Primary Structure of an Aliphatic Nitrile-Degrading Enzyme, Aliphatic Nitrilase, from *Rhodococcus rhodochrous* K22 and Expression of Its Gene and Identification of Its Active Site Residue†,‡

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ABSTRACT: Peptides obtained by cleavage of a *Rhodococcus rhodochrous* K22 nitrilase, which acts on aliphatic nitriles such as acrylonitrile, crotononitrile, and glutaronitrile, have been sequenced. The data allowed the design of oligonucleotide probes which were used to clone a nitrilase encoding gene. Plasmid pNK21, in which 2.05-kb sequence covering the region encoding the nitrilase was placed under the control of the *lac* promoter, directed overproduction of enzymatically active nitrilase in response to addition of isopropyl β-D-thiogalactopyranoside in *Escherichia coli*. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the cell extract showed that the amount of nitrilase was about 40% of the total soluble proteins, leading to the establishment of a simple purification of the nitrilase. The nucleotide sequence of the nitrilase gene predicts a protein composed of 383 amino acids (M_r = 42 275), including only one cysteine. The amino acid sequence homology between the *Rhodococcus* nitrilase and the *Klebsiella ozaenae* bromoxynil nitrilase [Stalker et al. (1988) *J. Biol. Chem. 263*, 6310–6314] was 38.3%, and a unique cysteinyl residue (Cys-170) in the former nitrilase was conserved at the corresponding position in the latter nitrilase. Cys-170 of the *Rhodococcus* nitrilase was replaced by Ala or Ser by site-directed mutagenesis. Both mutations resulted in the complete loss of nitrilase activity, clearly indicating that this cysteinyl residue is essential for the catalytic activity.

Nitriles are widely manufactured and extensively used by the chemical industry. Acetonitrile is used as a solvent, adiponitrile is a precursor by nylon-6,6, and acrylonitrile is produced as a precursor of acrylic fibers and plastics. Nitrile herbicides such as dichlobenil (2,6-dichlorobenzonitrile; commercial name Casoron), bromoxynil (3,5-dibromo-4-hydroxybenzonitrile; commerical name Brominal), ioxynil (3,5-diiodo-4-hydroxybenzonitrile; commercial name Bentrol), and Buctril (4-(octanoyloxy)-3,5-dibromobenzonitrile) are also widely used in wheat, barley, corn, berries, and rice fields. Since these nitriles contain a CN moiety, they are very toxic and are generally bio-undegradable compounds. These nitriles have been widely spread out in our environment in forms of industrial waste water and remaining agricultural chemicals if their release is not controlled, and finally they have threatened the environment. Therefore, a nitrile is an important target in terms of an environmental purification and preservation.

Some microorganisms have the ability to utilize nitriles as carbon and/or nitrogen sources. Nitrilase catalyzes the direct cleavage of nitriles to the corresponding acids and ammonia, whereas nitrile hydratase catalyzes the hydration of nitriles to amides. All nitrilases so far reported from *Pseudomonas* (Robinson & Hook; Hook & Robinson, 1964), *Nocardia* sp. NCIB 11215 (Harper, 1985) and NCIB 11216 (Harper, 1977a), *Fusarium solani* (Harper, 1977b), *Arthrobacter* sp. (Bandypadhyay et al., 1986), *Rhodococcus rhodochrous* J1 (Kobayashi et al., 1989), *Klebsiella ozaenae* (Stalker et al., 1988a), and *Alcaligenes faecalis* JM3 (Nagasawa et al., 1990) utilize benzonitrile and related aromatic nitriles as substrates. However, no nitrilases that act on aliphatic nitriles were reported.

Recently, we found the occurrence of a novel nitrilase which acts on aliphatic nitriles in *R. rhodochrous* K22 (Kobayashi et al., 1990a). Aliphatic nitriles such as acrylonitrile and glutaronitrile, which are not attacked by all other nitrilases, are suitable substrates for this nitrilase. The *R. rhodochrous* K22 nitrilase is also capable of hydrolyzing only one cyano group of glutaronitrile, whereas the selective monohydrolysis of this dinitrile by conventional chemical methods is difficult (Kobayashi et al., 1990b). Nitrilase is a promising catalyst in organic chemical processing (Kobayashi et al., 1990c) like nitrile hydratase which is applied for the industrial production of acrylamide from acrylonitrile (Nagasawa & Yamada, 1989). Nitrilase would be an interesting enzyme not only from the amelioration of the environmental pollution but also from bioconversion.

All nitrilases previously reported are, though some are resistant to some degree, sensitive to thiol reagents and are classified as sulfhydryl enzymes. However, no data have been published on the determination of the active amino acid in the nitrilase. There are also no reports on the gene cloning of a nitrilase except the bromoxynil nitrilase from *K. ozaenae*. Identification of the active site will certainly help to elucidate its unique substrate specificity and subsequently to understand its physiological functions. To pursue the protein engineering of nitrilase in order to clarify the mechanism by which the enzyme recognizes the substrate, we have here cloned an aliphatic nitrilase gene from *R. rhodochrous* K22 and constructed an overproducing strain. We also have attempted to identify its active site by site-directed mutagenesis.

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‡ The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank Nucleotide Sequence Databases under Accession Number D12583.

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MATERIALS AND METHODS

Bacterial Strains and Plasmids. *R. rhodochrous* K22, which produces a nitrilase that acts preferentially on aliphatic nitriles, was isolated from soil (Kobayashi et al., 1990a). *Escherichia coli* JM105 [Δ(*lac–pro*) *thi strA endA sbcB15 hsdR4* F′ *traD36 proAB lacI*^{q} *lacZ*Δ*M15*] (Maniatis et al., 1982) was the host for cloning the gene and for expressing the cloned gene. *E. coli* TG1 [Δ(*lac–pro*) *supE thi hsdD5* F′ *traD36 proAB lacI*^{q} *lacZ*Δ*M15*], which was provided from Amersham Corp. (U.K.), was the host for phage M13 propagation. The ampicillin-resistance plasmids pUC18 and pUC19 (Yanisch-Perron et al., 1985; Messing, 1983) containing the *E. coli lac* promoter were the cloning and expression vectors.

Media. *R. rhodochrous* K22 was cultivated as described previously (Kobayashi et al., 1990a). M13 phage was propagated, and the nitrilase gene was expressed in 2 × YT medium (Messing, 1983).

Enzymes and Chemicals. Lysyl-endopeptidase, V8 protease, isopropyl β-D-thiogalactopyranoside (IPTG),[1] and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were obtained from Wako Pure Chemicals (Tokyo, Japan). Restriction endonucleases and T4 DNA ligase were purchased from Takara Shuzo Co. Ltd. [γ-^{32}P]ATP (180 TBq/mmol), [α-^{32}P]dATP (15 TBq/mmol), and [α-^{32}P]dCTP (110 TBq/mmol) were from Amersham Corp. DEAE-Sephacel and a low-molecular-mass standard kit were obtained from Pharmacia (Sweden). Cellulofine GCL-2000 superfine was purchased from Seikagaku Kogyo Co. (Japan). Carboxypeptidase Y was purchased from Oriental Yeast Co. (Japan). All other chemicals used were reagent grade.

N- and C-Terminal Sequence Analyses. Nitrilase was purified from *R. rhodochrous* K22 as described previously (Kobayashi et al., 1990a), and the enzyme (1 mg in 1 mM potassium phosphate buffer, pH 7.5) was used directly for the NH_2-terminal sequence analysis by automated Edman (1967) degradation with an Applied Biosystems 470A gas-phase amino acid sequencer. The phenylthiohydantoin- (PTH-) amino acids were separated and identified by an on-line PTH analyzer (120A; Applied Biosystems Japan, Tokyo) with a PTH-C_{18} column.

The COOH-terminal amino acid residues were analyzed by carboxypeptidase digestion (Gladner & Neurath, 1953). Carboxypeptidase Y (0.41 nmol) was added to 15.2 nmol of sodium dodecyl sulfate- (SDS-) denatured enzyme in 1.2 mL of 0.1 M Tris–maleate buffer (pH 6.0), and the reaction mixture was incubated at 25 °C. Aliquots (0.2 mL) were withdrawn at various intervals and put into small tubes containing 40 μL of 30% (mass/vol) trichloroacetic acid. The released amino acids were quantitatively analyzed with an amino acid analyzer (Kyowa Semitsu, K101-AS, Mitaka, Japan).

Isolation of Peptide Fragments and Peptide Sequencing. Nitrilase was digested with (1) lysyl-endopeptidase, (2) *Staphylococcus aureus* V8 protease, or (3) CNBr. Each reaction was carried out as follows: (1) 5.98 mg of the nitrilase was treated with 1.2 μg of lysyl-endopeptidase in 1 mL of 0.01 M Tris-HCl (pH 7.6) at 37 °C for 24 h; (2) 5.98 mg of the nitrilase was treated with 54 μg of V8 protease in 1 mL of 0.01 M CH_3COONH_4/CH_3COOH (pH 4.0) at 28 °C for 22 h; (3) 0.59 mg of the nitrilase was treated with 1.43 mg of CNBr in 0.715 mL of 70% (by volume) formic acid at 20 °C for 20 h. Each reaction mixture was directly applied to high-performance liquid chromatography (HPLC) equipped with a ULTRON N-C_{18} (4.6 × 150 mm; Shinwa Kako, Kyoto, Japan) and eluted with a linear gradient of acetonitrile (0–60%, by volume) in the presence of 0.1% (by volume) trifluoroacetic acid at a flow rate of 1.0 mL/min. The peptides isolated were sequenced by automated Edman degradation.

Syntheses of Mixed Oligonucleotide Probes. Three mixed oligonucleotide (16–35 nucleotides) probes for the nitrilase, deduced from the amino acid sequences of the NH_2 termini and the internal fragments generated with lysyl-endopeptidase, V8 protease, or CNBr treatment, were synthesized according to the phosphoramidite method (Beacage et al., 1981) on a DNA synthesizer (381A; Applied Biosystems Japan, Tokyo). The oligonucleotides were purified by oligonucleotide purification cartridges (Applied Biosystems), labeled at their 5′ ends with T4 polynucleotide kinase and [γ-^{32}P]ATP and used as hybridization probes.

Oligonucleotide Screening on the Nitrilase Gene. A total DNA of *R. rhodochrous* K22 was prepared after lysis of cells (wet mass, 15 g) with lysozyme and *Achromobacter* peptidase followed by the method of Saito and Miura (1963). After digestion of the total DNA with several restriction endonucleases, Southern (1975) hybridization was carried out by using each of three mixed ^{32}P-labeled oligonucleotide probes with the following modifications. Hybridization was performed in 6× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate) containing 0.1% (mass/volume) SDS at 42 °C for 12 h, and the nitrocellulose filter was washed twice at 50 °C in 4× SSC containing 0.1% (mass/volume) SDS. A distinct 3.1-kb band hybridizing to a K1 probe was observed, when the total DNA was digested with *Sac*I. This fragment was recovered and ligated with T4 DNA ligase to linear molecules of pUC19 successively treated with *Sac*I and bacterial alkaline phosphatase. The ligated mixture was introduced by transformation into *E. coli* JM105, and ampicillin-resistant transformants were selected on 2× YT agar medium containing 50 μg/mL ampicillin. Colony hybridization with a ^{32}P-labeled K1 probe was carried out according to Grunstein and Hogness (1975). Briefly, a DNA-blotted nitrocellulose filter was prehybridized at 60 °C for 2 h in 4× SSC, 0.1% (mass/volume) SDS and 180 μg/mL salmon sperm DNA and then hybridized at 42 °C overnight in the same buffer containing the ^{32}P-labeled K1 probe. After being washed several times in 4× SSC (lower-stringency) at 50 °C, a plasmid DNA, designated as pNK10, was prepared from the positive colonies. As described later, the 3.1-kb fragment did not contain the whole nitrilase gene.

In order to obtain the entire gene, after digestion of the total DNA with several restriction endonucleases, Southern hybridization was performed by using the nick-translated (Rigby et al., 1977) 3.1-kb *Sac*I fragment from pNK10 as the probe. Hybridization was performed at higher stringency using a buffer containing 40% (by volume) formamide, 5× SSC, and 0.1% (mass/volume) SDS at 42 °C for 12 h. A single 6.0-kb band was detected when the total DNA was digested with *Bgl*II. The fragment was recovered and ligated with *Bam*HI-digested pUC19. Plasmid DNA containing the 6.0-kb *Bgl*II fragment, designated as pNK20, was prepared from a positive colony.

Nucleotide Sequencing. DNA fragments covering the nitrilase gene, which were obtained by specific restriction enzymes, were cloned into appropriate M13 vectors and

[1] Abbreviations: HPLC, high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; kb, kilobase(s); IPTG, isopropyl β-D-thiogalactopyranoside; PTH, phenylthiohydantoin; DEAE, (diethylamino)ethyl; CD, circular dichroism.

sequenced by the dideoxy chain-termination method (Sanger et al., 1977) using a sequencing kit from U.S. Biochemical Corp. (Cleveland, OH). Deoxy-ITP or 2′-deoxy-7-deaza-GTP was used as a substitute for dGTP during M13 sequencing, to minimize compression. The nucleotide sequence was determined in both orientations.

Site-Directed Mutagenesis. Site-directed mutagenesis was carried out according to the phosphorothioate-based strategy of Taylor et al. (1985), using a mutagenesis kit of Amersham Corp. To prepare single-stranded DNA, the *Sph*I–*Hin*dIII fragment of replicative-form M13mp19 was ligated with the 1.6-kb *Sph*I–*Hin*dIII fragment isolated from plasmid pNK10. After transformation into the competent *E. coli* TG1, the recombinant phages were screened initially for white plaques on plates containing IPTG and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Two oligonucleotides were synthesized for mutagenesis: 5′-GGCCTGAATTC*CTGGGAGCAC-3′ for replacement of Cys-170 by Ser and 5′-GGT-CGTGTCGGA*GGCCTGAATG*C*CTGGGAGCACT-TC-3′ for replacement of Cys-170 by Ala (the positions of the introduced *Eco*RI and *Stu*I restriction sites are underlined, respectively), where asterisks indicate mismatched bases. The mutants were initially screened by restriction enzyme mapping on replicative-form DNA prepared from several plaques and then by DNA sequencing. A 589-bp *Sph*I–*Xho*I fragment (nucleotide positions 587–1175) was excised from each of the mutated phage DNAs and inserted between the *Sph*I and *Xho*I sites of plasmid pNK21 instead of the parental fragment. The two mutant plasmids thus obtained were designated pNK21-170S and pNK21-170A, using the one-letter code for each substituted amino acid residue. The constructs were confirmed by restriction mapping.

Preparation of Crude Extracts from E. coli Transformants. Recombinant *E. coli* JM105 was cultured aerobically to full growth in 10 mL of 2× YT medium containing 50 μg/mL ampicillin in a 50-mL test tube at 37 °C, and then transferred to 100 mL of the same medium in a 500-mL shaking flask with IPTG added to a final concentration of 1 mM, to induce the *lac* promoter. After various culture periods, the cells were harvested by centrifugation, suspended in 3 mL of 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol, disrupted by sonication for 5 min (19 kHz, Insonator Model 201M; Kubota, Tokyo, Japan) and centrifuged at 12000*g* for 30 min. The resulting supernatants and pellets were used for the enzyme assay. SDS–PAGE was performed by the method of Laemmli (1970). Quantitation of the nitrilase on slab gels after separation by SDS–PAGE was carried out with a dual-wavelength TLC scanner CS-930 system (Shimadzu, Japan). Scanning was performed at 650 nm; a standard curve was obtained with varing amounts of the purified enzyme.

Purification of Nonmutant and Mutant Nitrilases from E. coli Transformants. A nitrilase-overproducing strain was constructed by transforming *E. coli* JM105 with plasmid pNK21 and it was cultured as described in Results. Cells of the transformants were harvested by centrifugation and subjected to the following simple procedures. All procedures were carried out at 0–4 °C. Throughout purification steps, potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol was used. Seven grams of wet cells was suspended in 0.1 M buffer, broken by sonication for 10 min, and centrifuged at 12000*g* for 30 min. The supernatant was dialyzed against 0.01 M buffer and applied to a DEAE-Sephacel column equilibrated with 0.01 M buffer and eluted by a 0.2–0.3 M linear KCl gradient, in 0.01 M buffer. The enzyme solution was applied to a Cellulofine GCL-2000 superfine column and eluted with 0.01 M buffer. Active fractions were pooled, precipitated with 60% saturated ammonium sulfate, and dialyzed against the above buffer.

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K1 probe
          12  13  14  15  16  17  18  19  20  21  22  23
     A.A. Lys Val Lys Val Ala Thr Val Gln Ala Glu Pro Val

       5' AAG GTC AAA GTI GCC ACC GTI CAG GCC GAG CCG GT  3'
            A   G   G           G               A

K2 probe
          353 354 355 356 357 358
     A.A. Tyr Glu Asn Ala Glu Ala

       5' TAT GAA AAT GCA GAA GC  3'
            C   G   C   T   G
                        C
                        G

K3 probe
          179 180 181 182 183 184
     A.A. Lys Tyr Met Met Tyr Ser

       5' AAA TAC ATG ATG TAC T   3'
            G   T           T
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FIGURE 1: Oligonucleotide probes for cloning of the nitrilase gene. The determined amino acid sequences (A.A.) and their corresponding codons are shown, together with the synthesized oligonucleotide probes. Inosine is denoted by I. Numbers indicate the position of the deduced amino acid sequence displayed in Figure 4.

Each mutant nitrilase, in which Cys-170 was replaced with Ala or Ser, was purified from each mutant nitrilase-overproducing transformant according to the same procedure used for the purification of the active nonmutant nitrilase described above.

Enzyme Assays. Nitrilase activity of *R. rhodochrous* K22 was assayed by the same method as previously described (Kobayashi et al., 1990a). The protein concentration was determined according to Bradford (1976). One unit of nitrilase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of crotonic acid/min under the above conditions. The specific activity was expressed as units/per milligram of protein.

Circular Dichroism Analysis. Circular dichroism (CD) measurements were carried out with a Jasco J-600 recording spectropolarimeter at 25 °C with a 1-mm light pathlength cell. The instrument was calibrated with (+)-10-camphor-sulfonic acid, $\Delta\epsilon = +2.42$ $M^{-1}\cdot cm^{-1}$ at 290.5 nm. In the calculation of the mean residue ellipticity, $[\theta]$, the mean residue mass was taken to be 110.4 for the protein of the nitrilase. The CD spectra were obtained at protein concentration of 0.107 mg/mL in the far-UV region (184–260 nm) under a nitrogen atmosphere.

Computer Analysis of Amino Acid Sequences. The DNA sequence was analyzed using the GENETYX sequence analysis program (Software Development Co., Tokyo, Japan). A search of the National Biomedical Research Foundation (NBRF) protein sequence data bank (George et al., 1986) for sequence similarities was carried out with the SEQFP algorithm of the Integrated Database and Extended Analysis System (IDEAS) (Wilber et al., 1983).

RESULTS

Cloning and Nucleotide Sequence of the Nitrilase Gene. With aid of the codon usage data of the *K. ozaenae* nitrilase, we synthesized oligonucleotides that corresponded to the determined amino acid sequences of the NH_2-terminus of the *R. rhodochrous* K22 nitrilase and its peptide fragments generated with lysyl-endopeptidase, V8 protease, or CNBr (Figure 1). In the design of synthetic oligonucleotides in which in some cases GTI was used for Val and G or C was

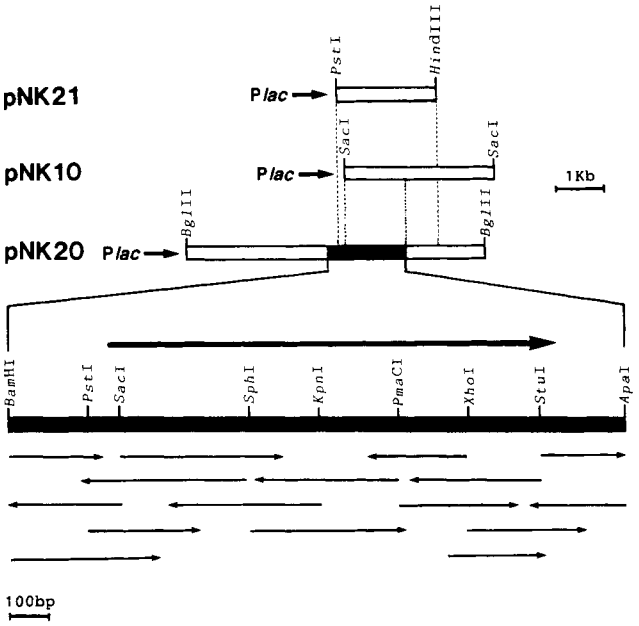

FIGURE 2: Restriction maps, sequencing strategy of plasmid pNK20, and the construction of plasmid pNK21 for expression of the nitrilase gene. Plasmid pNK10 contains a 3.1-kb fragment at the *Sac*I site of pUC19. Plasmid pNK20 contains a 6.0-kb fragment at the *Bam*HI site of pUC19. Arrows indicate the direction and extent of the sequence determination. Plasmid pNK21 contains a 2.05-kb fragment at the *Pst*I and *Hin*dIII sites of pUC18. The location and direction of the *lac* promoter are also indicated.

preferentially used in the third position of the codon, the following information was also used. (1) The G + C content of *R. rhodochrous* K22 DNA ranges from 67 to 70% mol (T_m) (Kobayashi et al., 1990a) and nitrile hydratase genes from *R. rhodochrous* J1 are characterized by the high frequency of G/C at the third nucleotide of the codon within the region (Kobayashi et al., 1991). (2) The use of inosine lessens the degeneracy of the pools used for hybridization (Maniatis et al., 1982). The K1, K2, and K3 probes were a 35-mer with 32 variants, a 17-mer with 64 variants, and a 16-mer with 8 variants, respectively. Southern hybridization using these probes against the *Sac*I-digested total DNA of *R. rhodochrous* K22 revealed that the K1 probe hybridized with a single 3.1-kb fragment. The K2 probe barely hybridized with this fragment, but the K3 probe did not. One explanation for these findings may be that it is sometimes difficult to establish conditions for hybridization with oligonucleotides and posthybridization washing (Maniatis et al., 1982). The nucleotide sequence corresponding to the NH_2-terminal four amino acids was not present in the 0.85-kb *Sac*I–*Xho*I region hybridizing with the K1 probe in the cloned 3.1-kb fragment (Figure 2). We then carried out further cloning of the entire nitrilase gene and got plasmid pNK20 containing a 6.0-kb *Bgl*II fragment (Figure 2). On the basis of the results of restriction analysis, we determined the nucleotide sequence of the *Bam*HI–*Apa*I fragment.

Figure 3 shows the 1653-bp nucleotide sequence. An open reading frame encoding 383 amino acids, which started with methionine and terminated with a TAG codon, also encoded the amino acid sequences corresponding precisely to those determined using the purified nitrilase. Comparison of the experimentally determined and the predicted overall amino acid compositions of the enzyme revealed good correlation for all residues. The molecular mass of the protein encoded by this coding sequence was calculated to be 42 275, which is almost identical with that of the enzyme subunit (M_r = 41 000) measured by SDS–PAGE (Kobayashi et al., 1990a). The COOH-terminal amino acid residue was quantitatively determined by carboxypeptidase Y digestion. The first residue released on the digestion was alanine, the amount of which was calculated to be 0.88–0.97 mol/mol of subunit. It is evident that the enzyme has a single COOH-terminal amino acid, alanine. The sequence of the COOH-terminal end of the enzyme was found to be -[Glu]-[Ala] on digestion with carboxypeptidase Y. The result was in good agreement with the corresponding amino acid sequence deduced from the nucleotide sequence.

The ATG codon appears to be the best translation initiation site because of its location close to the putative ribosome binding site (Figure 3), which is highly complementary with the 3′-end of 16S rRNA (Shine & Dalgarno, 1974). However, none of the consensus promoter sequences found in other prokaryotes (Rosenberg & Court, 1979) were observed in the upstream region. A strong hairpin structure (ΔG = –40.9 kcal/mol) just downstream of the termination codon of the nitrilase may serve as a *rho*-independent transcriptional termination signal. The overall G + C composition of the 1149 bp is 64.8% and that of the positions 1, 2, and 3 of the codons of the nitrilase are 64.0, 42.0, and 88.5%, respectively. The relatively high G + C content is reflected preferentially at the third codon position. The G + C content of the second codon position is considerably low. *Rhodococcus* is classified as a nocardioform-actinomycete in Bergey's manual (Goodfellow, 1986). Such a codon usage pattern is characteristic for genes with a high G + C content from various bacteria, such as *Thermus* (Kagawa et al., 1984) and *Streptomyces* of an actinomycete (Bibb et al., 1984).

The predicted amino acid sequence of *R. rhodochrous* K22 nitrilase was compared with that of *K. ozaenae* bromoxynil nitrilase, which catalyzes the hydrolysis of bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) to the corresponding acid (Stalker et al., 1988a) (Figure 4). There was a 38.3% match of amino acids in 347 overlapping residues between both nitrilases. The carboxyl-terminal 54 amino acid region (positions 330–383) is not well conserved, but significant similarity can be seen at the amino-terminal and the internal regions. It is noteworthy that *R. rhodochrous* K22 enzyme contains only one cysteinyl residue and this residue at positions 170 is conserved in the corresponding amino acid sequences of the bromoxynil enzyme.

Production of the Nitrilase Protein in E. coli. To overproduce the nitrilase in *E. coli*, a 2.05-kb *Pst*I–*Hin*dIII fragment was inserted between the *Pst*I and *Hin*dIII sites of pUC18, resulting in plasmid pNK21 (Figure 2), in which the nitrilase gene was under the control of the *lac* promoter. Culture conditions for the nitrilase production were investigated (Table I). When *E. coli* harboring pNK21 was cultured in the presence of IPTG at 37 °C, nitrilase activity was detected in the supernatant of the sonicated cell-free extracts but not in the pellet obtained at 12000*g*. Incubation at 37 °C was more effective than incubation at 28 °C for the nitrilase activity. Incubation for 12 h from the start exhibited higher nitrilase activity than incubation for 6 or 7 h. The level of nitrilase activity in the cell-free extract of *E. coli* containing pNK21 was 0.28 unit/mg, which was three times that in *R. rhodochrous* K22 (with crotononitrile as a substrate), when this transformant was cultured for 12 h at 37 °C in 2× YT medium in the presence of IPTG.

Then, the production of the nitrilase in the supernatant of the sonicated cell-free extracts was analyzed by SDS–PAGE (Figure 5). As judged by quantifying the gel track, the nitrilase formed seemed to correspond to about 40% of the

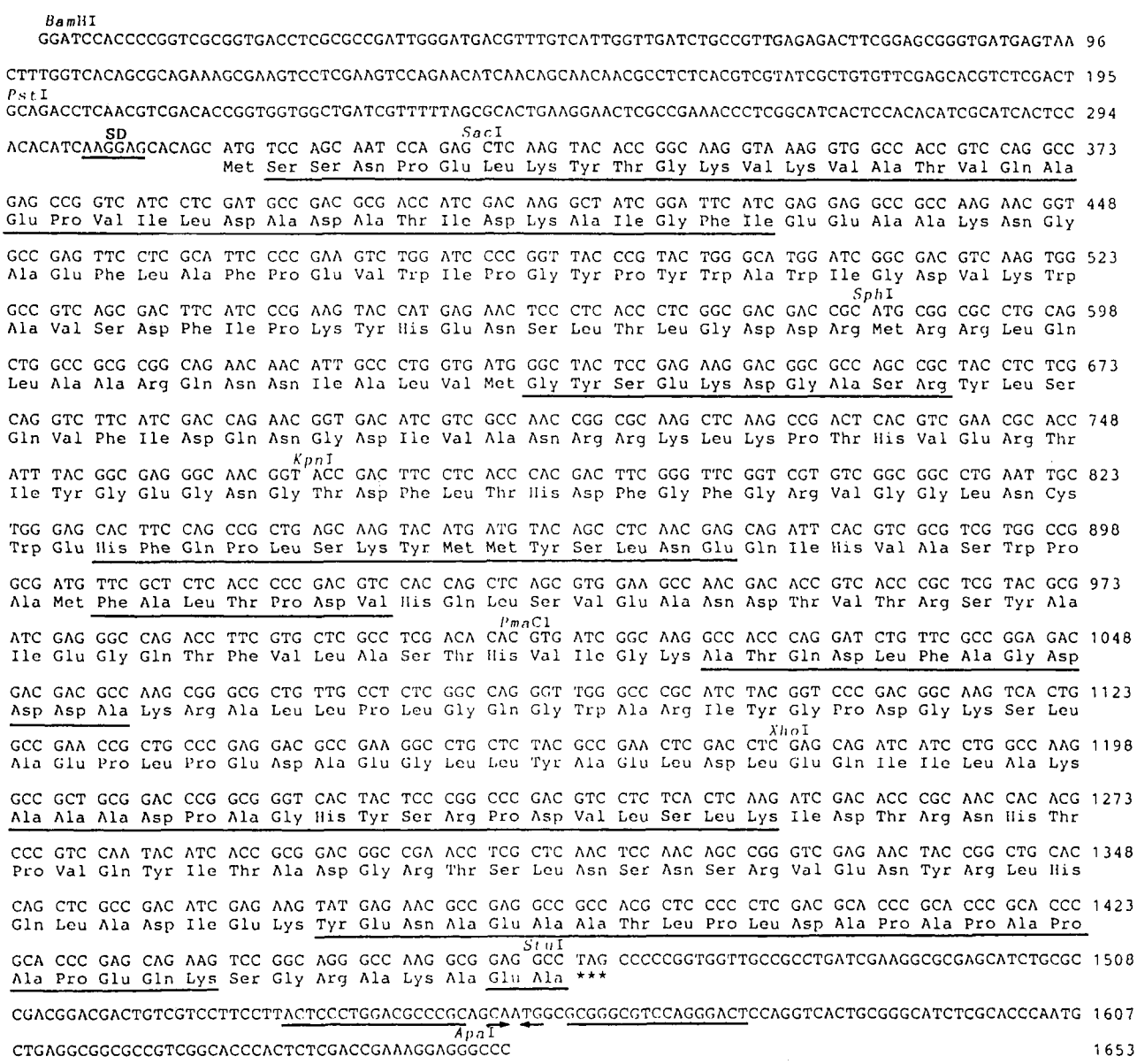

FIGURE 3: Nucleotide and amino acid sequence of the nitrilase gene. The underlined amino acid sequences were determined by Edman degradation. The carboxyl-terminal amino acid sequence was determined by carboxypeptidase Y. The potential ribosome-binding sequence is marked as SD, and a relevant stop codon is indicated by asterisks. An inverted repeat sequence downstream of the nitrilase gene is indicated by opposing arrows.

total soluble protein in response to addition of IPTG. From lysate after sonication, nitrilase was easily purified to give a single band on SDS–PAGE (Figure 5) with a purity increase of about 2.6-fold by two-step column procedures. The yield of the enzyme was usually more than 90%. The physicochemical properties such as specific activity and molecular mass were almost the same as those of the parental nitrilase.

Expression of Mutant Nitrilase Genes. We constructed two mutant enzymes in which Cys-170 was replaced with Ala or Ser. Each mutation was designed to minimize possible conformational change caused by the substitution. The oligonucleotides directing these site-specific mutations were also designed to create a new restriction site in the gene, and this site was used as a marker in the initial selection of the mutant. The identity of each mutant was confirmed by determining the complete nucleotide sequence of the mutant gene. The mutated enzymes were successfully overproduced in *E. coli* JM105. SDS–PAGE of cell-free extracts from the transformant cells harboring plasmids pNK21-170S or pNK21-170A, which were cultured under the same conditions used to overproduce the active nitrilase in the transformant carrying pNK21, gave a major protein band with a mobility identical to that of the nonmutated nitrilase. Both mutant proteins were purified to give a single band on SDS–PAGE (data not shown).

The specific activities of the mutant enzymes were less than the detection threshold, even when large amounts of enzymes were used in the reaction for 10 h. The Cys-170 to Ala or Ser mutations resulted in complete loss of activity, clearly indicating that this cysteine residue possessing a sulfhydryl group is essential for catalytic activity.

Their far-UV CD spectra (Figure 6) and molecular mass (data not shown) were virtually identical with those of the parental enzyme. Antiserum prepared against the parental nitrilase, which was purified from the cell-free extract of *R. rhodochrous* K22 (Kobayashi et al., 1990a), reacted completely with the purified C170S and C170A enzymes. These results indicate that the disappearance of enzymatic activities of mutants was not due to marked changes in tertiary structure. The α-helix and β-sheet contents of the nitrilase were calculated as 18.5% and 16.0%, respectively, by the matrix multiplication method using molecular CD values ($\Delta\epsilon$ units) of 39 points in

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K22  MSSNPELKYTGKVKVATVQAEPVILDADATIDKAIGFIEEAAKNGAEFLAFP   52
Bxn  MDTTFKAAAVQAEPVWMDAAATADKTVTLVAKAAAAGAQLVAFP   44
Ara  MSTVQNATPFNGVAPSTTVRVTIVQSSTVYNDTPATIDKAEKYIVEAASKGAELVLFP   58

K22  EVWIPGYPYWAW----IGDVKWAVSDFIPKYHENSLTLGDDRMRRLQLAARQNNIALV  106
Bxn  ELWIPGYPGFM-----LTHNQTETLPFIIKYRKQAIAADGPEIEKIRCAAQEHNIALS   97
Ara  EGFIGGYPRGFRFGLAVGVHNEEGRDEFRKYHASAIHVPGPEVARLADVARKNHVYLV  116

K22  MGYSEKDGASRYLSQVFIDQNGDIVANRRKLKPTHVERTIYGEGNGTDFLTHDFGFGR  164
Bxn  FGYSERAGRTLYMSQMLIDADGITKIRRRKLKPTRFERELFGEGDGSDLQVAQTSVGR  155
Ara  MGAIEKEGYTLYCTVLFFSPQGQFLGKHRKLMPTSLERCIWGQGDGSTIPVYDTPIGK  174

K22  VGGLNCWEHFQPLSKYMMYSLN-EQIHVASWPAMFALTPDVHQLSVEANDTVTRSYAI  221
Bxn  VGALNCAENLQSLNKFALAAEG-EQIHISAWPFTL-GSPVLVGDSIGAINQV---YAA  208
Ara  LGAATCWENRMPLYRTALYAKGIELYCA---PTAD-GSKEW-QSSMLHI------AI  220

K22  EGQTFVLASTHVIGKATQ----DLFAGD-DDAKRA-LLPLGQGWARIYGPDGKSLAEP  273
Bxn  ETGTFVLMSTQVVGP-TGIA-----AFEI-EDRYNPNQYLGGGYARIYGPDMQLKSKS  259
Ara  EGGCFVLSACQFCQRKHFPDHPDYLFTDWYDDKEHDSIVSQGGSV-ISPLGQVLAG-  276

K22  LPEDAEGLLYAELDLEQIILAKAAADPAGHYSRPDVLSLKIDTRNHTPVQYITADGRT  331
Bxn  LSPTEEGIVYAEIDLSMLEAAKYSLDPTGHYSRPDVFSVSINRQRQPAVSEVIDSNGD  317
Ara  PNFESEGLVTADIDLGDIARAKLYFDSVGYYSRPDVLHLTVNEHPRKSVTFVTKVEKA  334

K22  SLNSNSRVENYRLHQLADIEKYENAEAATLPLDAPAPAPAPEQKSGRAKAEA  383
Bxn  EDPRAACEPDEGDREVVISTAIGVLPRYCGHS  349
Ara  EDDSNK  340
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FIGURE 4: Comparison of the deduced amino acid sequences of the nitrilases from *R. rhodochrous* K22, *K. ozaenae*, and *Arabidopsis thaliana*. Three sequences were aligned by introducing gaps (hyphens) to maximize identities. Identical residues are enclosed by boxes. The conserved cysteine residue is highlighted in bold.

Table I: Nitrilase Activities of *E. coli* Transformants under Various Culture Conditions[a]

IPTG feeding time (h)	cultivation time (h)	cultivation temp (°C)	sp act. (unit/mg)
0[b]	6	28	0
0[b]	12	28	0.015
4[c]	7	28	0
4[c]	12	28	0.001
0[b]	6	37	0.008
0[b]	12	37	0.280
4[c]	7	37	0.018
4[c]	12	37	0.181

[a] The concentration of IPTG was 1 mM in 2× YT medium. [b] IPTG was added to 2× YT medium at the same time as culture started. [c] IPTG was added to 2× YT medium 4 h after the start of culture.

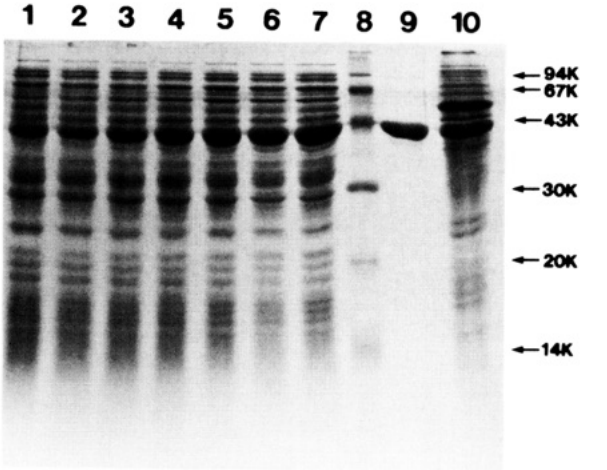

FIGURE 5: SDS–PAGE of the supernatant prepared from *E. coli* containing pNK21. Lanes 1, 2, 3, 4, 5, 6, 7, and 10 show supernatants of sonicates (80 μg of protein): Lane 1, *E. coli* JM105 containing pNK21, sample taken after a 6-h incubation at 37 °C with IPTG; lanes 2, 3, 4, 5, 6, and 7, as lane 1, but after 7-, 8-, 9-, 10-, 11-, and 12-h incubations, respectively; lane 8 was loaded with the molecular mass standards phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa); lane 9, purified nitrilase (27 μg of protein) from the sample derived from lane 7 as described in the text, as a control; Lane 10, cell-free extracts (82 μg of protein) of *R. rhodochrous* K22 cells cultured for 60 h with the feeding of 0.2% (by volume) isovaleronitrile at 48 h from the start, as a control.

the region between 184 and 260 nm (Compton & Johnson, 1986).

DISCUSSION

Nitriles are synthesized on a large scale as plastics, herbicides, and starting materials from other industrially important chemicals. Nitrile herbicides used remain in fields and pollute subterranean water and river. Industrial water including acrylonitrile etc. is discharged from petrochemical factories. Our fundamental analysis of nitrile degradation at the protein and gene levels is expected to lead to biological environmental improvement.

The *R. rhodochrous* K22 nitrilase that preferentially acts on aliphatic nitriles is distinct from the *K. ozaenae* nitrilase that is highly specific for benzonitrile derivatives with two meta-positioned halogen atoms such as bromoxynil and chloroxynil (3,5-dichloro-4-hydroxybenzonitrile) of herbicides (Stalker et al., 1988a,b). The former nitrilase formed active high-molecular-mass aggregates (M_r = 650 000), opposite from the latter enzyme (M_r = 72 000), which is a homodimer. On the basis of the sequence similarity between nitrilases from *R. rhodochrous* K22 and *K. ozaenae*, the structure of both enzymes may be basically similar. A large difference of amino acid sequence in carboxyl-terminal portions or a small difference of that in amino-terminal and internal portions between them seems to bring about a large difference in the substrate specificity and the subunit interaction to which the enzyme polymerization is probably ascribed.

We succeeded in overproduction of the nitrilase in *E. coli* (40% of the total soluble protein). This is consistent with the finding showing that the maximum level of cell-free nitrilase (0.28 unit/mg) (Table I) corresponded to 38% compared to the specific activity in the purified nitrilase from *R. rhodo-*

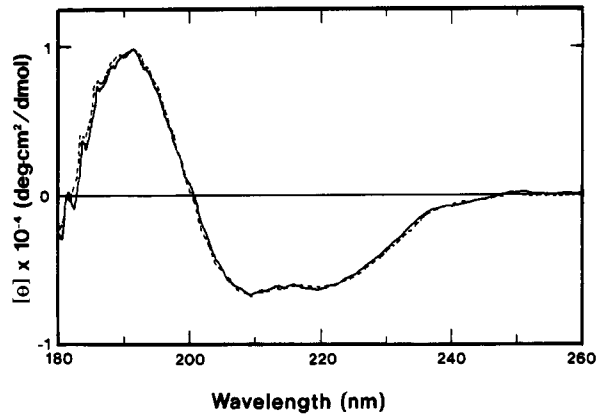

FIGURE 6: CD spectra of the nitrilase. Both C170S and C170A mutant enzymes were examined, and the spectrum of C170A (- - -) as a representative is shown in comparison with that of the parental enzyme (—). The enzyme concentration was 0.107 mg/mL in 5 mM potassium phosphate buffer (pH 7.5). All spectra were measured as described in Materials and Methods.

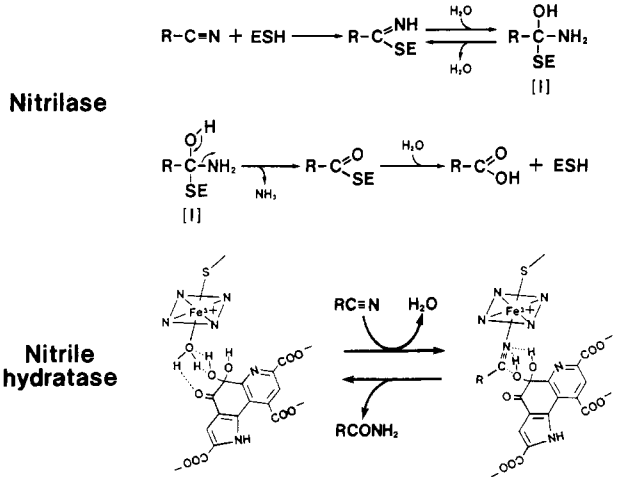

FIGURE 7: Comparison of the scheme for the nitrilase reaction mechanism with that for the nitrile hydratase reaction mechanism.

chrous K22 (0.737 unit/mg). This hyperproduction with the succeeding rapid enzyme purification provides us with the opportunity to investigate the role of a cysteine residue in the nitrilase. The *R. rhodochrous* K22 nitrilase contains a single cysteine residue per subunit, whereas both nitrilases from *R. rhodochrous* J1 (Kobayashi et al., 1989) and *K. ozaenae* have 4 cysteine residues per subunit. This unique Cys-170 residue in *R. rhodochrous* K22 was conserved in the corresponding amino acid sequences of the *Klebsiella* enzyme. Several other residues around Cys-170 are also well conserved. On the other hand, the other three cysteines (at positions 87, 324, 346) in the latter nitrilase are located in the nonhomologous region of the former enzyme.

Whereas all nitrilases previously reported were susceptible to thiol reagents, *R. rhodochrous* K22 exhibited relatively high resistance to the thiol reagents which strongly inhibited other nitrilases (Kobayashi et al., 1990a). However, the nitrilase activities of the purified mutated enzymes C170S and C170A, in which Cys-170 is replaced with Ser and Ala, respectively, have been lost, clearly demonstrating that Cys-170 should play important roles in this nitrilase reaction. Considering these findings, there is only one cysteine residue per subunit functioning in the active site, but this cysteine seems to be "buried" to not be easily inactivated with thiol reagents.

Both Harper (1977a) and Mahadevan and Thimann (1964) proposed a possible mechanism for the nitrilase (Figure 7): a nucleophilic attack on a nitrile carbon atom by a sulfhydryl group of the enzyme (ESH in Figure 7) leads to the formation of a tetrahedral intermediate ([I] in Figure 7) via enzyme–iminothiol ester. Ammonia is then removed from the intermediate to form an acylenzyme, followed by its hydrolysis to an acid. On the contrary, a hypothetical reaction mechanism for the nitrile hydratase from *Pseudomonas chlororaphis* B23 is proposed (Nagasawa & Yamada, 1989) (Figure 7): by the replacement of H_2O coordinated to the ferric center with nitrile, nitrile binds to the low-spin iron(III) site of the enzyme and is activated. The hydrated pyrroloquinoline quinone-like compound activates H_2O and hydrates the CN bond, leading to the production of the corresponding amide. Considering the remarkable difference in specific activity between the nitrilase and nitrile hydratase (Kobayashi et al., 1990a), each enzyme reaction seems to be catalyzed according to the completely different mechanism. Judging from the similarities of amino acid sequences between nitrilases from *R. rhodochrous* K22 and *K. ozaenae*, it is suggested that the nitrilase genes evolved from a common ancestral gene. In comparison with nitrilases from *R. rhodochrous* K22 and *K. ozaenae*, a homologous sequence was searched for among the protein sequence database of the NBRF. However, no homology was found. The amino acid sequences of the nitrilases were not similar to those of nitrile hydratases (Ikehata et al., 1989; Kobayashi et al., 1991; Nishiyama et al., 1991; Mayaux et al., 1990, 1991). Although both nitrilase and nitrile hydratase are classified as a group of nitrile-degrading enzymes, the two enzymes do not share a common ancestor.

Nitrilase is found in plants such as Cruciferae (cabbage group and radishi), Gramineae (grasses), and Musaceae (banana family) (Thimann & Mahadevan, 1964; Mahadevan & Thimann, 1964), and the plant nitrilase is regarded as a key enzyme for biosynthesis of a plant hormone, indole-3-acetic acid, from indole-3-acetonitrile which exists in Brassicaceae (Henbest et al., 1953). Another predominant biosynthesis route of indole-3-acetic acid has been found in which tryptophan is converted into indole-3-pyruvic acid or tryptamine followed by its transformation to indole-3-acetic acid via indole-3-acetaldehyde. Very recently, cDNA cloning of the nitrilase which converts indole-3-acetonitrile into indole-3-acetic acid from *Arabidopsis thaliana* was reported (Bartling et al., 1992). The predicted amino acid sequence of this plant nitrilase showed 34.1% and 33.9% homology with those of *R. rhodochrous* K22 and *K. ozaenae* nitrilases, respectively (Figure 4). As expected for a catalytically essential amino acid, Cys-170 in the *R. rhodochrous* K22 nitrilase is conserved in the *Arabidopsis* nitrilase. Therefore, studies on the nitrilase are significant from not only an applied standpoint such as biological environmental improvement and enzymatic transformation but also an academic point of view including the evolution of nitrilase in indole-3-acetic acid biosynthesis. Purification of a nitrilase acting on indole-3-acetonitrile and the molecular cloning of its gene from plants other than *Arabidopsis* are in progress.

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