | 1.06 ± 0.04         | ND             | 33      |
| H206N/D113A   | 5.79 ± 0.19 | 0.91 ± 0.07 | 1.07 ± 0.04         | ND             | 45      |

‡ ND, not determined.

No cooperativity was observed, and \( [S]_{0.5} = K_m \) and n = 1.

NM, not measurable.

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**Fig. 1.** Ribbon diagram of the zinc-binding domain in the T347G mutant of hAcy1. Glycine was modeled in place of a putative t-norleucine-ligand molecule and is shown in a ball-and-stick representation. Zinc ions are represented as gray spheres.
mutations resulted in 103- to 104-fold catalytic inactivations of alanine (H80A, D113A, E148A, E175A, and H373A). These mutated individually each of the five zinc-binding residues to metal-binding sites in the dinuclear zinc center of hAcy1, we compared with zinc 1 alone (49). In order to assess the importance of both zinc 2, and the enzyme exhibits 80% of its catalytic activity in the contrary, in AAP zinc 1 appears to be bound stronger than low (24), which may indicate a low affinity zinc-binding site. On crystal structure of PepT, the occupancy of the zinc 1 site was obtained by the Hill or Michaelis-Menten equation (see “Experimental Procedures”), respectively, increased up to 7-fold for the T347G, H80A, E148A, H373A, and H206N mutants. Compared with the above-mentioned changes in kcat, these differences are much lower, and their significance is less clear.

The Zinc Center and Glu147 in hAcy1—Attempts to determine metal-binding stoichiometries in enzymes of the Acy1/M20 family by spectroscopic methods frequently showed only one zinc atom per monomer (27, 27, 47, 48). Moreover, in the crystal structure of PepT, the occupancy of the zinc 1 site was low (24), which may indicate a low affinity zinc-binding site. On the contrary, in AAP zinc 1 appears to be bound stronger than zinc 2, and the enzyme exhibits 80% of its catalytic activity with zinc 1 alone (49). In order to assess the importance of both metal-binding sites in the dinuclear zinc center of hAcy1, we mutated individually each of the five zinc-binding residues to alanine (H80A, D113A, E148A, E175A, and H373A). These mutations resulted in 10^2- to 10^4-fold catalytic inactivations (Table V). No trends were observed in the half-saturation substrate concentrations and ΔG(H2O) values by mutations of zinc 1 or zinc 2 ligands, respectively. Mutation of the zinc-bridging residue Asp113 (Fig. 2), however, clearly led to the highest loss in overall stability among the five mutants. The results indicate that both zinc sites are essential for catalysis and suggest that only a fully intact zinc center confers optimal enzyme stability.

A conserved glutamic acid residue, Glu147 in hAcy1, in the immediate vicinity of the zinc center (Fig. 2) in the MH clan is thought to act as a general base during catalysis, accepting a proton from the zinc-bound water molecule. Replacing Glu147 with an alanine (E147A) or glutamine (E147Q) led to a 1000-fold decrease in enzyme activity. No activity was detectable, and no stability data could be obtained with the aspartate mutant (E147D). To understand this pronounced effect of the Glu-to-Asp mutation, we modeled this replacement in the structure of hAcy1 (not shown). Shortening the amino acid side chain in this position not only brings its acidic group away from the appropriate position for activation of the catalytic water molecule but also positions it closer to Asp348, possibly introducing an unfavorable interaction between the two residues.

Two Subdomains in the Lid Domain of PepV Mimic a Dimer—In contrast to the single-domain enzymes from the M28 family, Acy1/M20 family enzymes generally contain a second, smaller domain, the possible role of which in catalysis is not clear. As mentioned above, most of these enzymes exist as homodimers. In CGP2 and S. typhimurium PepT, the small domains were shown to mediate enzyme dimerization through side-by-side packing of their four-stranded β-sheets, thereby forming a contiguous extended eight-stranded sheet (23, 24). In contrast, L. delbrueckii PepV exists in a monomeric form (29). The crystal structure of this enzyme revealed that its small domain contains a central eight-stranded β-sheet. Here, we recognize that this domain can be subdivided into two subdomains, each composed of six β-strands. Subdomain 1 encompasses strands 1, 8, 9, 10, 11, and 12 and helices III and IV, whereas subdomain 2 includes strands 2, 3, 4, 5, 6, and 7 and...
helices I and II (Fig. 3). Whereas the resemblance of subdomain 1 to the dimerization domain of CPG2 was previously noted (29), we extend this observation by recognition that subdomain 2 also has the same topology as the dimerization domain of CPG2 (Fig. 3A) and of PepT (not shown). More importantly, we demonstrate that the two subdomains in the lid domain of

Catalytic Center in the Aminoacylase-1/M20 Family

Fig. 3. Structures of the small domains of enzymes from the Acy1M20 family. A, topology diagram for the lid domain in L. delbrueckii PepV and the dimerization domains from both monomers in Pseudomonas sp. CPG2. Subdomains 1 (gray) and 2 (white) of PepV show apparent similarity. However, strands 8 and 12 are only found in subdomain 1, and strands 3 and 7 are only found in subdomain 2. The β-sheet composed of the latter two strands is also present in the dimerization domain of CPG2. B, backbone trace superposition of subdomains 1 and 2 in the lid domain of PepV (blue) and the two associated dimerization domains in CPG2 (red and green). Known active site residues in PepV are shown in a stick representation, from left to right, Arg350, Asn217 (both carboxyl-terminal docking), and His269 (transition state stabilization). Corresponding residues from CPG2 are also shown. The enlargement above additionally shows the corresponding residues in PepT. Arg288 from CPG2 (red) and Arg280 from PepT (yellow) reside in the monomer, which superimposes with subdomain 1 of PepV. Asn275 and His229 from CPG2 (green) and His223 in PepT (purple) are recruited from the opposite monomer which superimposes with subdomain 2 of PepV. In the structure of CPG2, the side chain of His229 shows a rotation by about 90° relative to the other two structures and coordinates an additional interdimeric zinc ion in the protein crystal (not shown). C, multiple sequence alignment of the small domains in the PepV enzymes from L. delbrueckii (PEPV_LACDL) and Lactococcus lactis subsp. cremoris MG1363 (PEPV_LACLC) and from CPG2 (CBPG_PSES6), PepT (PEPT_SALTY), and hAcy1 (ACY1_HUMAN). Subdomain 1 and 2 in the lid domain of PepV are abbreviated s1 and s2, respectively. The alignment was assembled using an available alignment of the two PepV enzymes (15) and structure-based alignments of CPG2 to s1 in L. Delbrueckii (29) and CPG2 to PepT (24). The sequences of the dimerization domain in hAcy1 and CPG2 were aligned manually. Strands (s) and helices (h), as identified in the crystal structures of PepV, CPG2, and PepT, are printed in red and blue, respectively. Their numbering in s1 and s2 of L. delbrueckii PepV is indicated in the corresponding colors above the aligned sequences. Residues that interact with the bound transition state analog Asp(PO2CH2)AlaOH in the PepV structure are in yellow boxes. Greek letters indicate the sites of rearrangement generated by the insertion of s2 in the sequence of s1 and their sequel.
PepV mimic the arrangement of the two dimerization domains within the CPG2 and PepT dimers. The superposition of the entire small domain of PepV on the small domains of the CPG2 dimer (Fig. 3B) overlaps 196 C-α atoms of 230 with an r.m.s. deviation of 1.54 Å. In PepT, 208 C-α atoms out of 230 accordingly superimpose with an r.m.s. deviation of 1.62 Å. Two of three residues from the PepV lid domain, which interact with the bound transition state analog AspΨ[PO2CH2]AlaOH (29) (i.e. His269 in subdomain 2 and Arg350 in subdomain 1 of PepV) are also found in the dimerization domains of CPG2, PepT (Fig. 3B), and, by a multiple sequence alignment of small domains from the Acyl1/M20 family, also in hAcy1 (Fig. 3C). The third residue, Asn217 in subdomain 2, is missing only in PepT. His269 was assigned a role in transition state stabilization, and Arg350 and Asn217 were assigned roles in anchoring the free C terminus of the substrate (29). At the time of writing, these three residues were found to be conserved in 98, 89, and 72% of Acyl1/M20 family sequences, respectively (available on the World Wide Web at merops.sanger.ac.uk/). Beyond that, there is no identifiable sequence identity between subdomains 1 and 2 in PepV and any of the dimerization domains compared here (Fig. 3C), which reinforces the functional significance of the histidine, arginine, and, possibly, asparagine conservations.

Recently, Sintchak et al. (50) reported the case of a monomeric enzyme that is reminiscent of PepV. The crystal structure of class II ribonucleotide triphosphate reductase from Lactobacillus leichmannii revealed that a 130-amino acid insertion in the core αβ-barrel of the monomeric enzyme mimics a dimer. In the structures of related dimeric class I ribonucleotide reductases, each of the two subunits contributes one-half of a four-helix bundle, on top of which an allosteric effector-binding region is located. In the monomeric class II ribonucleotide triphosphate reductase, the 130-amino acid insertion provides the second half of this four-helix bundle. Thereby, allosteric control of substrate specificity in the monomer occurs, presumably, by the same mechanism as in the dimeric enzymes.

**Catalysis in hAcy1 Occurs at the Dimer Interface**—We also investigated the role of a conserved histidine in the small domain of the Acyl1/M20 family of enzymes (5). The respective His206 from the lid domain of L. delbrueckii PepV functions in the stabilization of the transition state (29). This residue corresponds to His229, His223, and His206 in the small domains of the dimeric homologs CPG2, PepT, and hAcy1, respectively (Fig. 3C). In the crystal structures of CPG2 and PepT, the two enzyme domains appear to be connected by flexible linkers and show an open conformation. In both structures, the histidine is solvent-exposed and is located 50 Å from the metal center in the same monomer and more than 8 Å from the metal center in the opposite monomer of the dimer. His229 in CPG2 coordinates an additional interdimeric zinc ion, which is further coordinated by Asp247 from another dimer within the crystallographic tetramer. His223 in PepT contributes to the binding of a putative sulfate ion in the crystal structure, which was suggested to occupy a binding site for the C-terminal carboxyl group of the tripeptide substrate (24). On the basis of these observations, His206 in hAcy1 was selected for mutation. A His-to-Asn mutation generated an enzyme with a 2000-fold decrease in $k_{cat}$ and a 10 °C reduced $T_m$ value (Table V). These effects on activity and $T_m$ are largely comparable with those observed upon mutation of any of the zinc-binding residues.

The detrimental effect on activity by the His-to-Asn mutation in position 206 of hAcy1, and the structural resemblance between the lid domain of PepV and the two associated dimerization domains in CPG2 (Fig. 3), discussed above, led us to hypothesize that the conserved histidine in the dimerization domain of dimeric Acyl1/M20 family enzymes contributes in trans to the active site. In order to test directly this hypothesis, we took advantage of the existence of two separate active sites within the dimer to design an enzyme complementation assay. Mixing two different mutants should lead upon equilibration to the formation of heterodimers. If two inactivating mutations act in trans, one catalytic site in these heterodimers harbors both mutations, whereas the other is functional (Fig. 4A). When equimolar amounts of the two mutants H206N and E147D were mixed together, a partial recovery of catalytic activity over time was observed, amounting to 11% of the wild-type activity (Fig. 4B). Similar recovery of activity was observed for the combination of H206N with E147Q. Variations
of the H206N/E147D and H206N/E147Q ratios yielded activity optima at approximately equimolar ratios (Fig. 4C), indicating that homo- and heterodimers formed with equal affinities and that the mutations did not interfere with dimerization itself. Equilibration of the H206N mutant with each of the metal-binding domain mutants at 1:1 ratios also led to recovery of catalytic activity, which reached between 3% (H206N/H373A) and 24% (H206N/E147Q) of the wild-type activity (Fig. 4D).

This is in the range of the calculated maximum theoretical value of 16.7% wild-type activity (Table V), which would be expected when the solution contains equal amounts of all three dimeric species, and when there is no interdependence between the two active sites in the dimer. Accordingly, similar half-saturation substrate concentrations as in the wild type for three selected heterodimer combinations, H206N with E147D, E147Q, and D113A, indicate unchanged substrate affinities. Notably, the complementation assay also showed that inactivation of the E147D mutant was not associated with irreversible unfolding of this protein. The active site stabilities in the three combinations, as measured by $T_m$, follow the order of the $\Delta G(H_2O)$ values for the three mutants E147D, E147Q, and D113A alone (Table VI). This probably reflects the impact of each of these mutations on the overall stability of the respective heterodimer with the H206N mutant.

A Revised Catalytic Site in the Acy1/M20 Family—The M28 family enzymes AAP and SGAP are believed to follow a zinc peptidase mechanism, which requires both metal ions for full activity (21, 51). This mechanism is assumed to be operational in all enzymes from the MH clan, which are therefore called co-catalytic metallopeptidases (2, 14). In support of this conclusion, the dinuclear zinc center and the putative general base residue, Glu147 in hAcy1, and in six available structures from the MH clan superimpose well (Fig. 2). Furthermore, the results from our mutational analysis of hAcy1 from the Acy1/M20 family corroborate the catalytic significance of a fully intact dicing center and Glu147. Previous nuclear magnetic relaxation studies of manganese-substituted porcine Acy1 in complex with t-butoxycarbonyl amino acid inhibitors indicated metal-inhibitor distances of at least 12 Å, which suggested not a catalytic but a purely structural role for the zinc center (52). We speculate that the binding of these competitive inhibitors to porcine Acy1 may not, however, be representative of catalytically competent enzyme-substrate complexes.

Previously, Wouters and Husain (2) argued that, among the co-catalytic exopeptidases, neither amino- nor carboxyl-terminal docking of the substrate would restrict peptidase phenotypes, because corresponding docking groups among these enzymes were found not to be conserved. However, among the known enzymes of the Acy1/M20 family (Table I), the presence of an aspartate as zinc 2 ligand is associated with aminopeptidase or dipeptidase rather than aminopeptidase or carboxypeptidase specificities. It remains to be shown whether this feature generally contributes to substrate specificity in this enzyme family, as suggested for the dipeptidase PepV (29). In hAcy1, the Glu-to-Asp mutation in the corresponding position 175 led to an over 1000-fold loss in enzyme activity in our standard assay (21), providing a rational explanation for the silencing of immunogenic sites in the zinc-binding domains of CPG2 (54) by humanization or for the engineering of the specificity of the human enzyme to perform the same function as CPG2 in ADEPT.

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