

# Further characterization of the putative human isopeptidase T catalytic site

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Received 12 August 2002; revised 9 October 2002; accepted 12 October 2002

First published online 23 October 2002

Edited by Judit Ovádi

**Abstract** The human isopeptidase T (isoT) is a zinc-binding deubiquitinating enzyme involved in the disassembly of free K48-linked polyubiquitin chains into ubiquitin monomers. The catalytic site of this enzyme is thought to be composed of Cys335, Asp435, His786 and His795. These four residues were site-directed mutagenized. None of the mutants were able to cleave a peptide-linked ubiquitin dimer. Similarly, C335S, D435N and H795N mutants had virtually no activity against a K48-linked isopeptide ubiquitin dimer, which is an isoT-specific substrate that mimics the K48-linked polyubiquitin chains. On the other hand, the H786N mutant retained a partial activity toward the K48-linked substrate, suggesting that the His786 residue might not be part of the catalytic site. None of the mutations significantly affected the capacity of isoT to bind ubiquitin and zinc. Thus, the catalytic site of UBPs could resemble that of other cysteine proteases, which contain one Cys, one Asp and one His.

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**Key words:** Isopeptidase T; Ubiquitin-specific processing protease; Ubiquitin

## 1. Introduction

Ubiquitin is a highly conserved 76 amino acid protein. The covalent addition of polyubiquitin chains to cellular proteins is specifically recognized for selective degradation by the proteasome [1]. Ubiquitination of proteins is a sequential enzymatic reaction involving an ubiquitin activating enzyme (E1), an ubiquitin conjugating enzyme (E2) and an ubiquitin ligase (E3) [2,3]. Polyubiquitin chains are attached to their substrate through an isopeptide bond between the C-terminal carboxylate of ubiquitin and the  $\epsilon$ -amino group of a specific lysine lateral chain of the substrate protein. In polyubiquitin chains, ubiquitin molecules are also linked to one another by an isopeptide bond through the ubiquitin K48 residue. Nevertheless, ubiquitination is more than a proteasomal targeting signal, and other lysines of ubiquitin besides Lys48 are used to form polyubiquitin chains [3].

The reversibility of ubiquitination is conferred by specific proteases called deubiquitinating enzymes which are subdivided into ubiquitin C-terminal hydrolases (UCHs) and ubiquitin-specific processing proteases (UBPs). UCHs are not related in primary sequence to UBPs. UBPs hydrolyze isopeptide bonds between ubiquitin and the substrate protein or within polyubiquitin chains [3]. Human isopeptidase T (isoT) is an UBP capable of binding ubiquitin that specifically disassembles free polyubiquitin chains linked through isopeptide bonds involving the K48 of ubiquitin into ubiquitin monomers [4,5]. The isoT is the only UBP known to require zinc binding to be active [6].

The UBPs are characterized by the presence of three conserved domains surrounding, respectively, one cysteine (Cys box), one aspartic acid (Asp box) and two histidine residues (His box) [7–10]. These residues are postulated to form the catalytic site of UBPs by analogy with other proteases. This idea has been reinforced, both in vivo and in vitro, by mutagenesis experiments showing that these residues are needed for deubiquitinating activity in UBPs toward a variety of generic substrates [5,11–19]. The inhibition of UBPs by covalent inhibitors such as ubiquitin aldehyde or ubiquitin vinyl sulfone strongly suggests the presence of a Cys residue in the active site, as found in UCHs [20–23]. Nevertheless, there is at present no direct evidence that these residues are effectively part of the catalytic site.

In this study, we mutagenized for the first time the four highly conserved residues of the putative catalytic site of the human isoT. Previous studies were done on other members of the UBP family using generic substrates that may have lacked high affinity for the UBP analyzed. We defined the importance of these residues in the isoT activity toward its specific substrate, and in the capacity of isoT to bind zinc and ubiquitin.

## 2. Materials and methods

### 2.1. IsoT site-directed mutagenesis

The C335S and D435N mutations were produced by reverse PCR (by the QuickChange<sup>TM</sup> method, Stratagene, La Jolla, CA, USA) using the complete pMPM-isoT-S wt (wild-type) plasmid [6] as a template. XL1-Blue bacteria were then electroporated using 1  $\mu$ l of the *DpnI*-treated PCR reaction. The entire isoT ORFs were verified by sequencing.

The H786N and H795N mutations were produced by a two-step PCR-based approach [5] from the pMPM-isoT-S wt plasmid as a template. The final product was cloned in the pMPM-isoT-S wt plasmid as a *Eco47III*/*FseI* fragment. The sequences encompassing the mutagenized *Eco47III*/*FseI* fragments were verified by sequencing.

### 2.2. IsoT expression and purification

Wt and mutated isoT were expressed in the MC1061 *Escherichia coli* strain, and cells were lysed as previously described [6]. IsoT was purified on an ubiquitin–Sepharose column. The loaded ubiquitin–

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**Abbreviations:** UBP, ubiquitin-specific processing protease; UCH, ubiquitin carboxyl-terminal hydrolase

Sephacrose column was washed with buffer A (50 mM Tris–HCl, pH 7.2, 0.2 mM EDTA, 5% glycerol, 1 mM DTT), buffer B (buffer A+0.5 M KCl), and once again buffer A. IsoT was eluted in buffer C (buffer A+3 M KSCN). Finally, isoT was dialyzed against 50 mM Tris–HCl, pH 7.2, 1 mM DTT and kept at  $-20^{\circ}\text{C}$  until use.

### 2.3. Substrates synthesis

IsoT activity was monitored using both linear and branched ubiquitin dimers as substrates.

The linear ubiquitin dimer was expressed from pMPM250-HUb<sub>2</sub> in the BL21(DE3) *E. coli* strain as a 6His-tagged protein [6], and purified on a Ni-NTA column (Qiagen, Hilden, Germany). The linear dimer is linked head-to-tail through a peptide bond.

The branched ubiquitin dimer was synthesized using human E1 and E2<sub>5K</sub> enzymes, and purified as previously described [6] but with the following modifications. Both human E1 and E2<sub>5K</sub> were expressed as recombinant proteins in the MC1061 *E. coli* strain harboring the pMPM201-E1 (a kind gift from L. Falquet) or the pMPM201-E2<sub>5K</sub> [6] plasmids. Cells were lysed as described for isoT except that 2 mM ATP was added to the crude extract, and human E1 was loaded onto an ubiquitin–Sephacrose affinity chromatography equilibrated in buffer A (50 mM Tris–HCl, pH 7.2, 5% glycerol, 5 mM MgCl<sub>2</sub>, 2 mM ATP). After washing with buffer B (50 mM Tris–HCl, pH 7.2, 1 M KCl, 5% glycerol) and buffer C (50 mM Tris–HCl, pH 7.2, 5% glycerol), E1 enzyme was eluted with buffer D (50 mM Tris–HCl, pH 7.2, 0.5 mM MgCl<sub>2</sub>, 2 mM AMP, 40  $\mu\text{M}$  Na<sub>2</sub>PPi, 5% glycerol), and further concentrated onto a DE52 column eluted with buffer A+0.5 M KCl without DTT. The E2<sub>5K</sub> catalyzes the synthesis of K48-linked ubiquitin dimers, in which one monomer is linked to the internal K48 of the other through an isopeptide bond [24,25].

### 2.4. IsoT activity assay

The enzymatic reaction was performed in a 15  $\mu\text{l}$  total volume. Indicated quantities of isoT were pre-incubated for 15 min at  $22^{\circ}\text{C}$  in 50 mM Tris–HCl, pH 7.2, 1 mM DTT. The linear and branched substrates were added to the reaction at a final concentration of 5.5  $\mu\text{M}$  (1.5  $\mu\text{g}$ ) and 5.8  $\mu\text{M}$  (1.5  $\mu\text{g}$ ), respectively, and then further incubated at  $37^{\circ}\text{C}$  for the indicated times. The reaction was stopped by adding 3  $\mu\text{l}$  of six-fold concentrated loading buffer and analyzed on a Tris–tricine gel by Coomassie staining.

### 2.5. Ubiquitin-binding assay

The binding of wt and mutated isoT to ubiquitin was assessed using an FPLC column where ubiquitin (Sigma, St. Louis, MO, USA) was covalently coupled to a HiTrap NHS-activated column (Amersham Biosciences, Buckinghamshire, UK). The column was equilibrated in buffer A (10 mM NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>, pH 7, 2 mM  $\beta$ -mercaptoethanol) and submitted to two consecutive gradients in buffer A: a 0–3 M KCl linear gradient followed by a 0–8 M urea linear gradient. The gradients were performed using a FPLC station (Amersham Biosciences).

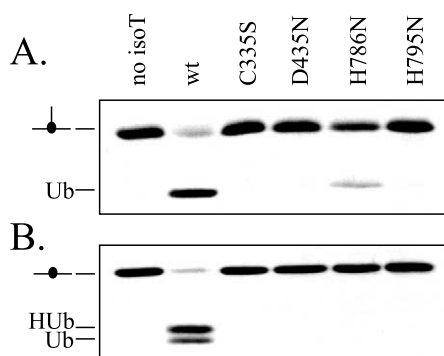


Fig. 1. IsoT activity tested on linear and branched ubiquitin dimers. Reaction products were analyzed on a Tris–tricine gel by Coomassie staining. The reaction was performed as previously described [6], under conditions that allowed almost complete cleavage of branched (A) and linear (B) ubiquitin dimers (i.e. a 15  $\mu\text{l}$  reaction containing 0.1  $\mu\text{M}$  of isoT, 1.5  $\mu\text{g}$  of substrate and performed at  $37^{\circ}\text{C}$  during 5 min).  $\bullet$ —, branched dimer;  $\bullet$ —, linear dimer. Ub, ubiquitin; HUb, 6His-ubiquitin.

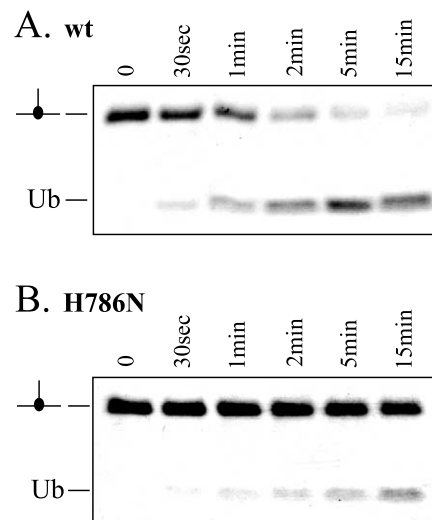


Fig. 2. Time-course reaction comparing the activity of wt and H786N isoT toward the branched ubiquitin dimer. Reaction products were analyzed on a Tris–tricine gel by Coomassie staining. The reaction was performed at  $37^{\circ}\text{C}$  in a volume of 15  $\mu\text{l}$  containing 0.1  $\mu\text{M}$  of wt (A) or H786N (B) isoT, 1.5  $\mu\text{g}$  of branched substrate, for the indicated times. Ub, ubiquitin.

ces). The urea concentration needed to elute isoT was defined as the timepoint of maximum absorbance at 278 nm of the elution profile.

## 3. Results

### 3.1. Relative importance of C335, D435, H786 and H795 residues in the enzymatic reaction

The activity of the isoT catalytic site mutants was measured using two types of substrates: linear and branched ubiquitin dimers (see Section 2). IsoT is known to cleave these two substrates [26] but in vitro, it has a preference for the branched one [4,26]. On one hand, the linear dimer is related to artificial ubiquitin fusion proteins, such as Ub– $\beta$ -galactosidase, which are classically used to analyze the deubiquitinating activity of UBPs. It is also related to naturally occurring precursors where ubiquitin is fused with ribosomal proteins or to another ubiquitin molecule in a ubiquitin polymer. On the other hand, the branched dimer mimics the specific in vivo substrate of the isoT enzyme, the K48-linked polyubiquitin chains [5]. For this reason, the branched dimer might enhance the sensitivity of the assay.

In a first approach the activities of the C335S (Cys box), D435N (Asp box), H786N and H795N (His box) mutants were tested according to the standard conditions used in the laboratory in which wt isoT almost completely cleaves both substrates (Fig. 1A,B). As expected none of the mutants of the four conserved residues of the putative catalytic site of isoT had detectable activity against the linear dimer (Fig. 1B). The C335S, D435N, and H795N mutants also showed no activity against the branched dimer (Fig. 1A). The H786N mutant was capable of cleaving the branched dimer, although to a lower extent compared to wt isoT (Fig. 1A).

To further study the enzymatic activity of H786N isoT, a time-course was performed at an enzymatic concentration of 0.1  $\mu\text{M}$  (Fig. 2). While wt isoT almost completely cleaved the branched dimer in 5 min (Fig. 2A), the H786N mutant cleaved more slowly since after 15 min of reaction less than

one half of the branched ubiquitin dimer was cleaved (Fig. 2B).

A more detailed analysis of the four mutants was carried out on both ubiquitin dimers (Fig. 3) to reveal if any activity could be detected with the C335S, D435N and H795N mutants. This was done by increasing the enzyme concentration until enzymatic activity could be observed. The enzymatic activity of the wt isoT toward branched dimer could be visualized in a 5 min reaction at an enzyme concentration of 0.02  $\mu$ M. All the mutants were capable of cleaving branched dimer, but only at much higher enzyme concentrations. The H786N mutant needed 0.1  $\mu$ M for the same level of reaction. D435N

and H795N mutants required a reaction time of 1 h and a concentration of 0.5  $\mu$ M to give a perceptible cleavage of the branched substrate, whereas the C335S mutant needed a reaction time of 1 h at a 1  $\mu$ M concentration. According to the enzymatic reactions presented here, the C335S mutation was the most drastic, followed by the D435N and H795N mutations which affect the deubiquitinating activity in a similar manner, and finally the H786N mutation which retained a partial but significant activity ( $C335S > D435N \approx H795N > H786N$ ). However we cannot exclude that other amino acid substitutions could affect the isoT activity differently. For example, a C335A mutation should remove any possible acti-

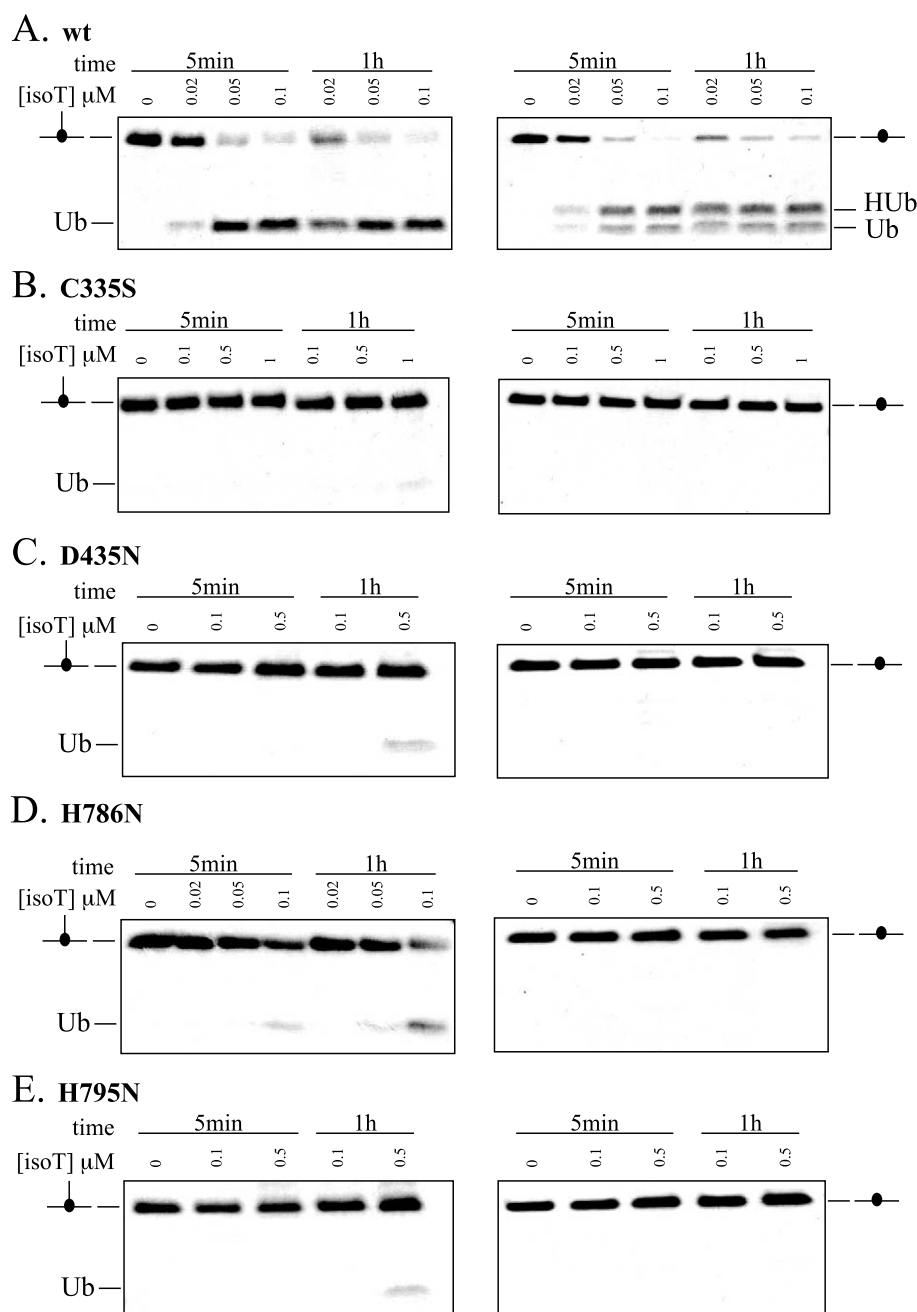


Fig. 3. Detailed analysis of the isoT mutants activity using a broader range of reaction conditions. Reaction products were analyzed on a Tris-tricine gel by Coomassie staining. The reaction was performed at 37°C in a volume of 15  $\mu$ l containing 1.5  $\mu$ g of branched substrate, the indicated concentrations of wt (A), C335S (B), D435N (C), H786N (D) and H795N (E) isoT, for the indicated times. The enzyme concentrations used to detect the enzymatic activity varied according to the mutant analyzed. Ub, ubiquitin; HUb, 6His-ubiquitin.

Table 1  
Zinc binding of isoT

IsoT	Zinc/isoT (mol/mol)
Wt	1.04 ± 0.05
C335S	1.0 ± 0.03
D435N	1.03 ± 0.02
H786N	1.04 ± 0.06
H795N	1.09 ± 0.06

IsoT zinc content was assessed by atomic absorption spectrophotometry (spectrophotometer Perkin Elmer 2380) as previously described [6], from recombinant isoT purified from *E. coli*. Results are expressed in mol/mol as mean ± S.D. of six separate experiments.

vation of the nucleophile. Moreover the pH optimum for enzymatic activity is perhaps altered in the C335S mutant compared to the wt enzyme. None of the mutants were capable of cleaving linear dimers even after 1 h reaction with 1 μM of enzyme; this confirms the higher activity of isoT for branched compared to linear dimer.

### 3.2. Zinc and ubiquitin binding are independent of the conserved catalytic residues

The isoT binds both zinc and ubiquitin. Zinc is required for the isoT activity [6], but its exact role in the enzymatic reaction is unknown. Since Cys, His and Asp residues are potential zinc ligands, the residues of the putative catalytic site could be involved in zinc binding, although these residues were never described as having the characteristic features of a zinc-binding site. To test this possibility, isoT zinc content was measured by atomic absorption spectrophotometry. Wt isoT contained one zinc as did the C335S, D435N, H786N and H795N mutants (Table 1).

To further study the effect of mutations in the putative catalytic site, an ubiquitin-binding assay was performed to determine if the loss of activity could not be a consequence of a defect in ubiquitin binding. The binding of isoT to ubiquitin was measured using a ubiquitin–Sepharose column submitted to a tandem KCl and urea linear gradients. None of the proteins were eluted in the KCl gradient, whereas the wt isoT and the C335S, D435N, H786N and H795N mutants were all eluted at approximately 5.8 M urea (Table 2), showing that the ubiquitin–isoT interaction was only disrupted at high urea concentrations, probably as a result of isoT denaturation.

Table 2  
IsoT binding to ubiquitin

IsoT	IsoT elution [urea] M
Wt	5.81 ± 0.08
C335S	5.67 ± 0.01
D435N	5.82 ± 0.03
H786N	5.60 ± 0.01
H795N	5.93 ± 0.03

IsoT affinity to ubiquitin was assessed by an urea gradient applied onto a ubiquitin–Sepharose column. Results are expressed in molar (M) as mean ± S.D. of three separate experiments.

Thus, the mutations of the conserved residues exclusively affect the catalytic activity of isoT but apparently not zinc nor ubiquitin binding.

## 4. Discussion

This study describes an in vitro analysis of the putative catalytic residues of an UBP using a specific substrate mimicking the natural substrate, instead of a generic substrate that lacked specificity for the UBP analyzed. This approach allowed us to study more precisely the importance of each residue in the reaction and to get more insight into the enzymatic mechanism of UBPs.

The only previous in vitro study of the four highly conserved amino acids of the UBPs was on the DUB-2A enzyme using a generic Ub–β-galactosidase chimeric substrate [13]. In this study, the four residues appeared to be critical for the deubiquitinating activity. Several other studies showed that the conserved cysteine and histidines are necessary for the enzymatic activity of various UBPs both in vivo and in vitro [5,11,12,14–19].

Since isoT mutants bind zinc and ubiquitin as efficiently as the wt isoT, it appears that defects in the catalytic site of the enzyme are probably at the origin of the loss of or decrease in activity observed in the isoT mutants. At the highest isoT concentration used, C335S, D435N, and H795N mutants are completely inactive toward linear ubiquitin dimers, and very slightly active toward branched ubiquitin dimers. This provides further evidence that these residues are necessary for enzymatic activity and constitute part of the catalytic site. Although inactive on the linear ubiquitin dimer, the H786N

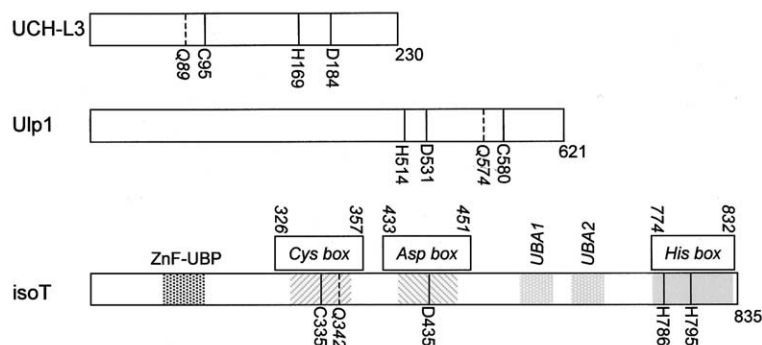


Fig. 4. Schematic representation of the conserved domains in human isoT. The catalytic site includes three domains named Cys, Asp and His boxes containing, respectively, the C335, D435, H786 and H795 residues in isoT. In human UCH-L3 and yeast Ulp1 the catalytic triad is composed respectively, of C95, H169, and D184 [32], and of C580, D531, and H514 [31]. Q89 in UCH-L3 [32], Q574 in Ulp1 [31] and potentially Q342 in isoT form the oxyanion hole. The UBA1, UBA2 [34] and the ZnF-UBP [37] domains constitute the putative ubiquitin-binding domains. The Cys and His boxes are defined according to the Pfam database (domains PF00442 and PF00443, respectively); the Asp box is defined according to [8].



mutant retains a significant capacity to cleave the branched ubiquitin dimer, a substrate that more closely mimics the specific substrate of isoT. This partial loss of the deubiquitinating activity is also observed in another UBP, the mouse Unp, in which the corresponding His was replaced by an Ala [27]. On the contrary, when replaced by an Arg, this His appeared to be critical for the *in vivo* function of the Fat Facets UBP in *Drosophila* [11]. However, it is unknown if the catalytic activity per se of this Fat Facets mutant is affected. Besides isoT [4,5], specific ubiquitinated substrates of Fat Facets [28] and USP7 [29] are also identified. So, it would be interesting to see if these UBPs have the same *in vitro* properties.

The capacity of the H786N mutant to cleave the branched ubiquitin dimers raises the possibility that the His786 residue is not part of the catalytic site even though this residue is conserved in all the known UBPs. Instead, it could be involved in the correct positioning of the substrate or in the structure of the catalytic site. Thus, the catalytic site of UBPs could be formed by one Cys, one Asp and only one His; it could resemble the catalytic site of the UCH and Ulp protease families that are involved, respectively, in ubiquitin and SUMO (an ubiquitin-like protein) recycling and/or maturation [23,30,31]. In these cysteine proteases, the nucleophilic cysteine is coordinated by a histidine general base, which is in turn stabilized by an aspartic acid. In isoT, the catalytic triad would be composed of Cys335, Asp435 and His795. Since, in Ulp and UCHs, the catalytic Asp residue is close to the His residue in the primary structure, a conserved Asp residue in the His box (Asp811) could be part of the catalytic site instead of the Asp435 (Fig. 4). Three conserved Asn residues (Asn330, Asn333 and Asn338) contained in the Cys box could also be potential catalytic residues in place of the Asp435 residue. In addition to the catalytic triad, UCH and Ulp enzymes contain a Gln as an oxyanion hole [32] and Fig. 4). In UBPs a Gln is fully conserved in the Cys box (Q342 in isoT) and could potentially play this role. Two other Gln are present in the Asp box (Q433 and Q434 in isoT), but they are less conserved. An extensive mutational analysis of these residues could reveal any role they might play in enzymatic activity.

The observation that none of the mutants of the isoT putative catalytic site are affected in their capacity to bind ubiquitin or zinc suggests that other parts of the isoT protein are involved in these functions. Two ubiquitin-binding sites were described in isoT [4,33], whereas isoT contains two copies of an UBA domain capable of binding ubiquitin [34,35]. It is not known if these UBA domains constitute one or two ubiquitin-binding sites. Besides the UBA domains, the ZnF-UBP/DAUP domain (PF02148 in Pfam database, [36]), which is involved in the binding of a histone deacetylase to ubiquitin [37], could also constitute one of the two binding sites. This ZnF-UBP domain could represent the veritable site of zinc binding since it is depicted as a putative zinc-binding site. In contrast, although being potential zinc ligands, the putative catalytic residues are not involved in zinc binding. These residues are conserved amongst all UBPs even though some UBPs are not zinc-binding proteins (T. Lacombe, unpublished data).

Although further investigations are necessary to fully understand the role of each catalytic residue in the enzymatic reaction, such as kinetic and affinity measurements, or crystallization experiments, this study allowed us to go another step forward in the understanding of the UBPs functioning.

**Acknowledgements:** We thank B. Angenieux, S. Carobbio and J. Della Torre for technical assistance, and L. Falquet for plasmid pMPM201-E1. We thank K. Tanner, M. Hochstrasser and P. Linder for critical reading of the manuscript. We are grateful to P. Linder and C. Georgopoulos for financial support. This work was supported by the Grant 3100-59430.99 from the Swiss National Foundation to J.M.G.

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