Mechanistic aspects of cyanogenesis from active-site mutant Ser80Ala of hydroxynitrile lyase from *Manihot esculenta* in complex with acetone cyanohydrin

HANSPETER LAUBLE,¹ BURKHARD MIEHLICH,¹ SIEGFRIED FÖRSTER,¹
HARALD WAJANT,² AND FRANZ EFFENBERGER¹

¹Institut für Organische Chemie der Universität Stuttgart, D-70569 Stuttgart, Germany

²Institut für Zellbiologie und Immunologie der Universität Stuttgart, D-70569 Stuttgart, Germany

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Abstract

The structure and function of hydroxynitrile lyase from *Manihot esculenta* (MeHNL) have been analyzed by X-ray crystallography and site-directed mutagenesis. The crystal structure of the MeHNL–S80A mutant enzyme has been refined to an R-factor of 18.0% against diffraction data to 2.1-Å resolution. The three-dimensional structure of the MeHNL–S80A–acetone cyanohydrin complex was determined at 2.2-Å resolution and refined to an R-factor of 18.7%. Thr11 and Cys81 involved in substrate binding have been substituted by Ala in site-directed mutagenesis. The kinetic measurements of these mutant enzymes are presented. Combined with structural data, the results support a mechanism for cyanogenesis in which His236 as a general base abstracts a proton from Ser80, thereby allowing proton transfer from the hydroxyl group of acetone cyanohydrin to Ser80. The His236 imidazolium cation then facilitates the leaving of the nitrile group by proton donating.

Keywords: Cyanogenesis; acetone cyanohydrin complex; site-directed mutagenesis; reaction mechanism

Hydroxynitrile lyases (HNLs) catalyze the decomposition of a variety of cyanohydrins into their corresponding carbonyl compounds and HCN. The release of toxic HCN is believed to play a central role in the defense mechanism of plants against herbivores (Seigler 1991). Several HNLs have been identified that can be differentiated by sequence similarity and enzymatic properties (Wajant and Effenberger 1996). The HNLs from *Hevea brasiliensis* (HbHNL) and *Manihot esculenta* (MeHNL) have received additional attention as biocatalysts for the reverse reaction, for example, the stereoselective addition of HCN to carbonyl compounds (Förster et al. 1996; Schmidt et al. 1996; Griengl et al. 1997; Effenberger 1999), and therefore represent the best-characterized HNLs. The three-dimensional

structures of HbHNL (Wagner et al. 1996; Zuegg et al. 1999) and MeHNL (Lauble et al. 2001) show a topology that is related to but still distinct from α/β hydrolases. The active-site residues are in an arrangement reminiscent of a catalytic triad, and a mechanism closely related to the base-catalyzed chemical addition of HCN to carbonyl compounds could be deduced (Lauble et al. 2001). The X-ray structure of MeHNL complexed with acetone and chloroacetone, respectively, shows that Ser80 is involved in binding the substrate carbonyl group, with participation of Thr11 and, to a lesser extent, Cys81, whereas His236 is proposed to act as a general base (Lauble et al. 2001). Although the importance of His236 in the catalytic mechanism is confirmed by site-directed mutagenesis, there is no structural evidence for the direct interaction of the scissile nitrile group of the natural substrate acetone cyanohydrin with the active-site residues.

Recently an HbHNL–rhodanide complex was reported with the rhodanide positioned to interact with His235 and Lys236 (both HbHNL numbering), respectively (Zuegg et

Reprint requests to: Hanspeter Lauble, Institut für Organische Chemie, Universität Stuttgart, Pfaffenwaldring 55, D-70569 Stuttgart, Germany; e-mail: PeterLauble@t-online.de; fax: +49-711-685-4269.

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al. 1999). The results obtained from a two-atom analog, however, can be misleading and difficult to interpret. Obtaining an HNL crystal structure of the acetone cyanohydrin complex represents a difficult challenge. This complex cannot be studied with the wild-type HNL because of catalytic interconversion. Because MeHNL-S80A is catalytically inactive (Wajant and Pfizenmaier 1996), the mutant enzyme–acetone cyanohydrin complex can be characterized independently. The stability of this complex at room temperature, however, is limited by the spontaneous decomposition of acetone cyanohydrin into acetone and HCN, making a crystallographic analysis of this complex impractical. However, less than 10% of acetone cyanohydrin decomposes over a period of 8 h at 100 K, as judged from absorption spectra (data not shown). Therefore, the best approach to obtaining structural data seemed to be a rapid binding of acetone cyanohydrin to the crystalline MeHNL-S80A mutant enzyme under physiological pH conditions at room temperature and then trapping the complex at cryogenic temperatures. To distinguish protein conformational changes that might occur because of the mutation from those that are induced by substrate binding, a substrate-free MeHNL-S80A structure was also determined at cryogenic temperatures.

To elucidate the role of various active-site residues involved in substrate binding, Thr11 and Cys81 were mutated to Ala and characterized. The precise substrate orientation with respect to the active-site residues as derived from X-ray structures in conjunction with the mutagenesis results is discussed in relation to the mechanism proposed for cyanogenesis.

Results

Crystal structure of the mutant MeHNL-S80A

The mutant enzyme was crystallized in a substrate-free form, and the three-dimensional structure was solved to a resolution of 2.1 Å by X-ray crystallography. Data processing and refinement statistics are summarized in Table 1. The refined structure of MeHNL-S80A consists of 4176 nonhydrogen protein atoms containing all 520 residues of both molecules in the asymmetric unit and 426 water molecules. The refinement of the final model converged to $R = 18.0\%$, $R_{\text{free}} = 21.9\%$ for 60,467 reflections in the resolution range 8–2.1 Å (Table 1). A Ramachandran plot shows that residues Ala80 and Arg129 of each subunit fell outside of the energetically favorable regions. Ala80 is shown clearly in the electron density map, whereas Arg129 deviates from ideal values because of a strong salt bridge to Glu156.

$2F_o - F_c$ and unbiased $F_o - F_c$ maps show clearly that the density was consistent with the mutated residue. The electron density map also showed three well-resolved water molecules in the active site of molecule A (W197, W5, W4)

Table 1. Data collection and refinement statistics

	MeHNL-S80A	MeHNL-S80A– acetone cyanohydrin
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)	106.2, 188.2	105.9, 187.8
Resolution range (Å)	20.0–2.1	15.0–2.2
No reflections	265,511	211,247
Unique reflections	63,336	52,736
Completeness (%)	96.7	97.1
Multiplicity	4.3	3.9
Intensities (<i>I</i> /σ)	8.8	8.1
R_{sym} (<i>I</i>) for all data ^a	6.5	6.8
Outer resolution shell		
Resolution range (Å)	2.2–2.1	2.3–2.2
Completeness (%)	98.4	98.5
Multiplicity	4.2	3.8
Intensities (<i>I</i> /σ)	3.4	3.4
R_{sym} (<i>I</i>) for all data ^a	14.1	13.5
Refinement statistics		
Number of protein nonH atoms		
	4176	4176
Water molecules	426	273
Acetone cyanohydrin molecules	—	4
Resolution range (Å)	8.0–2.1	8.0–2.2
No reflections ($F_o > 2.0\sigma F_o$)	60,467	50,187
No reflections for R_{free} ($F_o > 2.0\sigma F_o$)	6025	5233
R_{cryst} (%)	18.0	18.7
R_{free} (%)	21.9	23.8
RMS bond length (Å)	0.015	0.020
RMS bond angle (°)	2.8	2.8
Average B-values (Å ²)		
Protein	21	25
Water	34	35
Acetone cyanohydrin	—	34
Ramachandran plot (%) ^b	89.8; 8.9; 0.4; 0.9	89.5; 9.2; 0.4; 0.9

All crystallographic refinement was carried out using the program X-PLOR (Brünger 1992^b). Ten percent of all reflections were excluded from the refinement to follow R_{free} for cross-validation analysis (Brünger 1992^a). The molecular display and map-fitting program TOM, version 3.1 (Jones 1978) was used to examine and adjust the structures during the refinement process.

^a $R_{\text{sym}} = \sum_{hkl} \sum_i |I_i - \langle I \rangle| / \sum_i I_i$, 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. $R = 100 \sum_{hkl} |F_{\text{obs}(hkl)} - F_{\text{calc}(hkl)}| / \sum_{hkl} F_{\text{obs}(hkl)}$, 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.

and two in the active site of molecule B (X206, X2). Water molecule W197 forms hydrogen bonds to active-site His236 – NE2 (2.8 Å) and W4 (2.8 Å), and W4 is within hydrogen-bond distance to Thr11 – OG (2.8 Å) and W5 (2.6 Å), respectively. Analogous water molecules X206 and X2 bound to the active site of molecule B are located on similar positions. A water molecule comparable to W5 is not ob-

served in molecule B. The geometry of the close contacts indicates that active-site waters in the substrate-free form are involved in saturating the H-bonding properties of the catalytic residues. The hydration mode of the active sites observed for the mutant MeHNL substrate-free form differs from that of the HbHNL substrate-free form (Protein Data Bank [PDB] entry PDB 7YAS; Zuegg et al. 1999), showing a water-mediated hydrogen bond between Lys236 and His235 (both HbHNL numbering). Superposition of the two molecules in the asymmetric unit shows a root mean square (rms) deviation of 0.23 Å for all polypeptide backbone atoms and an rms deviation of 0.13 Å for all atoms of the active-site residues. Least-square superpositioning of the MeHNL-S80A mutant enzyme and the wild-type MeHNL-acetone complex structure shows rms deviation of 0.16 Å for all atoms of the active-site residues, except those in residue 80, and 0.16 Å for the polypeptide backbone atoms, respectively, showing clearly that neither the overall structure of the mutant enzyme nor the geometry of the active-site residues are affected by the mutation (PDB entry 1E89).

Crystal structure of the mutant

MeHNL-S80A-acetone cyanohydrin complex

The refined structure of the MeHNL-S80A-acetone cyanohydrin complex converged to $R = 18.7\%$ and $R_{\text{free}} = 23.8\%$ for 50,187 reflections in the resolution range 8–2.2 Å (Table 1). This model contains all 520 residues of both subunits in the asymmetric unit, 4 molecules of acetone cyanohydrin, and 273 water molecules. A Ramachandran plot shows that the stereochemistry of this model is basically identical to the substrate-free form reported above, with two outliers from the energetically favorable regions for each subunit, namely Ala80 and Arg129.

The electron density map (Fig. 1A) showed clearly that two molecules of acetone cyanohydrin are bound to the active sites of molecules A and B, respectively, and two additional molecules are bound on the surface of molecule A next to residues Thr137 and Gln49, respectively.

Refinement of the MeHNL-S80A-acetone cyanohydrin complex (Table 1), assuming full occupancy for the substrates in the active site, resulted in an averaged B-factor of 41 Å² for acetone cyanohydrin of molecule A and 52 Å² of molecule B, respectively. From the two molecules in the asymmetric unit, molecule A is chosen for comparison because it is the more well-ordered one. The higher B-factors for the substrates compared with the surrounding protein atoms (21 Å²) probably reflects the high flexibility of the bound substrate. To attribute the higher B-factors to the chemical instability of acetone cyanohydrin seems unlikely, because two additional acetone cyanohydrin molecules, bound on the surface of the protein, refine properly to an averaged B-factor of 26 Å².

Superposition of the two molecules in the asymmetric unit shows an rms deviation of 0.24 Å for all polypeptide backbone atoms and an rms deviation of 0.12 Å for all atoms of the active-site residues. The MeHNL-S80A-acetone cyanohydrin form and the MeHNL-S80A substrate-free form are very similar to each other and superimpose with an rms deviation of 0.18 Å for all polypeptide backbone atoms and with an rms deviation of 0.14 Å for all atoms of the active-site residues. This comparison reveals clearly the rigidity of the catalytic site architecture, even on substrate binding, which is most likely due to the hydrogen bonding network formed in this region. Comparison of the active sites of substrate-bound and substrate-free forms of MeHNL-S80A shows that the hydroxyl group of acetone cyanohydrin is positioned in the active site occupied in the substrate-free form by W4, whereas the position of the nitrile group is analogous to W197 (PDB entry 1E8D).

Substrate binding interaction in the

MeHNL-S80A-acetone cyanohydrin complex

In the active site of molecule A, the electron density for acetone cyanohydrin is well-resolved (Fig. 1A). The tetrahedral shape of the density with one axis clearly extended allows an unambiguous placement of the nitrile group. The active site of molecule B also shows continuous electron density, but the extended axes expected for the nitrile group were not as well-defined as in molecule A. This observation is in agreement with MeHNL-acetone and chloroacetone complexed structures, showing also less-resolved electron densities in the active site of molecule B. The hydroxyl group of acetone cyanohydrin was modeled into the densities using the orientation of the carbonyl oxygen in the MeHNL-acetone complex as reference. This treatment places the C1 methyl group into the apolar subsite formed by Ile12, Leu149, and Leu158. The C1 atom of acetone cyanohydrin is in van der Waals contacts to Leu149 – CD1 (3.8 Å) and Ile12 – CG1 (4.1 Å) (Fig. 1B). The C3 methyl group of acetone cyanohydrin is facing outward from the active-site residues into a hydrophobic region of the active-site channel. This second binding pocket is formed by Trp128, Leu153, and Ile210 and is lined by the residues His14 and Lys237. The C3 atom of acetone cyanohydrin interacts with the side chain of Trp128 – CH2 (3.6 Å), Phe211 – CE1 (3.6 Å), and Ile210 – CD1 (3.8 Å), respectively (Fig. 1B).

The hydroxyl group at C2 forms a hydrogen bond to the active-site residues Thr11 – OG (2.8 Å) and Cys81 – SG (3.4 Å) and also shows a weak van der Waals contact to Ala80 – CB (3.6 Å). The nitrile group of acetone cyanohydrin (labeled N5) points straight toward the side chain of His236, and the 2.6-Å electrostatic interaction between His236 – NE2 and N5 indicates clearly that His236 is perfectly positioned to donate a proton to leaving cyanide. N5

Fig. 1. Stereoview of the MeHNL-S80A active site in complex with acetone cyanohydrin. Molecule A of the asymmetric unit is shown. *A* $F_o - F_c$ electron density omit map, contoured at 4σ . *B* Intermolecular interactions between the substrate and selected active-site residues. (Red dashed lines) Hydrogen bonds; (black dashed lines) van der Waals interactions. *C* Superposition with the substrate binding site in the wild-type MeHNL-acetone complex (thin sticks). Possible hydrogen bonds predicted for acetone cyanohydrin to wild-type MeHNL residues are depicted (red dashed lines). Acetone cyanohydrin binds to the active site basically in the same way as acetone.

also shows van der Waals interactions to Thr11 – CG2 (3.4 Å), Leu158 – CD2 (3.4 Å), and Ala80 – CB (3.6 Å) (Fig. 1B).

A comparison of the MeHNL–S80A–acetone cyanohydrin complex and the wild-type MeHNL–acetone complex shows no conformational changes in the active sites. Superposition shows that both structures are basically identical, showing an rms difference of 0.14 Å for all backbone atoms and an rms deviation of 0.18 Å for all active-site residues. Acetone cyanohydrin and acetone are also oriented in each structure in the same manner (Fig. 1C).

Although the replacement of Ser80 by Ala in the S80A mutant eliminates the direct polar contact made by the hydroxyl group, the oxygen positions for cyanohydrin and acetone are nearly identical, as is the hydrogen bonding distance to Thr11 – OG (3.0 Å). This result may reflect the strength of the Thr11 hydrogen bonds involving these atoms on forming the two enzyme–substrate complexes.

Effects of mutations on catalytic activity

To further elucidate the role of Thr11 and Cys81 in the catalytic mechanism of MeHNL, two mutants were constructed: Thr11Ala and Cys81Ala. Both mutant proteins could be produced and purified, and gel filtration analysis (data not shown) indicates that these mutants were folded properly into a tetrameric structure similar to the native enzyme (Wajant and Pfizenmaier 1996). The specific activity of the Thr11Ala and the Cys81Ala mutants for the substrate acetone cyanohydrin was 3 units/mg and 67 units/mg, respectively, compared with 72 units/mg for the wild-type enzyme. In addition, the K_m value of Cys81Ala is 169 mM, indistinguishable from that of the wild-type (174 mM). Similar determination of the K_m value for the Thr11Ala mutant was not possible because of the poor enzymatic activity.

The observation that the kinetics of the Cys81Ala mutant were similar to those of the wild-type enzyme is consistent with the structure of the MeHNL–S80A–acetone cyanohydrin complex, showing that Cys81 plays a more indirect role by making hydrogen bonds to the substrate hydroxyl group (Fig. 1B). The mutation Thr11Ala, however, had a dramatic effect on enzyme properties and resulted in a 95% decrease of activity compared with the wild-type enzyme. The importance of Thr11 in catalysis is supported by the MeHNL–S80A–acetone cyanohydrin complex structure with a short hydrogen bonding contact of 2.8 Å between Thr11 – OG and the substrate hydroxyl group (Fig. 1B).

Discussion

One of the foremost issues that is crucial to the understanding of how hydroxynitrile lyases catalyze reactions is how the enzyme donates a proton to the leaving cyanide in cyanogenesis and activates hydrogen cyanide for the addition to

the carbonyl group in the reverse reaction. The 2.2-Å resolution X-ray structure of a mutant MeHNL–S80A enzyme in complex with the substrate acetone cyanohydrin addresses this issue directly. Although this mutant enzyme cannot turn over the substrate, MeHNL–S80A retains the ability to bind acetone cyanohydrin in the way it should exist in the active site before the first catalytic step.

The MeHNL–S80A–acetone cyanohydrin structure shows that the lack of catalytic activity of the mutant enzyme is not caused by an overall change of protein structure or by the loss in substrate binding. Comparison of the MeHNL–S80A–acetone cyanohydrin complex and MeHNL–acetone complex reveals that the substitution of Ser80 by Ala results in loss of hydrogen bonding to His236 (Fig. 1C). The function of this hydrogen bond is to initiate the catalytic process by deprotonation of Ser80 – OG, followed by proton transfer from the hydroxyl group of acetone cyanohydrin to Ser80 – OG (Fig. 2). These hydrogen-bonding interactions are possible only in wild-type MeHNL, showing that Ser80 is absolutely essential for catalytic activity. The binding mode revealed by the MeHNL–S80A–acetone cyanohydrin structure is also in agreement with site-directed mutagenesis experiments (Wajant and Pfizenmaier 1996).

The structure also verifies the important role of Thr11 – OG, which is involved directly in substrate binding by a 2.8-Å hydrogen bond to the acetone cyanohydrin hydroxyl group (Fig. 1B). This hydrogen bond should help orient the hydroxyl group in the correct position for catalysis. A similar functional role was assigned to Thr11 in the MeHNL–acetone complex structure (Lauble et al. 2001), in which the acetone carbonyl oxygen is within 2.9-Å hydrogen-bonding distance to Thr11 – OG. Substitution of Thr11 should result in loss of this hydrogen bond and great impairment of catalytic activity, as shown by site-directed mutagenesis: Thr11 to Ala mutation results in 95% decrease in catalytic activity compared with the wild type (see above).

The binding model presented here shows that the nitrile group of acetone cyanohydrin is clearly oriented toward His236 (Fig. 1B). The 2.6-Å electrostatic interaction between NE2 of His236 and the scissile nitrile group of the substrate indicates that His236 is capable of facilitating the leaving of the nitrile group by donating a proton to cyanide in the forward reaction and to deprotonate incoming hydrogen cyanide in the reverse reaction. The importance of His236 in the catalytic mechanism is confirmed further by site-directed mutagenesis, showing that a replacement of His236 by alanine results in a dramatic loss of enzymatic activity (Wajant and Pfizenmaier 1996). From the HbHNL–rhodanide crystal structure (Zuegg et al. 1999), Lys236 (HbHNL numbering) is positioned to interact with His235 (HbHNL numbering) through a rhodanide-mediated interaction, and consequently Lys236 was predicted to provide additional interaction with the nitrile group of acetone cyanohydrin. This proposal is inconsistent with structural data

Fig. 2. Catalytic mechanism of MeHNL. This mechanism is a modification of that proposed by Wajant and Pfizenmaier (1996) and is consistent with results discussed in the text.

presented here. Inspection of the MeHNL–S80A–acetone cyanohydrin structure shows that Lys237 (MeHNL numbering) is not within hydrogen-bonding or van der Waals distances to the nitrile group.

In the MeHNL–acetone cyanohydrin complex structure, His236 – ND1 is in close contact with Asp208 – OD2 (2.8 Å) (Fig. 1B), which is assumed to orient His236 in the correct position and tautomeric form, but it is not essential for catalytic activity. This conclusion is supported by an Asp208 to Ala mutation giving a mutant with still 30% catalytic activity compared with the wild-type enzyme (Wajant and Pfizenmaier 1996).

In summary, a mechanism in which His236 functions as a general base is obvious from our results. The alternative mechanism with the base Cys81 that deprotonates HCN, as suggested by Wagner et al. (1996), seems unlikely based on the presented structural studies. A direct involvement of Cys81 in the catalytic mechanism is also in contrast to site-directed mutagenesis experiments, showing that a Cys81 to Ala mutation in MeHNL leads to only 20% reduction of the reaction rate (see above).

Figure 2 shows schematically the proposed catalytic cycle of cyanogenesis. Substrate first enters the active site

and is anchored by its hydroxyl group via hydrogen bonds to Ser80 – OG and Thr11 – OG. His236 deprotonates Ser80 – OG, which then abstracts the proton from the OH group of acetone cyanohydrin. As investigated intensively (Bradbury and Carver 1984; Carver and Bradbury 1984), the general base function of His236 is enhanced by Asp208, and proton abstraction from acetone cyanohydrin is facilitated by its hydrogen bond to Thr11 – OG. The transfer of the hydroxyl proton occurs with the concomitant proton donation from the imidazolium cation of His236 toward the leaving nitrile group. Protonation of the nitrile group results in the products hydrogen cyanide and acetone, thus completing the catalytic cycle. The last step is the release of the products from the active site and the reconstitution of the catalytic residues.

Materials and methods

Site-directed mutagenesis, expression, and purification

MeHNL–S80A mutant enzyme was expressed and purified to homogeneity as described previously (Wajant and Pfizenmaier

1996). The protein was dialyzed against 10 mM sodium citrate (pH 4.8) and concentrated to 32 mg/mL.

Site-directed mutagenesis was performed to change Thr11 and Cys81 codons to Ala. The point mutations of interest were introduced into pQE4-MeHNLwt (Wajant and Pfizenmaier 1996) using the Quick Change Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's recommendations. In brief, complementary primers corresponding to nucleotides 35–77 and 247–286 of MeHNL (Hughes et al. 1994) containing the T11A and C81A point mutations (T11A: 5' GCA CAT TTT GTT CTG ATT CAC GCG ATT TGC CAT GGT GCA TGG 3'; C18A: 5' ATC ATT GTT GGT GAG AGC GCT GCA GGG CTG AAT ATT GC 3'; mutations bold and underlined) were extended during temperature cycling with Pfu DNA polymerase, giving mutated plasmids containing staggered nicks. The products were treated with *DpnI*, which selectively digests methylated (parental) and hemimethylated (semiparental) plasmids. The nicked, mutation-containing plasmids were then transformed into *Escherichia coli* XL1-Blue cells, and several clones were analyzed for the presence of the desired mutation. The introduced mutations created additional *Bst*UI (T11A) and *Hae*II (C81A) restriction sites, allowing the identification of mutant plasmids by restriction analysis. Finally, the mutants were controlled by sequence analysis using a modified chain-termination method (Sanger et al. 1977) with T7 DNA polymerase and ALF express DNA analysis system (Pharmacia). Expression and purification of mutant enzymes was the same as described for the wild type (Förster et al. 1996). All chemicals used were purchased from Sigma.

Enzyme kinetics

Cleavage of acetone cyanohydrin by MeHNL-wt, MeHNL-T11A, and MeHNL-C81A was measured as described by Selmar et al. (1987). Briefly, the amount of HNL that decomposes 1 μ mole of substrate in 1 min at room temperature at pH 5.2 in 100 mM sodium acetate is defined as 1 unit. Total protein for calculation of specific activities was determined with the BCA Protein Assay Reagent (Pierce) according to the manufacturer's recommendations. To determine the K_m values for MeHNL and mutants derived thereof for acetone cyanohydrin, the initial velocity for a wide range of substrate concentrations (5.5–219 mM) was determined in 100 mM sodium acetate (pH 5.5) at room temperature. The obtained data were analyzed according to the method of Michaelis and Menten (Palmer 1981). Accurate determination of V_{max} values was obtained from Lineweaver-Burk plots (Palmer 1981). The coefficient of determination value of the linear regressions was determined using Sigma Plot software (Jandel Scientific).

Crystallization and data collection

Diffraction-quality crystals of MeHNL-S80A were grown by the vapor diffusion hanging drop method under the conditions described for the wild-type protein (Lauble et al. 1999), but using sodium citrate buffer at pH 4.8 instead of pH 5.4 to extend the stability of the soaking component acetone cyanohydrin. Crystals of the MeHNL-S80A mutant belong, like the wild-type enzyme, to the tetragonal space group $P4_12_12$ (Table 1), with two molecules per asymmetric unit. For data collection, a single crystal was transferred into a stabilization buffer containing 100 mM sodium citrate (pH 4.8), 5% PEG 8000, 28% MPD and flash-cooled at 100 K. MeHNL-S80A-acetone cyanohydrin complex crystals (Table 1) were prepared by soaking a MeHNL-S80A crystal ($0.5 \times 0.5 \times 0.8$

mm) in a stabilization buffer containing 100 mM sodium citrate (pH 4.8), 5% PEG 8000, 28% MPD and a stepwise increasing acetone cyanohydrin concentration of 10 mM, 50 mM, 200 mM, 500 mM, and 1.0 M, respectively. To avoid problems due to the spontaneous decomposition of acetone cyanohydrin, the maximum soaking time for the crystal was 90 sec in each freshly prepared solution. The soaked crystal was flash-cooled at 100 K. Diffraction data were collected at 100 K on a 30-cm MAR research image plate using the synchrotron radiation at station PX11 ($\lambda = 0.902$ Å) at EMBL outstation, Hamburg, Germany. Exposure was 60 sec for 1° oscillation, and a total of 60° of data were collected. The crystal-to-detector distance was 200 mm, and the slits were set at 0.2 mm. The MeHNL-S80A mutant crystal and the MeHNL-S80A-acetone cyanohydrin complex crystal diffracted to 2.1 Å and 2.2 Å resolution, respectively. Raw data images were indexed using the program DENZO (Otwinowski 1993), and all data were scaled and merged using SCALEPACK (Otwinowski 1993). All intensities were truncated to amplitudes using programs from the CCP4 suite (Collaborative Computational Project, Number 4 1994).

Refinement

The starting model for the refinement of the MeHNL-S80A structure was the 2.2-Å resolution structure of the MeHNL-acetone complex (Lauble et al. 2001; PDB entry 1DWO). 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 Ser80 residue was substituted by alanine for molecule A and B in the asymmetric unit, and all water and ligands were removed from the structure. Rigid-body refinement was performed to allow rearrangement of molecules A and B in the asymmetric unit against each other. Subsequent cycles of positional refinement led to $R = 23.8\%$ and $R_{free} = 29.3\%$ for all data in the resolution range 8–2.5 Å. $2|F_o| - |F_c|$ and $|F_o| - |F_c|$ electron density maps were calculated and the protein model analyzed carefully. The engineered S80A mutation was clearly defined, and water molecules were added using the MeHNL-acetone complex structure as a guideline. Further refinement was continued using individual B-factors. The water molecules were checked in difference Fourier electron density maps, and only water molecules with B-factors better than 60 Å² were retained. This model was subjected to further rounds of positional, simulated annealing ($T = 3000$ K) and B-factor refinement including all data in the resolution range 8–2.2 Å. The final model contains 426 water molecules and refinement converged to $R = 18.0\%$ and $R_{free} = 21.9\%$ (Table 1). The geometric parameters of the model have been analyzed with PROCHECK (Laskowski et al. 1993) and are within the expected deviation (Table 1). A Ramachandran plot shows that the residues Ala80 and Arg129 of each subunit fell outside of the energetically favorable regions. Ala80 is located in the active site and shown clearly in the electron density map, whereas Arg129 deviates from ideal values because of strain from a strong salt bridge to Glu156. The coordinates have been deposited with the Protein Data Bank (PDB entry 1E89).

Initial refinement of the MeHNL-S80A-acetone cyanohydrin complex structure used the MeHNL-S80A substrate-free enzyme as a starting model. Five of the water molecules, located in the active sites of MeHNL-S80A, were removed before refinement to provide independent unbiased electron density for this region. Sub-

sequent cycles of refinement, following the strategy described above for the substrate-free enzyme, for all data between 8 Å and 2.2 Å led to $R = 19.3\%$ and $R_{\text{free}} = 24.0\%$. $2F_o - F_c$ difference Fourier electron density map was calculated and showed continuous electron density in the active sites of molecules A and B, respectively. These densities had a tetrahedral shape with one axis extended and were therefore modeled as acetone cyanohydrin. The map also showed two prominent peaks of positive density next to residues Thr137 and Gln49 of molecule A, which clearly could also be modeled as acetone cyanohydrin. The hydroxyl group of the latter was placed into the density in a way to perform the most favored hydrogen-bond contacts. For acetone cyanohydrin refinement the structure of the substrate was defined with bond lengths and angles from its INSIGHT II/DISCOVER (BIOSYM/Molecular Simulations Inc., Release 95.0, 1995) structure. The protein structure along with the water molecules and the four acetone cyanohydrins was refined against data between 8 and 2.2 Å. $2F_o - F_c$ maps were calculated and confirmed the fit of these models to the density. The refinement converged to $R = 18.7\%$ and $R_{\text{free}} = 23.8\%$ for all 50,187 reflections in the resolution range 8–2.2 Å. The Ramachandran plot of the MeHNL–S80A–acetone cyanohydrin complex is basically identical to that of MeHNL–S80A, showing two outliers in each subunit, namely Ala80 and Arg129, respectively (Table 1). The final coordinates have been deposited with the Protein Data Bank (PDB entry 1E8D).

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References

- Bradbury, J.H. and Carver, J.A. 1984. Conformational differences between various myoglobin ligated states as monitored by ^1H NMR spectroscopy. *Biochemistry* **23**: 4905–4913.
- Brünger, A.T. 1992a. Free R value: A novel statistical quantity for assessing the accuracy of crystal structures. *Nature* **355**: 472–475.
- . 1992b. *X-PLOR. Version 3.1. A system for X-ray crystallography and NMR*. Yale University, New Haven, CT.
- Carver, J.A. and Bradbury, J.H. 1984. Assignment of ^1H NMR resonances of histidine and other aromatic residues in met-, cyano-, oxy-, and (carbon monoxy)myoglobins. *Biochemistry* **23**: 4890–4905.
- Collaborative Computational Project, Number 4. 1994. The CCP4 suite: Programs for protein crystallography. *Acta Cryst.* **D50**: 760–763.
- Effenberger, F. 1999. Enzyme-catalyzed preparation and synthetic applications of optically active cyanohydrins. *Chimia* **53**: 3–10.
- Förster, S., Roos, J., Effenberger, F., Wajant, H., and Sprauer, A. 1996. The first recombinant hydroxynitrile lyase and its application in the synthesis of (S)-cyanohydrins. *Angew. Chem. Int. Ed. Engl.* **35**: 437–439.
- Griengl, H., Hickel, A., Johnson, D.V., Kratky, C., Schmidt, M., and Schwab, H. 1997. Enzymatic cleavage and formation of cyanohydrins: A reaction of biological and synthetic relevance. *Chem. Commun.* 1933–1940.
- Hughes, J., Carvalho, F.J.P., and Hughes, M.A. 1994. Purification, characterization, and cloning of α -hydroxynitrile lyase from cassava (*Manihot esculenta* Crantz). *Arch. Biochem. Biophys.* **311**: 469–502.
- Jones, T.A. 1978. A graphics model building and refinement system for macromolecules. *J. Appl. Crystallogr.* **11**: 268–272.
- Laskowski, R.A., MacArthur, M.W., Moss, D.S., and Thornton, J.M. 1993. PROCHECK: A program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* **26**: 282–291.
- Lauble, H., Decanniere, K., Wajant, H., Förster, S., and Effenberger, F. 1999. Crystallization and preliminary X-ray diffraction analysis of hydroxynitrile lyase from cassava (*Manihot esculenta*). *Acta Cryst.* **D55**: 904–906.
- Lauble, H., Förster, S., Miehl, B., Wajant, H., and Effenberger, F. 2001. Crystal structure of hydroxynitrile lyase from *Manihot esculenta* in complex with substrates acetone and chloroacetone: implications for the mechanism of cyanogenesis. *Acta Cryst.* **D57**: 194–200.
- Otwinowski, Z. 1993. *DENZO/SCALEPACK: An oscillation data processing program for macromolecular crystallography*. Yale University, New Haven, CT.
- Palmer, T. 1981. *Understanding enzymes*. Wiley, New York.
- Sanger, F., Nicklen, S., and Coulson, A.R. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**: 5463–5467.
- Schmidt, M., Hervé, S., Klempier, N., and Griengl, H. 1996. Preparation of optically active cyanohydrins using the (S)-hydroxynitrile lyase from *Hevea brasiliensis*. *Tetrahedron* **52**: 7833–7840.
- Seigler, D.S. 1991. Cyanide and cyanogenic glycosides. In *Herbivores: Their interactions with secondary plant metabolites*. (eds. G.A. Rosenthal and M.R. Berenbaum), pp. 35–77. Academic Press, New York.
- Selmar, D., Carvalho, F.J.P., and Conn, E.E. 1987. A colorimetric assay for α -hydroxynitrile lyase. *Anal. Biochem.* **166**: 208–211.
- Wagner, U.G., Hasslacher, M., Griengl, H., Schwab, H., and Kratky, C. 1996. Mechanism of cyanogenesis: The crystal structure of hydroxynitrile lyase from *Hevea brasiliensis*. *Structure* **4**: 811–822.
- Wajant, H. and Effenberger, F. 1996. Hydroxynitrile lyases of higher plants. *Biol. Chem.* **377**: 611–617.
- Wajant, H. and Pfizenmaier, K. 1996. Identification of potential active-site residues in the hydroxynitrile lyase from *Manihot esculenta* by site-directed mutagenesis. *J. Biol. Chem.* **271**: 25830–25834.
- Zuegg, J., Gruber, K., Gugganig, M., Wagner, U.G., and Kratky, C. 1999. Three-dimensional structures of enzyme-substrate complexes of the hydroxynitrile lyase from *Hevea brasiliensis*. *Protein Sci.* **8**: 1990–2000.