Structure of Ddi2, a highly inducible detoxifying metalloenzyme from Saccharomyces cerevisiae

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Cyanamide (H₂N-CN) is used to break bud dormancy in woody plants and to deter alcohol use in humans. The biological effects of cyanamide in both these cases require the enzyme catalase. We previously demonstrated that Saccharomyces cerevisiae exposed to cyanamide resulted in strong induction of DDI2 gene expression. Ddi2 enzymatically hydrates cyanamide to urea and belongs to the family of HD-domain metalloenzymes (named after conserved active-site metal-binding His and Asp residues). Here, we report the X-ray structure of yeast Ddi2 to 2.6 Å resolution, revealing that Ddi2 is a dimeric zinc metalloenzyme. We also confirm that Ddi2 shares structural similarity with other known HD-domain proteins. HD residues His-55, His-88, and Asp-89 coordinate the active-site zinc, and the fourth zinc ligand is a water/hydroxide molecule. Other HD domain enzymes have a second aspartate metal ligand, but in Ddi2 this residue (Thr-157) does not interact with the zinc ion. Several Ddi2 active-site point mutations exhibited reduced catalytic activity. We kinetically and structurally characterized analogous active-site mutations in Ddi2. A cyanamide soak of the Ddi2-T157V enzyme revealed cyanamide binding directly to the Zn²⁺ ion, having displaced the zinc-bound water molecule. The mode of cyanamide binding to Ddi2 resembles cyanamide binding to the active-site zinc of carbonic anhydrase, a known cyanamide hydratase. Finally, we observed that the sensitivity of ddi2Δ ddi3Δ to cyanamide was not rescued by plasmids harboring ddi2-H137N or ddi2-T157V variants, demonstrating that yeast cells require a functioning cyanamide hydratase to overcome cyanamide-induced growth defects.

Cyanamide (H₂N-CN) can be regarded as the amide of cyanic acid (HOCN) or equivalently as the carbamide of cyanic acid (H₂N-COOH). Cyanamide is bifunctional, being capable of reacting as a nucleophile at the amino group or as an electrophile at the cyano group. The hydration of cyanamide (H₂N-CN) to form urea (H₂N-CO-NH₂) is similar to reactions carried out by nitrile hydratase (NHase) enzymes, which catalyze the hydration of nitriles (R-CN) to the corresponding amides (R-CO-NH₂), although it is worth noting that nitrile hydratases do not convert cyanamide to urea (1). However, the soil fungus Myrothecium verrucaria and the budding yeast Saccharomycyes cerevisiae have both been shown to contain a cyanamide hydratase (CAH, EC 4.2.1.69) enzyme that converts cyanamide to urea (2, 3), and CAH belongs to the diverse HD-domain family of metalloproteins (3, 4).

Cyanamide is used as both a fertilizer and herbicide in agriculture and is toxic to plant cells because it causes oxidative stress via inhibition of catalase (5). Furthermore breakdown of cyanamide into nitroxy (HNO) by catalase inhibits aldehyde dehydrogenases (5). Plants apparently do not contain a homologous CAH gene, at least according to BLAST searches using the yeast Ddi2 protein sequence (3, 6). Treatment of perennial woody plants with cyanamide is known to break bud dormancy and has revealed the plant metabolic pathways that are activated in response to cyanamide treatment (7–9). These pathways involve activation of the jasmonate and cytokinin pathways via cyanide (cyanamide is first broken down into cyanide (HCN) and HNO by the action of catalase), plus activation of several cyanide detoxification and oxidative stress response enzymes. Interestingly, cyanamide is not only a substrate of catalase, it also functions as a competitive inhibitor (10–12). Oxidative stress in woody plants exposed to cyanamide presumably is initiated by cyanamide-inhibiting catalase and subsequent hydrogen peroxide accumulation (5, 7–10).

Cyanamide has been used as an alcohol deterrent, because in humans it is similarly metabolized to nitroxy and HCN by catalase; nitroxy then inhibits aldehyde dehydrogenase and thus interferes with the oxidation of acetaldehyde to acetate (10–12). Cyanamide is also known to be both a poor substrate and an inhibitor of carbonic anhydrase II (13, 14). Carbonic anhydrases are zinc metalloenzymes that hydrate carbon dioxide to carbonate and thereby help maintain physiological pH via carbonate/bicarbonate buffering. The linear arrangement of N–CN in cyanamide approximately resembles that in CO₂. Crystallographic studies have revealed, however, that cyanamide

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This article contains Tables S1 and S2 and Figs. S1–S5.

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3 The abbreviations used are: CAH, cyanamide hydratase; BLAST, basic local alignment search tool; Pde, phosphodiesterase; PDB, Protein Data Bank; MMS, methyl methanesulfonate; GST, glutathione S-transferase.
Biodegradation of cyanamide in microorganisms was first credited to the soil fungus *M. verrucaria* that specifically catalyzed the formation of urea from cyanamide but did not catalyze the conversion of other nitriles to their respective amides (2). *M. verrucaria* cyanamide hydratase was found to contain zinc, and the functional enzyme was assessed to be a hexamer of identical subunits. The deduced *Kₘ* for cyanamide was reported to be 27 mM for *M. verrucaria* CAH (2). We recently demonstrated that *S. cerevisiae* Ddi2, a homolog of *M. verrucaria* CAH (37% pairwise sequence identity), is also a bona fide CAH, enzymatically converting cyanamide to urea with kinetic parameters comparable with those of the *M. verrucaria* enzyme (3). Closely related orthologs of cyanamide hydratase identified by BLAST are all from fungi and appear to be limited to the Ascomycota and Basidiomycota.

Because cyanamide is synthesized by limited species of plants presumably as a chemical defense against fungi and weeds (15–17), it is not surprising that some microorganisms, especially those living in soil or associated with plants, have developed a cyanamide biodegradation pathway to utilize the nitrogen in cyanamide and to protect against oxidative stress related to cyanamide reaction with catalase.

The yeast Ddi2 CAH enzyme was originally brought to our attention because microarray studies demonstrate that *S. cerevisiae DDI2* expression is highly induced by the DNA alkylating agent methyl methanesulfonate (MMS) (18, 19). Recently, we demonstrated that Ddi2 is also induced upon adding dimethyl sulfate or cyanamide to the growth medium (3).

The *S. cerevisiae* Ddi2 protein is encoded by two identical duplicated genes *DDI2* and *DDI3* (two copies of *DDI2* orthologs were also found in *Aspergillus nidulans* and *Aspergillus oryzae*) and belong to the HD-domain superfamily of enzymes, reflecting the presence of key conserved histidine and aspartate residues in the primary sequence that bind metal ions involved in enzyme catalysis (3, 4). Henceforth, we will refer to Ddi2/DDI3 as Ddi2. Site-specific mutations of the conserved HD signature residues in Ddi2 resulted in the loss of CAH enzymatic activity, indicating that the core Ddi2 HD residues are required for enzyme stability and/or catalysis (3). HD domain proteins have a mostly helical structure and are either divalent metal-dependent phosphohydrolases (20–25) or oxidases (26, 27).

Hence Ddi2 and its fungal CAH orthologs represent a novel and distinct subgroup of HD domain proteins that display novel catalytic activity (2–4). To better understand the effects of cyanamide on *S. cerevisiae* physiology and the mechanism of cyanamide hydration, we here describe the crystallographic determination of the *S. cerevisiae* Ddi2 structure, identify conserved amino acid residues essential for catalysis, and deduce the mode of cyanamide substrate binding to the active-site Zn²⁺ atom in a Ddi2-T157V mutant enzyme. Furthermore, we demonstrate that only enzymatically active Ddi2 protein is capable of rescuing yeast growth defects in the presence of cyanamide.

**Structure of Ddi2: a HD-domain cyanamide hydratase**

The Ddi2 structure solution and refinement are described under “Experimental procedures.” The structure was refined using diffraction data to 2.6 Å resolution, and the model consists of 9 identical subunits of 225 residues (chains A–I), with each subunit being present in a symmetrical dimer (Fig. 1, Table 1, and Fig. S1). Chains A–G had significantly lower B factors than chains H and I, which exhibited overall weak electron density and high B-factors. The refined Ddi2 model showing zinc coordination and the deduced active site is depicted in Fig. 1. Secondary structure assignments for the Ddi2 polypeptide, sequence conservation in fungal homologs, and the location of active-site residues are summarized in Fig. 2. In each Ddi2 monomer, the zinc ion is coordinated by HD residues His-55, His-88, Asp-89, and Thr-157 near the zinc-binding site are depicted as stick models with nitrogen in blue and oxygen in red. B drawn as for A but showing the observed dimer. The panels were drawn with PyMOL (42).

**Results**

**Ddi2 is a dimeric zinc metalloenzyme**

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Structure of Ddi2: a HD-domain cyanamide hydratase

Table 1
Parameters of X-ray Zn-SAD data collection and native data/refinement for Ddi2

| Ddi2 zinc peak | Ddi2 native (6DK9) |
|----------------|------------------|
| Space group | P521 | P521 |
| a, c (Å) | 264.2, 119.2 | 263.6, 119.0 |
| a, β, γ (°) | 90, 90, 120 | 90, 90, 120 |
| Resolution (Å) (high) | 50–3.10 (2.21–3.10) | 50–2.60 (2.64–2.60) |
| Wavelength (Å) | 1.28146 | 1.12713 |
| Temperature (K) | 100 | 100 |
| Rmerge (%) | 17.5 (60.6) | 10.5 (69.5) |
| Rfree (%) | 10.1 (2.2) | 17.6 (2.9) |
| No. of reflections: total/unique | 1,475,844/168,856 | 1,111,859/144,816 |
| Redundancy | 8.7 (8.6) | 7.7 (7.7) |
| Completeness (%): total/high | 99.9 (100.0) | 99.0 (97.9) |

Refinement statistics

| | Ddi2 zinc peak | Ddi2 native (6DK9) |
|----------------|----------------|------------------|
| Resolution (Å) | 39.7–2.60 (2.63–2.60) | 98.7 (92.0) |
| Completeness (%) | 99.9 (100.0) | 99.0 (97.9) |
| Numbers of independent molecules | 9 | 9 |
| Rwork (%) | 20.1 (26.0) | 21.6 (28.9) |
| Rfree (%) | 21.6 (28.9) | 20.1 (26.0) |
| Root-mean-square deviation, bonds/angles (Å/°) | 0.004/0.81 | 0.004/0.81 |
| Wilson B factor | 43.9 | 43.9 |
| No. of protein atoms | 14,324 | 14,324 |
| No. of solvent atoms | 334 | 334 |
| Results in most favored region (%) | 97.3 | 97.3 |
| Residues in generously allowed regions (%) | 2.2 | 2.2 |
| Rotamer outliers (%) | 1.9 | 1.9 |

*a* The details of data collection and processing are provided under “Experimental procedures.”

*b* The values in parentheses correspond to the highest resolution shell.

| | Ddi2 zinc peak | Ddi2 native (6DK9) |
|----------------|----------------|------------------|
| Rmerge = Σhkl{Fobs(hkl) − |Fcalc(hkl)||Fobs(hkl) |, where Fobs(hkl) is the individual measurement of an hkl intensity, and |Fcalc(hkl)| is the observed and calculated amplitudes, respectively, for the structure factor F(hkl). |
| Rwork = Σhkl{|Fobs(hkl)| − |Fcalc(hkl)||Fobs(hkl) |, where Fobs(hkl) and |Fcalc(hkl)| are the observed and calculated amplitudes, respectively, for the structure factor F(hkl). |
| Rfree is the equivalent of Rwork for 5% of the reflections (randomly selected) that were not used in structure refinement. |

summarized in Fig. 3 and Table S2 (see also below). Conserved Ddi2 residues Phe-104, His-137, Gln-138, Thr-157, Asn-161, and Trp-186 form a pocket that is likely to be the active site (Figs. 1–4). Four water molecules (Wat-1 to Wat-4) were observed near the zinc site for chains A—G, and this was confirmed by noncrystallographic symmetry averaging of |F| – |F| omit electron density maps for the seven well-defined subunits in the structure (Fig. 4). The Wat-1 molecule is bound directly to the zinc ion in the absence of substrate; the remaining water molecules form hydrogen bonds with the side chains of Asp-89 and His-137 (Wat-2), Gln-138 and Asn-161 (Wat-3), and Thr-157 (Wat-4) (Fig. 4). Wat-4 also hydrogen-bonds to the backbone carbonyl groups of residues 153 and 154 in helix α8, one helical turn prior to residue Thr-157.

Dimerization of all Ddi2 molecules was observed in the crystal lattice and included molecule pairs A:A’, B:C, D:E, F:G, and H:H (Fig. S1). Residues involved in dimerization include: residues 99–103, helix α6 (residues 104–118), helix α7 (residues 123–135), a large surface loop including residues 136–147, and a short 310 helix between residues 203 and 207, making up a buried surface area of 2700 Å²/monomer (Figs. 1 and 2). There are numerous van der Waals contacts and hydrogen bonds at the dimer interface. Many of the polar residues making hydrogen bonds at the dimer interface are highly conserved in Ddi2 sequences, including residues Thr-100, Lys-101, Ser-103, Asp-125, Glu-132, Asp-139, Tyr-145, Trp-203, and Sedr-207 (Fig. 2). Note that active-site residues His-137 and Gln-138 are proximal to the dimer interface (Fig. 1).

**Ddi2 shares overall structural similarity with HD-domain family proteins**

Structural overlays of the Ddi2 polypeptide onto other known HD domain proteins (Fig. 3 and Fig. S2 and Table S2) identified residues 51–161 of Ddi2 as the core HD motif, including five core helices (here labeled αA–αE) of the HD domain region and in some cases a sixth helix at the N terminus preceding helix αA (depicted as αN) (Fig. 3 and Table S2). Briefly the conserved HD-domain helices in Ddi2 are as follows: αN corresponds to α2, αA corresponds to α3, αB corresponds to α4, αC corresponds to α6, αD corresponds to α7, and αE corresponds to α8 (Fig. 3 and Table S2). Most structurally characterized HD domain superfamily members overlaid reasonably well with the core HD-domain fold of Ddi2 (20–27). RMS differences on backbone atoms ranged between 1.6 and 2.4 Å for 79 equivalent residues, including conserved helix αN (Table S2). Structural similarity searches with FATCAT (29) and BLAST (6) searches of the PDB confirmed the similarity of Ddi2 to known HD domains.

A few HD domain proteins stood out as having the most informative structural similarity to Ddi2. They are YpgQ (PDB code 5DQW), a bacterial nucleotide phosphohydrolase (25), PA4781 (PDB code 4R8Z), a cyclic di-GMP phosphodiesterase (24), and phosphodiesterase 2A (Pde2A) (PDB code 4D08) (30) (Fig. 3 and Fig. S2). For instance, there is a conserved salt-bridge interaction between helices αA (Arg-58 in Ddi2) and αE (Asp-160 in Ddi2) that is observed in PA4781 (PDB code 4R8Z) and YpgQ (PDB code 5DQW) (24, 25) (Fig. 3 and Fig. S2). The side-chain carboxylate of Asp-160 of Ddi2 and the equivalent residue in the above-mentioned HD-domain proteins form a hydrogen bond with the ND1 side-chain nitrogen of metal-coordinating residue His-55 and likely helps orient the side chain of His-55 to coordinate its metal ion.

Ddi2 is somewhat unusual because it contains three C-terminal helices (α10, α14, and α11) that form a partial lid over the active-site entrance; these helices are not found in most other HD-domain family members (Fig. 3). However, two of the HD domain proteins noted above (YpgQ and Pde2A) (25, 30) do have two C-terminal helices arranged in a hairpin fashion that resemble helices α10 and α11 of Ddi2. In particular, the C-terminal region of Pde2A aligns reasonably well with the Ddi2 polypeptide in BLAST searches.

Of particular note, a second aspartate ligand found in most HD-domain proteins on helix αE is missing in Ddi2, being substituted by threonine (Thr-157), and Thr-157 does not coordinate the zinc metal ion, although it is nearby. The placement of a threonine residue rather than an aspartate at this position in the Ddi2 sequence is very likely important for the Zn²⁺ ion specificity of the enzyme and permits the binding of a water/hydroxide molecule at the fourth ligand site (Fig. 4).

**Kinetic and substrate-binding studies of Ddi2 active-site mutants**

We determined the pH optimum for the WT Ddi2 reaction to be 7.5 using our standard enzyme assay operating under sat-
### Structure of Ddi2: a HD-domain cyanamide hydratase

![Sequence Alignment]

**Sc_Ddi2**

| Sequence | Score |
|----------|-------|
| Sc_Ddi2  | ![Sequence Alignment] |

**Mv_Cah**

| Sequence | Score |
|----------|-------|
| Mv_Cah   | ![Sequence Alignment] |

**Fg_FG1132.1**

| Sequence | Score |
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| Fg_FG1132.1 | ![Sequence Alignment] |

**An_ANS5411.2**

| Sequence | Score |
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| An_ANS5411.2 | ![Sequence Alignment] |

**Eg_FG6421.2**

| Sequence | Score |
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| Eg_FG6421.2 | ![Sequence Alignment] |

**Ca_Ca019.9024**

| Sequence | Score |
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| Ca_Ca019.9024 | ![Sequence Alignment] |

**Ao_BAE61376.1**

| Sequence | Score |
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| Ao_BAE61376.1 | ![Sequence Alignment] |

**Ao_BAE655603.1**

| Sequence | Score |
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| Ao_BAE655603.1 | ![Sequence Alignment] |

**Dh_DEHA2A01694p**

| Sequence | Score |
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| Dh_DEHA2A01694p | ![Sequence Alignment] |

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**Sc_Ddi2**

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Figure 2. Multiple sequence alignment of fungal orthologs of Ddi2. The sequence alignment was calculated using ClustalW (43), and the figure was prepared using ESPript 3.0 (44), based on the refined Ddi2 atomic coordinates. Secondary structures are depicted as follows: α represents α-helix, β represents β-sheet, and γ represents 3₁₀-helix. HD residues coordinating the zinc ion are indicated by black stars. The source organism abbreviations are given in the left-hand column. Sc, S. cerevisiae; Mv, M. verrucaria; Fg, Fusarium graminearum; An, A. nidulans; Ca, Candida albicans; Ao, A. oryzae; Dh, Debaryomyces hansenii. Identical residues are colored red. Conserved HD residues are highlighted by black stars, and residues chosen for site-specific mutagenesis are indicated by blue ovals. Residues involved in dimerization are highlighted by green triangles.

Figure 3. Superposition of the Ddi2 and YpgQ HD-domain motifs. A, schematic ribbon depiction of Ddi2 chain D (conserved HD motif in blue, helix α-N in magenta, and other segments in green). The secondary structures are labeled as in Fig. 2. B, superposition of the HD motif of Ddi2 (blue) onto the HD motif of YpgQ (orange, PDB code 5DQV), highlighting the conserved five conserved α-helices: αA–αE (in this case a sixth N-terminal helix α-N (magenta) is also conserved). The panels were drawn using PyMOL (42).

Figure 4. Active site of Ddi2 and cyanamide binding in the T157V mutant. A, NCS symmetry averaged |Fo|–|Fc| omit electron density map for chains A–G of the refined model (density for chains H and I is significantly weaker), using chain D as the reference subunit, with active-site solvent molecules omitted from the model (magenta, density displayed at 10.0 o), showing the difference electron density near the active site (chain D). Conserved solvent molecules Wat-1–Wat-4 (red spheres) have been refined into the difference density, B, as in A, but showing the cyanamide (CYA) soak for the Ddi2-T157V mutant. The placed cyanamide substrate is depicted as a stick model. Side chains of residues are colored by element: oxygen in red, carbon in green, and nitrogen in blue. Bonds to the zinc ion are represented as yellow dashes. The zinc ion (cyan) and water molecules (red) are represented as spheres. The NCS maps were calculated using the NCS averaging option in Coot (40) and drawn with PyMOL (42).
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levels of substrate, and we resorted to using 100 μg of mutant enzyme to measure its kinetic parameters (typically 1 μg of Ddi2 is used in the assay). Ddi2-H137N exhibited a near WT $K_m$ value (33 ± 60 mM) but a significantly reduced value of $k_{\text{cat}}$ (0.0040 ± 0.0029 s⁻¹). Ddi2-T157V exhibited a $K_m$ value of 25 ± 6 mM, comparable with the WT enzyme but a more modestly compromised turnover number, $k_{\text{cat}} = 1.3 ± 0.1$ s⁻¹ (Table 3). We conclude from these measurements that the H137N mutant is severely compromised in catalysis, whereas the T157V mutant binds substrate as efficiently as the WT enzyme and is moderately catalytically impaired.

**Binding of cyanamide at the Ddi2 active site**

To observe substrate binding in Ddi2 crystals, we utilized the T157V and H137N mutants, because extensive soaking and co-crystallization experiments of the WT Ddi2 protein with cyanamide or cyanide yielded negative results. Crystals of Ddi2-H137N and Ddi2-T157V were briefly (30 s) soaked in cryo-buffer containing 0.2–0.3 M cyanamide before flash-freezing in liquid nitrogen for X-ray diffraction experiments. Diffraction data to 3.0 Å resolution were collected for the Ddi2-H137N mutant crystals; the Ddi2-T157V mutant crystals diffracted to 2.90 Å resolution (Table 2). The structures were solved by rigid-body and maximum-likelihood refinement (see “Experimental procedures”), because the unit cell dimensions and crystal morphology were isomorphous to the WT Ddi2 crystals (Table 2). Analysis of the H137N mutant structure revealed that the Asn-137 side chain rotated away from the position of His-137 in the WT enzyme and made hydrogen bonds to the side chain of nearby residue Thr-157 (Fig. S4). In addition, there was no electron density observed for cyanamide at the active site.

In contrast, the cyanamide soak of the T157V mutant revealed electron density consistent with cyanamide binding directly to the zinc for seven of the nine independent active sites in the crystal structure. To objectively evaluate cyanamide binding, we averaged the $|F_o| - |F_c|$ difference electron density for subunits A, B, C, E, F, and G onto subunit D (Fig. 4) for the cyanamide soak data. Real-space refinement of a cyanamide molecule into the 7-fold averaged difference electron density demonstrated that cyanamide bound directly to the zinc atom and hydrogen-bonded to the side chains of Asp-89 and His-137, thereby displacing the bound water molecules Wat-1 and Wat-2 observed in the apo-enzyme structure (Fig. 4). This observation seems to rule out a catalytic role for the zinc-bound water molecule observed in the apo-enzyme structure, because it otherwise would have been a candidate for the attacking nucleophile in the hydration reaction. Binding of cyanamide to the active site of the T157V mutant is consistent with our kinetic measurements, because the T157V mutant exhibited a near WT $K_m$ value for substrate but a 10-fold lower $k_{\text{cat}}$ value, thereby permitting the trapping of bound substrate (Fig. 5). In addition, we observed a change in side chain orientation at residue 157 upon substituting threonine with valine. In the WT enzyme, Thr-157 hydrogen-bonds directly to the Val-157 side chain, possibly affecting the catalytic rate of hydration. For the WT enzyme, Thr-157 hydrogen-bonds to Wat-3 and the backbone carbonyl oxygen atoms of residues 153 and 154 (Fig. 4). In the T157V mutant, these H-bonds are no longer possible, and the valine side chain adopts a presumably lower energy conformation ($\chi^1 = 180$) in which the CG1 methyl group now points almost directly at the zinc atom (Figs. 4 and 6). This small structural change results in a small displacement of the zinc ion away from the CG1 atom of Val-157, and a more dramatic displacement of the zinc-bound water molecule at the active site (Wat-1), presumably as a result of repulsive van der Waals forces (Fig. 6 and Fig. S4). This suggests the position of the zinc-bound cyanamide would be similarly displaced in the T157V mutant by steric clashes with the Val-157 side chain, possibly affecting the catalytic rate of hydration. For the WT enzyme, Thr-157 hydrogen-bonds directly to Wat-3 that in turn hydrogen-bonds to Wat-4. Wat-4 would sit above the nitrile group of cyanamide in a Michaelis complex, almost within van der Waals distance of the substrate nitrile carbon. Hence not only is Wat-4 a candidate for the attacking water nucleophile in the hydra-
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Table 2
X-ray crystallographic data collection and refinement stats for Ddi2 mutants

| Complex                | Ddi2-H137N (6DKD) | Ddi2-T157V (6DKA) | Ddi2-T157V cyanamide (6DKC) |
|------------------------|-------------------|-------------------|-----------------------------|
| Space group            | P321              | P321              | P321                        |
| a, c (Å)               | 264.6, 119.3      | 263.6, 119.0      | 263.4, 119.2                |
| α, β, γ (°)            | 90, 90, 120       | 90, 90, 120       | 90, 90, 120                 |
| Resolution (Å) (high)  | 50–3.00 (3.05–3.00) | 50–2.90 (2.95–2.90) | 50–2.90 (2.95–2.90)        |
| Wavelength (Å)         | 1.0246            | 1.0246            | 1.0246                      |
| Temperature (K)        | 100               | 100               | 100                         |
| Rsym (%)              | 9.2 (56.8)        | 10.5 (69.5)       | 11.4 (63.0)                 |
| I/σ(I) (%)            | 20.9 (2.7)        | 17.6 (2.9)        | 16.1 (2.2)                  |
| No. of reflections: total/unique | 614,102/614     | 538,150/102,026   | 671,645/101,420             |
| Redundancy            | 6.6 (6.6)         | 5.3 (5.2)         | 6.6 (6.5)                   |
| Completeness (%): total/high | 96.5 (97.8)     | 97.0 (98.8)       | 96.8 (98.4)                 |

Refinement statistics

| Complex                | Ddi2-H137N (6DKD) | Ddi2-T157V (6DKA) | Ddi2-T157V cyanamide (6DKC) |
|------------------------|-------------------|-------------------|-----------------------------|
| Resolution (Å)         | 39.8–3.00 (3.07–3.00) | 39.3–2.90 (2.96–2.90) | 39.3–2.90 (2.96–2.90)        |
| Completeness (%)       | 96.5 (97.0)       | 97.0 (99.0)       | 96.7 (97.0)                 |
| Numbers of independent molecules | 9             | 9                 | 9                           |
| Rwork (%)              | 19.4 (27.5)       | 19.3 (23.1)       | 19.8 (27.2)                 |
| Rfree (%)              | 22.0 (32.5)       | 21.5 (25.7)       | 23.0 (30.3)                 |
| Root-mean-square deviation, bonds/angles (Å/°) | 0.006/0.95 | 0.006/0.86 | 0.006/0.84 |
| Wilson B factor        | 66.2              | 53.5              | 55.6                        |
| No. of protein atoms   | 16,186            | 16,317            | 16,338                      |
| No. of solvent atoms   | 178               | 248               | 265                         |
| Results in most favored region (%) | 96.3            | 97.5              | 97.1                        |
| Residues in generously allowed regions (%) | 3.3           | 2.0               | 2.2                         |
| Rotamer outliers (%)   | 3.9               | 2.4               | 1.8                         |

The details of data collection and processing are described under "Experimental procedures."
and transformed it into yeast minator sequences into a yeast multicopy plasmid (YEPlac195).

The inhibition of catalase and concomitant H$_2$O$_2$ buildup, as well as catalase activity. The rapid induction of yeast $DDI2$ upon exposure to cyanamide would presumably remove the cyanamide before significant amounts of cellular catalase could undergo cyanamide inhibition. Hence the presence of Ddi2 would protect yeast cells from the unwanted buildup of H$_2$O$_2$ resulting from cyanamide inhibition of catalase.

Given that $ddi2$ orthologs are widespread in yeasts and fungi, it is interesting to speculate on their likely role in cellular physiology and metabolism. The cellular response to cyanamide treatment in woody shrubs, for the breaking of bud dormancy, may provide valuable clues (7–9). Both transcriptome studies and physiological studies indicate that cyanamide exposure induces cyanide degradation pathways and that cellular H$_2$O$_2$ levels rise because of the inhibition of catalase by cyanamide (7–9). Increased H$_2$O$_2$ levels resulting from catalase inhibition appear crucial for bud break in woody perennials and also induce expression of several oxidative stress response proteins (7–9).

Diatoms of Ddi2 is Asn-161, which hydrogen-bonds to cyanamide and a nearby water molecule. This residue is valine in bacterial homologs. Because valine has a hydrophobic side chain, it cannot form a hydrogen bond with solvent or cyanamide as Asn-161 does in Ddi2. However, the bacterial homologs of Ddi2 will likely bind Zn$^{2+}$, because although Thr-157 is mutated to alanine or valine in bacterial homologs, it is not an aspartate in any of the sequences, as would be expected based on known HD motifs in other HD-domain proteins.

$ddi2$-$H137N$ and $ddi2$-$T157V$ are unable to rescue yeast $ddi2{\Delta}$ $ddi3{\Delta}$ mutant phenotype

To assess the functional implications of Ddi2 enzyme activity in vivo, we cloned the $DDI2$ gene including promoter and terminator sequences into a yeast multicopy plasmid (YEPlac195) and transformed it into yeast $ddi2{\Delta}$ $ddi3{\Delta}$ ($ddi2/3{\Delta}$) double mutant cells. As expected, the $ddi2/3{\Delta}$ double mutant displayed an increased growth defect in the presence of cyanamide in a gradient plate assay (Fig. 7). Yeast transformants harboring the YEpuDDI2 plasmid carrying the WT $DDI2$ gene rescued the $ddi2/3{\Delta}$ double mutant sensitivity to growth on cyanamide, exhibiting growth comparable with WT levels. In sharp contrast, the same plasmid containing the $ddi2$-$H137N$ or $ddi2$-$T157V$ mutant did not rescue growth defects of the $ddi2/3{\Delta}$ strain grown in the presence of cyanamide (Fig. 7), strongly suggesting that the efficient hydrolysis of cyanamide by Ddi2 plays important roles in its biological function in vivo.

### Discussion

#### Implications for the presence of cyanamide hydratases in yeasts

Given that Ddi2 orthologs are widespread in yeasts and fungi, it is interesting to speculate on their likely role in cellular physiology and metabolism. The cellular response to cyanamide treatment in woody shrubs, for the breaking of bud dormancy, may provide valuable clues (7–9). Both transcriptome studies and physiological studies indicate that cyanamide exposure induces cyanide degradation pathways and that cellular H$_2$O$_2$ levels rise because of the inhibition of catalase by cyanamide (7–9). Increased H$_2$O$_2$ levels resulting from catalase inhibition appear crucial for bud break in woody perennials and also induce expression of several oxidative stress response proteins (7–9). Catalases also break down cyanide to cyanide in vivo.

### Table 3

| Protein       | $K_m$  | $k_{cat}$ | $K_m/k_{cat}$ |
|---------------|--------|-----------|---------------|
| Ddi2 wildtype | 0.0173 | 9.8 (0.11)| 566 (19)      |
| Ddi2-T157V    | 0.025  | 1.3 (0.11)| 53 (14)       |
| Ddi2-H137N    | 0.033  | 0.0040   | 0.12 (0.24)   |

* 100 μg of pure Ddi2-H137N was used in the enzyme assay, whereas 1 μg of purified Ddi2 wildtype or Ddi2-T157V was used.

#### Structure of Ddi2: a HD-domain cyanamide hydratase

Figure 6. Analysis of cyanamide binding in the Ddi2 T157V mutant. A, superposition of chain D of WT Ddi2 (green) and Ddi2-T157V (cyan) showing active-site residues, solvent molecules, or bound cyanamide. B, view depicting the active site in chain D but viewing from the top right corner in A. The zinc ion is represented as a green (WT) or cyan (T157V) sphere, water molecules are represented as light red spheres, and cyanamide (CYA) is depicted as a stick model. The side chains of active-site residues are colored by element: oxygen is red, carbon is green, and nitrogen is blue. The panels were drawn with PyMOL (42).
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Other possible enzymatic activities for Ddi2

Not only can carbonic anhydrase function as a cyanamide hydratase, it is also known to function as an esterase using its zinc-bound water molecule to hydrolyze esters (14). Given that Ddi2 also contains a zinc-bound water/hydroxide molecule, perhaps Ddi2 also has an esterase function. Ddi2 is known to be strongly induced by exposure to MMS; therefore, it seems reasonable to suppose that MMS is hydrolyzed and therefore metabolically inactivated by the zinc-bound water molecule in Ddi2. Modeling suggests that MMS could easily fit into the Ddi2 active site near the zinc-bound water molecule (not shown). It is also relevant that most HD-domain family members have esterase functions, being phosphodiesterases. Further studies on the enzymology of Ddi2 and the effects of cyanamide on yeast physiology and metabolism will shed light on the roles of CAH enzymes in these organisms.

Ddi2 has unique metal coordination geometry as compared with other HD-domain proteins

The structure of yeast Ddi2 adds to the diversity of HD-domain family metalloenzymes and their respective chemistries because Ddi2 binds zinc and carries out a simple hydration reaction of cyanamide, yielding urea as the product. The substrate cyanamide where the nitrile N2 atom of the substrate would be difficult to reconcile with the requirement for His-137 in substrate binding and catalysis, because there really is no other obvious place for cyanamide to bind at the active site than directly to the zinc, as we observe.

Together, these observations provide compelling evidence that the pocket containing the zinc site is the actual active site of Ddi2. Given that the pH optimum for Ddi2 is 7.5, it is reasonable to assume that His-137 does not carry a positive charge at pH 7.5, but the cyanamide N1 amino group may carry a positive charge via protonation. Hence His-137 more than likely functions to correctly position the substrate for catalysis via hydrogen bonding.

The current structural model of Ddi2-T157V with bound cyanamide has a relatively low resolution of 2.90 Å for definitive mechanistic studies, and hence we are unable to discriminate the exact chemical nature of cyanamide binding to the zinc ion (N1 or N2) at this resolution. Therefore, the possibility of cyanamide binding to the zinc ion via the N1 amino group cannot currently be ruled out. More concrete mechanistic inferences on the Ddi2 family of enzymes will have to await higher resolution structures of trapped substrates or inhibitors.

In summary, we have solved and refined the structure of S. cerevisiae Ddi2. The enzyme is a zinc-containing member of the HD domain superfamily and forms tightly associated dimers. Using crystallography, we also captured the bound substrate cyanamide in a catalytically compromised T157V mutant. Overall, the mode of cyanamide binding in Ddi2 is reminiscent of the binding of cyanamide to carbonic anhydrase. We also identified residues His-137, Gin-138, and Asn-161 as being important for substrate binding and/or catalysis because of their proximity to the zinc site or via interactions with the substrate. Finally, yeast lacking a functional cyanamide hydratase exhibit growth defects in the presence of cyanamide, strongly suggesting that oxidative and chemical stress brought on by cyanamide are harmful to yeast, and Ddi2 provides protection from these stresses.

Experimental procedures

Cloning of the DDI2/3 gene into a bacterial overexpression system

The cloning of DDI2 into a PGEX-6P1 GST-fusion vector has been described previously (3). The cloning process adds an 8-residue tag to the N terminus of the cleaved recombinant Ddi2 protein (GPLGPSEF). To clone DDI2 into a C-terminally His6-tagged expression vector, the DDI2 ORF was similarly amplified from yeast genomic DNA using primers 5’-GGCCCTGCAAGG-

Figure 7. Rescue of cyanamide sensitivity of a ddi2 ddi3Δ mutant by WT Ddi2 or its active-site mutant derivatives. WT strain BY4741 and its ddi2Δ ddi3Δ mutant derivative WXY3149 were transformed with plasmids as indicated and used for a gradient plate assay as described under “Experimental procedures.” The plates were incubated for 48 h before taking the photograph. Only a single representative clone for each strain is shown on the plates. The arrow points in the direction of increasing cyanamide concentration.

Experimental procedures

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**Structure of Ddi2: a HD-domain cyanamide hydratase**

Recombinant plasmids pGEX-DDI2 or pET-DDI2-His₆ were transformed into E. coli strain BL21 (DE3), and protein expression/purification was carried out as previously described (3). For GST-fused Ddi2, a further ion-exchange purification step was carried out after GST tag removal and prior to kinetic assays or protein crystallization. Partially purified Ddi2 was dialyzed into 20 mM Tris-HCl, pH 8.0, containing 20 mM NaCl before loading onto a Source Q anion-exchange column (GE Healthcare) and was eluted using a salt gradient from 0.02–2 M NaCl in 20 mM Tris-HCl, pH 8.0. Eluted protein fractions were analyzed by SDS-PAGE. Fractions containing highly purified Ddi2 were combined and concentrated to 10 mg/ml by using UltraSpin (EMD Millipore) microconcentrators with a molecular mass cutoff of 10 kDa. Concentrated Ddi2 was dialyzed into storage buffer containing 20 mM bis-Tris propane, pH 7.0, 150 mM NaCl, and 0.01 mM ZnCl₂.

Similarly, C-terminally His-tagged Ddi2 was purified from E. coli BL21 (DE3) cells transformed with pET-DDI2-His₆ using nickel–nitrilotriacetic acid affinity chromatography. The cell lysate was equilibrated into buffer containing 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20 mM imidazole and was then loaded onto a nickel–Sepharose FF column (GE Healthcare) and washed extensively prior to elution in the same buffer containing an additional 500 mM imidazole.

**Urease-based enzymatic assay of cyanamide hydratase activity**

Steady-state kinetic studies on purified recombinant WT Ddi2 and active-site point mutants thereof (T157V and H137N) were carried out as previously described (3). In the case of measuring the pH dependence of the cyanamide hydratase reaction, the urease and glutamate dehydrogenase reactions were carried out separately as previously described (3). To monitor the depletion of cyanamide in solutions containing purified Ddi2-His₆ or point mutants thereof, cyanamide was added to a final concentration of 2 mM to a solution of 3.8 μM Ddi2 enzyme, and the solution was incubated at room temperature for 2 h. The cyanamide concentration was monitored using a colorimetric assay as described (31). Briefly, 100 μl of reaction mixture was added to 500 μl of PBS, followed by adding 400 μl of 0.1 M sodium carbonate-bicarbonate buffer, pH 10.4, and 200 μl of a 4% sodium pentacyanoammine-ferroate (II) (TCI Chemicals) solution as the color reagent. After reaction in the dark for 10 min, A₅₃₀ nm was measured to determine the remaining cyanamide concentration. The decrease of A₅₃₀ nm after incubation indicates the consumption of cyanamide in solution, reflecting the activity of cyanamide hydratase. In addition, initial velocity measurements were carried out for each mutant as described in Ref. 3, and no urea was detected after 20 min. Kinetic parameters and standard errors were calculated using the nonlinear least squares fit to the Michaelis–Menten equation as implemented in Prism/GraphPad.

**Protein crystallization and cryoprotection**

Crystals of Ddi2 or mutants thereof were grown by the hanging-drop vapor diffusion method at 20 °C using 0.8–2.0 μl of protein solution (8–11 mg/ml) mixed with an equal volume of precipitant solution. Initial crystallization hits were found using various commercial sparse-matrix crystallization kits, including Wizard I and Wizard II (Rigaku), and the ammonium sul-
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fate suite (Qiagen). Single crystals of Ddi2 grew in solutions containing 1.1–1.3 M ammonium sulfate, 0.2 M arginine, 0.1 M N-morpholino ethane sulfonic acid, pH 5.2–6.0. Crystals took 2–3 weeks to grow to a size that allowed X-ray diffraction data to be recorded. Cryoprotection was attained by the sequential addition of mother liquor solution supplemented with 24–26% (v/v) glycerol to drops containing crystals, followed by subsequent flash-freezing in liquid nitrogen or a stream of liquid nitrogen boil-off vapor. Substrate soaking was performed by addition of 0.20–0.35 M cyanamide to the cryo-buffer, followed by subsequent flash-freezing in liquid nitrogen.

X-ray data collection and structure solution/refinement

Crystals of Ddi2 belong to the space group P321, a = b = 264.4 Å, c = 119.2 Å. This space group is relatively rare for proteins (0.5% of PDB entries) and lacks a translational symmetry element along the principal 3-fold axis, but the presence of a 3-fold screw axis (space groups P3, P3121, P310, and P32) was clearly ruled out by the diffraction data based on the presence of numerous strong (0, 0, l) reflections for l ≠ 3n from l = 3 up to l = 43. In addition, indexing in lower symmetry Laue groups (e.g. 3) did not improve the merging statistics and indexing in 31m dramatically worsened the merging statistics. Furthermore, analysis of intensity statistics effectively ruled out the presence of merohedral twinning. We confirmed that P321 was the correct space group using Pointless (34, 35), which yielded a probability of 0.998 that the space group was indeed P321 with no likely alternate choices. The structure of Ddi2 was solved to 3.2 Å resolution in space group P321 using the single-wavelength anomalous dispersion method (36). Three isomorphous zinc K-edge peak wavelength (Å = 1.28146 Å) data sets were collected under identical conditions (each data set was from a different crystal). Each data set consisted of 500 frames of 0.2° oscillations of 4 s at a crystal to film distance of 320 mm. All data frames from the three crystals were simultaneously scaled and merged within Denzo/HKL2000 and Scalepack (37) (Table 1). Anomalous pairs were scaled independently in this process because both I1 and I2 were at least 8-fold redundant overall. Using Solve (38), 10 candidate zinc sites were located and refined using anomalous scattering differences to a resolution of 8.0 Å resolution with an overall figure of merit of 0.28. Subsequent heavy atom substructure refinement and phasing in PHENIX-Autosol yielded nine stable sites and these were used to calculate initial phases and electron density maps to 3.2 Å resolution (39). The figure of merit after heavy atom refinement was 0.13 to 3.2 Å resolution. Solvent flattening and automated model building using PHENIX-Autosol improved phases significantly and generated an interpretable electron density map and partial model with the correct zinc ligands placed in most of the nine subunits (39). Structure building and refinement were then performed using COOT (40) and PHENIX (39). The final model was refined against native data to 2.6 Å resolution, and the unit cell contained 77% solvent. X-ray diffraction data on all Ddi2 crystals or point mutations thereof were collected using the bending magnet Beamline 08-B1 at the Canadian Light Source. The data collection strategy was identical to that used for the zinc single-wavelength anomalous dispersion peak data sets, and all indexing, scaling, and merging of diffraction intensities were carried out as described above. Further manipulations of the resultant diffraction intensities were performed using the CCP4 suite of programs (41). Structural figures were prepared using PyMOL (42).

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