Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 4260–4264, May 1991
Biochemistry

Isolation and properties of a nitrile hydratase from the soil fungus *Myrothecium verrucaria* that is highly specific for the fertilizer cyanamide and cloning of its gene

(cyanamide hydratase/gene expression)

URSULA H. MAIER-GREINER*, BRIGITTE M. M. OBERMAIER-SKROBRANEK*, LYDIA M. ESTERMAIER*, WERNER KAMMERLOHER*, CHRISTIAN FREUND*, CHRISTOPH WÜLFING*, ULRIKE I. BURKERT*, DAGMAR H. MATERN*, MICHAEL BREUER†, MANFRED EULITZ†, Ö. IRFAN KÜFREVIOGLU*, AND GUIDO R. HARTMANN*‡

*Institut für Biochemie, Ludwig-Maximilians-Universität, D-8000 Munich 2, Federal Republic of Germany; and †Institut für Klinische Molekularbiologie, Gesellschaft für Strahlen- und Umweltforschung mbH, D-8000 Munich 70, Federal Republic of Germany

Communicated by Eric E. Conn, February 5, 1991 (received for review December 14, 1990)

ABSTRACT **A protein was purified from crude extracts of the soil fungus *Myrothecium verrucaria* by gel filtration and hydrophobic chromatography to homogeneity; this protein catalyzed the stoichiometric hydration of the fertilizer cyanamide to urea with high substrate specificity. This cyanamide hydratase (urea hydro-lyase; EC 4.2.1.69) contained zinc and consisted of six identical subunits with M_r = 27,700. It was partially sequenced. The protein was detectable only when the fungus was grown on cyanamide as the sole nitrogen source. Genomic DNA from the fungus was cloned, and the gene encoding the enzyme was mapped with an oligonucleotide probe derived from the amino acid sequence within a 25,800-base-pair DNA region. The subunit of the enzyme is encoded by a 795-base-pair DNA sequence containing a 63-base-pair intron. A cDNA clone containing the intronless gene with an open reading frame encoding a sequence of 244 amino acids expressed the enzyme in active form in *Escherichia coli* with excellent yield.**

Cyanamide ($H_2N—C\equiv N$) in aqueous solution or in the form of its calcium salt is used as a fertilizer in agriculture. It provides ammonia to the soil by its metabolic conversion. It has the additional advantage of acting as an effective herbicide. Therefore it has to be applied prior to sowing. In view of the present interest in the ecological effects of widely used chemicals, we became interested in the metabolic conversion of this not naturally occurring compound. Chemically, cyanamide belongs to the class of nitriles. In spite of the relatively rare occurrence in nature of compounds containing the nitrile group, enzymes that hydrate this group to the corresponding amide (nitrile hydratases) have been frequently found in various bacteria (1–5) and also in plants (6). In 1973 Stransky and Amberger (7) described the occurrence of an inducible enzymatic activity in extracts of the soil fungus *Myrothecium verrucaria*, which hydrates the nitrile group of cyanamide with formation of urea (Eq. 1)

$$H_2N—C\equiv N + HOH \rightarrow H_2N—CO—NH_2. \quad [1]$$

In this communication we describe the purification to homogeneity and the characterization of this cyanamide hydratase (urea hydro-lyase; EC 4.2.1.69). The amino acid sequence of the enzyme was partially determined and compared with the nucleotide sequence of the cyanamide hydratase gene locus *cah*.§ After modification, the eukaryotic gene could be expressed with good efficiency in *Escherichia coli*.

EXPERIMENTAL PROCEDURES

Growth of Fungi. *M. verrucaria* (DSM no. 2087) was obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, F.R.G.) and grown as described (7) but scaled up. From 4 liters of medium, 10–25 g (fresh weight) of mycelium was harvested after 10 days. The rapidly filtered mycelium was stored at −70°C.

Cyanamide Hydratase Assay. The assay is based on the decrease in cyanamide concentration during incubation with the enzyme (7). The enzyme (0.05–0.2 units) was incubated with 20 mM cyanamide in 5 mM sodium phosphate buffer (pH 7.7) (total volume = 0.25 ml) at 37°C for 15–60 min, depending on the activity of the enzyme. The cyanamide concentration was determined by a colorometric assay at 530 nm in 20 μl of the incubation mixture before and after enzyme addition (8). One unit of cyanamide hydratase metabolizes 1 μmol of substrate per min under these conditions.

Purification of Cyanamide Hydratase. Twenty grams of the frozen mycelium, after addition of 10% (vol/vol) 5 mM phosphate buffer (pH 7.7), was rapidly thawed at 37°C and disrupted by sonication, under cooling, at intervals for a total of 3 min. The homogenate was cleared by centrifugation at 48,000 × *g* for 1 h. Seventeen milliliters of the supernatant was applied (8 ml/h) to a Sephadex G-200 column (3 × 60 cm) that had been equilibrated with 5 mM phosphate buffer (pH 7.7). Forty-five to 50 ml of the most active 1.3-ml fractions was combined. To 8 ml of this eluate [containing about 1.5 mg of protein (9)], ammonium sulfate (pH 7.7) was added to a final concentration of 1 M, and the solution was then applied (<6 ml/h) to a phenyl-Superose HR 5/5 column (Pharmacia LKB) equilibrated with 1 M ammonium sulfate in 5 mM phosphate (pH 7.7). The enzymatic activity was slowly eluted (≤0.3 ml/h) with a linear gradient of 0 to 1 M ammonium sulfate in 5 mM phosphate buffer (pH 7.7) at room temperature. Cyanamide hydratase appeared as a single sharp peak at 90 mM ammonium sulfate. The molar concentration of the homogeneous enzyme can be determined by its absorption at 280 nm assuming a molar absorption coefficient of $3.3 \times 10^5\ M^{-1} \cdot cm^{-1}$, which is calculated (10) from the molar content of tryptophan, tyrosine, and cysteine in the

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

‡To whom reprint requests should be addressed at: Institut für Biochemie, Ludwig-Maximilians-Universität, Karlstrasse 23, D-8000 Munich 2, F.R.G.

§The sequence reported in this paper has been deposited in GenBank data base (accession no. M59078).

enzyme. The highly purified enzyme can be precipitated with 1.3–2.5 M ammonium sulfate and stored at 4°C or in liquid nitrogen (but not at −20°C) in 5 mM phosphate buffer (pH 7.7) for at least 2 months without significant loss of activity.

Preparation of Antibodies. Polyclonal antibodies were prepared from rabbits according to standard techniques. The antibodies were further purified by adsorption to a blot of electrophoretically separated cyanamide hydratase and subsequent desorption (11).

Carbohydrate Determination. An 84-μg sample of highly purified cyanamide hydratase was used in a colorimetric assay for glycoproteins (12). Fifty micrograms of β-D-fructofuranosidase (invertase) served as a positive control.

Substrate Specificity. The incubation was carried out as described for the determination of cyanamide hydratase activity but with higher enzyme concentrations (40–70 enzyme units/ml) and longer incubation times (300–1500 min). The substrate concentration was 20 mM (except for the acetonitrile concentration, which was 50 mM). The disappearance of the various substrates was determined either directly or indirectly by the formation of their hydration products according to the references cited: cyanide [formation of formamide (13)], formamide (13), cyanate [formation of ammonia (14)], dicyandiamide (15), creatine (16), acetonitrile (formation of acetamide measured by the increase in absorption at 230 nm), cyanourea [determined with the assay for urea (17), the end-product biuret does not interfere], urea (17), formylcyanamide and acetylcyanamide (formation of the corresponding substituted urea, measured by the decrease of the absorption at 250 nm).

Amino Acid Sequence Analysis. Three hundred micrograms of protein was dissolved in 0.5 ml of 85 mM *N*-methylmorpholine/F_3CCOOH, pH 8.0. Endoproteinase Lys-C (Boehringer Mannheim) was added at an enzyme-to-substrate ratio of 1:40 and incubated for 1 h at 37°C. The mixture of peptides was then lyophilized. Separation and amino acid sequence analysis were performed as described by Eulitz *et al.* (18), with the exception that for separation of the peptides a Vydac reversed-phase C_{18} column (Muder & Wochele, Berlin) was used at a flow rate of 1.3 ml/min.

Isolation of DNA. For the preparation of high molecular weight DNA from *M. verrucaria*, freshly grown and lyophilized mycelium was mixed 1:1 with alumina (Alcoa 305; Serva) and ground with a pestle in a mortar to break up the cells. DNA was then extracted as described (19).

Recombinant DNA Techniques. Standard DNA manipulations were carried out as described (20, 21). For the construction of a genomic library, high molecular weight DNA from *M. verrucaria* was partially digested with the restriction enzyme *Sau*3A, and fragments of 9 to 23 kilobase pairs were separated in a 5–25% NaCl gradient (22). The fragments were cloned in phage vector λEMBL3 (Promega) and packaged using Packagene λ DNA packaging extracts (Promega). Recombinant phages were selected in *E. coli* NM 539 (Promega). Hybridization of plaques and bacterial colonies was carried out as described (23), with a 5′ end-labeled 17-nucleotide-long oligonucleotide mixture (see *Results*) or with a 1104-base-pair (bp) *Hpa* II fragment containing the cyanamide hydratase gene (see *Results*). The latter was labeled with the random-primed DNA labeling kit (Boehringer Mannheim). DNA fragments cloned in pUC19 were sequenced with the pUC sequencing kit (Boehringer Mannheim). Isolation of mRNA from *M. verrucaria* and cDNA synthesis was carried out with the appropriate kits from Pharmacia LKB.

RESULTS

Induction of Cyanamide Hydratase. When *M. verrucaria* is grown on 20 mM nitrate as the nitrogen source, concentrations of cyanamide as low as 0.2 mM exert an inhibitory effect on its growth. However, when the fungus is incubated for several days with 20 mM cyanamide as the sole nitrogen source, strong growth was observed. Only under these conditions is cyanamide hydratase activity detectable in the crude cell extract (7). Chemically related compounds that can also serve—at least after adaptation of the cells—as the sole nitrogen source, such as dicyandiamide, cyanourea, guanidinourea [H_2N—C(NH)—NH—CO—NH_2], urea, formamidine [HC(NH)—NH_2], or formamide (HCO—NH_2), do not induce cyanamide hydratase activity. KOCN cannot serve as a nitrogen source. These observations confirm and extend previous observations (7) on the high specificity of the induction process. To check for the presence of an inactive precursor of the enzyme in uninduced cells, crude cell extracts were fractionated by gel electrophoresis and tested with polyclonal antibodies against cyanamide hydratase for cross-reactivity in a Western blot. No reaction was observed, in contrast to controls with cell extracts from induced cells.

Purification of Cyanamide Hydratase. The enzyme was purified from the extract to homogeneity in only two steps as described in *Experimental Procedures* (Table 1). Electrophoretic analysis in a denaturing polyacrylamide gel showed a single protein band and a single spot when analyzed in two dimensions (24). There is no evidence for the presence of carbohydrate chains in the protein (determined as in ref. 12). Analysis by atomic absorption spectroscopy clearly demonstrates a zinc content of 0.15 ± 0.02 μg/ml at an enzyme concentration of 0.6 μM, which corresponds to a ratio of 3.9 ± 0.9 mol of zinc per mol of the hexameric enzyme. Mg, K, Ca, Cr, Mn, Fe, Co, Ni, Cu, and Cd were not present above background (≤3.3 ng/ml). Purification of the enzyme was about 30-fold with an overall yield of ≈20%. The specific activity of the homogeneous enzyme suggests that cyanamide hydratase accounts for ≈3% of the total soluble protein in induced *M. verrucaria*.

Molecular Weight of Cyanamide Hydratase. The molecular size of the active enzyme was determined by gel filtration on a Sephadex G-200 column. The activity eluted as a single peak between catalase (M_r = 240,000) and aldolase (M_r = 158,000), at a position corresponding to a molecular weight of ≈170,000. During electrophoresis in a denaturing polyacrylamide gel, the homogeneous enzyme migrated with an apparent molecular weight of 27,700 ± 2000. These findings suggest that the active enzyme consists of six identical subunits.

Enzyme Kinetics and Substrate Specificity. The activity of the purified enzyme was greatest at pH 7.7 and 55°C, as reported for the activity in the crude preparation (7). Cyanamide is stoichiometrically converted to urea as shown by the quantitative formation of urea and by the simultaneous equimolar disappearance of cyanamide. It exhibits simple single-order saturation with respect to cyanamide, with a K_m of 27 mM. The turnover number of the enzyme at a saturating concentration of cyanamide (200 mM) and at 37°C is ≈144/sec, with the assumption that all protein applied represents active enzyme.

Other compounds closely related chemically to cyanamide, such as formylcyanamide (H—CO—NH—CN), acetylcyana-

Table 1. Purification of cyanamide hydratase

Sample	Volume, ml	Protein, mg	Total units	Specific activity, units/mg of protein
Crude extract	8.8	48.40	184	3.8
Gel filtrate	8.0	1.44	82	56.9
Hydrophobic chromatography eluate	3.0	0.33	41	124

Table 2. Inhibitors of cyanamide hydratase activity

Compound added	Final concentration, mM	Activity, % control
None	—	100
$AgNO_3$*	0.1	26
p-Chloromercuribenzoate*	0.1	29
Iodoacetamide*	1	13
EDTA†	10	10
o-Phenanthroline†	2	16
Urea	20	85
Dicyandiamide	20	74
NaN_3	20	74
Hydroxylamine	20	51
Sodium thiocyanate	15	32
Potassium cyanate	10	15
Potassium cyanide	1	15

*The enzyme was preincubated with the compound for 10 min.
†Preincubation with the chelating agents was carried out for 15 min; the pH during both preincubation and incubation was pH 8.3.

mide (H_3C—CO—NH—CN), acetonitrile (H_3C—CN), cyanate (O—CN^-), cyanide (CN^-), dicyandiamide (H_2N—C(NH)—NH—CN), cyanourea (H_2N—CO—NH—CN), formamide (H_2N—CO—H), urea (H_2N—CO—NH_2), or creatine, were not hydrated to any detectable extent, even when the enzyme concentration was increased in the assay by a factor of at least 50 and the incubation time was simultaneously extended 20-fold or more (see *Experimental Procedures*). Obviously, the enzyme is extremely specific for the hydration of cyanamide.

Inhibitors. The highest activity of cyanamide hydratase was measured in the presence of anionic buffers and low ionic strength. Cationic buffers and higher ionic strength reduce the activity. Compounds that react with thiol groups, such as Ag^+, *p*-chloromercuribenzoate, and iodoacetamide (Table 2), show a strong inhibitory effect on cyanamide hydratase. This indicates that at least some of the four cysteine residues present in the enzyme (see below) may be essential for activity. The enzymatic activity is sensitive to chelating agents such as EDTA or *o*-phenanthroline, pointing to a functional role of zinc in the protein. Compounds that depress the activity of pyrroloquinoline quinone-dependent enzymes (4, 25), such as phenylhydrazine or semicarbazide, do not inhibit cyanamide hydratase. Reagents containing structural elements of cyanamide, such as urea, dicyandiamide, azide ($^-$N—N≡N), hydroxylamine (H_2N—OH), thiocyanate (NaS—CN), or cyanate reduce the activity (Table 2). The most potent inhibitor among them is cyanide, where 0.3 mM is sufficient to depress the reaction rate by half in the presence of 20 mM cyanamide. Malonate or bicarbonate, which strongly influence the activity of cyanase (26), were without effect on cyanamide hydratase.

Amino Acid Sequence Determination. A homogeneous preparation of cyanamide hydratase was digested with the endoproteinase Lys-C, and the resulting mixture of oligopeptides was separated by HPLC on a reversed-phase column (18). The stepwise degradation of the eluted peptides by an automatic gas-phase sequencer resulted in seven amino acid sequences comprising a total of 125 amino acids (Fig. 1). This is about half of the total amino acid sequence of the enzyme, as predicted from the molecular weight of the subunit.

1 CGGCTCTTATACGACATTGATTAGAAATGCAATTGACGCAAAATGGAAGTTATACGACAAGCGCAATAATGGATTGAGCACTTATCGAACACGTACGTCATAGTGAACAT 110

111 TGGCCACTGTTGTCTCTGGTCCCCTCAAACGTATAACCCTTATTGAGGACTTGGGTATATATACACGCACTATCCGTCATGTTTCACGAAAGGTCGACCTCATACTCCTA 220

221 CGTTTCTCAAACTAGTACGATCCTACTTCCTCGCTTATCTGCTCTAAACGATTCAACAAGATGTCTTCTTCAGAAGTCAAAGCCAACGGATGGACTGCCGTTCCAGTCAG 330
M S S S E V K *A N G W T A V P V S*

331 CGCAAAGGCCATTGTTGACTCCCTGGGAAAGCTTGGTGATGTCTCCTCATATTCTGTGGAAGATATCGCGTTCCCTGCGGCAGACAAACTTGTTGCCGAGGCACAGGCCT 440
A K A I V D S L G K L G D V S S Y S V E D I A F P A A D K L V A E A Q A

441 TTGTGAAGGCCCGATTGAGTCCCGAAACCTACAATCACTCCATGCGCGTTTTCTACTGGGGTAAGTCATGCCGTGCTGCGTGCCTGACATAATTCCAAATTCTCACAAAT 550
F V K A R L S P E T Y N H S M R V F Y W

551 ATTTTCCAACAAGGAACCGTCATCGCGAGACGTTTACTTCCCGAGCAAGCTAAAGACTTGTCTCCAAGTACATGGGCACTGACATGTCTTCTGCATGACGTTGGTACTGC 660
G T V I A R R L L P E Q A K D L S P S T W A L T C L L H D V G T A

661 GGAGGCATACTTTACATCTACACGAATGTCCTTCGATATTTACGGTGGCATTAAGGCTATGGAGGTGCTCAAGGTCCTTGGGAGTAGCACCGACCAGGCTGAGGCTGTTG 770
E A Y F T S T R M S F D I Y G G I K A M E V L K *V L G S S T D Q A E A V*

771 CCGAGGCCATCATTCGTCATGAGGATGTGGGGGTAGATGGCAACATCACATTCCTCGGTCAGTTGATCCAGCTGGCTACGCTTTATGACAATGTCGGGGCCTACGATGGG 880
A E A I I R H E D V G V D G N I T F L G Q L I Q L A T L Y D N V G A Y D G

881 ATTGATGATTTTGGTAGCTGGGTTGATGACACCACACGCAACAGTATCAACACGGCATTCCCACGACATGGTTGGTGTTCTTGGTTTGCCTGCACGGTTCGTAAGGAAGA 990
I D D F G S W V D D T T R N S I N T A F P R H G W C S W F A C T V R K E E

991 AAGTAACAAGCCTTGGTGCCACACAACGCATATCCCTCAGTTCGATAAACAGATGGAAGCGAACACTTTGATGAAGCCTTGGGAGTAACTCTGAGTAAGCAGAGAATATT 1100
S N K P W C H T T H I P Q F D K *Q M E A N T L M K* P W E

1101 TAGCCGGGTAGCTATAGATGAATCTGGACAAATTCAGGCACATTTGGTTTCACGATACAGGTATTGGAAATAGCTTGCAGGAAGGTATCATGTCAACACCATGTATTTTA 1210

1211 TTTTCCCTCGGTGATTTAAATGGGG 1235

FIG. 1. Sequence of the 1235-bp-long DNA region from *M. verrucaria*, containing the coding region for cyanamide hydratase. Codon-specified amino acids are displayed below the sequence. The sequences of the isolated and analyzed peptides [amino acid positions 8–19, 20–26, 28–46 (amino acid at position 35 was not identified), 47–56, 57–87, 131–165 (peptide could be sequenced only up to position 165), and 233–241] are shown in italics. The oligonucleotide complementary to the underlined DNA sequence (nucleotides 1040–1056) was used as probe for plaque hybridization.

Cloning of the Cyanamide Hydratase Gene. For the construction of a genomic library, high molecular weight DNA from *M. verrucaria* was prepared and partially digested with the restriction endonuclease *Sau*3A. The fragments were cloned into the *Bam*HI restriction site of the phage vector λEMBL3.

The mixed oligonucleotide probe

5′-GTR TTN GCY TCC ATY TG-3′,

where N = A, T, G, or C; R = G or A; and Y = T or C, was constructed from one of the sequenced peptides (Fig. 1) and used for plaque hybridization. From 38,000 recombinant phage clones, 6 independent clones were identified. The inserts of these phages were mapped by digestion with the restriction enzymes shown in Fig. 2. The six inserts comprised partially overlapping DNA regions within a totally mapped region of 25,800 base pairs (Fig. 2).

By subcloning one of the phage inserts into the plasmid pUC19, a strongly hybridizing 1104-bp-long *Hpa* II fragment (Fig. 1, nucleotides 1–1104) and a 366-bp-long *Hae* III fragment (Fig. 1, nucleotides 869–1235) were obtained and sequenced. The two fragments overlap in a region of 235 bp so that in all 1235 bp of *M. verrucaria* DNA were sequenced (Fig. 1).

Furthermore, a λgt10 cDNA gene bank was constructed from poly(A)-mRNA isolated from freshly grown *M. verrucaria* mycelium. It proved to be essential that the culture medium contain sufficient cyanamide (≈20 mM) up to the moment of harvest in order to obtain a preparation with sufficient mRNA specific for cyanamide hydratase. The 1104-bp *Hpa* II fragment from the genomic library was then used as a probe to isolate a cloned 900-bp fragment from the cDNA gene bank. It was recloned in the *Eco*RI site of pUC13 and also sequenced completely. Except for the absence of nucleotides 501–563, the sequence was identical to the corresponding genomic region from nucleotide 235 to nucleotide 1197 (Fig. 1).

Structure of the Cyanamide Hydratase Gene. The genomic *Hpa* II fragment contains a long reading frame beginning at nucleotide 236 after a stop codon and ending at nucleotide 1075, which is interrupted only by a stop codon at position 529. The latter is located within the region from nucleotide 501 to nucleotide 563, which is characterized by its absence in the cDNA clone. Furthermore it interrupts the coding region for the isolated oligopeptide Ala-57 → Lys-87 at Gly-74. For all other sequenced oligopeptides, continuous coding regions are found within the reading frame (Fig. 1). These characteristics suggest that the region from nucleotide 501 to nucleotide 563 is an intron in the structure of the cyanamide hydratase *cah* gene. The first AUG codon at the 5′ end after an in-frame stop codon occurs at position 281 (Fig. 1). The open reading frame ends at a stop codon at position 1075 and codes (with the omission of the intron sequence) for a protein of 244 amino acids [M_r (calc) = 26,969], in excellent agreement with the observed size of the cyanamide hydratase subunit.

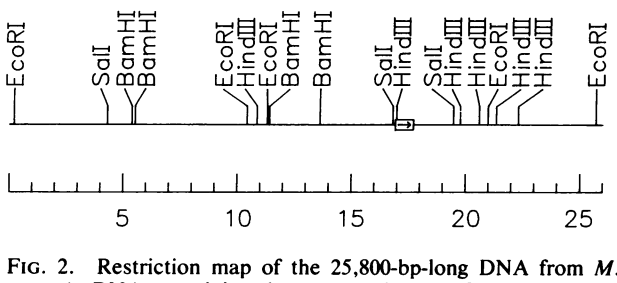

FIG. 2. Restriction map of the 25,800-bp-long DNA from *M. verrucaria* DNA containing the structural gene of cyanamide hydratase (open bar). The reading frame of the gene runs from left to right. The map was derived from six independent λ phage clones containing the following inserts [approximate position on the scale (in kilobase pairs) is given]: 1–18.0, 5.6–23.9, 6.0–19.1, 6.4–25.8, 7.2–19.3, and 9.3–23.9.

Expression of the Enzyme in *E. coli*. As the coding sequence of the *cah* gene was not in-frame with the *lacZ* gene in the pUC13 recombinant clone described above, it was necessary for expression to modify the sequence upstream of the presumed ATG start codon. This was achieved by using the polymerase chain reaction with the recombinant plasmid as template and two chemically synthesized oligonucleotides as primers. Oligonucleotide I consisted of 47 nucleotides with the sequence

ribosome binding site — stop — start

5′-GGCCGGCTGCAGTAAGGAGGAATAACATATGTCTTCTTCAGAAGTCA-3′.

Pst I — *Nde* I

It contained a *Pst* I recognition site, a ribosome binding site, and a TAA stop codon in-frame with the *lacZ* gene in pUC19 as well as a *Nde* I recognition site and 19 nucleotides of the cyanamide hydratase structural gene starting with the ATG start codon 9 nucleotides downstream of the ribosome binding site. In the design of this construct, base pair sequences that could form a strong hairpin secondary structure were carefully avoided. Such structures around the ribosome binding site may strongly reduce translation (27). Oligonucleotide II consisted of 30 nucleotides with the sequence

5′-GATATCTACTTAGACCTGGACGTCGGCCGG-3′,

Pst I

which contains 18 nucleotides of the gene (complementary to nucleotides 1112–1129 in Fig. 1) at a distance of 36 nucleotides downstream from the end of the reading frame at nucleotide 1075 and, in addition, a *Pst* I recognition site. The product of the polymerase chain reaction was ligated into the plasmid pUC19/PstI and transformed into *E. coli* DK1.

Extracts prepared by sonication of bacteria with the recombinant plasmid exhibited cyanamide hydratase activity that was highest several hours after cessation of cell division at 37°C. Upon longer growth, the enzymatic activity as well as the recombinant protein (as shown by the loss of cross-reaction with antibodies against the fungal enzyme) disappeared rapidly. As revealed by gel electrophoretic analysis, 90% of the cyanamide hydratase protein was found in the supernatant of the centrifuged cell extract. The enzymatic activity in the crude extract was stable at 4°C for at least 3 days. The specific enzymatic activity in crude extracts of recombinant *E. coli* was 15 times higher than in extracts from induced *Myrothecium*. The cyanamide hydratase could be purified to homogeneity by the same purification scheme as for the fungal extracts. The overall yield of purified recombinant protein from a 1-liter culture was 15 mg with about 3300 units of cyanamide hydratase activity. The relative molecular mass of the subunit and of the undissociated enzyme was identical with that of the protein from *Myroth-*

ecium. Similarly, the K_m for cyanamide and the dependence of the activity on pH, temperature, and ionic strength were the same.

DISCUSSION

The experiments reported clearly show that the degradation of the fertilizer cyanamide by the soil fungus *M. verrucaria* is due to action of a single soluble enzyme [M_r (calc) = 161,814] that converts cyanamide to urea. Cyanamide hydratase occurs in the fungus in combination with urease (C. Klaus, U.H.M.-G., and G.R.H., unpublished results). Thus the enzyme is able to degrade the highly toxic cyanamide to an essential nutrient. The extensive synthesis of cyanamide hydratase after adaptation is not the result of gene amplification (U.I.B. and G.R.H., unpublished results), but rather the consequence of an increase in transcription, since the specific transcript disappears immediately after the consumption of cyanamide. Induction of transcription in eukaryotic cells is considered to be regulated by proteins that are able to bind an inducer, such as steroids, with high specificity (28). Consequently, one would expect the existence of another protein in *Myrothecium* that specifically binds cyanamide. With the homogeneous enzyme, we could not confirm some properties reported in ref. 7 for the activity in the crude extract, such as stimulation by 1 mM Mn^{2+}, the K_m for cyanamide, or the range of substrates. Cyanamide hydratase shows an extremely narrow substrate specificity. Chemically related compounds do not act as substrates, but—if at all—as inhibitors. In contrast, many bacterial nitrile hydratases metabolize a rather broad spectrum of nitriles (29). Indeed, the occurrence of such a specific enzyme in *Myrothecium* as the result of a similarly specific induction is rather surprising in view of the fact that the chemically reactive cyanamide has never been found in nature and is only discussed as a constituent of the prebiotic environment (30). No similarity is found with the only other sequenced nitrile hydratase subunit (5). Therefore, cyanamide hydratase seems to be unrelated to the nitrile hydratase from *Rhodococcus* sp. N 774 (5). Cyanamide is toxic not only to uninduced *Myrothecium* (7) but also to plants, which is the basis for its herbicidal activity. The discovery of the high substrate specificity of cyanamide hydratase, the elucidation of its simple structure as a homohexameric polypeptide, and the isolation of its gene offer the interesting possibility of transforming it into plant cells with the hope of obtaining plants resistant to the herbicidal action of this fertilizer. Thus it would no longer be necessary to apply it before sowing. This could lead to a distinct reduction in the amount of cyanamide required for fertilizing without loss of its effectiveness as a nitrogen source.

We thank R. J. Youngman (SKW Trostberg) for a continuous supply of frozen *M. verrucaria*, A. Mertz and E.-L. Winnacker (Martinsried) for oligonucleotide synthesis, B. Adelmann-Grill and K. Kühn (Martinsried) for the preparation of antibodies, P. Heinrich (München) for many valuable suggestions and PCR experiments, H. Hartl and H.-P. Boehm for spectroscopic analysis of the enzyme by atomic absorption, and C. Jung, K. Merkel, and H. Sieber for competent technical assistance. O.I.K. was supported by a stipend of the Deutscher Akademischer Austauschdienst (Bad Godesberg), and U.H.M.-G. was supported by the Fonds der Chemischen Industrie (Frankfurt). We gratefully acknowledge the financial support of this work by SKW Trostberg.

1. Asano, Y., Fujishiro, K., Tani, Y. & Yamada, H. (1982) *Agric. Biol. Chem.* **46,** 1165–1174.
2. Nagasawa, T., Ryuno, K. & Yamada, H. (1986) *Biochem. Biophys. Res. Commun.* **139,** 1305–1312.
3. Nagasawa, T., Nanba, H., Ryuno, K., Takeuchi, K. & Yamada, H. (1987) *Eur. J. Biochem.* **162,** 691–698.
4. Nagasawa, T., Takeuchi, K. & Yamada, H. (1988) *Biochem. Biophys. Res. Commun.* **155,** 1008–1016.
5. Endo, T. & Watanabe, I. (1989) *FEBS Lett.* **243,** 61–64.
6. Castric, P. A., Farnden, K. J. F. & Conn, E. (1972) *Arch. Biochem. Biophys.* **152,** 62–69.
7. Stransky, H. & Amberger, A. (1973) *Z. Pflanzenphysiol.* **70,** 74–87.
8. Steller, W. A., Frederick, J. B. & Morgan, P. W. (1965) *J. Agric. Food Chem.* **13,** 329–330.
9. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
10. Gill, S. C. & von Hippel, P. H. (1989) *Anal. Biochem.* **182,** 319–326.
11. Smith, D. E. & Fisher, P. A. (1984) *J. Cell Biol.* **99,** 20–28.
12. Allen, G. (1989) in *Laboratory Techniques in Biochemistry and Molecular Biology*, eds. Burdon, R. H. & van Knippenberg, P. H. (Elsevier, Amsterdam), Vol. 9, p. 199.
13. Fry, W. & Miller, R. (1972) *Arch. Biochem. Biophys.* **151,** 468–474.
14. Anderson, P. M. (1980) *Biochemistry* **19,** 2882–2888.
15. Vilsmeier, K. (1982) *Z. Pflanzenernähr. Bodenkd.* **145,** 503–505.
16. Wahlefeld, A.-W. & Siedel, J. (1985) in *Methods of Enzymatic Analysis*, eds. Bergmeyer, H. U., Bergmeyer, J. & Grassl, M. (VCH, Weinheim, F.R.G.), Vol. 8, pp. 488–514.
17. Polacco, J. C. (1976) *Plant Physiol.* **58,** 350–357.
18. Eulitz, M., Breuer, M. & Linke, R. P. (1987) *Biol. Chem. Hoppe-Seyler* **368,** 863–870.
19. Raeder, U. & Broda, P. (1985) *Lett. Appl. Microbiol.* **1,** 17–20.
20. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1987) *Current Protocols in Molecular Biology* (Wiley, New York).
21. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
22. Kaiser, K. & Murray, N. E. (1985) in *DNA Cloning: A Practical Approach*, ed. Glover, D. M. (IRL, Oxford), pp. 1–47.
23. Benton, W. D. & Davis, R. W. (1977) *Science* **196,** 180–182.
24. O'Farrell, P. H. & O'Farrell, P. Z. (1977) *Methods Cell. Biol.* **16,** 407–420.
25. Nagasawa, T. & Yamada, H. (1987) *Biochem. Biophys. Res. Commun.* **147,** 701–709.
26. Anderson, P. M. & Little, R. M. (1986) *Biochemistry* **25,** 1621–1626.
27. Tomich, S. C., Olson, E. R., Olson, M. K., Kaytes, P. S., Rockenbach, S. K. & Hatzenbuhler, N. T. (1989) *Nucleic Acids Res.* **17,** 3179–3197.
28. Beato, M. (1989) *Cell* **56,** 335–344.
29. Ingvorsen, K., Yde, B., Godtfredsen, S. E. & Tsuchiya, R. T. (1988) in *Cyanide Compounds in Biology*, Ciba Foundation Symposium 140, eds. Evered, D. & Harnett, S. (Wiley, Chichester, U.K.), pp. 16–31.
30. Schimpl, A., Lemmon, R. M. & Calvin, M. (1965) *Science* **147,** 149–150.