Zinc Is Required for the Catalytic Activity of the Human Deubiquitinating Isopeptidase T†

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ABSTRACT: Two recombinant human isopeptidase T isoforms, ISOT-S and ISOT-L, differing by an insertion of 23 amino acids in ISOT-L, were previously classified as thiol proteases. Both contain one Zn^{2+}-binding site of high-affinity, which is part of a cryptic nitrilo-triacetate-resistant pocket (site 1). A second Zn^{2+} site (site 2) was disclosed when both isoforms of the holoenzyme were incubated with an excess of Zn^{2+}. The firmly bound Zn^{2+} of site 1 could be removed either slowly by dialysis against 1,10-phenanthroline at pH 5.5 or rapidly by treatment at pH 3.0 in the presence of 6 M urea followed by gel filtration at neutral pH. Zn^{2+} in site 1, but not in site 2, is essential for proteolytic activity because apoproteins were inactive. Inhibition of the catalytic activity was not due to a loss of ubiquitin binding capacity. CD spectra of both isoforms disclosed no major structural differences between the apo- and holoenzymes. The reconstitution of apoenzyme with Zn^{2+} under nondenaturing conditions at pH 5.5 completely restored enzymatic activity, which was indistinguishable from the reconstitution carried out in urea at pH 3.0. Thus, both human ISOTs are either thiol proteases with a local structural Zn^{2+} or monozinc metalloproteases that might use in catalysis a Zn^{2+}-activated hydroxide ion polarized by Cys335.

Polyubiquitin chains, in which the C-terminal glycine of a first ubiquitin molecule is linked by an isopeptide bond to the ϵ-lysine-48 of the next ubiquitin, specifically target the modified protein to an important intracellular protein degradation process involving the proteasome in virtually all eukaryotic cells. Enzymes specifically designed to disassemble such chains are called deubiquitinating enzymes (DUBs)[1] or isopeptidases. DUBs can be divided into two families: the ∼20−80 kDa UCHs (ubiquitin C-terminal hydrolases or UCH-1) and the ∼40−200 kDa UBPs (ubiquitin-specific processing proteases) (reviewed in refs *2*−*4*). Members of both families are activated by thiol compounds (*5*) and are inactivated by thiol-blocking reagents such as iodoacetamide (*6*) or NEM (*N*-ethylmaleimide) (*7*) but not by inhibitors of other classes of peptidases (*6, 7*). Both are inhibited by ubiquitin aldehyde through a mechanism that is analogous to the mechanism of inhibition of thiol proteases by peptide aldehydes involving the reversible formation of a hemithioacetal (*8, 9*) or are modified by ubiquitin vinyl sulfone (*10*). Furthermore, mutants of a well-defined conserved cysteine abolished activity in numerous UCHs and UBPs, emphasizing the role of this conserved cysteine as important in the active site (*11, 12*). Therefore, both families of enzymes were considered as thiol proteases (reviewed in ref *13*). However, there are no sequence homologies between UCHs and UBPs nor any apparent sequence homology to any other proteases (*2*−*4, 13*). UBPs are characterized by the presence of several consensus sequences, in which a cysteine and a histidine residue have been postulated to be involved in the active site of the enzyme (*1, 4, 14*−*16*) by analogy with the catalytic dyad (cysteine and histidine) of thiol proteases (*13*) and UCHs (*17*). Despite divergent amino acid sequences, UCHs have a very weak structural homology with the papain-like cysteine protease family (*17*). However, there is no structural homology between the UBPs and any other protease family (*11, 13*).

The UBP deubiquitinating enzymes play an essential role in the disassembly of polyubiquitin chains released during proteolysis of various targeted protein substrates by the 26S proteasome (*12, 16, 18*). On the basis of sequence homology, two human ubiquitin specific isopeptidase T isoforms (EC 3.1.2.15; swissprot|P45974) were shown to belong to the UBP family (*1, 19*), as well as orthologous enzymes in the

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; branched multiubiquitins, ubiquitin polymers linked by an isoamide bond ($G_{76}-\epsilon K_{48}$); DEAE, diethylaminoethyl; DTT, dithiothreitol; DUB, deubiquitinating enzymes; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(2-aminoethyl)tetraacetic acid; FPLC, fast protein liquid chromatography; ICP-MS, inductively coupled plasma mass spectrometry; ISOT-S and ISOT-L, the short and long isoforms of deubiquitinating isopeptidase T; linear multiubiquitins, ubiquitin polymers linked by a peptide bond ($G_{76}-M_1$); MES, 2-(*N*-morpholino)ethanesulfonic acid; NTA, nitrilo-triacetate; OP, 1,10-*ortho*-phenanthroline; PCR, polymerase chain reaction; pLF-ISOT-S and pLF-ISOT-L, plasmids containing cDNA encoding the S and L isoforms, respectively; RT-PCR, reverse transcriptase PCR; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; UBA, ubiquitin-associated domain; UBP, ubiquitin-specific protease; UCH, ubiquitin C-terminal hydrolase.

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genome of yeast (*12*), slime mold (*20*), fruit fly (*21*), nematode (*22*), mouse, rice, or mouse-ear cress (*23*). Alternative splicing of the human ISOT gene in exon 15 leads to an insertion of 23 amino acids in ISOT-L, the high molecular weight form, as compared to ISOT-S, the low molecular weight form (*24*). Whether both isoforms may display different functions and may be differentially expressed in various human tissues remains to be investigated. Native ISOT-S, isolated from human red blood cells, was shown to hydrolyze both the $G_{76}-\epsilon K_{48}$ isopeptide bonds in branched ubiquitin polymers (*18*) and the conventional $G_{76}-M_1$ peptide bonds in "head-to-tail" polyubiquitins (*25*), although with a marked preference for isoamide over classical peptide bonds (*4*, *19*). The isozyme ISOT-L, the existence of which was predicted in a human brain cDNA library, has not yet been isolated and purified, although Northern blot analysis disclosed a high level of a 3.3 kb transcript in brain and a low level in heart, lung, spleen, and skeletal muscle (*24*).

Amino acid sequences of ISOT-S and ISOT-L (*1*) predict several possible metal ion-binding motifs (*26*, *27*), particularly Zn^{2+}, which is known to bind mainly the side chains of histidine $\gg$ glutamic acid $>$ aspartic acid $=$ cysteine residues (*28*). Moreover, the insert in ISOT-L is itself a putative Zn^{2+} binding site because it contains a CxxH motif. Preliminary experiments on the inhibition of enzymatic activity by 1,10-phenanthroline (OP) confirmed the possible occurrence of a divalent cation in the molecule (*1*).

The finding in this work that 1 mol of Zn^{2+} is bound to 1 mol of "native" ISOT-S prompted us to compare the relative Zn^{2+}-binding properties of both isoforms and the possible role of Zn^{2+} in catalysis. For this purpose, both isoforms were expressed in *Escherichia coli* and purified. Here, we report that both isoforms contain one poorly accessible Zn^{2+}-binding site of high-affinity, called site 1, of which the Zn^{2+} removal required unusual combinations of OP, pH, and urea concentration. A second Zn^{2+}-binding site, called site 2, was disclosed under particular conditions in excess of free Zn^{2+} or during the reconstitution of apoenzymes at neutral pH rather than at acidic pH. The presence of Zn^{2+} (or Ni^{2+}) in site 1, but not in site 2, is essential for proteolytic activity, whereas Zn^{2+} binding to a third set of low affinity sites leads to complete inhibition of the enzyme. To our knowledge, this is the first time that a member of the large DUB/UBP family is described as containing Zn^{2+} that is required for enzymatic activity.

MATERIAL AND METHODS

Cloning and Expression. The cDNA obtained from human brain mRNA (*1*) was recloned in the pMPM201 expression vector (gift of Dr. Matthias Mayer) (*29*) using an Nco I site created at the first ATG of the coding sequence and a Sac II site at the 3′ end originating from vector pBluescript. The resulting vector (pLF-ISOT-L) expressed the recombinant protein under the control of AraC repressor/activator of the ara operon (*29*). The cDNA sequence of the shorter isoform (*19*) was obtained by RT-PCR using total RNA from HL-60 cell line (*30*). Only the region surrounding the deletion was amplified using specific primers: I9-R for reverse transcription and I1-F and I11-R, for PCR (*1*). The PCR product (1080 bp) was digested by Sse 8387 I and Eco 47 III and recloned into pLF-ISOT-L, digested by the same enzymes, leading to pLF-ISOT-S. Sequencing of the insert (510 bp) confirmed the deletion and disclosed no mutations.

The plasmid was transformed into *E. coli* strain MC1061 (F^- araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac)X74 rpsL-(Str^R) hsdR2(r_k^-, m_k^+) mcrA mcrB1;) (*31*). A single colony was used to inoculate 100 mL of LB medium containing 100 μg/mL ampicilline and cells were grown at 37 °C overnight. The culture was 1:10 diluted in LB + ampicilline and grown for 1 h at 37 °C before induction by 1 mM arabinose L (+) overnight at 20 °C. The cells were harvested by centrifugation at 3000 × g for 10 min, and the pellets were snap frozen in ethanol/dry ice. The pellet was thawed in ice-cold lysis buffer (1× PBS; 50 mM EDTA, pH 8.0; 1 mM PMSF; 28 mM β-mercaptoethanol; 100 μg/mL lysozyme; 0.8 μg/mL pepstatin A) and left on ice for 15 min. Cells were lysed by sonication (Cell Disruptor B-30, Branson Sonic Power Co). Insoluble material was removed by centrifugation at 20 000 g for 20 min at 4 °C. The supernatant was filtered through a 0.45 μm membrane and purified by ubiquitin−Sepharose affinity chromatography as described (*25*, *32*).

Site-Directed Mutagenesis of Active Site Cys335Ser. The plasmid DNA encoding the short isoform (pLF-ISOT-S) was mutagenized by PCR with two complementary primers containing a serine codon instead of the cysteine-335 codon and will be described in detail elsewhere (T.L. and J.-M.G., unpublished data). The recombinant protein was expressed and purified as described above, with the exception that a new ubiquitin−Sepharose column was made and used for the affinity purification.

Zn^{2+}-Binding Studies. Detection of the low affinity Zn^{2+} binding sites was carried out at room temperature by equilibrium gel filtration according to the method of Hummel−Dryer (*33*). A Sephadex G-25 column (0.7 × 40 cm) was equilibrated in 50 mM Tris-HCl, pH 7.5, containing 150 mM KCl and varying concentrations of Zn^{2+}. Fractions of 0.8 mL were collected. Zn^{2+} concentration was determined in the eluant by atomic absorption with a Perkin-Elmer atomic absorption spectrophotometer model 2380. For atomic absorption measurements, 1 mM EDTA was added to all solutions including the standards (Zinc Standard Solution, Merck) to normalize quenching effects. Relative errors of protein-bound Zn^{2+} measurements were estimated to be 5−10%. The lower limit of sensitivity of Zn^{2+} detection was 0.1 μM. Protein concentration was measured from the UV absorption spectrum using molar extinction coefficients at 278 nm of 90 232 and 88 705 M^{-1} cm^{-1} for ISOT-L and ISOT-S, respectively, based on the content of aromatic residues (*34*). To adjust for equal concentrations of apo- and holoenzymes, aliquots of proteins were denatured in 8 M guanidine-HCl prior to spectroscopic analyses. Occasionally, the protein concentration was determined by the Bradford assay (*35*) with pure protein as standard. The concentration of free and metal-bound complexes was iteratively calculated with a speciation computer program (Eqcal for Windows, version 1.1, Biosoft 1996) (*36*).

Inductively Coupled Plasma Mass Spectrometry (ICP/MS). Human red blood cell ISOT-S was purified as described (*25*) and equilibrated by ultrafiltration in 50 mM Tris-HCl buffer, pH 7.2, containing 1 mM DTT (buffer A). One milliliter of protein (1 mg/mL) and buffer were lyophilized in siliconized plastic tubes, which were prewashed with MilliQ water.

Metal ions were qualitatively and quantitatively estimated by ICP/MS (Hewlett-Packard, model 4500) by Trace-Analytics SA, Morges, Switzerland, and in the Department of Inorganic Chemistry, University of Geneva (*37*).

Zn^{2+} Removal and Reconstitution. A stringent requirement for efficient Zn^{2+} removal was that all solutions were made nominally Zn^{2+} free by using MilliQ water and Chelex 100 resin (Bio-Rad); glassware was excluded. Zn^{2+} removal under denaturing conditions was carried out as follows: 5−15 μM of the isoenzymes dissolved in 50 mM Tris-HCl, pH 7.2, containing 500 mM NaCl and 1 mM DTT, were treated with 6 M freshly deionized urea and glacial acetic acid was added to reach pH 3.0 (about 1.3−1.6 M final concentration). The reaction mixture was left for 10 min in ice prior to neutralization and gel filtration on a Sephadex G-25 SF column (0.8 × 25 cm) equilibrated in 50 mM Tris-HCl, 1 mM DTT, pH 7.2 (buffer A), yielding the apoprotein. Zn^{2+} removal under nondenaturing conditions was achieved by a 1 week dialysis of the holoenzyme with 20- and 100-fold molar excesses of OP over the protein concentration in 0.1 M MES at pH 5.5, followed by an additional week of dialysis in 0.1 M MES at pH 5.5 to remove the chelator.

For Zn^{2+} reconstitution with restoration of enzymatic activity, as well as for substitution of Zn^{2+} in the not readily accessible site 1 with Cd^{2+} (acetate), Co^{2+} (chloride), Cu^{2+} (sulfate), and Ni^{2+} (sulfate), the apoproteins (4−10 μM in 500−800 μL) in 6 M urea containing 0.2 N acetic acid, pH 3.0, were incubated on ice for 10 min with a 3-fold molar excess of metal, neutralized with 3 M Tris base, and run through Sephadex G-25 SF column equilibrated in buffer A. Alternatively, the apoenzymes in 0.1 M MES, pH 5.5, were incubated with a 3-fold molar excess of metal over the protein concentration and processed as above.

Synthesis of Linear and Branched Ubiquitin Dimer Substrates. A six-histidine tag fused to the N-terminal extremity of the linear dimeric ubiquitin substrate was cloned in pMPM-A4 vector (gift of Dr. Matthias Mayer) and expressed in MC1061 *E. coli* strains under the control of AraC repressor/activator of the *ara* operon (*29*). After induction with 2 mM arabinose L(+) for 3 h at 37 °C, bacterial pellets were lysed with lysozyme and sonicated as described above for the ISOT preparation; the resulting soluble fraction containing the tagged linear dimeric ubiquitin substrate was purified by Ni-NTA column chromatography (Qiagen) and eluted according to manufacturer's instructions. Further purification was achieved using a MonoS FPLC column (Pharmacia), as described below for the purification of the branched ubiquitin dimer.

Branched dimeric ubiquitin substrate was synthesized in vitro according to Chen and Pickart (*38*) with the following modifications: E1 enzyme was purified from human outdated reticulocytes as previously described (*1*). E1 enzyme was concentrated on Centricon50 (Amicon) and shown to be >95% pure by SDS−PAGE analysis (*39*). $E2_{25K}$ enzyme was cloned by RT-PCR from calf thymus with primers based on the published sequence (*40*) into the expression vector pMPM201 (gift of Dr. Matthias Mayer) (*29*) and expressed in MC1061 *E. coli* strains under the control of AraC repressor/activator of the *ara* operon with 2 mM arabinose L(+) for 4 h at 37 °C. Bacterial pellets were lysed with lysozyme and sonicated as described above for the ISOT preparation. The supernatant, following ultracentrifugation, was further purified by Q-Sepharose chromatography and differential precipitation with 40% and 60% (w/v) $(NH_4)_2SO_4$. After dialysis against buffer B (50 mM Tris-HCl, pH 7.2, 1 mM EDTA, 1 mM DTT), the recombinant $E2_{25K}$ enzyme was finally purified on MonoQ FPLC and Superose 12 FPLC (Pharmacia) chromatography in buffer B. Purity was 90−95% according to SDS−PAGE analysis (*39*). Large scale synthesis of branched dimeric ubiquitin substrate was performed for 2 h at 37 °C in a 2.5 mL total volume containing a final concentration of 300 nM of E1 enzyme, 4.8 μM of $E2_{25K}$ enzyme, 0.6 U/mL of inorganic pyrophosphatase, 0.6 U/mL of creatine kinase, 10 μM creatine phosphate, 2 mM ATP, 5 mM $MgCl_2$, and 2 mg/mL of ubiquitin. Reaction was stopped by the addition on ice of a final concentration of 52 mM acetic acid and 36.5 mM ammonium acetate, pH 4.5. Branched dimers of ubiquitin were separated from ubiquitin monomers, trimers, and tetramers by FPLC chromatography on MonoS column equilibrated in 50 mM ammonium acetate, pH 4.5, 50 mM NaCl and eluted with a linear 50−500 mM NaCl gradient. The yield was 33.4% with a contamination by tetrameric ubiquitin amounting to less than 5%.

Both substrates were made Zn^{2+}-free by treatment in 6 M urea containing 0.2 N acetic acid at pH 3.0, followed by ultrafiltration on a SR3 Centricon (Amicon) and several washes at pH 3.0 until no Zn^{2+} could be detected by atomic absorption. Subsequently, the retentate was brought to a 2.2−2.4 mg/mL final concentration in MilliQ water.

Enzymatic Tests. The deubiquitinating activity was monitored by preincubation for 15 min at room temperature of ∼80−160 ng of the isozymes in buffer A (15 μL total volume), followed by addition of 1−1.5 μg of linear or branched dimeric ubiquitin and further incubation at 37 °C for 5 min, as described previously (*25*). The reaction was stopped by the addition of 15 μL of 2-fold concentrated sample buffer (*40*), boiled for 5 min, and analyzed on a Tris-tricine gel (*41*). Enzymatic activity was semiquantitated by densitometric scanning of the Coomassie blue stained bands of substrates and products.

Quantitative Binding to Ubiquitin−Sepharose Affinity FPLC Column. A ubiquitin−Sepharose (3 mg/mL HiTrap NHS-activated Sepharose FPLC column, Pharmacia, Uppsala, Sweden) and control Sepharose columns, previously equilibrated in 10 mM phosphate buffer, 1 mM β-ME, pH 7.0 (buffer B), were injected at 0.5 mL/min flow rate with either wild-type or Cys335Ser mutant holoenzyme-S or apoenzyme-S (300−750 μg in 0.5−1 mL of buffer B) with continuous monitoring of absorbance at 280 nm. After an isocratic washing in buffer B and a subsequent washing with a gradient of 0−3 M KCl in buffer B, proteins were eluted with a 0−8 M urea concentration gradient in buffer B. The maximal urea concentration needed to elute the proteins was defined at the timepoint of maximum absorbance reading at 280 nm because the elution profile follows a bell-curved shape. Unknown impurities present in urea caused sometimes major shifts in the baseline absorbance at 280 nm during the gradient elution.

CD and Fluorescence Spectroscopy Analyses. CD spectra were collected on a Jasco J-715 spectropolarimeter equipped with a stress plate modulator continuously purged with dry nitrogen. Calibration was carried out using (+)-10-camphor sulfonic acid in an aqueous solution. At least four spectra

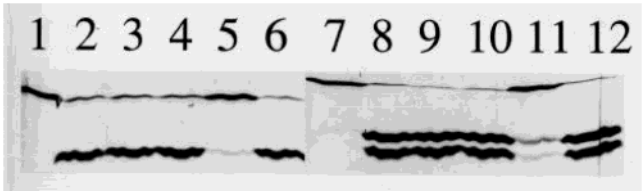

FIGURE 1: Inhibition of the wild-type ISOT-S from red blood cells by ion chelators. The cleavage activity of the 100 kDa wild-type ISOT-S toward dimeric ubiquitin substrates was analyzed by Tris-tricine SDS−PAGE in the presence of 1 mM of ion chelators: (lanes 1−6) branched dimeric ubiquitin; (lanes 7−12) linear dimeric ubiquitin; (lanes 1 and 7) substrate alone; (lanes 2 and 8) no chelator; (lanes 3 and 9) EDTA; (lanes 4 and 10) EGTA; (lanes 5 and 11) OP; (lanes 6 and 12) the nonchelating isomer 1,7-phenanthroline. Note that upon digestion of the linear dimeric ubiquitin substrate, two ubiquitin bands appear the size of which differs by an N-terminal six-histidine tag.

of the far- (180−260 nm) and near-UV (250−340 nm) range were accumulated over 20 min at 0.5 nm intervals, and the appropriate buffer blanks were subtracted. One centimeter cells were used for the collection of near-UV CD spectra and 0.01 cm cells for far-UV CD spectra. Samples at 876 (ISOT-S) and 962 μg/mL (ISOT-L) contained 10 mM Tris-HCl, 1 mM DTT, pH 7.2, and the same sample of apo- and holoenzyme was used for far-UV, near-UV, and fluorescence spectra on the same day. Mean residue molar ellipticities (deg cm^2/dmol) were calculated on the basis of the short or long isoform concentration, the number of residues in the recombinant protein, and the cell path length. Independent secondary structure predictions were performed by deconvolution of the far-UV CD spectra according to Johnson (*42*) with 22 CD reference spectra of proteins with known 3-D structure. The accuracy of the prediction was evaluated by superimposition of the original CD spectra with that of the reconstructed CD spectra (*42*), and the coefficient of variation was found to be less than 5%.

Fluorescence spectroscopy was carried out on a Perkin-Elmer LS 5B fluorospectrophotometer. Tryptophan fluorescence emissions were collected following excitation at 280 nm (slit 5 nm), and samples were diluted at 50 μg/mL in 10 mM Tris-HCl, 1 mM DTT, pH 7.2, with or without 8 M guanidine-HCl in 5 mm microcuvettes.

RESULTS

Identification of the Native S Isoform as a Zn^{2+}-Binding Protein. Examination of the primary structure of two isoforms of isopeptidase T suggests that they contain several motifs for a metal binding site (*1*), which are typical of metalloproteins (*26*, *27*). The effect of the chelating agents OP, EDTA, and EGTA, known to inhibit various metal-dependent enzymatic activities (*43*), were therefore assayed. Only OP was found to be inhibitory toward cleavage of both linear and branched polyubiquitin substrates (Figure 1). The nature of metals bound to red blood cell ISOT-S was then determined by ICP-MS. Zn^{2+} is the predominant cation identified (0.9 mol/mol of protein) followed by Ni^{2+} and Cu^{2+}, with 0.5 and 0.2 mol/mol of enzyme, respectively.

Zn^{2+} Content of Recombinant ISOT-S and ISOT-L Enzymes. Because only limited amounts of pure "native" ISOT-S can be purified from human red blood cells, the two recombinant DUBs were expressed in *E. coli* with a yield of 4−6 mg of protein per liter of culture. After purification as described in Material and Methods, both ISOT-S and ISOT-L displayed a single major band on SDS−PAGE and automated Edman degradation confirmed the expected N-terminal sequence. Direct measurements by atomic absorption in carefully controlled Zn^{2+}-free conditions disclosed one high affinity Zn^{2+} binding site (called site 1; Table 1) for both the S and L isoforms. Site 1 is likely to be common to S and L isoforms (Table 1). Treatment of both enzymes with or without 6 M urea at pH 7.2 followed by gel filtration over Sephadex G-25 SF column in 50 mM Tris-HCl, pH 7.2, containing 1 mM DTT (buffer A) was used to remove adventitious Zn^{2+} from the enzyme preparation. Note that DTT is a known Zn^{2+} chelator with binding constant up to 10^{12} M^{-1} (*44*, *45*).

Table 1: Zinc Content and Enzymatic Activity of Holoenzyme vs Apoenzyme of S and L Isoforms of Isopeptidase T[a]

ISOT-S		
treatment	Zn^{2+}/protein	activity, %
	Holoenzyme	
6 M urea, pH 7.2	0.90 ± 0.05 (3)	100
	Apoenzyme	
6 M urea, pH 3.0	0.02 ± 0.003 (4)	0
4 M urea, pH 3.0	0.13	nd
2 M urea, pH 3.0	0.10	nd
6 M urea, pH 4.0	0.33	nd
6 M urea, pH 5.0	0.56	nd
OP, pH 5.5	<0.01[b]	0
OP removal, pH 5.5	0.10[c] (2)	10
ISOT-L		
treatment	Zn^{2+}/protein	activity, %
	Holoenzyme	
6 M urea, pH 7.2	0.94 ± 0.07 (6)	100
	Apoenzyme	
6 M urea, pH 3.0	0.01 ± 0.002 (2)	0

[a] The holoenzyme was treated in 6 M urea at pH 7.2 and filtered through Sephadex G-25 SF in 50 mM Tris-HCl, pH 7.2, buffer containing 1 mM DTT without denaturing agent. The apoenzyme was prepared in 6 M urea, pH 3.0, unless otherwise stated, and run over Sephadex G-25 SF as for the holoenzyme. Alternatively, the apoenzyme was prepared by dialysis in 0.1 M MES buffer, pH 5.5, with or without 1 mM OP. The zinc content, determined by atomic absorption, is given as the mean ratio of moles of Zn^{2+}/mole of protein ± SE for the number of experiments given in parentheses. The enzymatic activity was assayed with linear dimeric ubiquitin substrate and was estimated relative to that of the untreated holoenzyme. Further details are given in Material and Methods. [b] After dialysis for 7 days in 0.1 M MES, pH 5.5, buffer containing 1 mM OP. [c] After dialysis for 7 days in 0.1 M MES, pH 5.5, buffer containing 1 mM OP and an additional 7 days of dialysis in 0.1 M MES, pH 5.5, to remove OP.

Zn^{2+} Removal, Substrate Binding, and Enzymatic Properties of the Holoenzyme vs Apoenzyme. Different procedures were tried to remove Zn^{2+} from the enzymes by using chelators, denaturation, acidification, or a combination of them. When 5−25 μM of ISOT-S and -L were incubated overnight in buffer A at 4 °C with a 12.5-fold molar excess of OP followed by gel filtration on a G-25 SF column equilibrated in the same buffer, both enzymes were shown to bind one Zn^{2+} atom per molecule (data not shown). When 6 M urea was added under the same conditions, both proteins recovered from gel filtration still contained 0.65 mol of Zn^{2+} atom per mole protein, suggesting that denaturation at neutral pH partially triggers Zn^{2+} release from the protein by OP. Treatment of 10 μM of holoenzyme isoforms with a 500-fold molar excess of NTA or dipicolinic acid in buffer A

followed by gel filtration in the same buffer containing 1 μM NTA or dipicolinic acid was also ineffective (data not shown). In contrast, when holoenzyme was treated for 3 weeks at 4 °C (in buffer A, in the presence of 6 M urea) with a 10-fold molar excess of OP, NTA, or EDTA, Zn^{2+} was totally removed from the protein after gel filtration. However, SDS−PAGE analysis showed that the apoprotein was found intact after OP treatment but degraded after NTA or EDTA treatment (data not shown). Zn^{2+} binding affinity of site 1 was estimated at $K_a > 10^{13}$ M^{-1} with a speciation program (EqCal for Windows, see Material and Methods) on the basis of the known concentration of the OP chelator, which was used for the equilibrium dialysis experiments described above. Note that this estimation is supported by the fact that Zn^{2+} of site 1 is *not* chelated by DTT ($K_a > 10^{12}$ M^{-1}) because the enzyme is fully active in 1 mM DTT (10 000−fold molar excess to free zinc and ISOT concentrations).

A much faster protocol to remove efficiently the endogenous metal ions was carried out by a combination of urea and low pH (Table 1). Treatment of holoproteins for 10 min on ice with a solution of acetic acid at pH 3.0, containing either 2, 4, or 6 M urea (final concentration), followed by gel filtration on Sephadex G-25 SF in buffer A, disclosed the presence of 0.10, 0.13, and 0.02 mol of remaining Zn^{2+} per mole of enzyme, respectively (Table 1). Similar experiments at pH 5 or 4 in the presence of 6 M urea led to 0.56 and 0.33 mole of remaining Zn^{2+} per mole of protein, respectively (Table 1). The data suggest that the site for the tightly bound Zn^{2+}, referred to as site 1, must be unfolded by denaturation at pH 3.0 to quickly release the metal ion from the protonated ligands. Denaturation with 6 M urea at neutral pH did not release Zn^{2+} (Table 1). Denaturation in acidic urea is, however, not compulsory for Zn^{2+} removal because a week-long dialysis at pH 5.5 (without urea) was sufficient to completely remove Zn^{2+} from site 1 in the presence of both 20- and 100-fold excess of OP over the protein concentration, followed by an additional week of dialysis in 0.1 M MES at pH 5.5 to remove OP. Apoproteins remained fully soluble at pH 5.5 and contained 0.01−0.10 Zn^{2+}/molecule (Table 1). By contrast, substitution of OP by NTA or EDTA was ineffective for Zn^{2+} release, suggesting that Zn^{2+} is presumably bound in a hydrophobic pocket.

In the standard protocol of Zn^{2+} removal described under Material and Methods, the apoproteins contained no more than 0.02 Zn^{2+} (Table 1) and the recovery of soluble apoproteins invariably ranged from 85% to 90% after gel filtration. Because the 30 kDa ubiquitin carboxyl-terminal hydrolase (UCH) enzyme−ubiquitin complex was known to be stable at neutral pH in 5 M urea (*5*), quantitative binding to and elution with urea from ubiquitin−Sepharose affinity column was tested to determine whether apoproteins of ISOT still recognize their substrate. The apoenzymes of either the wild-type (Figure 2A, data not shown for ISOT-L) or the active-site-Cys335Ser mutant of ISOT-S (Figure 2B) bind as strongly to ubiquitin−Sepharose conjugate as the corresponding holoproteins. Virtually no enzyme remains in the unbound fractions or was eluted from the column during a 0−3 M KCl concentration gradient washing step (data not shown). In a linear gradient of 0−8 M urea, the wild-type holoform of ISOT-S was eluted from the ubiquitin−Sepharose affinity column with 5.6−5.8 M urea whereas the wild-type apoform was eluted with 5.2−5.4 M urea (Figure 2A). Comparable results were obtained with the holo- (6.1 M urea) and apoforms (5.4 M urea) of the active-site-Cys335Ser mutant of ISOT-S (Figure 2B), suggesting that the substitution did not affect substrate binding. By contrast with ubiquitin−Sepharose beads, most of the enzymes were found in the flow-through fraction of control Sepharose beads (data not shown). Residual weakly nonspecific bound apo- or holoenzyme was subsequently completely eluted from control Sepharose beads with KCl.

Despite substrate binding, the wild-type apoforms of ISOT-S (Figure 3A) and -L (data not shown) were devoid of enzymatic activity for the linear dimeric ubiquitin substrate and exhibited very weak activity for the branched ubiquitin substrate, irrespective of the denaturing or nondenaturing character of the Zn^{2+} removal method used. The loss of enzymatic activity is unlikely to be the consequence of a lack of direct interaction between the ubiquitin substrate and Zn^{2+} ion in the enzyme−substrate complex. Because the enzyme−substrate affinity did not change significantly upon Zn^{2+} removal (Figure 2A,B), the binding of the ubiquitin substrate does not require Zn^{2+} in the precatalytic enzyme−substrate complex. Moreover, monomeric, dimeric, or multimeric ubiquitin substrates were unable to bind Zn^{2+} directly by atomic absorption or $^{65}Zn^{2+}$ blot (data not shown).

Role of Zn^{2+} on the Structural Properties of the Holoenzyme vs Apoenzyme. Far-UV CD spectroscopy analysis of the apo- and holoenzyme of both isoforms showed a maximum ellipticity at 191 nm and two minima at 208 and 215 nm, which are indicative of a mixed α-helical and β-sheet secondary structures. Metal-free apoenzyme and Zn^{2+}-complexed holoenzyme of both the S and L isoforms showed virtually identical CD spectra, suggesting that the release of Zn^{2+} did not substantially affect the isopeptidase T secondary structure (Figure 4A). Deconvolution of the CD spectra by the variable selection and self-consistent method of Johnson (*42*) predicted, respectively, 20% and 21% α-helices, 13% and 12% β-sheet, and 16% and 17% β-turns, as well as 51% and 49% random coils for the apo- and holoenzyme of ISOT-S, while 22% and 24% α-helices, 12% and 11% β-sheets, and 16% and 16% β-turns, as well as 50% and 49% of random coils were predicted for the apo- and holoenzymes of ISOT-L, respectively. Near-UV CD spectra of the apoenzymes showed a prominent band at 264 nm, while spectra of the holoenzymes showed a prominent band at 294 nm (Figure 4B). This change in the aromatic region of the CD spectrum suggests a change of the local structural environment of the aromatic residues upon complexation with Zn^{2+}. The small change in the CD spectra is poorly compatible with Zn^{2+} playing a scaffolding role to build up the enzymatically active tertiary conformation.

Tryptophan fluorescence spectra of the apo- and holoenzymes confirmed the observations made by near-UV CD spectroscopy. Removal of Zn^{2+} in the apoenzyme induced a quench of the fluorescence to 66% of the holenzyme's intensity maxima centered at 334−338 nm ($n = 5$), as well as a slight blueshift of 3−4 nm, suggesting that the indole side chains of some tryptophan residues in the apoenzyme are exposed to the aqueous buffer (Figure 4C). Therefore, Zn^{2+} in the holoenzyme may confer a hydrophobic environment to several tryptophan residues. By contrast, the spectrum of the native apoenzyme markedly differed from

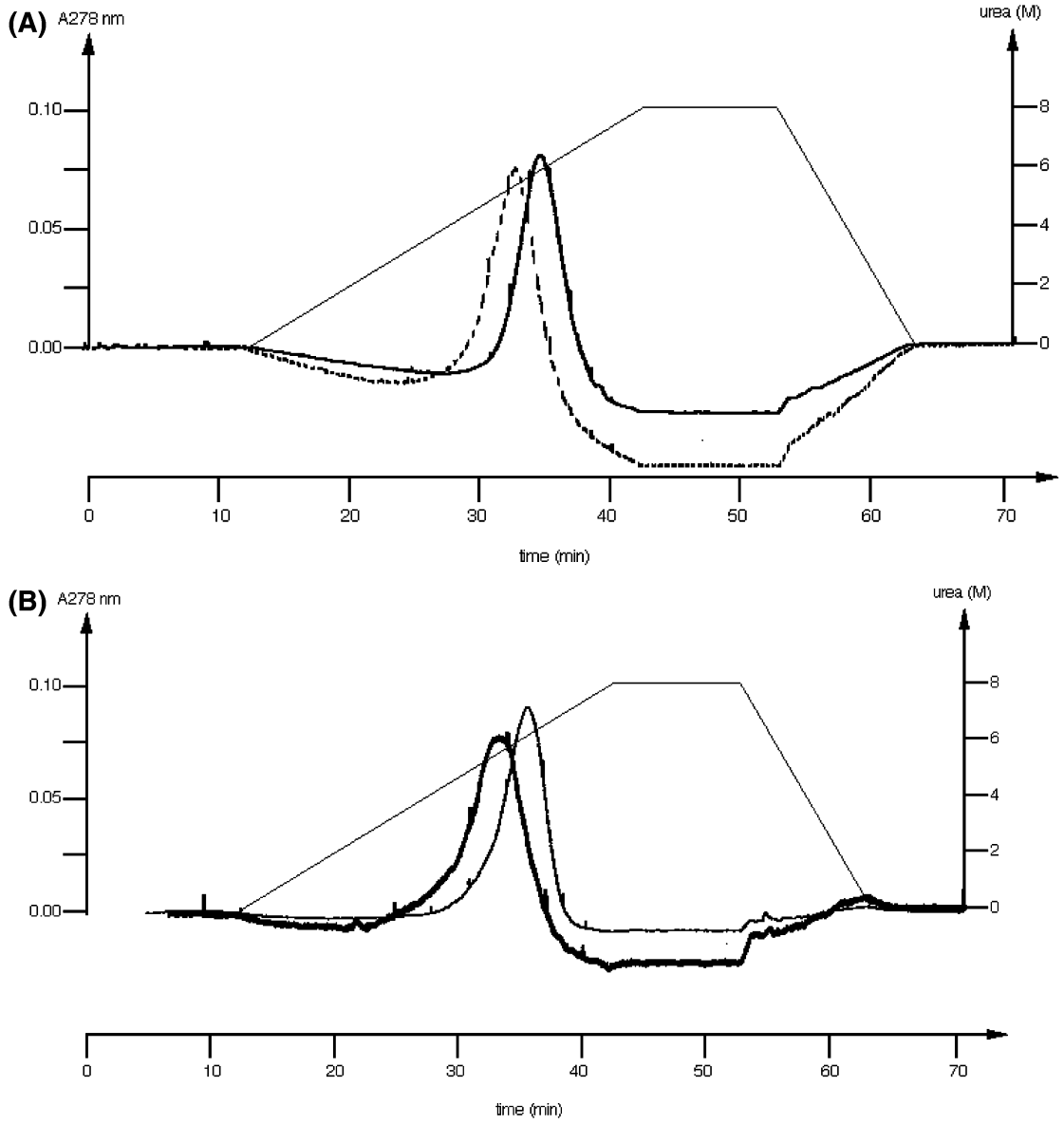

FIGURE 2: Effect of Zn^{2+} on the enzyme−substrate affinity: urea denaturation of holo- and apo-ISOT-S isoform on ubiquitin−Sepharose affinity column. Control Sepharose (data not shown) or ubiquitin−Sepharose affinity FPLC columns were loaded at 20 °C with 300−750 μg of holo- or apoprotein ISOT-S isoform in buffer B, washed in buffer B alone or in buffer B containing 0−3 M KCl (see Material and Methods for more details). To evaluate the effect of Zn^{2+} on the enzyme−substrate affinity, elution of holo- or apoproteins was performed at 0.5 mL/min with a linear gradient of 0−8 M urea concentration with continuous monitoring of absorbance at 280 nm. Panel A shows the elution of wild-type ISOT-S as a function of urea concentration: holoenzyme (solid line); apoenzyme (dotted line). Panel B shows the elution of active site Cys335Ser mutant ISOT-S as a function of urea concentration: holoenzyme (solid line); apoenzyme (bold line).

the spectra obtained with the apo- and holoenzymes denatured in 8 M guanidine-HCl, suggesting that the structure of the apoenzyme is not destroyed upon Zn^{2+} removal. It is likely that the blueshift-induced Zn^{2+} removal correlates with a slightly increased rigidity of the tertiary structure.

Binding of Additional Atoms of Zn^{2+} by Both S and L Isoforms of the Holoenzyme. When 10 μM solutions of both isozymes (containing one tightly bound Zn^{2+} atom per molecule) were incubated in the presence of 50 μM free Zn^{2+} solution at 4 °C and pH 7.2 for 48 h and subjected to gel filtration on Sephadex G-25 in buffer A, two Zn^{2+} atoms per molecule were detected (Table 2). In contrast to the first tightly bound Zn^{2+} of site 1, the second Zn^{2+} atom could be removed upon treatment with NTA at neutral pH, suggesting that its site, referred to as site 2, is much more solvent-accessible than site 1 (Table 2). Nevertheless, its affinity for the protein must be at least 10^8 M^{-1} because the detectable free Zn^{2+} concentration in buffer A is below 0.1 μM. This accessible Zn^{2+} plays no significant role in catalysis because both S and L isoforms containing either one or two Zn^{2+} atoms per molecule exhibited indistinguishable proteolytic activity (Figure 3B).

Both isozymes possess additional loosely bound Zn^{2+} sites because upon increasing the free Zn^{2+} concentration from 10 to 50 μM the enzymatic activity was inhibited and was almost completely lost at 50 μM free Zn^{2+} (Figure 3C). Their stoichiometry and affinity were quantified by equilibrium gel filtration according to Hummel−Dryer (*33*), which disclosed, in addition to sites 1 and 2, six and seven additional Zn^{2+}-binding sites for the S (Figure 5A) and L isoform (Figure 5B), respectively, with a dissociation constant of 5.8 μM and a Hill coefficient of 1, as calculated

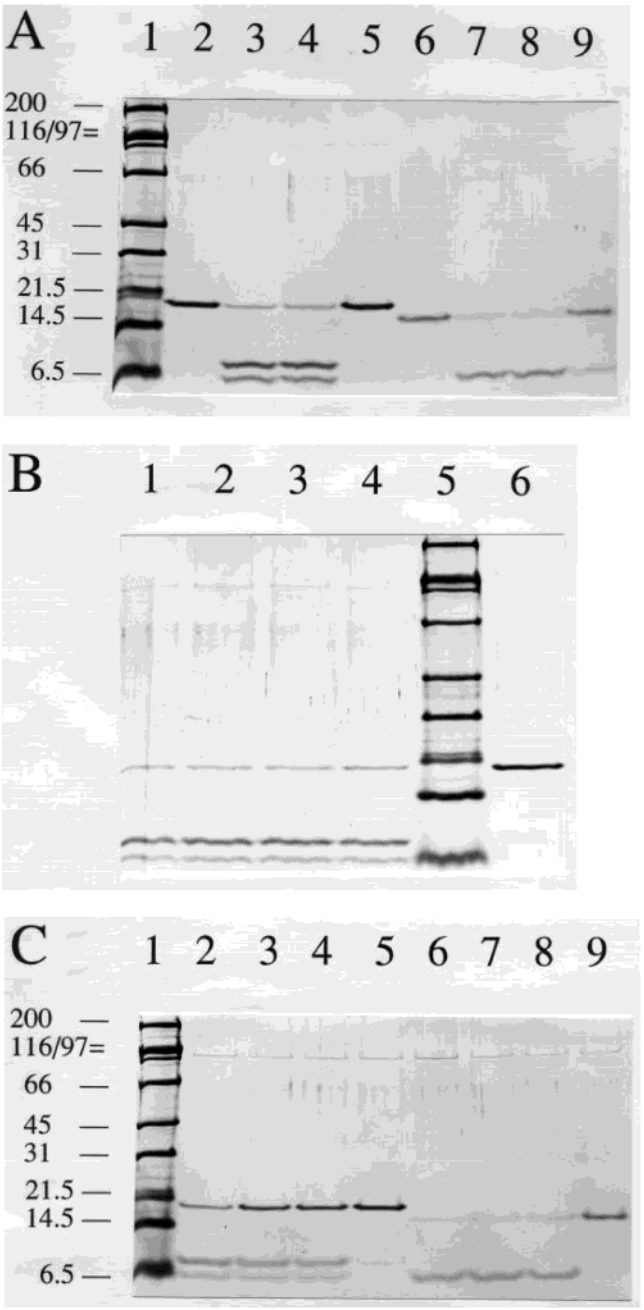

FIGURE 3: Enzymatic activities of holoenzymes and apoproteins. All enzymatic incubations were carried out by incubating 80−160 ng of enzyme with 1 μg of linear or branched dimeric ubiquitin for 5 min at 37 °C and analyzed by Tris-tricine SDS−PAGE. Panel A shows the activity of ISOT-S: (lane 1) Mr markers; (lanes 2−5) digestion of linear ubiquitin dimer with (2) substrate alone, (3) untreated enzyme, (4) holoenzyme treated with 6 M urea pH 7.2, (5) apoenzyme; (lanes 6−9) digestion of branched dimeric ubiquitin with (6) substrate alone, (7) native enzyme, (8) holoenzyme treated with 6 M urea pH 7.2, (9) apoenzyme. Panel B shows the activity of ISOT-L and ISOT-S comprising each an additional Zn^{2+} atom/molecule towards linear dimeric ubiquitin: (lane 1) L isoform with two Zn^{2+}; (lane 2) L isoform with one Zn^{2+}; (lane 3) S isoform with two Zn^{2+}; (lane 4) S isoform with one Zn^{2+} atom/molecule; (lane 5) same Mr markers as in panels A and C; (lane 6) linear substrate alone. See text and Table 2 for details. Panel C shows the enzymatic inhibition of ISOT-S by $ZnCl_2$, tested with linear dimeric ubiquitin (lanes 2−5) and branched ubiquitin dimer (lanes 6−9) substrate: (lane 1) Mr markers; (lanes 2−5 and 6−9) 0, 10, 20, and 50 μM free Zn^{2+}, respectively.

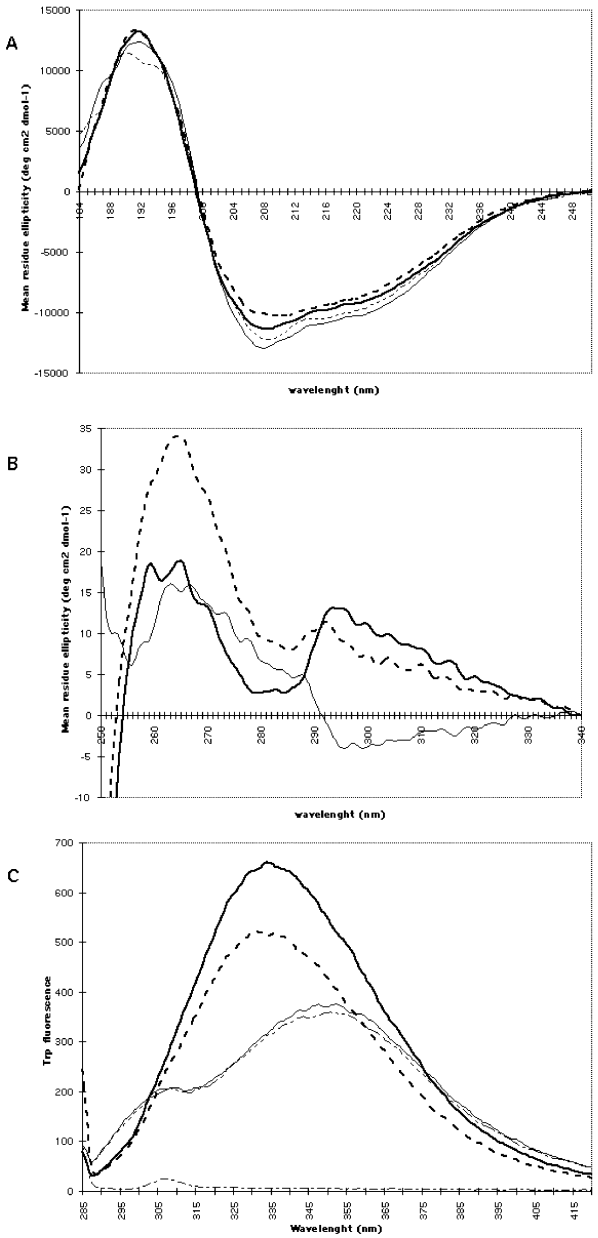

FIGURE 4: Structural analyses of apo- and holoenzymes by CD and fluorescence spectroscopy. Panel A shows the effect of Zn^{2+} removal on the secondary structure of S and L isoforms of isopeptidase T. Far-UV CD spectroscopy of apoenzymes (dotted lines) or holoenzymes (solid lines), each from both the S (bold lines) and L isoforms (thin lines) are presented. Blank buffer was subtracted and data were subjected to a seven-point line smoothing. Panel B shows the effect of Zn^{2+} removal on the tertiary structure of ISOT-S. Near-UV CD spectroscopy of apo- (bold dotted line) and holoenzyme (bold continuous line) of S isoform are presented. Difference spectra (thin continuous line) were obtained by subtracting the apoenzyme spectra from the holoenzyme spectra. Data were processed as above. Panel C shows the changes in the aqueous environment of tryptophan residues upon Zn^{2+} release from ISOT-S. Tryptophan emission fluorescence spectra of native apoenzyme (bold dotted line), native holoenzyme (bold continuous line), guanidine-HCl-denatured apoenzyme (thin dotted line) and guanidine-HCl-denatured holoenzyme (thin continuous line) are presented. The appropriate blank buffer, with or without 8 M guanidine-HCl, was subtracted. The blank buffer containing 8 M guanidine-HCl is shown as a control. Light backscattering around 287 nm was a regular feature, only observed with the native apoenzyme, which is typical for a slight state of protein aggregation.

Table 2: Binding of a Second Zinc Atom to S and L Isoforms of Isopeptidase T Holoenzyme[a]

ISOT-S			
sample Zn^{2+}/protein	treatment	Zn^{2+}/protein	activity, %
1.0	none	1.0	100
1.0	50 μM Zn^{2+}	2.0	100
1.1	50 μM NTA	1.0	100
2.0	none	1.8	100
2.0	80 μM NTA	1.0	100
ISOT-L			
sample Zn^{2+}/protein	treatment	Zn^{2+}/protein	activity, %
1.0	none	1.0	100
1.0	50 μM Zn^{2+}	2.1	100
1.1	50 μM NTA	1.0	100
2.1	60 μM NTA	1.0	100

[a] Holoenzymes (1 Zn^{2+}/mol) were first treated with a 5-fold molar excess of Zn^{2+} at pH 7.2 for 48 h at 4 °C and filtered through a Sephadex G-25 SF column equilibrated in Zn^{2+}-free 50 mM Tris-HCl, pH 7.2, buffer containing 1 mM DTT. Both isoforms loaded with 2 Zn^{2+} ions/molecule were then incubated with a 9−10-fold molar excess of NTA for 30 min at 4 °C, followed by gel filtration at neutral pH. Zn^{2+} was determined by atomic absorption. Activity was assayed on both linear and branched dimeric ubiquitin substrates and was expressed relative to that of the untreated holoenzyme.

as in ref (*46*). Because the insert of the L isoform contains a CxxH motif, we tested whether amino acid ligands located within this insert could play a role in the supernumerary Zn^{2+}-binding site. The corresponding synthetic peptide itself was unable to bind Zn^{2+}, as measured by equilibrium gel filtration and by MALDI-TOF mass spectrometry (data not shown). Because the limit of detection by our atomic absorption equipment was 0.1 μM Zn^{2+}, no reliable measurements could be made below 0.1 μM of free Zn^{2+} concentration. Similarly, at high free Zn^{2+} concentration (i.e., over 200 μM), no obvious protein saturation with Zn^{2+} could be observed (Figure 5A,B), which is likely due to the nonspecific binding of Zn^{2+} on both isoforms. The theoretical calculated isotherms (best fit of data; solid lines in Figure 5A,B) were derived from Scatchard plots (insert in Figure 5A,B), which suggests a maximal number of bound Zn^{2+} atom/molecule of 8 and 9 for the S and L isoforms, respectively.

Metal Ion Reconstitution and Restoration of Enzymatic Activity. When S and L isoforms of apoenzyme were incubated at neutral pH (buffer A) for 48 h at 4 °C with 50 μM Zn^{2+} without urea, followed by gel filtration in buffer A, 1.25 mol of Zn^{2+} were bound to the enzymes (Table 3, condition A). Apparently, Zn^{2+} was located in the exchangeable site 2 only because (i) this ion could be easily removed by NTA (data not shown) and (ii) the Zn^{2+}-containing enzymes were found to be completely inactive (Table 3, condition A). However, when the apoproteins were submitted to the reconstitution protocol in urea at acidic pH, both isozymes contained about 1 mol of Zn^{2+} per mole of protein (Table 3, condition B) and both regained close to 100% of their original activity toward linear and branched dimeric ubiquitin. Denaturation in 6 M urea is not necessarily required to change the level of metal occupancy of site 1 because reconstitution of holoenzymes (1.1−1.3 Zn^{2+}/molecule) concomitant with full restoration of enzymatic activity could also be achieved at pH 5.5 under nondenaturing conditions (data not shown). Partial reconstitution with 10% or 50% of Zn^{2+} leads to 10% or 50% of the original activity, respectively (data not shown).

The S isoform could be reconstituted by other cations than Zn^{2+} in acidic urea. At a molar ratio of 3:1 cation to protein, 0.7−1.1 mol of Co^{2+}, Cd^{2+}, Cu^{2+}, or Ni^{2+} was bound to the enzyme (Table 3, condition B). Only Ni^{2+} restored almost 90% of the original activity of the Zn^{2+} enzyme. Poor restoration of activity was found with Cu^{2+} (10%), whereas Co^{2+}- and Cd^{2+}-reconstituted proteins were inactive. Thus, it appears that the binding of Zn^{2+} or Ni^{2+}, presumably to site 1, requires opening of a pocket favored by acidic conditions to achieve the correct liganding of the metal in its binding site, leading to full restoration of catalytic activity. The 10-fold reduction in activity of the Cu^{2+}-bound molecule compared with the Zn^{2+}-bound enzyme could be attributed to fine differences in the charge distribution due to the difference in the ionic radii of the two metals (0.72 and 0.74 Å, respectively), while almost 90% activity remained with the Ni^{2+}-bound enzyme, although Ni^{2+} (0.69 Å) is smaller than Zn^{2+} (0.74 Å). Other factors, such as differences in the coordination environments for each type of metal, may contribute to the loss of activity because Co^{2+} and Zn^{2+} have identical ionic radii and Cd^{2+} is larger than Zn^{2+}.

DISCUSSION

The S and L Isoforms of the Human ISOT are Monozinc Metalloproteases. The aim of this investigation was to determine the effects of Zn^{2+} on the hydrolysis of linear and branched dimeric ubiquitins and to compare the Zn^{2+}-binding properties of two ISOT-S (short) and ISOT-L (long) isoforms. The L isoform differs from the S isoform by an insertion of 23 amino acids, which comprises a $C_{641}xxH_{643}$[2] putative metal-binding motif. Despite this difference, both isozymes show indistinguishable Zn^{2+}-dependent enzymatic profiles and substrate selectivities. Thus, the role of this insert is presently not elucidated, and the functional difference between the isoforms is not apparent.

The interaction of both isozymes with Zn^{2+} is rather complex and suggests three distinct categories of sites: (i) A cryptic site 1 binds or releases Zn^{2+} either instantaneously in 6 M urea at acidic pH or gradually at pH 5.5 provided OP is present but is insensitive to NTA, EDTA, EGTA, or dipicolinic acid. (ii) A site 2 binds Zn^{2+} with high affinity and can be emptied with NTA. Whereas metal occupancy of site 1 appears to be essential for catalysis, Zn^{2+} in site 2 does not influence catalytic activity; when site 1 is empty, filling of site 2 does not restore activity. (iii) Six and seven low-affinity Zn^{2+}-binding sites (K_d around 5.8 μM) are found in S and L isoforms, respectively. Binding of free Zn^{2+} to the third category of low-affinity sites leads to enzymatic inhibition, which is complete at 50 μM free Zn^{2+}. The inhibition of enzymatic activity due to Zn^{2+} binding to low-affinity sites has been reported for several metallopeptidases (*43, 47*).

The tightly bound Zn^{2+} of site 1 cannot efficiently be removed by chelators such as OP, EDTA, EGTA, NTA, or dipicolinic acid at neutral pH. In the presence of 6 M urea

[2] Numbering according to ISOT-L sequence as in ref (*1*).

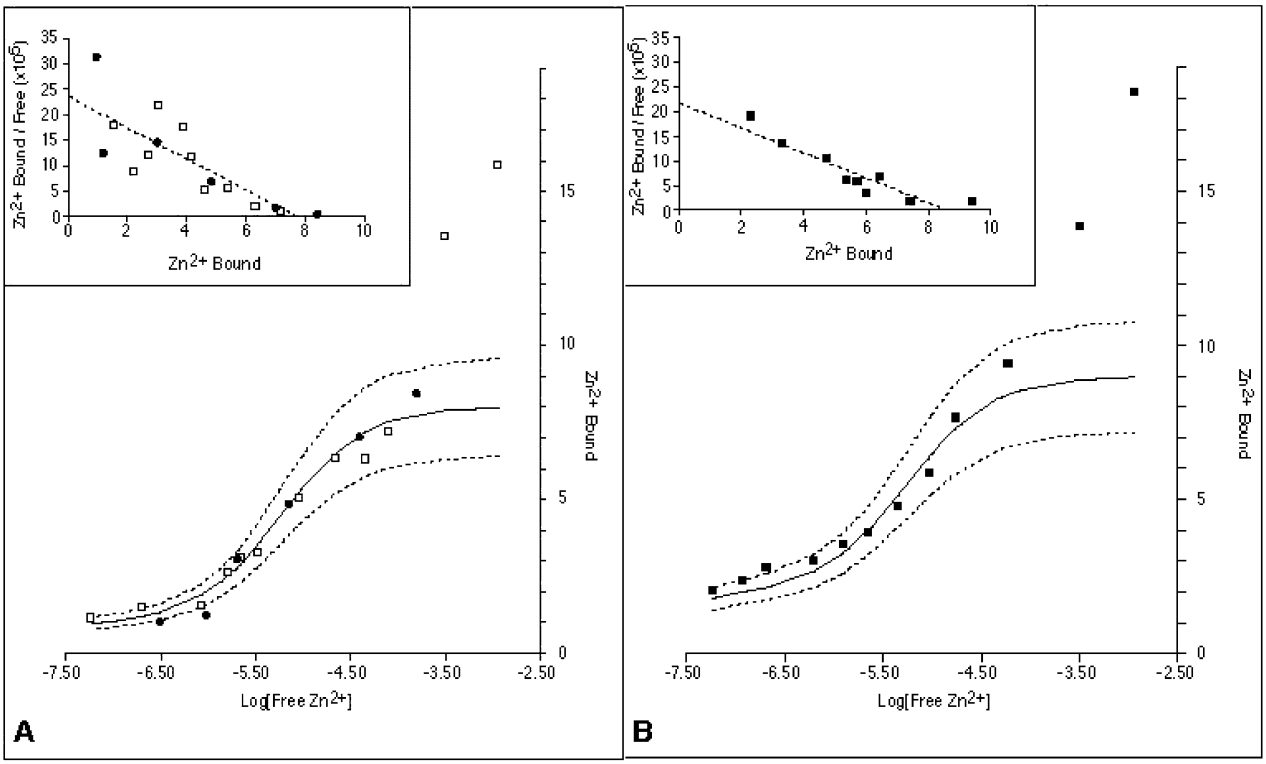

FIGURE 5: Zn^{2+} binding to S and L isoforms as determined by the Hummel−Dryer method: (●) ISOT-S purified from human erythrocytes; (□) ISOT-S; (■) ISOT-L. In panel A, the solid line (● and □) is the theoretical isotherm calculated for seven sites of low affinity. In panel B, the solid line (■) is the isotherm calculated for eight sites of low affinity. Dashed lines represent relative errors (estimation due to the limiting protein concentration). Inserts in panels A and B show Scatchard plots of the data.

at pH 7.2 for as long as 18 h, OP released 0.35 atom of Zn^{2+} per molecule, while NTA was still ineffective. Because under these conditions the free Zn^{2+} concentration was around 10^{-13} M (calculated with a speciation program) (*36*), this site either has a very high affinity for Zn^{2+} ($K_d < 10^{-14}$ M, assuming a fast equilibrium following a classical binding isotherm) or is inaccessible to solvent at pH 7.2, that is, the kinetic barrier for binding or dissociation of the metal ion is so high that no changes are observed in a relative short time span (*43*). The latter hypothesis is more likely because (i) it is unlikely that metalloenzymes have a higher affinity than metallothioneins, the strongest Zn^{2+}-binding proteins with a K_d of 1.4×10^{-13} M (*48*, *49*), (ii) Zn^{2+} can only be removed from site 1 provided the proteins are dialyzed against OP either for 1 week at pH 5.5 in the absence of urea or for 3 weeks at pH 7.2 in the presence of 6 M urea, and (iii) reconstitution of metal ions to site 1 is inefficient at neutral pH, leading to inactive apoenzyme and filling of site 2. The kinetic barrier, reported also in the case of other Zn^{2+}-binding proteins (*43*), prevents moderately fast equilibration between bound and free Zn^{2+} and thus an adequate estimation of the affinity. Interestingly, acidification in the presence of 6 M urea renders the cryptic site 1 more accessible because metal depletion was progressively more advanced at pH 5 and 4 and complete at pH 3.0, suggesting that increased protonation of protein Zn^{2+} ligands released the Zn^{2+} atom from site 1 by competition with H^+. Urea is obviously required to keep the enzymes soluble at pH 3.0 because it did not release Zn^{2+} nor abolish ubiquitin binding at neutral pH. Site 1 seems to constitute a hydrophobic pocket because at pH 5.5 slow Zn^{2+} removal is facilitated by the hydrophobic chelator OP but not by acidic chelators such as NTA, EDTA, EGTA, or dipicolinic acid. The reason OP could remove Zn^{2+} from site 1 while NTA, EDTA, EGTA, or dipicolinic acid could not might be related to the type of coordination needed to chelate the metal. Therefore, the tightly bound Zn^{2+} in site 1 would not be accessible to a charged molecule, whereas a neutral hydrophobic molecule such as OP has access, however, only at pH 5.5 or in 6 M urea at neutral pH.

Zn^{2+} is Required for Enzymatic Activity. The presence of Zn^{2+} in site 1 is essential for the enzymatic activity of isopeptidase T: the metal-free form is inactive, whereas the reconstituted holoenzymes are as active as the original Zn^{2+}-bound proteins. Partial reconstitution with Zn^{2+} leads to partial restoration of activity. Surprisingly, even though OP was shown to inhibit enzymatic activity of isopeptidase T within minutes (*1*), Zn^{2+} was not removed from the protein at neutral pH. The enzyme inhibition cannot be attributed to nonchelating properties of OP such as its hydrophobicity because 1,7-phenanthroline, a nonchelating analogue, was not inhibitory. We surmised that a ternary enzyme−Zn^{2+}−OP complex is responsible for the time- and concentration-dependent inhibition, probably because of steric hindrance by OP bound to Zn^{2+}, and that a long time (7 days at pH 5.5 or 20 days in neutral urea) is required for the dissociation of this ternary complex and subsequent Zn^{2+}−OP release from the enzyme.

Both isozymes also bind a second Zn^{2+} ion in site 2, which could easily be removed by NTA. The affinity of site 2 for Zn^{2+} can be estimated as between 10^8 and 10^{11} M^{-1} (assuming a fast equilibrium reaction). In contrast to site 1, the occupancy of site 2 had no modulating or reconstitutive effect on enzymatic activity. Interestingly, the Zn^{2+}-depend-

Table 3: Metal Content and Enzymatic Activity in Metal-Reconstituted S and L Isoforms of Isopeptidase T Holoenzyme at Different pH Conditions

Condition A[a]			
metal (pH 7.2)	treatment	Zn^{2+}/protein	activity, %
50 μM Zn^{2+}	48 h mixing	1.25[b]	0
0.5 μM Zn^{2+}	18 h dialysis	1.24[c]	0

Condition B[d]			
metal (pH 3.0)	metal/protein (ICP-MS)	Zn^{2+}/protein	activity, %
Zn^{2+}	nd	0.98 ± 0.11 (8)	100 (8)
Cd^{2+}	0.94	<0.029	0 (2)
Cu^{2+}	1.11	<0.028	10 (2)
Co^{2+}	0.78	<0.026	0 (2)
Ni^{2+}	0.66	<0.036	90 (2)
Ni^{2+}	0.80 ± 0.05 (4) (AA)	nd	90 (2)

[a] A solution of apoenzyme in 6 M urea at pH 3.0 was first neutralized and treated at pH 7.2 with 0.2−7-fold molar excess of Zn^{2+}. Excess metal was then removed by dialysis or gel filtration at neutral pH. The activity was assayed with both linear and branched dimeric ubiquitin substrates as compared to that of untreated zinc-containing holoenzyme. [b] Treated with 7-fold molar excess of free zinc concentration at pH 7.2 for 48 h at 4 °C followed by G-25 SF gel filtration in zinc-free buffer A. [c] Dialysis for 18 h at 4 °C in stepwise decreasing urea concentration buffer containing 0.5 μM zinc at pH 7.2 followed by a final 4 h dialysis in zinc-free buffer A. [d] Solutions of apoenzyme prepared in 6 M urea at pH 3.0 were treated with a 3-fold molar excess of Zn^{2+}, Cd^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+} ions; after neutralization and removal of excess urea and metal by gel filtration at pH 7.2, metal content was determined by ICP-MS or atomic absorption (AA) and Zn^{2+} contamination in the reconstituted samples was detected by atomic absorption (AA). The mean ratio of metal/protein ± SE and the activity relative to that of the untreated holoenzyme are shown with the number of experiments given in parentheses.

ent β-lactamase from *Bacillus cereus* was also shown to bind two Zn^{2+} atoms, although only one was reported to be required for activity (*50*). The picture of the diversity of the interaction of Zn^{2+} with both isopeptidases is completed by the presence of a third set of several low-affinity sites ($K_d \approx 5.8$ μM); occupancy by loosely bound Zn^{2+} leads to complete inhibition of the enzymatic activity.

Using our particular reconstitution procedure implying acidic urea, not only Zn^{2+} but also Co^{2+}, Cd^{2+}, Cu^{2+}, and Ni^{2+} could be introduced in both isoforms. Of these cations, only Ni^{2+} restored the enzymatic activity to almost the same extent as Zn^{2+}, which thus presumably also binds to site 1. Because Co^{2+}, Cd^{2+}, and Cu^{2+} do not restore enzymatic activity, it could be speculated that they either bind to site 1 but yield an inactive state or fill site 2, obviously not involved in catalysis. More detailed studies are needed to settle this question. Even though coincidental, it is noteworthy that the native S isoform of isopeptidase from red blood cells contains Ni^{2+} as the second most important cation associated with the enzyme. Because in most organisms, Ni^{2+} is far less abundant than Zn^{2+}, the physiological importance of this functional substitution in vitro remains obscure.

How do these different Zn^{2+} sites control the activity of these enzymes in vivo? Because of the relative abundance of metallothioneins, the cytosolic Zn^{2+} concentration is exceedingly low, that is, <0.1 nM. However, thanks to an ingenious mechanism based on redox-modulated changes in their Zn^{2+} affinity, metallothioneins can provide or withdraw Zn^{2+} from transcription factors and metalloenzymes (*48*, *51*, *52*). Thus, the cellular redox state likely regulates also the state of occupancy of Zn^{2+} sites in this enzyme, including the loosely bound Zn^{2+} sites. Ultimately, the redox state of the cells may control the recycling of ubiquitin and protein turn-over as suggested in many neurodegenerative diseases, including Alzheimer's (*53*, *54*) and Parkinson's diseases, in which increased levels of Zn^{2+} in the brain have been reported (*55*, *56*).

Proposed Models of Catalysis. On the basis of sequence analysis comparison (*1*, *19*), both isoforms of the human ISOT were previously classified as thiol protease members of the UBP family (reviewed in refs *2*−*4*). However, we have shown in this study that both isoforms of the human ISOT have a catalytic requirement for Zn^{2+}. We propose here two putative models of catalysis to assess the role of the zinc.

The first model proposes that zinc participates in a local structure in the vicinity of the active site. The active site of ISOT is composed of three distinct cooperating entities, which may or may not overlap as in many metalloenzymes: the catalytic effector's site, the Zn^{2+}-binding site 1, and the substrate binding site (*57*), which is probably composed of two UBA domains (*58*−*60*). The latter remains functional because the ubiquitin binding capacity of the apoenzyme was nearly indistinguishable from that of the holoenzyme, suggesting that Zn^{2+} does not play a significant part in maintaining the *global* structural stability of the protein and Zn^{2+} removal does not render the protein markedly unstable. Moreover, all of our spectroscopy and fluorimetry data indicate that Zn^{2+} of site 1 is clearly not essential for maintaining the *global* structure of the enzyme. Near-UV CD and fluorimetry only point to quantitatively small changes in the internal protein organization, as evidenced by minor changes in the environment of aromatic residues, which are compatible with an enhanced rigidity of the tertiary structure upon Zn^{2+} removal. If Zn^{2+} was structural, it is clearly limited. Indeed, the apoproteins both elute from the ubiquitin−Sepharose affinity column slightly earlier than the holoproteins, suggesting a slight weakening of the affinity for substrate or slight enhanced succeptibility to denaturation by urea or both. Our data are compatible with Zn^{2+} playing a *local* structural role and affecting only the active site of this thiol protease.

The second model of catalysis proposes that zinc is the only catalytic effector in this enzyme. The lack of any significant structural changes, as mentioned above, could also point to a Zn^{2+} playing a direct catalytic role because of the following: (i) Zn^{2+} of site 1 could be removed with OP and subsequently substituted by Zn^{2+} under *nondenaturing* conditions at pH 5.5, which resulted in full restoration of activity at neutral pH. If Zn^{2+} was structural, one should expect an incomplete restoration of activity at neutral pH because the activity is nil at pH 5.5. (ii) Complete restoration of enzymatic activity upon reconstitution with Zn^{2+} was equally effective in urea at pH 3.0 or in nondenaturing conditions at pH 5.5, suggesting that denaturation, Zn^{2+} removal, and reconstitution did not irreversibly denature the structure of the enzyme. (iii) The loss of activity in apoenzymes is solely due to a deficiency of hydrolysis unrelated to substrate binding because the apparent affinities of the wild-type holo- and apoenzymes for the ubiquitin affinity column were nearly identical and comparable to those

obtained with the active-site-Cys335Ser mutant of ISOT-S, suggesting almost identical structures between the holo- and apoenzymes. Substrate binding without subsequent hydrolysis was shown in this study with both the wild-type apoprotein and the active-site-Cys335Ser mutant of ISOT-S and has also been reported for a deficient Zn^{2+}-binding mutant of insulin-degrading enzyme (*61*) and the α subunit of mitochondrial processing protein (*47*). This would argue for the reclassification of these two enzymes as metalloprotease, and not thiol protease, in classic mechanistic terms. Thimet oligopeptidase (EC 3.4.24.15), an enzyme that was at one time thought to be a cysteine proteinase, is now classified as a metalloproteinase. To our knowledge, Zn^{2+}-containing metallopeptidases do without exception contain Zn^{2+} that is catalytic (three protein−Zn^{2+} ligands and a fourth ligand activating water) rather than structural (four protein−Zn^{2+} ligands) (*26*−*28*). By analogy with Zn^{2+} metallopeptidases, a putative reaction mechanism for the catalytic activity of ISOT might involve a Zn^{2+} atom that is coordinated to three protein−Zn^{2+} ligands and a water molecule. Because the ubiquitin-aldehyde inhibitor is thought to react with the active site Cys335 in ISOT (*9*), we propose that the negative charge of the active site Cys335 could play a role in catalysis by polarizing the Zn^{2+}-coordinated water molecule. This proposal is supported by the fact that replacement of Cys335 by a Ser abolished enzymatic activity but did not modify Zn^{2+} binding (T. L. and J.-M. G., unpublished results). We surmised that the functional consequence of modifying the side chain of Cys335 with thiol-blocking reagents or by site-directed mutagenesis would be a suppression of the nucleophilicity of the Zn^{2+}-coordinated water molecule.

Putative Zn^{2+}-Binding Motif. The location of the Zn^{2+}-binding site 1 of ISOT is of major interest. The three-dimensional structure of a UBP family member has not been elucidated nor is there any sequence homology of an UBP member with any thiol- or metalloprotease, but one can compare the sequence of ISOT with those of known Zn^{2+}-binding motifs in metalloproteins. Insulin-degrading enzyme (IDE; EC 3.4.24.56; swissprot|P14735) is a monozinc metalloprotease with three protein−Zn^{2+} ligands (His107, His111, Glu188) (*62*−*64*) in which chelating agents EDTA and OP or thiol-blocking reagents such as *N*-ethylmaleimide inhibit its catalytic activity (*65*). A similar Zn^{2+}-binding site also exists in ISOT (His232, His236, and Glu312). His232 and His236 of ISOT are also part of a putative metal-binding domain called the ZnF_UBP domain in Smart database (SM00290) or Zn_UBP domain in Interpro database (IPR001607), or the DAUP domain shared with a subset of UBPs, histone deacetylase HDAC-6 (*11*), BRCA-1 associated protein-2, and several ubiquitin ligases. Thus, it appears that this motif is not always associated with deubiquitinating activity. Whether the Zn^{2+} atom of ISOT or other UBPs is bound in such a site remains to be investigated.

In conclusion, we have shown that two members of the UBP family, the human ISOT-S and ISOT-L, are Zn^{2+}-dependent proteases, thereby expanding the diversification of mechanisms of ubiquitin substrate proteolysis in this family. It is impossible to clarify at this moment whether the Zn^{2+} in site 1 plays a local structural or direct catalytic role. Further studies of ISOT will help to further ascertain the role of Zn^{2+} in catalysis.

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