Structure determinants of substrate specificity of hydroxynitrile lyase from *Manihot esculenta*

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Abstract

Tryptophan 128 of hydroxynitrile lyase of *Manihot esculenta* (MeHNL) covers a significant part of a hydrophobic channel that gives access to the active site of the enzyme. This residue was therefore substituted in the mutant MeHNL-W128A by alanine to study its importance for the substrate specificity of the enzyme. Wild-type MeHNL and MeHNL-W128A showed comparable activity on the natural substrate acetone cyanohydrin (53 and 40 U/mg, respectively). However, the specific activities of MeHNL-W128A for the unnatural substrates mandelonitrile and 4-hydroxymandelonitrile are increased 9-fold and ~450-fold, respectively, compared with the wild-type MeHNL. The crystal structure of the MeHNL-W128A substrate-free form at 2.1 Å resolution indicates that the W128A substitution has significantly enlarged the active-site channel entrance, and thereby explains the observed changes in substrate specificity for bulky substrates. Surprisingly, the MeHNL-W128A–4-hydroxybenzaldehyde complex structure at 2.1 Å resolution shows the presence of two hydroxybenzaldehyde molecules in a sandwich type arrangement in the active site with an additional hydrogen bridge to the reacting center.

Keywords: Substrate specificity; active-site tunnel mutant; crystal structure

Hydroxynitrile lyases (HNLs) constitute a diverse family of enzymes that catalyze the stereospecific cleavage of a wide range of cyanohydrins into aldehydes or ketones and hydrogen cyanide (cyanogenesis) (Wajant and Effenberger 1996), which have recently become the subject of intensive structural studies (Wagner et al. 1996; Zuegg et al. 1999; Lauble et al. 2001a,b). Apart from the interesting aspects of their catalytic function, HNLs are of practical importance as biocatalysts for the reverse reaction of cyanogenesis, that is, the stereoselective addition of HCN to carbonyl compounds (Scheme 1).

Although the HNL from *Manihot esculenta* (MeHNL) accepts a broad range of carbonyl compounds as substrates (Förster et al. 1996), MeHNL-catalyzed cyanohydrin for-

Scheme 1.

mation is limited by bulky substituents with respect to conversion and *ee*-value, as shown, for example, for 3-phenoxybenzaldehyde, an important starting material for pyrethroids (Wajant and Effenberger 1996; Bühler 2000).

On the basis of the X-ray structure of MeHNL (Lauble et al. 1999), first attempts have been undertaken to correlate the substrate specificity with structural features of the three-dimensional structure of the enzyme. The X-ray structure of

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wild-type MeHNL revealed that the active site of the enzyme is only accessible via a narrow tunnel in the surface of the protein (Lauble et al. 2001a). The tunnel entrance is capped by Trp 128, which, therefore, might be a primary determinant for substrate transport into the active site. To investigate the functional importance of Trp 128 for substrate specificity, this residue was substituted by alanine. Whereas the catalytic activity of this mutant toward acetone cyanohydrin was almost unchanged, the conversion of mandelonitrile and 4-hydroxymandelonitrile was significantly increased. To address this change in substrate specificity of the mutant enzyme, we have determined the X-ray crystal structure of MeHNL-W128A in complex with 4-hydroxybenzaldehyde at 2.1 Å resolution. Additionally, the three-dimensional structure of MeHNL-W128A was determined at 2.1 Å resolution in a substrate-free form to characterize any structural alterations that might have resulted from the mutation.

Results and Discussion

Kinetic characterization and X-ray structure of MeHNL-W128A

Wild-type MeHNL and MeHNL-W128A were expressed in *Escherichia coli* and purified by anion exchange chromatography to homogeneity (>95%) (Wajant et al. 1996). The purified enzymes were characterized with respect to their enzymatic activities by use of acetone cyanohydrin, mandelonitrile, and 4-hydroxymandelonitrile as substrates.

The MeHNL-W128A mutant shows a 30% reduced relative specific activity for the natural substrate acetone cyanohydrin compared with the wild-type enzyme (Table 1). However, MeHNL-W128A is much more potent in cleavage of mandelo- and 4-hydroxymandelonitrile than wild-type MeHNL. Thus, MeHNL-W128A mutant exerts a 9-fold increase in specific activity for mandelonitrile and a nearly 450-fold increase in specific activity for 4-hydroxymandelonitrile. Hence, Trp 128 of MeHNL has a significant impact on enzyme activity for bulky substrates. In accordance with the specific activities for the three substrates (Table 1), the K_m value of MeHNL-W128A for acetone

cyanohydrin is slightly higher than that for the wild-type enzyme, whereas the K_m values for the sterically demanding substrate mandelonitrile is seven times lower (Table 1). For 4-hydroxymandelonitrile, a K_m value of wild-type MeHNL could not be determined. Due to the slow conversion rate of the substrate, the enzyme activity was below the detection limit at low substrate concentrations.

To assess the structural implications of the Trp 128 substitution on substrate specificity, the X-ray structure of substrate-free MeHNL-W128A was determined at 2.1 Å resolution and refined with an R value of 19.2% and a corresponding R_{free} value of 23.4%. The structure contains all 520 residues of both molecules in the asymmetric unit. Like the wild-type structure, the Ramachandran plot showed Ser 80 and Arg 129 in disallowed regions. Ser 80 is an active-site residue and clearly shown in the electron density maps, whereas Arg 129 deviates from ideal values because of a strong salt bridge to Glu 156. A summary of the X-ray data and refinement statistics for the MeHNL-W128A structure is listed in Table 2.

Superposition of the MeHNL-W128A structure with the wild-type MeHNL–acetone complex shows root mean square (rms) deviations of 0.1 Å for all backbone atoms and 0.2 Å for all atoms of the active-site residues participating in the substrate hydrogen-bonding network (Thr 11, Ser 80, Asp 208, and His 236), whereas for all atoms of the 11 residues (Leu 121, Leu 122, Phe 125, Tyr 133, Met 147, Leu 149, Leu 153, Leu 158, Leu 179, Ile 210, and Phe 211) forming the wall of the substrate tunnel, the rms deviation is 0.2 Å. This comparison clearly shows that the replacement of Trp 128 by Ala does not alter the overall structure of the active-site tunnel or that of the catalytic residues. This observation is in agreement with kinetic data showing that the specific activity of the MeHNL-W128A catalyzed cleavage of acetone cyanohydrin is very similar to that of the wild-type enzyme, indicating that the interaction between the substrate and the active-site residues is intact. Molecular surface calculations on MeHNL-W128A substrate-free form, however, reveal that the substitution of W128 turns the narrow blocked-off tunnel of the wild-type enzyme into a wide-open cleft (Fig. 1A). As a result of this change, a hydrophobic area of ~280 Å² becomes exposed. It is obvi-

Table 1. Relative specific activities and K_m values for wild-type MeHNL and MeHNL-W128A

Substrate	Rel. specific activity		K_m value (mM)	
	Wild-type MeHNL	MeHNL-W128A	Wild-type MeHNL	MeHNL-W128A
Acetone cyanohydrin	1	0.7	67	150
Mandelonitrile	1	9	30	4.5
4-Hydroxymandelonitrile	1	445	—	0.625

Table 2. Data collection and refinement statistics

	W128A-MPD	W128A-hydroxybenzaldehyde
Crystals and diffraction data		
Space group	P4 ₁ 2 ₁ 2	P4 ₁ 2 ₁ 2
Unit cell (<i>a</i> , <i>b</i> , <i>c</i> in Å)	106.4, 188.4	106.3, 188.8
Resolution range (Å)	20.0–2.10	20.0–2.10
No. reflections	254,472	415,040
Unique reflections	63,450	63,477
Completeness (%)	99.9	99.4
Average multiplicity	4.5	6.5
Intensities (<i>I</i> /σ _{<i>I</i>})	21.1	19.2
R _{sym} (<i>I</i>) for all data ^a	4.2	5.2
Outer resolution shell		
Resolution range (Å)	2.17–2.10	2.17–2.10
Completeness (%)	99.4	99.9
Average multiplicity	4.4	6.4
Intensities (<i>I</i> /σ _{<i>I</i>})	5.2	5.1
R _{sym} (<i>I</i>) for all data ^a	14.1	15.4
Refinement statistics		
Number of protein nonH atoms	4160	4160
Water molecules	250	271
4-hydroxybenzaldehyde molecules	—	4
Resolution range (Å)	8.0–2.1	8.0–2.1
No. reflections (<i>F</i> _o > 2σ <i>F</i> _o)	54134	60760
No. reflections for R _{free} (<i>F</i> _o > 2σ <i>F</i> _o)	5408	6079
R _{cryst} (%)	19.2	19.7
R _{free} (%)	23.4	24.4
RMS bond length (Å)	0.015	0.015
RMS bond angles (°)	2.9	2.9
Average B-values (Å ²)		
Protein	26.0	27.0
Water	31.0	37.0
4-hydroxybenzaldehyde		
productive binding mode	—	41.0
second molecule	—	53.0
Ramachandran plot (%) ^b	89.0; 9.7; 0.4; 0.9	88.6; 10.1; 0.4; 0.9

All crystallographic refinement was carried out using the program X-PLOR (Brünger 1992b). A total of 10% of all reflections were excluded from the refinement to follow R_{free} for cross-validation analysis (Brünger 1992a).

^a R_{sym} = $\sum_{hkl} \sum_i |I_i - \langle I \rangle| / \sum_i I_i$, where *I_i* is the intensity of the *i*-th reflection (*hkl*) or a symmetry-related reflection and $\langle I \rangle$ is the scaled mean intensity. The summation is over all measured reflections.

^b Order: most favored; additionally allowed; generously allowed; disallowed regions; R = $100 \sum_{hkl} |F_{(obs,hkl)} - F_{(calc,hkl)}| / \sum_{hkl} F_{(obs,hkl)}$, where *F*_(obs) and *F*_(calc) are observed and calculated structure amplitudes, respectively, and the summation is over all reflections *hkl*. R_{cryst} and R_{free} are calculated using the working and free reflection sets, respectively.

ous that this structural modification could have an impact on the accessibility of the active site by larger substrates.

The MeHNL-W128A substrate-free form contains one water molecule in the active site of molecule A and B, respectively, which is hydrogen bonded to Ser 80-OG (2.6 Å), His 236-NE2 (2.6 Å), and 2-methyl-2,4-pentanediol (MPD)-O4 (2.7 Å). The MeHNL-W128A active site shows well-resolved electron density of MPD, present during crystallization. MPD is bound to the active site in the typical *cis* conformation and shows a hydrogen bond to the active-site water (see above) and a weak hydrophobic interaction of C5 with Ile 12-CG1 (3.9 Å) and Ile 149-CD1 (3.9 Å).

X-ray structure of MeHNL-W128A in complex with 4-hydroxybenzaldehyde

The crystal structure of MeHNL-W128A mutant enzyme in complex with 4-hydroxybenzaldehyde was determined at 2.1 Å resolution and refined with R = 19.7% and R_{free} = 24.4% (Table 2). In the active site of molecules A and B, the electron density for 4-hydroxybenzaldehyde is well resolved, clearly indicating the orientation of the molecule (Fig. 1B). The carbonyl oxygen forms hydrogen bonds to Ser 80-OG (2.3 Å), Thr 11-OG (2.6 Å), and to the active-site water X122 (2.8 Å). The phenyl ring shows van

Fig. 1. (A) Molecular surface drawing of wild-type MeHNL (*left*) and MeHNL-W128A-4-hydroxybenzaldehyde complex (*right*). The enzymes are viewed toward the active-site tunnel entrance. The surface of Trp 128 (*left*) and Ala 128 (*right*) is colored yellow, showing the completely covered entrance of the wild-type enzyme and the wide-open substrate binding site of the mutant enzyme. Stereoview of the MeHNL-W128A active site in complex with 4-hydroxybenzaldehyde. (B) $|F_o - F_c|$ electron density omit map contoured at 4σ . (C) Intermolecular interactions of the substrate molecules and selected active-site residues. Hydrogen bonds are shown as red broken lines, van der Waals interactions as black broken lines.

der Waals contacts with four tunnel residues including C2 with Ile 12-CG1 (3.7 Å) and Leu 149-CD2 (3.8 Å), C3 with Ile 12-CD1 (3.7 Å), Met 147-SD (3.7 Å) and Leu 149-CD2 (3.8 Å), and C6 with Cys81-SG (3.7 Å). The hydroxyl group forms no hydrogen bonds with protein residues. The binding of a second molecule 4-hydroxybenzaldehyde into the active site of molecules A and B is a most surprising result. The $2F_o - F_c$ electron density map (Fig. 1B) clearly shows the position and orientation of the second molecule, that could be modeled in such a way that its hydroxyl group is stabilized at the base of the active-site cavity forming a hydrogen bond to the active-site water X122 (2.8 Å). The phenyl ring of the second molecule makes van der Waals contacts to three tunnel residues. Most specifically, C2 interacts with Ile 210-CG1 (3.4 Å), C3 with Ile 210-CD1 (3.9 Å), C4 with Leu 149-CD2 (4.0 Å) and Leu 153-CD2 (4.0 Å), and C5 with Leu 149-CD2 (3.8 Å) and Ile 210-CD1 (4.0 Å). The carbonyl group points toward the tunnel entrance and interacts with the main-chain oxygen of Phe 125 (3.1 Å). These interactions place the phenyl ring of the second molecule in a sandwich type mode to the first one, thereby allowing aromatic stacking interactions. The distance between the two aromatic planes is ~3.8 Å. The two molecules bind in a head-to-tail arrangement and are bridged by the active-site water molecule, which is also hydrogen bonded to the catalytic residues His 236-NE2 (2.7 Å) and Ser 80-OG (3.0 Å).

Figure 1C represents schematically the binding of 4-hydroxybenzaldehyde to the active site of MeHNL-W128A mutant enzyme, including hydrogen bonding and van der Waals interactions of the two molecules and the protein. The average B factor for the productive mode bound 4-hydroxybenzaldehyde in the active site of molecules A and B is 41 Å², whereas the average B factor for the second 4-hydroxybenzaldehyde is 53 Å² (Table 2). The higher B factor for the latter probably reflects an occupancy of <1.0, which was the value for occupancy used in the refinement.

Superposition of the MeHNL-W128A-hydroxybenzaldehyde structure onto the MeHNL-W128A substrate-free structure shows an rms deviation of 0.5 Å for all atoms. In the complexed MeHNL-W128A structure, the carbonyl oxygen O1 is hydrogen bonded to the active-site water X122, replacing the MPD 4-hydroxyl group interaction observed in the substrate-free form. The active-site water X122 is hydrogen bonded to His 236-NE, as observed in the substrate-free complex, and additionally to the 4-hydroxyl group of the second hydroxybenzaldehyde molecule. Although the position of water X122 does not seem appreciably altered by the binding of two carbonyl substrates, Ser 80 undergoes a conformational change upon hydroxybenzaldehyde binding. Ser 80 reorients in a manner making a hydrogen bond between Ser 80-OG and His 236-NE, thereby favoring the abstraction of a proton by the general base His 236 as required in the catalytic mechanism (Lauble et al.

2001a,b). In addition, Ser 80-OG makes an unusually short hydrogen bond to the carbonyl oxygen O1 (2.3 Å). As a consequence of the Ser 80 reorientation, the hydrogen bond of OG to the substrate-free active-site water is lost and the hydrogen bond between the hydroxyl group of Ser 80 and Thr 11 lengthens from 3.0 to 3.6 Å, thereby allowing a new interaction of Thr 11-OG to the carbonyl oxygen of hydroxybenzaldehyde, increasing the total number of possible hydrogen bonds at the active site by two compared with the substrate-free complex.

Superposition of the MeHNL-W128A-hydroxybenzaldehyde complex onto wild-type MeHNL-acetone structure (Lauble et al. 2001a) shows an rms deviation of 0.2 Å for all backbone atoms and an rms deviation of 0.2 Å for active-site residues. The comparison shows that hydroxybenzaldehyde binds to the MeHNL-W128A active site, as does acetone, by interaction of the carbonyl oxygen with Ser 80-OG and Thr 11-OG, respectively. A surprising difference of the hydroxybenzaldehyde complex is the 90° rotation of the carbonyl plane with respect to that of acetone. The difference in the orientation means that the phenyl ring of 4-hydroxybenzaldehyde points perpendicular toward the tunnel entrance, whereas the corresponding methyl group in acetone lies more parallel to the entrance. The carbonyl oxygen, however, maintains basically the same position seen for the carbonyl oxygen in the MeHNL-acetone complex. The comparison also shows that the carbonyl oxygen of hydroxybenzaldehyde is additionally hydrogen bonded to the active-site water X122, a water molecule not observed in the wild-type MeHNL-acetone complex. The active-site water is also present in the MeHNL-W128A substrate-free form, in MeHNL-S80A substrate-free form (Lauble et al. 2001b), and in the wild-type MeHNL-chloroacetone structure (Lauble et al. 2001a).

In conclusion, the MeHNL-W128A-hydroxybenzaldehyde complex reveals a largely open-ended S2 pocket, thereby facilitating the accommodation of larger hydrophobic substrates most significantly demonstrated by the presence of two hydroxybenzaldehyde molecules in the active site.

The facilitated access to the active site of the MeHNL-W128A mutant enzyme compared with the wild-type enzyme could be proved. From specific activity and K_m value, a negligible steric influence could be deduced for the natural substrate acetone cyanohydrin. On the contrary, a reduced steric hindrance in the mutant MeHNL-W128A seems to cause clearly improved K_m values and increased specific activities for the bulkier substrates mandelo- and 4-hydroxymandelonitrile. Superposition of the MeHNL-W128A-hydroxybenzaldehyde complex onto the wild-type MeHNL-acetone crystal structure (Lauble et al. 2001a) shows that binding of hydroxybenzaldehyde into the wild-type MeHNL active site would result in a steric clash of the 4-hydroxyl group with the indole side chain of Trp 128. The

substrate preference for the mutant enzyme, however, is not exclusively determined by size exclusion. Because the specific activity, as well as the K_m value, for 4-hydroxymandelonitrile are clearly improved in comparison with mandelonitrile, the defined arrangement of a second molecule 4-hydroxybenzaldehyde in the active site of MeHNL-W128A seems to have an additional influence on the catalytic mechanism. From the experimental results, however, it is difficult to estimate the influence of the additional hydrogen bridge or the aromatic π -stacking on the unusually high substrate specificity for 4-hydroxymandelonitrile.

Materials and methods

Expression and purification of MeHNL-W128A

E. coli XL1-Blue (Stratagene) was used for the propagation of plasmids. Site-directed mutagenesis of MeHNL were achieved with the pQE4-MeHNLwild-type expression plasmid (Wajant and Pfizenmaier 1996). The W128A mutation was introduced into pQE4-MeHNLwild type using the Quick Change Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's recommendations with two complementary primers corresponding to nucleotides 383–435 of MeHNL (5'-AAGCTTTTGGAGTCGTTTCCTGCGAGAGACACAGAGTATTTACGTTTCAC-3') containing the mutation of interest (bold underlined). Wild-type MeHNL and MeHNL-W128A were expressed in an *E. coli* strain M15[pREP4] as described previously (Wajant et al. 1996). Purification of the recombinant proteins were achieved by use of anion exchange chromatography and gel exclusion chromatography (Wajant et al. 1996).

Enzyme assays and enzyme kinetics

Cleavage of acetone cyanohydrin by wild-type MeHNL and MeHNL-W128A was measured as described by Selmar et al. (1987). For acetone cyanohydrin, the amount of HNL that decomposes 1 μ mole of the substrate in 1 min at room temperature (pH 5.2) in 100 mM sodium acetate is defined as 1 unit. MeHNL-catalyzed cleavage of mandelonitrile was determined according to Hanefeld et al. (1999), whereas the cleavage of 4-hydroxymandelonitrile was determined as described by Seely et al. (1966). Total protein for calculations of specific activities was determined with the BCA Protein Assay Reagent (Pierce) according to the manufacturer's recommendations.

For determination of the K_m -values for wild-type MeHNL and MeHNL-W128A mutant enzyme for acetone cyanohydrin, mandelo- and 4-hydroxymandelonitrile, the initial velocity was determined over a substrate concentration of 1–55 mM for acetone cyanohydrin, 1–17 mM for mandelonitrile, and 1–1000 μ M for 4-hydroxymandelonitrile, respectively, in sodium acetate (pH 5.2) at room temperature. The obtained data were analyzed according to the method of Michaelis and Menten (Palmer 1981). Accurate determination of v_{max} values were obtained from Lineweaver-Burk plots (Palmer 1981).

Crystallization and data collection

For crystallization, the protein was dialyzed against 10 mM sodium citrate (pH 5.4) and concentrated to 32 mg/ml. Crystals of

MeHNL-W128A mutant enzyme were grown by the vapor-diffusion hanging-drop method under the conditions described previously (Lauble et al. 1999) and belong to the tetragonal space group $P4_12_12$ (Table 2), with two molecules per asymmetric unit and are isomorphous to wild-type MeHNL. For data collection of the MeHNL-W128A substrate-free form, a single crystal was transferred into a stabilization buffer containing 100 mM sodium citrate (pH 5.4), 5% PEG 8000, 28% MPD, and flash cooled at 100 K.

MeHNL-W128A-hydroxybenzaldehyde complex crystals were prepared by soaking a MeHNL-W128A crystal in 100 mM sodium citrate (pH 5.4), 8% PEG 8000, and 28% MPD containing 400 mM 4-hydroxybenzaldehyde for 1 h. The soaked crystal was flash cooled at 100 K. Diffraction data were collected on a 30-cm MAR research image plate using the synchrotron radiation at station PX11 ($\lambda = 0.902$ Å) at EMBL outstation, Hamburg, Germany. The exposing dose was 10,000 per frame using 1° oscillation. The crystal-to-detector distance was 200 mm, and the slits were set at 0.2 mm. MeHNL-W128A mutant crystal diffracted to 2.1 Å resolution. Mosaicity of the frozen crystals was in the range of 0.2°. Raw data images were indexed and integrated using the program DENZO (Otwinowski 1993), and all data scaled and reduced with the programs from the CCP4 suite (Collaborative Computational Project, Number 4 1994). The final statistics on the data set are given in Table 2.

Refinement

The starting model for the refinement of both structures was the coordination set of the refined structure of the MeHNL-acetone complex (PDB entry 1DWP) (Lauble et al. 2001a) without water molecules and ligands. This crystal structure contains two protein molecules in the asymmetric unit (A and B) and consists of 262 amino acids for molecule A and 258 amino acids for molecule B. The extra four amino-terminal residues observed for molecule A are part of the seven residues encoded by the multiple cloning site of the expression vector used for the overexpression of MeHNL (Wajant and Pfizenmaier 1996). The Trp 128 residue was substituted by alanine. To account for the pronounced anisotropic diffraction properties of MeHNL crystals (Lauble et al. 1999), an anisotropic correction was included in the refinement. The electron density maps were displayed using the graphic program XTALVIEW (McRee 1999), and the atomic model for hydroxybenzaldehyde and MPD were generated and geometrically minimized with INSIGHT II/DISCOVER (BIOSYM/ Molecular Simulations Inc., Release 95.0, 1995). Each structure was refined using the same protocol, starting with a rigid-body refinement to allow rearrangement of molecules A and B in the asymmetric unit against each other. Subsequent rounds of positional refinement proceeded in steps, each step including data shells of increasing resolution. When the resolution was extended to 2.5 Å, $2|F_o| - |F_c|$ electron density maps were calculated and each protein model analyzed. The engineered W128A mutation was clearly defined, and water molecules were added using the MeHNL-acetone complex structure as a guideline. Each model was subjected to further rounds of positional, simulated annealing ($T = 3000$ K) and B-factor refinement including, in the last step, all data in the resolution range 8–2.2 Å. For each structure, $2|F_o| - |F_c|$ and $|F_o| - |F_c|$ electron density maps were calculated to check the water molecules. Water molecules with B factors better than 60 Å² were retained. The electron density map for the MeHNL-W128A substrate-free form shows one well-resolved water molecule in the active site of molecule A, two in the active site of B, and additionally continuous electron density that could clearly be attributed to the crystallization compound MPD. Analysis of the $2|F_o| - |F_c|$

map calculated for MeHNL-W128A-hydroxybenzaldehyde shows clear electron density for the aldehyde in the active site of molecules A and B. Electron density is also observed for an active-site water molecule in both subsites. An additional extraneous electron density in the active site of both molecules A and B, showing basically the same long plane shape as hydroxybenzaldehyde, was observed and therefore modeled as 4-hydroxybenzaldehyde. Both MeHNL-W128A mutant structures along with the water molecules were refined against their diffraction data between 8–2.1 Å resolution. $2F_o - |F_c|$ maps were calculated to confirm the fit of these models to the active-site density. The refinement of the MeHNL-W128A substrate-free structure and the MeHNL-W128A-hydroxybenzaldehyde complex structure converged to $R = 19.2\%$, $R_{\text{free}} = 23.4\%$ and $R = 19.7\%$, $R_{\text{free}} = 24.4\%$, respectively (Table 2). The geometric parameters of both models were analyzed with PROCHECK (Laskowski et al. 1993) and are within the expected deviation (Table 2). A Ramachandran plot shows that the residues Ser 80 and Arg 129 of each subunit fell outside of the energetically favorable regions. Ser 80 is located in the active site and shown clearly in the electron density map, whereas Arg 129 deviates from ideal values because of strain from a salt bridge to Glu 156. The two molecules in the asymmetric unit are very similar for both structures and superimpose with rms differences of ~ 0.7 Å for all atoms. Molecule A of each structure is used for analysis in this paper. The final coordinates of the MeHNL-W128A structures have been deposited with the Protein Data Bank (PDB entry 1EB8 for MeHNL-W128A, PDB entry 1EB9 for MeHNL-W128A-hydroxybenzaldehyde complex).

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