Molecular Cloning of Acetone Cyanohydrin Lyase from Flax
(Linum usitatissimum)

DEFINITION OF A NOVEL CLASS OF HYDROXYNITRILE LYASES*

(Received for publication, September 6, 1996, and in revised form, November 13, 1996)

Klaus Trummler and Harald Wajant‡
From the Institute of Cell Biology and Immunology, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany

Acetone cyanohydrin lyase from Linum usitatissimum is a hydroxynitrile lyase (HNL) which is involved in the catabolism of cyanogenic glycosides in young seedlings of flax. We have isolated a full-length cDNA clone encoding L. usitatissimum HNL (LuHNL) from a cDNA expression library by immunoscreening. LuHNL cDNA was expressed in Escherichia coli and isolated from the respective soluble fraction in an active form which was biochemically indistinguishable from the natural enzyme. An open reading frame of 1266 base pairs encodes for a protein of 45,780 kDa. The derived amino acid sequence shows no overall homologies to the to date cloned HNLs, but has significant similarities to members of the alcohol dehydrogenase (ADH) family of enzymes. In particular, the cysteine and histidine residues responsible for coordination of an active site Zn²⁺ and a second structurally important Zn²⁺ in alcohol dehydrogenases are conserved. Nevertheless, we found neither alcohol dehydrogenase activity in LuHNL nor HNL activity in ADH. Moreover, well known inhibitors of ADHs, which interfere with the coordination of the active site Zn²⁺, fail to affect HNL activity of LuHNL, suggesting principally different mechanisms of cyanohydration and alcohol oxidation. Interestingly, LuHNL like ADH and Prunus serotina (PsHNL) possesses an ADP-binding βαβ unit motif, pointing to the possibility that the non-flavoprotein PsHNL and the flavoprotein LuHNL have developed from two independent lines of evolution of a common ancestor with an ADP-binding βαβ unit.

Hydroxynitrile lyases (HNLs) catalyze the decomposition of cyanohydrins (α-hydroxynitriles) into the corresponding aldehyde or ketone and cyanide (1). All HNLs described so far are found in cyanogenic plants. In these plants, HNLs are involved in the catabolization of cyanogenic glycosides during cyanogenesis or in the metabolization of these compounds during seedling development (1–4). In the presence of high concentrations of HCN and aldehydes or ketones, HNLs can be used as biocatalysts for the stereoselective synthesis of a wide array of cyanohydrins, important building blocks in the pharmaceutical and fine chemical industries (5).

In recent years the HNLs from Prunus serotina (PsHNL) (6), Sorghum bicolor (ShHNL) (7), Manihot esculenta (MeHNL) (8), and Hevea brasiliensis (HbHNL) (9) have been molecularly cloned. Analysis of the cDNA-derived amino acid sequences revealed that these enzymes belong to three different classes of HNL. While MeHNL and HbHNL share 74% identity (9), no sequence homologies can be found to PsHNL or ShHNL. The lack of sequence homologies between the cloned HNLs correlates with the fundamental differences between these enzymes with regard to molecular weight, subunit composition, glycosylation, FAD content, and substrate specificity.² The flavoprotein PsHNL has moderate homologies to various other flavoproteins, especially to various types of dehydrogenases and oxidases (6). In particular, a stretch of 27 amino acid residues near the N terminus of PsHNL fulfill Wierenga’s rule for an ADP-binding βαβ unit (6), while MeHNL and HbHNL show homologies to two proteins of unknown function from rice (9). However, the most intriguing homologies are found for ShHNL, namely, that this HNL possesses up to 50% homology over the whole sequence to serine carboxypeptidases, which belong to the structurally well investigated group of αβ hydrolase fold enzymes (5, 7). In particular, sites critical for function and structural integrity of serine carboxypeptidases are conserved, suggesting that ShHNL is also an αβ hydrolase fold enzyme. All αβ hydrolase fold enzymes have a “nucleophile-histidine-acid” catalytic triad found in common with the subtilisin and chymotrypsin class of serine proteases (10). In all these enzymes, the nucleophile is part of the consensus motif Gly-X-Ser/Cys-X-Gly/Ala-Gly/Ala (10). There is functional evidence by site-directed mutagenesis for the use of a catalytic triad by MeHNL and HbHNL, as well (9, 11). Moreover, the order of the catalytic triad residues in primary sequence suggests that these HNLs also belong to the αβ hydrolase fold group of enzymes despite having no sequence homologies to ShHNL (11).

Here we describe the molecular cloning of LuHNL, which, like MeHNL and HbHNL, has acetone cyanohydrin as its natural substrate. However, in contrast to these presumed αβ hydrolase fold enzymes, LuHNL was found to be structurally related to the alcohol dehydrogenase class of enzymes. In particular, amino acid residues of ADHs important for structural integrity or coordinating Zn²⁺ are conserved in LuHNL. However, despite having all the conserved residues responsible for Zn²⁺ binding, LuHNL neither exerts ADH activity nor is inhibited by reagents interfering with Zn²⁺ coordination in liver ADH.

EXPERIMENTAL PROCEDURES

Plant Material— Seeds of Linum usitatissimum L. were obtained from Frank AG (Herrenberg, Germany). Seeds were germinated for...
The cotyledons of the seedling were cut and stored at 2°C or used immediately for enzyme purification and RNA isolation. Chemicals—Except where noted, chemicals were purchased from Sigma (Deisenhofen, Germany). Chromatography resins, AutoRead sequencing kit, and cDNA synthesis kit were obtained from Pharmacia LKB Biotechnology Inc. (Freiburg, Germany). Lambda ZAP DNA was from Stratagene, and the bicinchoninic acid protein assay kit was from Pierce.

Enzyme Assays—The activity of LuHNL was measured as described by Selmar et al. (12). The amount of HNL which decomposes 1 mmol of acetone cyanohydrin in 1 min under the conditions described in Selmar et al. (12) is defined as 1 unit. Total protein for calculation of specific activities was determined with the bicinchoninic acid protein assay reagent from Pierce according to the manufacturer’s recommendations. Enzyme assays for liver alcohol dehydrogenase (Sigma) were essentially performed as described by Dunn and Bernhard (13). For inhibition studies, enzymes were incubated in the appropriate reaction buffer containing the respective reagent for 30 min at room temperature. The remaining activity was then determined as described above.

Purification of LuHNL and Immunization—Purification of LuHNL from flax seedlings and the immunization procedure were performed as described previously (14). Purification of recombinant LuHNL was performed as follows. E. coli cells expressing LuHNL were harvested by centrifugation, resuspended in binding buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl), and disrupted by sonication on ice. The supernatant was cleared by centrifugation and applied on a 1-ml Ni-nitrilotriacetic acid Superflow column (Quiagen, Minden, Germany). After washing with 10 ml of binding buffer, bound proteins were eluted with a 30-ml gradient of 0–0.5 M imidazole in binding buffer.

FIG. 1. Nucleotide and derived amino acid sequence of Lu-HNL. The stop codon determining the end of the reading frame is marked with an asterisk (*). Two putative polyadenylation signals are underlined (lower). Amino acid sequences of LuHNL peptides published by Albrecht et al. (22) are printed in boldface italics. An ADP-binding beta unit motif is underlined (lower).

FIG. 2. Homologies of LuHNL to other proteins. A, homologies revealed by computer alignment of deduced LuHNL amino acid sequence with amino acid sequences of alcohol dehydrogenase from Solanum tuberosum (StADH), Caenorhabditis elegans (CeADH), and Mus musculus (MmADH) are indicated by asterisks (*). Homologies solely among the ADHs are indicated by dots (•). Residues important for coordinating Zn2+ or maintaining overall structure in ADHs are indicated by arrows. B, sequence alignment of ADP-binding beta units in LuHNL, PsHNL, and horse liver ADH. Within the consensus fingerprint sequence the asterisk (*) indicates basic or hydrophilic residues (K, R, H, S, T, Q, N); ● represents small hydrophobic residues (A, I, L, V, M, C), and f represents the acidic residues D and E. The glycine residues within the ADP-binding fold are highly conserved.
Experimental Procedures.

were also determined. Assays were performed as described under “Experimental Procedures.” Three positive clones, containing the lacZ part were identified. The open reading frame encodes for purified LuHNL by SDS-PAGE (21). In particular, clones were further characterized.

DNA Sequencing and Analysis—Sequencing of double-stranded DNA templates was achieved by a modified chain-termination method (17) using T7 DNA polymerase and the ALF express DNA analysis system (Pharmacia). For priming, T3 and T7 primer or specific oligonucleotides based on the preceding sequences were used. Obtained DNA and amino acid sequences were compiled and analyzed using the HUSAR (Heidelberg UNIX Sequence Resources) software.

SDS-PAGE and Immunoblotting—Proteins were separated on 15% (w/v) polyacrylamide gels according to Laemmli (18) and either silver stained according to Blum et al. (19) or transferred to nitrocellulose as described by Towbin et al. (20). The immunoblots were further handled as described elsewhere (14).

RESULTS AND DISCUSSION

Cloning and Nucleotide Sequence of HNL from L. usitatissimum—A size selected (800–2500 base pairs) cDNA expression library in lambda ZAP comprising 5 × 10^6 plaque-forming units per packing reaction (85% recombinants) was used without further amplification for screening with anti-LuHNL antisera. About 100,000 plaques were screened as described under “Experimental Procedures.” Three positive clones, containing inserts of 1.3, 1.4, and 1.5 kilobase pairs in length were identified, converted into plasmid and used for further analysis. Both strands of the cDNAs were sequenced, and a common open reading frame of 422 amino acid residues downstream of the lacZ part was identified. The open reading frame encodes for a protein with a predicted molecular mass of 45,780 which is in good accordance with the molecular mass of 42,000 estimated for purified LuHNL by SDS-PAGE (21). In particular, the open reading frame contained sequences near the start methionine corresponding to the N-terminal sequence of LuHNL determined by Albrecht et al. (22) by Edman degradation. Two potential polyadenylation signals (AATAAA) occur at 1429 and 1507. The nucleotide sequence and the predicted amino acid sequence are shown in Fig. 1.

Protein Sequence Analysis—Searching for homologies using HUSAR and the TFASTA algorithms revealed that LuHNL shares up to 40% homology over the whole molecule with members of the zinc-containing alcohol dehydrogenase family of enzymes (Fig. 2). Of great interest is the fact that there are no significant homologies of LuHNL to the other HNLs cloned so far, remarkable residues which are structurally or functionally important in alcohol dehydrogenases are conserved (Fig. 2). Like LuHNL, alcohol dehydrogenases consist of two identical subunits (23). For ADHs it has been shown by resolution of the three-dimensional structure that each subunit is divided into two domains separated by the deep active site cleft (23). In ADHs one of these domains binds the coenzyme (coenzyme binding domain). The other domain binds two zinc ions (catalytic domain), one of which is located in the active-site pocket, whereas the other one is located on a loop outside the catalytic center (23). All residues responsible for Zn²⁺ binding in ADHs are conserved in LuHNL (Fig. 2A). Three glycines (LuHNL: Gly²⁴, Gly²⁵, and Gly³⁶) of ADHs which are invariant because of lack of space for a side chain in hydrophobic cores are also conserved (Fig. 2A). What is more, analyzing the deduced LuHNL amino acid sequence according to Wierenga et al.’s rules (24) revealed a ADP-binding βαβ-unit motif comprising residues 219–248 (Fig. 2A). Based on analysis of several FAD- and NAD-binding proteins, Wierenga et al. (24) have defined 11 residues within a 29–31 amino acid motif, which are necessary to allow the sequence folding into an ADP-binding βαβ unit. The ADP-binding βαβ unit motif of LuHNL, compared in Fig. 2B with those of other ADP-binding proteins, matched exactly with the consensus sequence and is therefore very likely to prove to be folded as an ADP-binding βαβ unit (Fig. 2B). Nevertheless, LuHNL catalyzes no net oxidation or reduction, suggesting that this fold is rather of structural than of catalytic

### Table I

Comparison of enzymatic properties of LuHNL and liver ADH

|           | LuHNL | Liver ADH |
|-----------|-------|-----------|
| HNL activity (units/mg) | 40    | <0.1      |
| ADH activity (units/mg) | <0.1  | 20        |

Inhibition of enzyme activity (%) by:

- Phenanthroline
  - 1 mM: <5 91
  - 10 mM: <5 >95
  - 20 mM: <5 >95
- DFP, 10 mM: <5 93
- DFP, 10 mM: 30 <5

### Footnotes

a Acetone cyanohydrin (8.4 mM) as substrate.
b Alcohol (50 mM) as substrate; cofactor NAD⁺ (30 μM).

c 

Accetone Cyanohydrin Lyase from Flax (L. usitatissimum)

Expression of recombinant LuHNL in induced and noninduced E. coli transfected with a LuHNL expression plasmid are analyzed by SDS-PAGE on 15.5% acrylamide gels (A) and Western blotting using anti-LuHNL antisera (B). Purified His₆-tagged recombinant LuHNL and LuHNL isolated from flax seedlings were analyzed by SDS-PAGE (C). The kinetic parameters of both were determined for acetone cyanohydrin as substrate. Experimentally determined rate values (v, μmol/min/mg) for the dissociation of acetone cyanohydrin to acetone and HCN were plotted against initial substrate concentration (S, mM) (D). For calculation of Kᵣ, a Lineweaver-Burk plot of the data was used giving a value of 1.9 mM. The coefficient of determination value of the first order regression was 0.98 (inset).
importance. Taking into account the above mentioned conservation of structurally important residues between LuHNL and ADHs, we propose that the overall structure of LuHNL is quite similar to that of ADHs.

Inhibition Studies—Given the complete conservation of cysteine and histidine residues required for coordination of the active Zn$^{2+}$ in alcohol dehydrogenases, we proposed two questions. First, has LuHNL a side dehydrogenase activity or have ADHs a side HNL activity and second, are the above mentioned conserved residues functionally involved in LuHNL-catalyzed acetone cyanohydrin cleavage? As shown in Table I, we found no ADH activity with LuHNL in a standard ADH assay; likewise, we found no indication for hydroxynitrile lyase activity in a commercially available liver alcohol dehydrogenase preparation. As mentioned above, one of the two coordinated Zn$^{2+}$ of each ADH subunit is located in the active site and functionally involved in catalysis. Therefore both o-phenanthroline, which forms complexes with Zn$^{2+}$, and diethyl pyrocarbonate, which modifies histidine side chains, are potent inhibitors of ADH activity (23). Taking into account that the above mentioned residues responsible for Zn$^{2+}$ coordination are conserved between LuHNL and ADHs, we inquired into a putative involvement in LuHNL-catalyzed cyanohydrin cleavage of these residues. Surprisingly, we found no influence of these inhibitors on LuHNL activity even after extended preincubation and at concentrations 10 to 20 times higher than those used for significant inhibition of ADH activity (Table I). The competitive inhibitor o-phenanthroline forms a ADH-Zn$^{2+}$-o-phenanthroline complex with the active-site Zn$^{2+}$ of ADHs (25) leading to reversible inactivation of these enzymes. The lack of inhibition of HNL-activity by this agent therefore suggests that Zn$^{2+}$ is not directly involved in LuHNL-catalyzed cyanohydrin cleavage.

However, a structural role of Zn$^{2+}$ ions in LuHNL cannot be ruled out by these data. LuHNL was poorly inhibited by high concentrations (10 mM) of the serine-modifying reagent diisopropyl fluorophosphate (Table I), whereas HNLs having a catalytic triad were almost completely inhibited by this compound in the low millimolar range (7, 11). Hence, involvement of a serine residue in LuHNL catalysis is rather unlikely.

Functional Expression of LuHNL—To investigate the biochemical properties of recombinant LuHNL, we cloned LuHNL cDNA in the inducible procaryotic expression vector pQE10 (Quiagen) and expressed the protein in E. coli (Fig. 9). Recombinant LuHNL was purified by affinity chromatography on a Ni-nitrilo triacetic acid Superflow column with a linear gradient from 0 to 0.5 M imidazole in binding buffer. The purification process yielded a pure enzyme preparation with a specific activity of 40 units/mg which is in accordance with the specific activity (34 units/mg) described for the natural enzyme. The recombinant enzyme exhibited a Michaelis-Menten kinetic with a $K_m$ for acetone cyanohydrin of 1.9 mM and $V_{max}$ of 71 μmol/min/mg (Fig. 3), which again matched well with the values published for the natural enzyme by Xu et al. (21) ($K_m$ = 2.5 mM). In contrast to other HNLs, the substrate specificity of LuHNL has not yet been studied in detail. Nevertheless, preliminary data of Albrecht et al. (22) suggested that LuHNL acts preferentially on aliphatic (R)-cyanohydrins.

Phylogeny of Hydroxynitrile Lyases of Higher Plants—Previously, HNLs were divided into two fundamental classes according to their FAD content. However, comparison of the amino acid sequence of various recently cloned HNLs (PsHNL, SbHNL, MeHNL, HbHNL) (6–9) revealed sequence homologies to other proteins, suggesting the existence of at least three phylogenetically independent groups of HNLs. One group formed by HbHNL and MeHNL, sharing 74% identity, exerts significant homologies to two proteins of as yet unknown function from rice (9). The other two groups are defined by SbHNL and PsHNL, respectively. While sequence analysis of the SbHNL revealed extensive homologies to serine carboxypeptidases, which belong to the structurally well investigated group of α/β hydrolase fold enzymes (5, 7), the flavoprotein PsHNL shows moderate homologies to various flavoproteins, especially to dehydrogenases and oxidases. Remarkably, we found no sequence homologies of LuHNL with the HNLs from cassava (MeHNL) and rubber tree (HbHNL), despite a common natural substrate (acetone cyanohydrin). This lack of sequence homology is consistent with the discrepancy in biochemical properties of these enzymes (Table II). Therefore, we propose here that LuHNL defines a fourth group of HNLs. LuHNL, like PsHNL, has an ADP-binding 80 β unit motif matching strikingly with the conserved sequence defined by Wierenga et al. (24) for this fold. Otherwise, there are no overall sequence similarities between these two HNLs, suggesting that they have evolved independently from two different lines of evolution from an ancestral...
ADP-binding $\beta\alpha\beta$ unit protein (Fig. 4). There is no indication of a phylogenetic relationship of LuHNL or PsHNL to the $\alpha\beta$ hydrolase fold enzymes. Taking into account the lack of similarity in biochemical properties of the, to date, noncloned HNLs from *Ximenia americana* (26) and *Phlebodium aureum* (5) and the above discussed HNLs, it is likely that additional, phylogenetically defined groups of HNLs exist (Fig. 4).

Acknowledgments—We gratefully acknowledge the technical assistance of Ute Emerich, Dr. Heiner Böttinger for immunization of mice, and Prof. Klaus Pfizenmaier for helpful discussion of the manuscript. We also thank James Parker for reading and correcting the manuscript.

REFERENCES

1. Conn, E. E. (1981) in *The Biochemistry of Plants* (Stumpf, P. K., and Conn, E. E., eds) Vol. 7, pp. 479–500, Academic Press, New York
2. Selmar, D., Lieberei, R., and Biehl, B. (1988) *Plant Physiol.* 86, 711–716
3. Lieberei, R., Selmar, D., and Biehl, B. (1985) *Plant Syst. Evol.* 150, 49–63
4. Swain, E., and Poulton, J. E. (1994) *Plant Physiol.* 106, 437–445
5. Wajant, H., and Effenberger, F. (1996) *Biol. Chem.* 377, 611–617
6. Cheng, I.-P., and Poulton, J. E. (1993) *Plant Cell Physiol.* 34, 1139–1143
7. Wajant, H., Mundry, K.-W., and Pfizenmaier, K. (1994) *Plant Mol. Biol.* 26, 735–746
8. Hughes, J., Carvalho, F. J. P. de C., and Hughes, M. A. (1994) *Arch. Biochem. Biophys.* 311, 496–502
9. Hasslacher, M., Schall, M., Hayn, M., Griendl, H., Kohlwein, S. D., and Schwab, H. (1996) *J. Biol. Chem.* 271, 5884–5891
10. Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remmington, S., Silmann, I., Schrag, J., Sussman, J. L., Vescheuren, K. H. G., and Goldmann, A. (1992) *Protein Eng.* 5, 197–211
11. Wajant, H., and Pfizenmaier, K. (1996) *J. Biol. Chem.* 271, 25630–25634
12. Selmar, D., Carvalho, F. J. P., and Conn, E. E. (1987) *Anal. Biochem.* 166, 208–211
13. Dunn, M. F., and Bernhard, S. A. (1971) *Biochemistry* 10, 4569–4575
14. Wajant, H., Riedel, D., Benz, S., and Mundry, K.-W. (1994) *Plant Sci.* 103, 145–154
15. Logeman, J., Schell, J., and Willmitzer, L. (1987) *Anal. Biochem.* 163, 16–20
16. Young, R. A., and Davis, R. W. (1983) *Proc. Natl. Acad. Sci. U.S.A.* 80, 1194–1198
17. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463–5467
18. Laemmli, U. K. (1970) *Nature* 227, 680–685
19. Blum, H., Beier, H., and Gross, H. J. (1987) *Electrophoresis* 8, 93–99
20. Towbin, H., Staehlin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76, 4350–4354
21. Xu, L. L., Singh, B. K., and Conn, E. E. (1988) *Arch. Biochem. Biophys.* 263, 256–263
22. Albrecht, J., Jansen, I., and Kula, M.-R. (1993) *Biotechnol. Appl. Biochem.* 17, 191–203
23. Brandén, C.-I., Jorvall, M., Eklund, M., and Puragren, B. (1975) in *The Enzymes* (Boyer, P., ed) Vol. XI, pp 103–190, Academic Press, New York
24. Wierenga, R. K., Terpstra, P., and Hol, W. G. J. (1986) *J. Mol. Biol.* 187, 101–107
25. Drumm, D. E., and Vallee, B. L. (1970) *Biochimistry* 9, 4078–4086
26. Kuroki, G. W., and Conn, E. E. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86, 6978–6981
27. Wajant, H., Förster, S., Selmar, D., Effenberger, F., and Pfizenmaier, K. (1995) *Plant Physiol.* 109, 1231–1238