A Stereoselective Cobalt-Containing Nitrile Hydratase

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ABSTRACT: Nitrile hydratase from *Pseudomonas putida* NRRL-18668 has been purified and characterized. The purified enzyme catalyzes the hydration of 2(*S*)-(4′-chlorophenyl)-3-methylbutyronitrile at least fifty times faster than that of 2(*R*)-(4′-chlorophenyl)-3-methylbutyronitrile. This enzyme is a member of the class of nitrile hydratase that contains cobalt. Visible absorption and CD spectra suggest the cobalt exists as a non-corrin low-spin Co^{3+} ion in a tetragonally-distorted octahedral ligand field. Chemical reduction of the native enzyme results in a species with the EPR signature of a low-spin Co^{2+} complex. Like the other cobalt-containing nitrile hydratases, this enzyme is relatively stable, maintaining its activity below 35 °C, and it shows a broad activity optimum between pH 7.2 and 7.8. The structural genes for this enzyme have been cloned and sequenced. The deduced amino acid sequences for the α and β subunits show 48−63% and 35−41% homology, respectively, to other sequenced nitrile hydratases. In particular, the cysteine residues in the α subunit that have been suggested to coordinate the metal ion in the iron-containing nitrile hydratases [Brennan, B. A., Cummings, J. G., Chase, D. B., Turner, I. M., Jr., & Nelson, M. J. (1996) *Biochemistry* 35, 10068−10077] are conserved in this enzyme, suggesting that this nitrile hydratase, like the enzyme from *Rhodococcus rhodochrous* J1, is a member of a newly described class of metalloenzymes with Co^{3+}−thiolate ligation [Brennan, B. A., Alms, G., Nelson, M. J., Durney, L. T., & Scarrow, R. C. (1996) *J. Am. Chem. Soc. 118*, 9194−9195].

Nitrile hydratases are bacterial metalloenzymes that catalyze the hydration of nitriles to amides (Nagasawa & Yamada, 1989). They have attracted substantial interest as catalysts for commercial processes. For example, *Rhodococcus rhodochrous* J1 that contains nitrile hydratase is used in a multi-kiloton scale commercial synthesis of acrylamide from acrylonitrile (Kobayashi et al., 1992; Nagasawa et al., 1993). These enzymes are also of interest as novel metalloenzymes; the iron nitrile hydratases contain Fe^{3+} in a biologically unprecedented mixed sulfur/nitrogen/oxygen coordination environment (Nagasawa et al., 1986, 1987, 1988, 1991; Sugiura et al., 1987; Nagamune et al., 1990; Nelson et al., 1991; Jin et al., 1993; Brennan et al., 1996b; Scarrow et al., 1996; Doan et al., 1996), and the metal environment in one cobalt nitrile hydratase has been shown to be very similar, including at least two cysteine thiolate−Co^{3+} bonds (Brennan et al., 1996a).

Nitrile hydratases generally exhibit very broad substrate specificity, an important characteristic for their development potential as industrial catalysts (Nagasawa & Yamada, 1989; Cohen et al., 1990). However, there are many commercially important processes in which hydration of one enantiomer of a racemic mixture of nitriles is desirable. The enantioselectivity of biological nitrile hydration generally has been probed in whole-organism systems rather than with purified enzymes, and occasionally some selectivity has been reported (Kakeya et al., 1991; Cohen et al., 1992; Beard et al., 1993; Blakey et al., 1995; Martínková et al., 1996). These studies are frequently complicated by the presence of amidases that catalyze the hydrolysis of the product amides by reactions that may or may not be enantioselective. One of the clearest examples of an enantioselective nitrile hydratase in a whole-cell system is that of *Rhodococcus equi*. In that case 49% of (*R*,*S*)-2-(4′-methoxyphenyl)propionitrile was converted to the corresponding (*S*)-acid with 87% enantiomeric excess, leaving behind 44% of the (*R*)-nitrile with >95% enantiomeric excess (Martínková et al., 1996). Presumably an amidase converted the nitrile hydratase product (*S*)-amide to the acid. To date, however, no purified nitrile hydratase has been reported to be enantioselective.

We have purified the nitrile hydratase from *Pseudomonas putida* NRRL-18668, a strain that carries out the enantioselective hydration of 2(*S*)-(4′-chlorophenyl)-3-methylbutyronitrile ((*S*)-CPIN)[1] (Fallon et al., 1997). We have shown that this enzyme is a member of the class of cobalt-containing nitrile hydratases and that the enantioselectivity in nitrile hydration shown by the cells is intrinsic to the enzyme. We also have cloned and sequenced the structural genes for this enzyme, and we report here a comparison of the derived amino acid sequences of the subunits to those of other nitrile hydratases.

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[1] Abbreviations: CPIN, 2-(4′-chlorophenyl)-3-methylbutyronitrile; CPIAm, 2-(4′-chlorophenyl)-3-methylbutyramide; EDBTC, buffer containing 20 mM Tris, 40 mM sodium butyrate, 5 mM dithiothreitol, and 2 mM EDTA, adjusted to pH 7.3 at 22 °C; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); PCR, polymerase chain reaction; TAPS, ([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)-1-propanesulfonic acid.

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EXPERIMENTAL PROCEDURES

Growth of P. putida NRRL-18668. The media used for the growth of *P. putida* NRRL-18668 contained the following: 60 mM potassium phosphate, pH 7.2, 20 g/L of glucose, 0.18 mM ferric citrate, 0.1 mM cobaltic citrate, 2.2 mM $NaHSO_4$, 2.0 mM $MgSO_4$, 0.8 mM $MnCl_2$, 0.8 mM boric acid, 0.2 mM $NiSO_4$, 0.2 mM $CuSO_4$, 0.2 mM $ZnSO_4$, 0.08 mM $NaMoO_4$, 0.08 mM KBr, 0.06 mM KI, 0.05 g/L of yeast extract, and 34 mM butyronitrile. 1 mL of a stock that had been stored in 15% glycerol at −80 °C was used to inoculate 1 L of culture media in a 2 L shake flask, and the culture was grown with shaking at 28 °C for 70 h. The 1 L culture was used to inoculate a 40 L New Brunswick Scientific Mobile Pilot Plant fermenter containing 30 L of culture media at 30 °C, and the 30 L culture was used to inoculate a 400 L New Brunswick Scientific Fermentation System containing 270 L of media, also at 30 °C. In the latter two growths the pH was maintained automatically at 7.0 by addition of 50% (w/v) NaOH solution or concentrated H_3PO_4. The 300 L culture was monitored turbidometrically, and the culture was harvested when $OD_{660} \approx 8.7$ (33 h after inoculation). Approximately 3 h before harvest, 500 mL of butyronitrile was added to the media to stimulate the production of nitrile hydratase. Cells were obtained by centrifugation, and the paste was frozen as pellets in liquid nitrogen. The yield was 8.4 g (wet weight) of cell paste/L. Frozen cell paste was stored at −80 °C.

Purification and Assay of Nitrile Hydratase from P. putida NRRL-18668. Nitrile hydratase activity was measured by following the hydration of methacrylonitrile spectrophotometrically in 0.1 M potassium phosphate, pH 7.0, at 25 °C ($\Delta\epsilon_{224} = 3.4$ mM^{-1} cm^{-1}) (Brennan et al., 1996b). All enzyme purification steps were carried out at 4 °C in a buffer containing 20 mM Tris, 40 mM sodium butyrate, 5 mM dithiothreitol, and 2 mM EDTA, adjusted to pH 7.3 at 22 °C (EDBTC), unless otherwise stated. Nitrile hydratase activity was liberated from the cells by thawing 420 g of frozen cell paste in 2 L of EDBTC. Cell fragments were sedimented by centrifugation, the supernatant was decanted, and the pellet was resuspended in EDBTC. The cell debris were again sedimented, and the supernatants were combined. Solid ammonium sulfate was added to 45% saturation, and solids were removed by centrifugation. The supernatant was brought to 60% saturation by addition of solid ammonium sulfate, and the precipitated protein was separated by centrifugation. The precipitate was dissolved in 230 mL of EDBTC and dialyzed against three 1 L changes of the same buffer. Solids were removed by centrifugation and the supernatant was applied to a DEAE cellulose column (5 × 20 cm) equilibrated with EDBTC. After the column was washed with 500 mL of EDBTC, nitrile hydratase was eluted with a 2 L gradient of 0−0.35 M NaCl in EDBTC. The fractions of highest specific activity were combined, and solid potassium phosphate was added to a final concentration of 5 mM phosphate. This solution was applied to a hydroxylapatite HTP column (2.5 × 20 cm) equilibrated with 5 mM potassium phosphate in EDBTC. After the column was washed with 500 mL of that buffer, nitrile hydratase activity was eluted with a 1 L gradient of 0.005−0.1 M potassium phosphate in EDBTC. Fractions of highest specific activity were combined, and solid ammonium sulfate was added to 25% of saturation. This solution was loaded onto a phenyl-Sepharose column (2.5 × 70 cm) equilibrated with 25% saturated ammonium sulfate in EDBTC. The column was washed with 550 mL of that buffer and eluted with a logarithmic gradient of 300 mL of 25%−0% saturated ammonium sulfate in EDBTC, followed by a further 1 L of EDBTC. The fractions of highest specific activity were combined, concentrated to approximately 150 mg/mL, and loaded onto two sequential Sephacryl S-400 columns (1 × 100 cm) equilibrated with 0.25 M NaCl in EDBTC. The columns were eluted with that buffer, and the fractions of highest specific activity were combined and stored at −20 °C.

Hydration of CPIN. Stock suspensions of (*R*)- and (*S*)-CPIN were made up at 10 mM in 20 mM KP_i, pH 7.2, and stirred in sealed vessels for 2 days to ensure homogeneity. Reactions were initiated by addition of 100 μg of purified enzyme per mL of reaction volume. Aliquots (0.5 mL) were removed, and the reaction was quenched by addition to 0.5 mL of 0.2 M HCl. The substrate and product (2-(4′-chlorophenyl)-3-methylbutyramide; CPIAm) were extracted into methylene chloride, which was dried with sodium sulfate and evaporated to dryness. The CPIN and CPIAm were redissolved in 0.1% trifluoroacetic acid in acetonitrile, and analyzed by HPLC using a Vydac C_{18} reverse-phase column eluted with 0.1% trifluoroacetic acid in 48% (v/v) acetonitrile in water. The concentrations of CPIN and CPIAm were determined by comparison of the peak integrals to standard curves. Total recoveries ranged from 50% to 80%; the insolubility of the substrate and product in water prevented higher recoveries. Control experiments showed that the recovery of (*S*)- and (*R*)-CPIN and (*S*)- and (*R*)-CPIAm were identical within experimental error, so the incomplete recoveries did not affect the conclusions of the experiment.

Estimation of the Holoprotein Molecular Weight. Purified *P. putida* NRRL-18668 nitrile hydratase was chromatographed on either a Pharmacia Superdex 200 column or a Sephacryl S-400 column eluted with 0.25 M NaCl, 40 mM sodium butyrate, 20 mM HEPES, pH 7.0, using a Pharmacia FPLC to control the elution rate. The columns were calibrated using sweet potato amylase (200 000 Da), alcohol dehydrogenase (150 000 Da), bovine serum albumin (67 000 Da), carbonic anhydrase (29 000 Da), and cytochrome *c* (12 400 Da). The enzyme also was chromatographed on a Sepharose CL-6B column eluted with 0.15 M NaCl, 10 mM phosphate, pH 7.4, calibrated with the same proteins. In both cases the void volume was determined from the elution of Blue Dextran (2 000 000 Da), and the molecular weight of the nitrile hydratase was estimated by comparison of its elution volume/void volume to those of the standards.

Peptide Sequencing. Nitrile hydratase subunits were separated on a Vydac C_{18} reversed-phase column attached to a Hewlett Packard HP-1090 HPLC. The enzyme sample was dialyzed against 0.1% trifluoroacetic acid in water and applied to the column. The column was eluted with an increasing gradient of acetonitrile in 0.1% trifluoroacetic acid in water. Only two peaks with substantial absorbance at 280 nm were observed. The N-terminal sequences of these peptides were obtained by use of a Beckman LF3000G gas phase protein sequencer.

Peptide Digests

TPCK-Treated Trypsin. Samples of the two subunits were dissolved in 0.1 mM ammonium bicarbonate, pH 7.8, and

2% (w/w) TPCK-treated trypsin (Pierce) was added. The solution was incubated overnight at 37 °C.

Cyanogen Bromide. Samples of the two subunits were lyophilized, and a 20-fold excess (w/w) cyanogen bromide in 70% formic acid was added. The samples were allowed to stand overnight in a vacuum desiccator at room temperature.

AspN Protease. Samples of the peptide were taken up in 20 mL of 50 mM potassium phosphate, pH 8.0, and 20 mL of a solution of 2 mg of AspN protease in 50 mL of water were added. These samples were incubated at 37 °C for 16 h.

In every case, digested samples were taken to dryness and redissolved in neat trifluoroacetic acid, the peptides were separated by HPLC, and samples of well-resolved peaks in the chromatograms were taken for sequencing as above.

Amino Acid Analysis. Purified nitrile hydratase was hydrolyzed by treatment with 6 N HCl for 24 h at 100 °C, and the total amino acid composition determined using a Beckman System 6300 amino acid analyzer.

UV/Vis and CD Spectroscopy. UV/vis spectra were obtained using an HP 8451a diode array spectrophotometer. CD spectra were obtained using an Aviv model 62DS spectrometer.

EPR Spectroscopy. Reduced nitrile hydratase was prepared by addition of a 20-fold excess of reduced methyl viologen, prepared by addition of sodium dithionite to methyl viologen in EDBTC, to a sample of enzyme prepared under argon. The sample was frozen after 5 min on ice and stored in liquid nitrogen. EPR spectra were obtained using a Brucker EM-200 spectrometer with an Oxford Instruments ESR900 liquid helium cryostat. The conditions were microwave frequency, 9.4 GHz; modulation amplitude, 1.0 mT; microwave power, 2 mW; T, 20 K; scan rate, 0.48 mT/s.

Cobalt Quantitation. Samples of nitrile hydratase in EDBTC were added to 1.0 M nitric acid (ultrapure nitric acid (J. T. Baker)) and heated in closed containers at 90 °C for 24 h. The liquid was allowed to evaporate, and the samples were taken up in 0.5 mL of 30 mM nitric acid. The concentration of cobalt in each sample was quantified in triplicate using a Perkin-Elmer 3300 atomic absorption spectrophotometer.

Cloning of Nitrile Hydratase Genes from P. putida NRRL-18668. Media and standard procedures used in molecular biology have been described (Maniatis et al., 1989). Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA). PCR (Mullis & Faloona, 1987) conditions were as follows: 100 ng of target, 1 μM each primer, 200 μM each of dATP, dCTP, dGTP, and dTTP, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 25 units/mL of Amplitaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT). PCR parameters were as follows: 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, 40 cycles. Plasmids M13mp18 (Yanish-Perron et al., 1985) and lambda ZAPII (Stratagene, La Jolla, CA) were used as cloning vectors. *Escherichia coli* strain XL1-Blue (Bullock et al., 1987) was used as the host for cloning and M13 propagation. 2 × YT medium was used for propagation of M13 phage, and LB medium was used for general *E. coli* propagation.

Nucleotide Sequencing. Nucleotide sequences were determined by the dideoxy chain-termination method (Sanger et al., 1977) from single-stranded M13 vectors and double-stranded pBluescript plasmids, using the Sequenase Kit (United States Biochemicals, Cleveland, OH) and [α-^{35}S]-dATP (New England Nuclear, Boston, MA). The sequence reported here has been assigned GenBank accession number U89363.

Table 1: Purification of Nitrile Hydratase from *P. putida* NRRL-18668

step	total protein (mg)	total activity (units)	specific activity (units/mg)	yield (%)
1. cell-free extract	8966	255 460	28.5	100
2. ammonium sulfate	1257	220 620	176	86
3. DEAE cellulose	195	158 700	813	62
4. hydroxylapatite	105	133 130	1263	52
5. phenyl-Sepharose	83	107 590	1300	42
6. Sephacryl S-400	54	86 010	1590	34

RESULTS

Purification of Nitrile Hydratase. The purification protocol used here for nitrile hydratase from *P. putida* NRRL-18668 was based on that used for the nitrile hydratase from *Rhodococcus* sp. R312 (formerly *Brevibacterium* R312 (Briand et al., 1994; Nagasawa et al., 1986; Brennan et al., 1996b). A summary of a typical purification is shown in Table 1. The protein appeared to be >95% pure on the basis of visual examination of heavily overloaded lanes in Coomassie Blue-stained SDS−PAGE gels. From 204 g (wet weight) of frozen cell paste we obtained approximately 54 mg of purified protein. The purified protein comprises two subunits as seen both by SDS−PAGE and by reversed-page HPLC of protein denatured in 0.1% trifluoroacetic acid (data not shown).

Cloning of Nitrile Hydratase Genes from P. putida NRRL-18668. A series of degenerate 21-mer oligonucleotide primers based on the nitrile hydratase amino acid sequence obtained from peptide sequences were designed and synthesized for use as PCR primers on genomic DNA isolated from *P. putida* NRRL-18668. The resulting amplified products were subjected to Southern blot analysis using [α-^{32}P]dCTP-labeled *R. rhodochrous* J1-L nitrile hydratase gene as a probe. One strongly hybridizing fragment of size 0.7 kb (generated from primers 5′-GGAATTCGAYCAYCAYCAYG-3′ and 5′-GGAATTCTTYTCCCARTCRTA-3′) was subcloned into the vector M13 and sequenced. The deduced amino acid sequence from this 0.7 kb fragment was compared to that of the purified protein, and to those of other known nitrile hydratases, and the comparison confirmed that this fragment was part of the *P. putida* NRRL-18668 nitrile hydratase gene.

Genomic DNA isolated from *P. putida* NRRL-18668 was partially digested with restriction enzymes *Eco*RI and *Xho*I and size-selected by agarose gel electrophoresis based on Southern blotting using the 0.7 kb DNA fragment described above as a probe. A lambda ZAPII library was constructed from digested genomic DNA of size range approximately 6.0−7.0 kb. The library was screened with the 0.7 kb DNA fragment, and one positively hybridizing phage clone with an insert of 6.5 kb was isolated, converted to a pBluescript plasmid, and restriction mapped (Figure 1).

Nucleotide Sequence of the Nitrile Hydratase Genes from P. putida NRRL-18668. We determined the nucleotide sequence of a 1.4 kb region of the 6.5 kb cloned *P. putida* NRRL-18668 insert encoding the nitrile hydratase genes (Figure 2). Sequence analysis confirmed that the nitrile

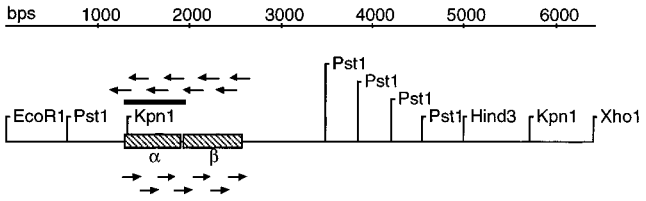

FIGURE 1: Restriction map of the 6.5 kb *P. putida* NRRL-18668 fragment and sequencing strategy for nitrile hydratase genes. Locations of the α and *β* NHase subunits are indicated by hatched boxes. The solid bar indicates the PCR product obtained with degenerate primers. The nucleotide sequence was obtained from both strands by walking along the insert using specific oligonucleotide primers as indicated by arrows.

hydratase coding region comprises two open reading frames, one encoding the α subunit and consisting of 210 amino acids (M_r 22 982), and the other encoding the *β* subunit and consisting of 217 amino acids (M_r 24 108). A comparison to nitrile hydratase genes from other organisms showed nucleotide homologies ranging from 53.8% to 64.3% for the α subunit and from 47.6% to 54.4% for the *β* subunit. The genes are organized 5′-α,*β*-3′, with ten base pairs separating the two. Of the other nitrile hydratase genes examined, four are organized 5′-α,*β*-3′, with separations ranging from 2 to 296 base pairs, while three are organized 5′-*β*,α-3′, with separations ranging from 1302 to 1366 base pairs. As in other organisms harboring nitrile hydratase genes, the *P. putida* NRRL-18668 nitrile hydratase genes appear to be part of an operon with potential coding sequences located both upstream and downstream of the nitrile hydratase genes.

Characterization of Nitrile Hydratase from P. putida NRRL-18668. The molecular weight of the intact protein was estimated to be 94 kDa by gel chromatography on Superdex 200, and 54 kDa by chromatography on Sephacryl S-400 and Sepharose CL-6B. The holoprotein thus appears to exist in α*β* and $(\alpha\beta)_2$ forms, both of which may be active. The elution profile for the Superdex 200 column showed evidence for a small amount of α*β*, and the profile of the Superose CL-6B column showed evidence for a small amount of $(\alpha\beta)_2$. The nitrile hydratase from *Rhodococcus* sp. R312 migrated with an apparent molecular weight of 83 kDa [appropriate for $(\alpha\beta)_2$] on both the Superdex 200 and Sephacryl S-400 columns, with no evidence for α*β*, in agreement with published results (Nagasawa et al., 1986). The concentration of α*β* units in a sample of nitrile hydratase from *P. putida* NRRL-18668 was determined from the total amino acid concentration, and the amino acid composition of the protein was predicted by the gene sequence. This and the OD_{280} of the same sample were used to calculate $\epsilon_{280} = 1.7 \pm 0.1$ $(mg/mL)^{-1}$ cm^{-1}.

All nitrile hydratases that have been examined are metalloenzymes, containing either iron or cobalt. We analyzed the purified nitrile hydratase from *P. putida* NRRL-18668 by atomic absorption spectroscopy and found 0.9 cobalt/α*β* dimer, analogous to the 1 mol of iron/α*β* dimer seen in the *Rhodococcus* sp. R312 and *Rhodococcus* sp. N-771 enzymes (Jin et al., 1993; Odaka et al., 1996) and the 1 mol of cobalt/α*β* dimer found for the *R. rhodochrous* J1 enzyme (Brennan et al., 1996a).

The electronic absorption and CD spectra of the enzyme are shown in Figure 3. The absorption spectrum does not have substantial intensity at wavelengths longer than 350 nm. Were the cobalt iron present as a cobalt−corrin complex, substantial absorption between 22 200 and 18 200 cm^{-1} (450 and 550 nm) with an extinction coefficient of approximately 8000 M^{-1} cm^{-1} would be expected. Therefore the nitrile hydratase from *P. putida* NRRL-18668 is a non-corrin cobalt enzyme, analogous to the nitrile hydratase from *R. rhodochrous* J1 (Brennan et al., 1996a). The CD spectrum does show several partially resolved features in the visible region. Gaussian deconvolution of the CD spectrum yields peaks at 17 402, 20 267, 22 564, 28 441, 30 254, and 32 894 cm^{-1} (575, 493, 443, 352, 331, and 304 nm). Ligand field transitions in octahedral complexes may have significant intensity in the CD spectrum but are formally forbidden in the absorption spectrum. Therefore it is reasonable to assign the lower energy features in the CD spectrum (whose corresponding peaks in the absorption spectrum are weak) to d−d transitions of the cobalt ion. We speculate that the higher energy features in the CD spectrum at 304 and 331 nm (whose corresponding peaks in the absorption spectrum are strong) reflect ligand-to-metal charge transfer transitions.

Temperature Stability and pH- and Temperature-Dependence of the Catalytic Activity. Studies of the temperature stability and pH- and temperature-dependencies of catalytic activity were carried out using methacrylonitrile as the substrate. At 25 °C and pH 7.0, $K_M = 16 \pm 5$ mM and $V_{max} = 1250 \pm 30$ s^{-1} for this substrate. The temperature stability of the enzyme was assessed by incubating samples in 50 mM potassium phosphate buffer containing 40 mM sodium butyrate at pH 7.2 at various temperatures for an hour. Aliquots were taken for assay with 10 mM methacrylonitrile periodically. The activity is stable under these conditions up to about 35 °C (data not shown); after incubation at 50 °C for 20 min approximately 60% of the activity remains, and above 50 °C activity is quickly lost. The nitrile hydratase from *P. putida* NRRL-18668 is more stable than those from either *Pseudomonas chlororaphis* or *Rhodococcus* sp. N-771, which lose activity at temperatures above 30 and 40 °C, respectively (Nagasawa et al., 1987; Nagamune et al., 1990), and is comparably stable to the high molecular weight nitrile hydratase from *R. rhodochrous* J1 (Nagasawa et al., 1991). This trend in stability parallels the identity of the metal ion in the enzyme, with the cobalt-containing nitrile hydratases being more stable than the iron-containing enzymes.

The temperature-dependence of activity was assessed by adding enzyme kept on ice to 0.1 M potassium phosphate, pH 7.0, equilibrated at the appropriate temperature, and immediately assaying activity with 10 mM methacrylonitrile. Until the thermal instability of the protein becomes a factor above 40 °C, the activity increases approximately linearly above 5 °C (data not shown). This is in good agreement with the behavior of the other nitrile hydratases studied, with the temperature at which activity begins to decrease higher than for the iron-containing enzymes (Nagasawa et al., 1987; Nagamune et al., 1990), and about the same as the high molecular weight cobalt nitrile hydratase (Nagasawa et al., 1991).

The pH-dependence of enzyme activity is shown in Figure 4, expressed normalized to the activity at pH 7.2, 25 °C. Stock samples of enzyme were kept on ice in 0.1 M potassium phosphate, pH 7.0, and added to assay buffer containing 10 mM methacrylonitrile at 25 °C, made up with the following components: 0.2 M sodium acetate (pH 4.8−

FIGURE 2: Nucleotide and deduced amino acid sequences of *P. putida* NRRL-18668 nitrile hydratase genes. Double-underlined nucleotide sequences indicate potential ribosome binding sites. Single-underlined amino acid sequences were determined by peptide sequencing as described in Experimental Procedures.

5.7); 0.2 M sodium bicarbonate (pH 5.4−6.9); 0.2 M sodium phosphate (pH 6.6−7.8); 0.2 M Tris (pH 7.5−8.7); 0.2 M sodium borate (pH 8.4−9.3); 0.2 M TAPS (pH 8.1−9.0). Note that borate appears to have a deleterious effect on the activity. Nitrile hydratase from *P. putida* NRRL-18668 has a broad activity maximum between pH 7.2 and 7.8, with the activity decreasing sharply at higher pH, and more gradually to lower pH. The pH optimum is generally similar to that observed for the *Rhodococcus* sp. N-771 and *P. chlororaphis* enzymes (Nagasawa et al., 1987; Nagamune et al., 1990). The cobalt-containing enzyme from *R. rhodochrous* J1 shows a similarly broad pH maximum, but at lower pH, between 6 and 7 (Nagasawa et al., 1991).

Stereoselectivity of Hydration of CPIN. The purified protein catalyzes the hydration of (*S*)-CPIN at a much faster rate than it does (*R*)-CPIN (Figure 5). The reaction of 10 mM (*S*)-CPIN at room temperature was nearly complete after 3 h; no (*S*)-CPIN could be detected and the production of

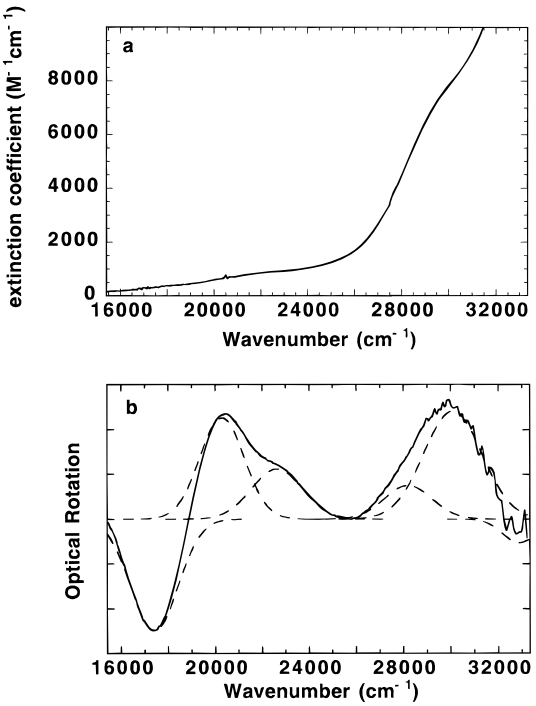

FIGURE 3: Absorption spectrum (a) and CD spectrum (b) of nitrile hydratase from *P. putida* NRRL-18668. The concentration of the sample used for b was 60 mg/mL; (—) data, (- - -) Gaussian curves fitted to the data.

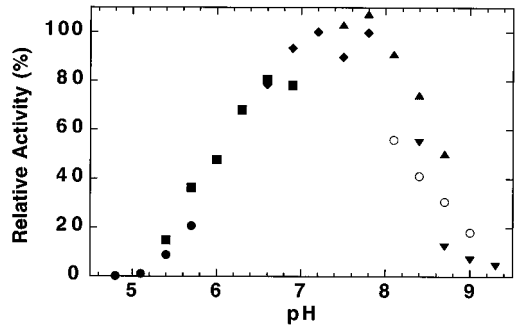

FIGURE 4: pH dependence of the activity of nitrile hydratase from *P. putida* NRRL-18668. Activity normalized to pH 7.2. Buffer: 0.2 M sodium acetate (●); 0.2 M sodium bicarbonate (■); 0.2 M sodium phosphate (◆); 0.2 M Tris (▲); 0.2 M sodium borate (▼); 0.2 M TAPS (○).

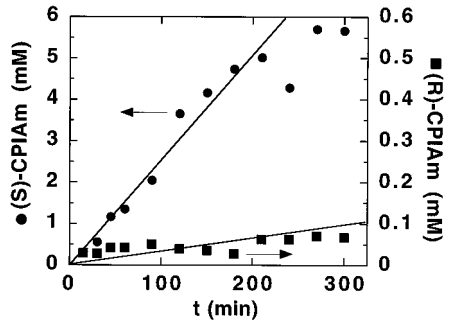

FIGURE 5: Hydration of (*S*)- and (*R*)-CPIN by *P. putida* NRRL-18668 nitrile hydratase. Assay conditions are described in the text; (●) (*S*)-CPIN; (■) (*R*)-CPIN.

(*S*)-CPIAm was complete. Under the same conditions there was no significant change in the amount of (*R*)-CPIN in the solutions, and only a small amount of (*R*)-CPIAm was detected. Comparable results were obtained by analysis of the hydration of racemic CPIN using chiral HPLC (data not shown), but the analysis is complicated by product inhibition of the hydration of (*R*)-CPIN by enzymatically generated (*S*)-CPIAm. Comparison of the approximate rates of the reactions (lines in Figure 5) suggests that the *S* enantiomer is hydrated approximately 200 times faster than the *R* enantiomer; however, the data are not very precise. We conservatively estimate that (*S*)-CPIN is hydrated at least 50 times faster than (*R*)-CPIN under these conditions.

DISCUSSION

Nitrile hydratases have been isolated and characterized from a number of bacteria. This is the first one to be shown to carry out stereoselective substrate hydration, a characteristic that enhances its potential as a catalyst for bioprocesses. In addition, this enzyme is an example of the class of nitrile hydratase that contains non-corrin cobalt, the only class of enzyme known to have naturally occurring Co^{3+} (Brennan, et al., 1996a).

Comparison of Amino Acid Sequences of Nitrile Hydratases. Comparisons of the amino acid sequences of the α and β subunits of nitrile hydratases were made using Pileup (Wisconsin Group, Version 8, Genetics Computer Group). Comparison of the *P. putida* NRRL-18668 nitrile hydratase α subunit shows amino acid identities ranging from 48.1% to 62.9%, with 37 amino acids conserved among all eight enzymes. The *P. putida* NRRL-18668 nitrile hydratase β subunit shows less homology to the others, with amino acid identities ranging from 35.0% to 40.9% and 28 amino acids conserved among all eight enzymes. The level of identity between the subunit amino acid sequences of the *P. putida* NRRL-18668 nitrile hydratase and the other nitrile hydratases is not unusual in these enzymes; for example, the two different nitrile hydratases found in *R. rhodochrous* J1 show 51.2% identity in their α subunits and 36.9% identity in their β subunits. A maximum parsimony analysis of the DNA sequences suggests that both the α and the β gene from *P. putida* NRRL-18668 are evolutionarily most closely related to those from *R. rhodochrous* J1 and most distantly related to those from *Rhodococcus* sp. N-774. The *P. putida* NRRL-18668 α gene appears to be more closely related to its nearest relative than does the *P. putida* NRRL-18668 β gene. This is consistent with the generally higher similarity observed among nitrile hydratase α subunits than among β subunits.

Of particular interest in the primary structures of nitrile hydratases is the highly conserved region from residues 111−120 in the α subunit (numbered according to *P. putida* NRRL-18668) that contains the sequence −VCTLCSC− (Figure 6). X-ray absorbance spectroscopy has shown the presence of at least two sulfur ligands to the metal ion in both the iron containing nitrile hydratase from *Rhodococcus* sp. R312 (Nelson et al., 1991; Scarrow et al., 1996) and the cobalt-containing nitrile hydratase from *R. rhodochrous* J1 (Brennan et al., 1996a). The conservation of the −VC(S/T)LCSC− sequence in all sequenced nitrile hydratases and the lack of other conserved cysteine residues suggest that at least two of these three cysteines are coordinated to the metal ion (Nelson et al., 1991). Although we have no direct evidence, it is reasonable that the cobalt−thiolate coordination exists in the *P. putida* NRRL-18668 nitrile hydratase as well, and that at least two of the cysteines in this sequence are the sulfur donor ligands. Inferential support for this idea comes from the absorption in the 300−350 nm region of the UV/vis spectrum. Synthetic low-spin Co^{3+}−thiolate

```
          111      120
   J1l  VVCTLCSCYP
  Rhod  IVCTLCSCYP
 18668  FVCTLCSCYP
   J1h  VVCTLCSCYP
  N774  IVCSLCSCTA
  Rery  IVCSLCSCTA
   B23  IVCSLCSCTN
 Ctest  ICCSLCSCTA
Consensus --C-LCSC--
```

FIGURE 6: Alignment of amino acid sequences for a portion of the nitrile hydratase α subunit. Alignments and consensus determined using PILEUP and PRETTY programs in the Wisconsin Sequence Analysis Package, Version 8.1. Consensus indicates residues identical among all sequences. N774, *Rhodococcus* sp. N-774 (Hashimoto et al., 1991); Rery, *R. erythropolis* JCM6823 (Duran et al., 1993); B23, *P. chlororaphis* (Nishiyama et al., 1991); J1l, *R. rhodochrous* J1 low molecular weight enzyme (Kobayashi et al., 1991); Rhod, *Rhodobacter* sp. (Mayaux et al., 1991); 18668, *P. putida* NRRL-18668 (this work); J1h, *R. rhodochrous* J1 high molecular weight enzyme (Kobayashi et al., 1991); Ctest, *Comomonas testosteroni* (Cerbelaud et al., 1995).

complexes show ligand-to-metal charge transfer bands at about 280 nm (Elder et al., 1978; Lydon et al., 1982). Perhaps the bands at 304 and 331 nm in the CD spectrum of nitrile hydratase from *P. putida* NRRL-18668 have $S\rightarrow Co^{3+}$ charge transfer character. Similar bands are seen in the CD spectrum of nitrile hydratase from *R. rhodochrous* J1 (M. J. Nelson, unpublished) which is known to contain cobalt−thiolate bonds (Brennan et al., 1996a).

The nitrile hydratases known to contain cobalt (*R. rhodochrous* J1, *P. putida* NRRL-18668) have threonine in the −VC(S/T)LCSC− sequence (position 113, *P. putida* NRRL-18668 numbering), whereas those known to contain iron (*P. chlororaphis*, *Rhodococcus* sp. N-774) have serine. It has been suggested previously that the primary determinants for metal binding and substrate specificity reside on the α subunit (Duran et al., 1993; Odaka et al., 1996). We hypothesize that there are two classes of nitrile hydratase α subunit: those that contain threonine in the cysteine-rich region and bind cobalt, and those that contain serine and bind iron.

We do not have much direct evidence as to the structure of the cobalt site in nitrile hydratase from *P. putida* NRRL-18668. The energies and number of the four lower energy transitions in the CD spectrum are reminiscent of CD spectra of low-spin Co^{3+} in a tetragonally-distorted octahedral ligand field (Figgis, 1966; Wakayama et al., 1983; Okamoto et al., 1987). The assignment of the cobalt ion to low-spin Co^{3+} is also indirectly supported by EPR data. The native protein is EPR silent, as expected for low-spin Co^{3+} (d^6; $S = 0$). Upon reduction with sodium dithionite and methyl viologen the spectrum in Figure 7 appears. This spectrum arises from low-spin Co^{2+} (d^7; S = 1/2); simulation of the spectrum gives $g_{1,2,3} = 2.222, 2.222, 1.995$ and $^{59}Co A_{1,2,3} = 26, 9, 99$ G; this may be compared to $g_{1,2,3} = 2.378, 2.206, 1.998$ and $^{59}Co A_{1,2,3} = 58, 11, 97$ G for the reduced form of the nitrile hydratase from *R. rhodochrous* J1 (Brennan et al., 1996a). There is at least a subtle difference in the coordination environments of the cobaltous ions in these two proteins that is apparent in the differences in their EPR spectra. The spectra of the reduced cobalt nitrile hydratases are very similar to those of low-spin Co^{2+}−porphyrin complexes (Wang et al., 1977; Wagner et al., 1981). Co^{2+}−porphyrin complexes have an A_{1g} ground state, the unpaired electron is in the d_{z^2} orbital, and axial N-donor ligands manifest themselves by resolvable

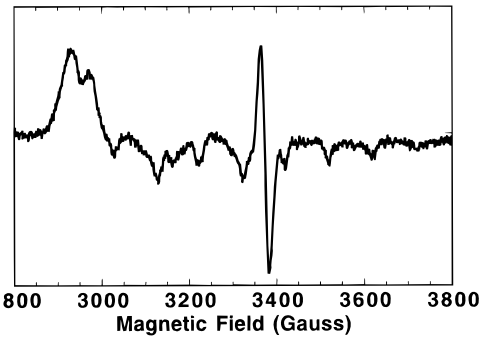

FIGURE 7: EPR spectrum of reduced nitrile hydratase from *P. putida* NRRL-18668. Native enzyme was reduced with methyl viologen and sodium dithionite. The sharp feature at 3370 gauss is the reduced methyl viologen radical.

^{14}N superhyperfine splittings in the highest field (g_3) feature of the spectrum (Wagner et al., 1981). Interestingly, there is no resolved ^{14}N hyperfine splitting in the g_3 features of the spectra of the reduced nitrile hydratases from either *R. rhodochrous* J1 or *P. putida* NRRL-18668. The absence of such resolved splittings may indicate that there is no nitrogen donor ligand (e.g., his, lys) on the g_3 axis in the reduced protein. ENDOR experiments to address the structure of the Co^{2+} form are in progress.

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