

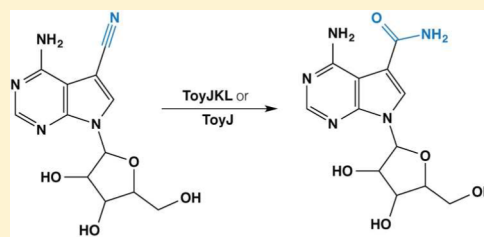
The Alpha Subunit of Nitrile Hydratase Is Sufficient for Catalytic Activity and Post-Translational Modification

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S Supporting Information

ABSTRACT: Nitrile hydratases (NHases) possess a mononuclear iron or cobalt cofactor whose coordination environment includes rare post-translationally oxidized cysteine sulfenic and sulfinic acid ligands. This cofactor is located in the α -subunit at the interfacial active site of the heterodimeric enzyme. Unlike canonical NHases, toyocamycin nitrile hydratase (TNHase) from *Streptomyces rimosus* is a unique three-subunit member of this family involved in the biosynthesis of pyrrolopyrimidine antibiotics. The subunits of TNHase are homologous to the α - and β -subunits of prototypical NHases. Herein we report the expression, purification, and characterization of the α -subunit of TNHase. The UV–visible, EPR, and mass spectra of the α -subunit TNHase provide evidence that this subunit alone is capable of synthesizing the active site complex with full post-translational modifications. Remarkably, the isolated post-translationally modified α -subunit is also catalytically active with the natural substrate, toyocamycin, as well as the niacin precursor 3-cyanopyridine. Comparisons of the steady state kinetic parameters of the single subunit variant to the heterotrimeric protein clearly show that the additional subunits impart substrate specificity and catalytic efficiency. We conclude that the α -subunit is the minimal sequence needed for nitrile hydration providing a simplified scaffold to study the mechanism and post-translational modification of this important class of catalysts.



Nitrile hydratases (NHases) catalyze the industrially important conversion of nitriles to amides under mild conditions and are a rare example of metalloenzymes that ligate trivalent cobalt or iron in noncorrin or nonheme environments.^{1,2} The active site complexes of both metal forms are similar in many respects: they are low spin with low redox potentials, and they share the same set of ligands, including three cysteine sulfurs, two of which are post-translationally oxidized, one to cysteine sulfenic acid and the other to cysteine sulfinic acid. The modified cysteine residues are necessary for activity.³ Along with the closely related thiocyanate hydrolase, NHases are the only known enzymes to employ these unusual modifications in metal binding and possibly catalysis.⁴

The mechanisms of post-translational cysteine modification and nitrile hydration remain unknown, but the importance of the β -subunit in each of these processes is implied by the fact that all known NHases possess a β -subunit or homologous subunits that are intimately involved with the active site complex. A highly conserved hydrogen bonding network links the two subunits,⁵ including bonds between the modified cysteine residues in the active site of the α -subunit and two β arginine residues, which when mutated drastically reduce activity or abolish it altogether.⁶

Uematsu and Suhadolnik first described the enzymatic activity of toyocamycin nitrile hydratase (TNHase) in *Streptomyces rimosus*, where it is involved in catalyzing the conversion of the nucleoside antibiotic toyocamycin to sangivamycin.⁷ We cloned this NHase in the course of studies involving the biosynthesis of pyrrolopyrimidine nucleoside

antibiotics.⁸ Sequence comparisons revealed that the TNHase encoded by the *toyJ*, *toyK*, and *toyL* genes is homologous to the family of nitrile hydratases. ToyJ is homologous to the α -subunit of NHase and ToyK and ToyL to the C- and N-terminal halves, respectively, of the β -subunit of NHase. However, unlike all previously reported NHase proteins that have two subunits, the *Streptomyces* protein is heterotrimeric (see Figure 1).⁹ ToyJKL is also homologous to thiocyanate hydrolase, which shares with ToyJKL a composition of three different subunits.¹⁰

Herein we report the expression and purification of recombinant ToyJKL and ToyJ and demonstrate that ToyJ alone is sufficient for the formation of the post-translationally modified, catalytically active NHase.

EXPERIMENTAL PROCEDURES

Cloning of ToyJ and ToyJKL. Plasmids containing all three subunits were prepared from those constructed and described previously.⁸ For expression of His₆-ToyJ, *toyJ* was cloned into the *Nde*I and *Xho*I sites of pET28a. ToyJKL, where ToyL was His₆-tagged was prepared by coexpression of *toyJ*, *toyK*, and *toyL* in *Escherichia coli* as follows. ToyJ was expressed by cloning *toyJ* into the *Nde*I/*Xho*I sites of pACYCDuet-1. ToyK was expressed by introducing *toyK* into the *Nde*I/*Xho*I

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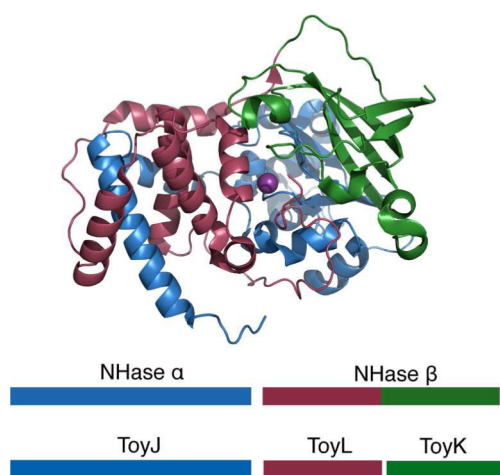


Figure 1. Structure of the two-subunit cobalt nitrile hydratase from *Pseudonocardia thermophila* (PDB 1IRE)²⁰ showing the active site metal closely associated with both subunits. The single β -subunit is depicted in both red and green showing where it is homologous to ToyK and ToyL.

sites of pCDFDuet-1. ToyL was expressed from a plasmid containing *toyL* in the *NdeI/XhoI* sites of pET28a.

Growth and Expression of ToyJ and ToyJKL. Expression plasmids were introduced into *E. coli* BL21(DE3) by electroporation and plated on Lennox broth (LB) agar containing 34 $\mu\text{g}/\text{mL}$ kanamycin for ToyJ alone or 34 $\mu\text{g}/\text{mL}$ kanamycin, 34 $\mu\text{g}/\text{mL}$ streptomycin, and 34 $\mu\text{g}/\text{mL}$ chloramphenicol for expression of ToyJKL. All fermentations contained these same concentrations of antibiotics. A single colony was used to inoculate 0.1 L LB medium which was grown overnight at 37 °C. The overnight culture was distributed evenly among six Fernbach flasks containing 1 L LB medium and grown to an $\text{OD}_{600\text{ nm}} \approx 0.5$ at which point the temperature was lowered to 30 °C, and protein expression was induced by addition of isopropyl- β -D-thiogalactopyranoside to a final concentration of 0.1 mM. The cultures were supplemented at the time of induction to 0.05 mM CoCl_2 . Cells were pelleted at 5000g after 8 h. The pellets were frozen in liquid nitrogen and stored at -80 °C.

Purification of ToyJ and ToyJKL. All steps were carried out at 4 °C. Cell paste (~ 15 g) was resuspended in 0.05 M potassium phosphate buffer containing 0.05 M imidazole and 1 mM phenylmethylsulfonylfluoride at pH 7.4. Cells were lysed with a Branson sonifier for a total of 15 min with 3 s bursts at 50% amplitude interspersed with 6 s pauses to equilibrate temperature. Insoluble components were removed by centrifugation at 18000g. Clarified lysate was loaded onto a 5 mL HisTrapHP column (GE Healthcare). Protein was eluted with a linear gradient from 0.05 M potassium phosphate buffer containing 0.05 M imidazole at pH 7.4 to one containing 0.5 M imidazole over 25 mL. Fractions containing TNHase were identified by an orange color and by SDS-PAGE, pooled, and concentrated using an Amicon Vivaspin Turbo centrifugal concentrator (PES 10K membrane). The protein was exchanged into 0.05 M HEPES-NaOH (pH 7.5) using a Bio-Rad 10DG column. Protein aliquots were frozen in liquid N_2 and stored at -80 °C.

Determination of Protein Concentration and Metal Content. Protein concentration was determined using the Bradford method (BioRad) with BSA as a standard. Garratt

Callahan (Burlingame, CA) performed ICP-OES to determine metal content. Amino acid analysis was conducted by the Molecular Structure Facility at the University of California, Davis. Empirically determined correction factors of 0.3 for ToyJ and 0.55 for ToyJKL were found to correlate the Bradford assay results to the amino acid analysis.

Activity Assays. The concentration of active enzyme was estimated to be that of cobalt content of the sample as determined by ICP-OES. Reactions to determine protein activity contained 0.05 M potassium phosphate (pH 6.5), varying concentrations of toyocamycin (Berry and Associates) from 0.1 to 2.5 mM and varying concentrations of 3-cyanopyridine from 0.05 to 0.75 M. The reactions were initiated by addition of 10 μM ToyJ or 10 nM ToyJKL and quenched at various times by addition of 30% (w/v) trichloroacetic acid to a final concentration of 5% (w/v). The products sangivamycin or nicotinamide and residual toyocamycin or 3-cyanopyridine were quantified by HPLC using an Agilent Zorbax Eclipse XDB-C-18 (4.6×50 mm) with an isocratic elution of 80% 0.1 M triethylammonium acetate (pH 6.8) and 20% methanol at a flow rate of 0.75 mL/min. Sangivamycin elutes at 2.3 min, and toyocamycin elutes at 3.4 min. Nicotinamide elutes at 1.3 min, and 3-cyanopyridine eluted at 2.3 min.

Thiocyanate degradation was assayed using the colorimetric method of Bowler modified as follows.¹¹ Potassium thiocyanate (1.5 mM) in 0.05 M HEPES-NaOH pH 7.0 was incubated with 0.25 mM ToyJ or 0.25 mM ToyJKL for 24 h. Control reactions contained buffer only. Samples were quenched by diluting 10 μL of the reaction mixture into 90 μL of 0.5 M FeNO_3 and 0.5 M HNO_3 . Precipitated protein was removed by centrifugation. Residual thiocyanate concentration in the \pm enzyme was compared using the absorbance of the iron thiocyanate complex at 455 nm. Solutions of potassium thiocyanate were used as a calibration standard. To ensure that the protein had not lost activity over the course of the reaction, an aliquot of the overnight mixture containing enzyme was also assayed with toyocamycin, and the rate of formation of sangivamycin was compared to an aliquot of freshly thawed enzyme to ensure that the protein was active.

UV-Vis Spectroscopy. UV-visible spectra were obtained using an Agilent 8453 UV-visible system.

Mass Spectrometry of ToyJ. Mass spectra were obtained using an LTQ Velos Orbitrap in the positive mode. ToyJ was prepared by exchanging 0.1 mL aliquots into deionized water using a nick column (GE Healthcare). This was lyophilized and resuspended in a 7:3 (v/v) solution of 0.1% trifluoroacetic acid and methanol to a final concentration of 0.1 mM. The solution was infused directly using an Advion Nanomate in LC Coupler at a flow rate of 200 nL/min with an applied voltage of 2.0 kV. Resolution was set to 100000 from 400 to 1800 m/z with 4.361 ms ion injection time with two microscans.

Chemical Reduction and EPR of ToyJKL and ToyJ. ToyJKL in 0.05 M potassium phosphate (pH 7.4) was lyophilized and resuspended in anaerobic water and 20% glycerol in a Coy anaerobic chamber. It was reduced by addition of sodium dithionite to a final concentration of 10 mM; the final concentration of cobalt was 0.5 mM as determined by ICP-OES. EPR samples of this were frozen in liquid nitrogen anaerobically.

Controls containing as isolated enzyme contained 0.15 mM ToyJ or 0.22 mM ToyJKL in 0.05 M HEPES-NaOH (pH 7.4) with 20% glycerol (v/v).

EPR spectra were obtained using a Bruker Elexsys E500 spectrometer at the microwave frequency of 9.335 GHz, microwave power of 2 mW, and the magnetic field modulation amplitude of 0.5 mT. The measurement temperature was 30 K.

Determination of Oligomeric State with Size Exclusion Chromatography. The oligomeric states of ToyJ and ToyJKL were determined using analytical size exclusion chromatography with a HiPrep 16/60 Sephacryl S200 column (GE Healthcare) and gel filtration protein standard (BioRad #151-1901) as indicated in Figures S1 and S2. The enzymes and standards were injected into the column pre-equilibrated with 0.02 M HEPES-NaOH (pH 7.4) and 0.15 M NaCl and eluted over 0.15 L at 0.1 mL/min. Elution volumes were determined using UV traces and SDS-PAGE analysis.

RESULTS AND DISCUSSION

Recombinant ToyJKL with ToyL N-terminally His₆-tagged was purified from *E. coli* grown in LB medium supplemented with CoCl₂ (50 μ M). Purified ToyJKL is orange and has a UV-visible spectrum that is typical of other cobalt-containing nitrile hydratases, which also exhibit low intensity shoulders at 314 and 429 nm (blue trace in Figure 2).¹² The protein

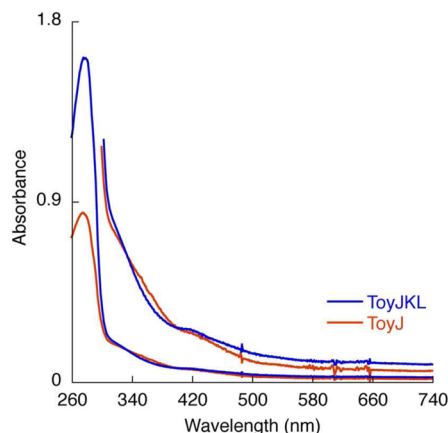


Figure 2. Comparison of UV-visible spectra of ToyJ and ToyJKL.

concentration and metal content of several preparations were determined by amino acid analysis and by ICP-OES, the results of which revealed there are 0.6 ± 0.2 cobalt atoms per heterotrimer (Supporting Figure S1, Supporting Information) in the recombinant heterologously expressed protein.

To delineate the role of each subunit in metal insertion, cysteine oxidation, and nitrile hydration, we explored if the α -subunit, which is the site of cobalt insertion and post-translational modification, could be expressed in a soluble and active form in the absence of the ToyK and ToyL subunits. Indeed, N-terminal His₆-tagged α -subunit, ToyJ, was readily expressed in the absence of the other two subunits. Gel filtration chromatography shows that ToyJ is monomeric (Supporting Figure S2, Supporting Information). The UV-visible spectrum of purified ToyJ is very similar to that of holo ToyJKL (orange trace in Figure 2). Amino acid analysis and ICP-OES revealed that the protein contains 1.1 ± 0.1 equiv of cobalt per monomer. Iron was not detected in any of the preparations. In the context of the cobalt content of ToyJKL, we propose that the metal preference of ToyJKL, ToyJ, and possibly the other homologous NHases derives solely from interactions with the α -subunit.

To our knowledge, this is the first report of successful expression and purification of a holo α -subunit of a nitrile hydratase. Therefore, ESI-MS was used to further confirm the presence of the post-translational modification, which was inferred from the similarities between the UV-visible spectra of ToyJ and ToyJKL. An Orbitrap Velos mass spectrometer was used because it affords the sensitivity and resolution to clearly differentiate between protein that is fully or partially modified. Deconvolution of the mass envelope (Supporting Figure S3, Supporting Information) shows that the most abundant species is consistent with the mass of ToyJ that lacks the starting methionine, as expected for a bacterial recombinant protein. Remarkably, the protein contains three additional oxygens and a cobalt. The mass of the most abundant species obtained from global deconvolution of the m/z envelope shown is 23353.086 amu for $[M + H]^+$. The theoretical mass corresponding to fully modified ToyJKL is 23352.9724 for $[M + H]^+$ as predicted using the QualBrowser Xtract program that is part of the Xcalibur 2.1.0 SP1.1160 operating software for the Orbitrap instrument. We note the molecular formula used to calculate the theoretical mass of modified ToyJ is that of the neutral molecule wherein three protons have been removed to accommodate the charge of the trivalent cobalt atom. The difference between the observed and calculated values is 0.114 amu, which is within the 10 ppm error of the instrument. The mass spectra of some preparations of ToyJ occasionally exhibit an additional m/z envelope corresponding to ToyJ with only two oxygens (see Figure 3). This is consistent with partial modification or loss of the sulfenic acid oxygen.¹³

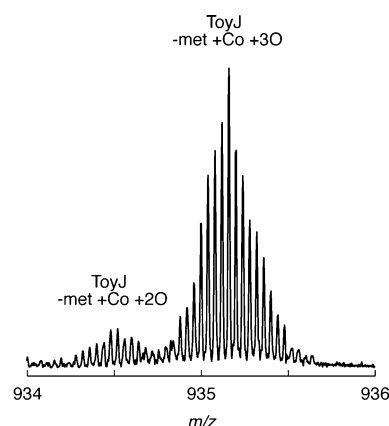


Figure 3. MS of ToyJ. The +25 charge state was selected and isolated in an Orbitrap Velos with a mass width of 1.6 and resolution of 100000.

Since several residues in the β -subunit of NHases have been previously shown to be intimately involved with the active site complex,¹⁴ we used electron paramagnetic resonance (EPR) to explore whether the absence of the β -subunit could affect the charge and spin state of the active site cobalt. EPR of ToyJKL and ToyJ as purified did not show any paramagnetic species (red trace in Figure 4), which suggests that the cobalt in each protein is in the low spin trivalent state. ToyJKL chemically reduced with dithionite (black trace in Figure 4) exhibits an EPR spectrum consistent with a low spin Co(II) center. The EPR spectrum of the reduced protein bears a striking similarity to that of Co(II) in corrin complexes, revealing the remarkable ability of the unique noncorrin set of ligands in NHases to enforce a low spin state.¹⁵ It was not possible to reduce ToyJ to

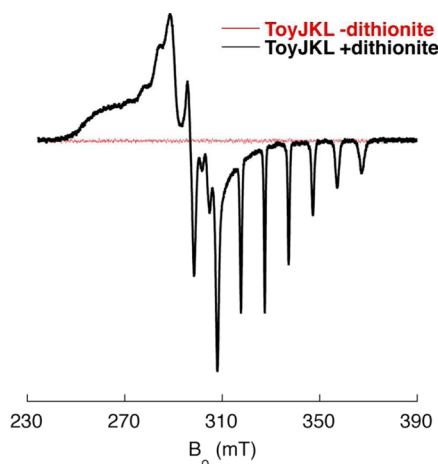


Figure 4. EPR of ToyJKL. EPR of ToyJKL. The EPR spectrum of ToyJKL (0.5 mM) chemically with sodium dithionite (10 mM) to the Co(II) state is shown in black. By comparison, no EPR signal is observed in the absence of dithionite as shown in the red trace with 0.22 mM ToyJKL. Experimental conditions: microwave frequency, 9.335 GHz; microwave power, 2 mW; magnetic field modulation amplitude, 0.5 mT; temperature, 30 K.

obtain the corresponding spectra for comparison as the protein quickly precipitated upon the addition of dithionite, presumably as a result of degradation of the active site complex, and only high spin Co(II) was detected in such samples.

Having established that ToyJ is sufficient for the biogenesis of the active site complex, we tested whether it is active against the natural substrate, toyocamycin. ToyJ catalyzes the time- and enzyme-dependent conversion of toyocamycin to the amide sangivamycin, which are readily separated via HPLC (Supporting Figures S4 and S5, Supporting Information). We carried out a steady-state kinetic comparison of ToyJ and ToyJKL with toyocamycin as substrate (shown in Figure 5 and summarized in Table 1). Maximal activity of ToyJ with toyocamycin is 360-fold lower than the wild type, and the K_M for the substrate is increased at least 500-fold. Since ToyJ does not saturate under the conditions of the assay, the fit provides a lower limit for this value. Nevertheless the data clearly show that the ToyK and ToyL subunits impart $\geq 10^5$ to the overall catalytic efficiency. Control experiments demonstrate that the activity of ToyJ requires intact protein, as boiled ToyJ is inactive (Supporting Figure S6, Supporting Information). At high substrate

Table 1. Kinetic Parameters of ToyJKL and ToyJ Catalyzed Hydrations of Toyocamycin and 3-Cyanopyridine

protein	substrate	k_{cat} (s^{-1}) ^a	K_M (mM)	k_{cat}/K_M ($mM^{-1} s^{-1}$)
ToyJKL	toyocamycin	159 ± 2	$2.8 \times 10^{-2} \pm 1 \times 10^{-3}$	5.7×10^3
ToyJ	toyocamycin	0.44 ± 0.04	15 ± 2	3.0×10^{-2}
ToyJKL	3-cyanopyridine	79 ± 5	99 ± 2	8.0×10^{-1}
ToyJ	3-cyanopyridine	35 ± 3	$1.1 \times 10^3 \pm 0.1 \times 10^3$	3.2×10^{-2}

^aAll reported activities are corrected for the cobalt content of the protein as determined by ICP-OES.

concentrations, the activity of ToyJKL deviates from the ideal Michaelis–Menten fit (Supporting Figure S7, Supporting Information). Controls show that this is not the result of DMSO used to solubilize the substrate. At this point we cannot rule out that the deviation results from other factors. The three-dimensional structure of ToyJKL is not known, but all published NHase homologues are dimeric, and one cannot exclude the potential for cooperativity.

To determine if TNHase is a promiscuous enzyme as with other nitrile hydratases, we compared the turnover of ToyJ and ToyJKL with 3-cyanopyridine, which is a common substrate and is turned over to nicotinamide.¹⁶ Both ToyJKL and ToyJ accept 3-cyanopyridine as substrate, the difference in k_{cat}/K_M values being only 25-fold (Supporting Figures S8 and S9, Supporting Information, and Table 1). This difference is orders of magnitude smaller than that with toyocamycin, which supports the notion that the ToyKL subunits are responsible for substrate specificity.

Yamanaka et al. recently reported that the closely related thiocyanate hydrolase could be engineered to hydrate nitriles by mutating arginine residues in the γ - and β -subunits to phenylalanine and tryptophan, respectively.¹⁷ We tested ToyJ alone for activity with thiocyanate. Even in the presence of substantial concentrations of enzyme and thiocyanate (0.25 mM ToyJ or ToyJKL and 1.5 mM thiocyanate), we were unable to detect any thiocyanate degradation after 24 h (Supporting Table S1, Supporting Information). We note that under these conditions, the protein retained 80–90% of the starting activity with toyocamycin, indicating that absence of activity was not due to protein denaturation. This observation

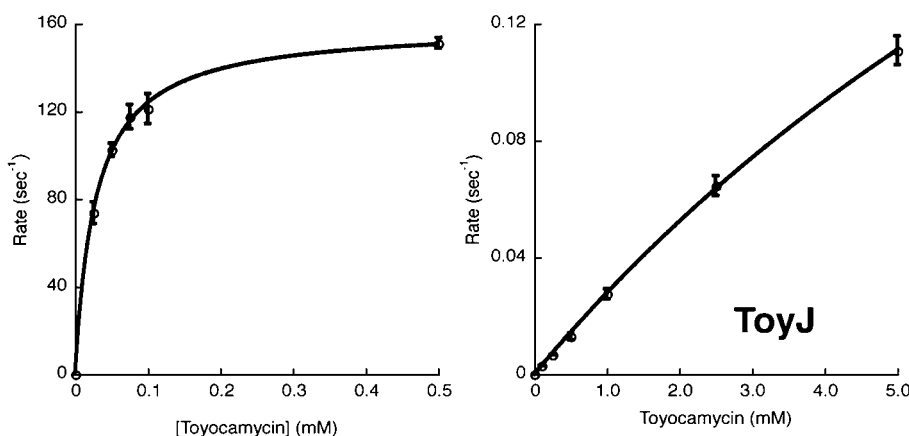


Figure 5. Michaelis–Menten plots of ToyJKL- and ToyJ-catalyzed conversion of toyocamycin to sangivamycin.

suggests that the substrate preference of TNHase for nitriles derives at least in part from the α -subunit

The activity of the α -subunit of a nitrile hydratase distinguishes among possible mechanisms of nitrile hydration by adding significant weight to those that rely solely on residues derived from the α -subunit. The mechanisms proposed by Hashimoto et al.¹⁸ and Martinez et al.¹⁹ involve mainly α -subunit residues and particularly the cysteine-sulfenate oxygen, known to be necessary for activity, to act as a general base in activating a water molecule to attack the metal bound nitrile or as the nucleophile itself, respectively.

This study provides insight into the vast differences in the catalytic efficiency between model complexes and NHases. The activity of ToyJ demonstrates what is possible in a single molecule with a significantly truncated hydrogen bonding network relative to the complete enzyme. Further studies on the contributions of each subunit will help to elucidate and model the significant catalytic efficiency found in NHases.

■ ASSOCIATED CONTENT

● Supporting Information

Figures of analytical size exclusion with ToyJKL and ToyJ; ESI-MS of ToyJ; HPLC trace of sangivamycin and toyocamycin; ToyJ activity at various enzyme concentrations; comparison of activity with ToyJ and boiled ToyJ; ToyJKL activity in hydrating toyocamycin and in hydrating 3-cyanopyridine and ToyJ activity in hydrating 3-cyanopyridine. Table of turnover of ToyJ and ToyJKL with thiocyanate. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

■ REFERENCES

- (1) Kobayashi, M., and Shimizu, S. (1996) Metalloenzyme Nitrile Hydratase: Structure, Regulation, and Application to Biotechnology. *Nat. Biotechnol.* 16, 733–736.
- (2) Kovacs, J. A. (2004) Synthetic Analogues of Cysteine-Ligated Non-Heme Iron and Non-Corrinoid Cobalt Enzymes. *Chem. Rev.* 104, 825–848.
- (3) Murakami, T., Nojiri, M., Hiroshi, N., Odaka, M., Masafumi, Y., Naoshi, D., Takio, K., Nagamune, T., and Endo, I. (2000) Post-Translational Modification is Essential for Catalytic Activity of Nitrile Hydratase. *Protein Sci.* 9, 1024–1030.

- (4) Arakawa, T., Kawano, T., Kataoka, S., Katayama, Y., Kamiya, N., Yohda, M., and Odaka, M. (2007) Structure of Thiocyanate Hydrolase: A New Nitrile Hydratase Family Protein with a Novel Five-Coordinate Cobalt(III) Center. *J. Mol. Biol.* 366, 1497–1509.
- (5) Nakasako, M., Odaka, M., Yohda, M., Dohmae, N., Takio, K., Kamiya, N., and Endo, I. (1999) Tertiary and Quaternary Structures of Photoreactive Fe-Type Nitrile Hydratase from *Rhodococcus* sp. N-771: Roles of Hydration Water Molecules in Stabilizing the Structures and Structural Origin of the Substrate Specificity of the Enzyme. *Biochemistry*. 38, 9887–9898.
- (6) Piersma, S. R., Nojiri, M., Tsujimura, M., Noguchi, T., Odaka, M., Yohda, M., Inoue, Y., and Endo, I. (2000) Arginine 56 Mutation in the β Subunit of Nitrile Hydratase: Importance of the Hydrogen Bonding to the Non-Heme Iron Center. *J. Inorg. Biochem.* 80, 283–288.
- (7) Uematsu, T., and Suhadolnik, R. L. (1974) *In Vivo* and Enzymatic Conversion of Toyocamycin to Sangivamycin by *Streptomyces rimosus*. *Arch. Biochem. Biophys.* 162, 614–619.
- (8) McCarty, R. M., and Bandarian, V. (2008) Deciphering Deazapurine Biosynthesis: Pathway for Pyrrolopyrimidine Nucleosides Toyocamycin and Sangivamycin. *Chem. Biol.* 15, 790–798.
- (9) Blackwell, E. A., Dodds, E. D., Bandarian, V., and Wysocki, V. H. (2011) Revealing the Quaternary Structure of a Heterogeneous Noncovalent Protein Complex through Surface-Induced Dissociation. *Anal. Chem.* 83, 2862–2865.
- (10) Katayama, Y., Narahara, Y., Inoue, Y., Amano, F., Kanagawa, T., and Kuraishi, H. (1992) A Thiocyanate Hydrolase of *Thiobacillus thioautotrophicus*. *J. Biol. Chem.* 267, 9170–9175.
- (11) Bowler, R. G. (1944) Determination of Thiocyanate in Blood Serum. *Biochem. J.* 38, 385–388.
- (12) Nojiri, M., Nakayama, H., Odaka, M., Yohda, M., Takio, K., and Endo, I. (2000) Cobalt-Substituted Fe-type Nitrile Hydratase of *Rhodococcus* sp. N771. *FEBS Lett.* 465, 173–177.
- (13) Nagashima, S., Nakasako, M., Dohmae, N., Tsujimura, M., Takio, K., Odaka, M., Yohda, M., Kamiya, N., and Endo, I. (1998) Novel Non-Heme Iron Center of Nitrile Hydratase with a Claw Setting of Oxygen Atoms. *Nat. Struct. Biol.* 5, 347–351.
- (14) Endo, I., Nojiri, M., Tsujimura, M., Nakasako, M., Nagashima, S., Yohda, M., and Odaka, M. (2001) Fe-Type Nitrile Hydratase. *J. Inorg. Biochem.* 83, 247–253.
- (15) Pilbrow, J. R. (1982) EPR of B12-Dependent Enzyme Reactions and Related Systems, in *B12 Vol. 1 Chemistry* (Dolphin, D., Ed.) pp 431–462, John Wiley & Sons, New York.
- (16) Nagasawa, T., Mathew, C. D., Mauger, J., and Yamada, H. (1988) Nitrile Hydratase-Catalyzed Production of Nicotinamide from 3-Cyanopyridine in *Rhodococcus rhodochrous* J1. *Appl. Environ. Microbiol.* 54, 1766–1769.
- (17) Yamanaka, Y., Arakawa, T., Watanabe, T., Namima, S., Sato, M., Hori, S., Ohtaki, A., Noguchi, K., Katayama, Y., Yohda, M., and Odaka, M. (2013) Two Arginine Residues in the Substrate Pocket Predominantly Control the Substrate Selectivity of Thiocyanate Hydrolase. *J. Biol. Sci. Bioeng.* 116, 22–27.
- (18) Hashimoto, K., Suzuki, H., Taniguchi, K., Noguchi, T., Yohda, M., and Odaka, M. (2008) Catalytic Mechanism of Nitrile Hydratase Proposed by Time-resolved X-ray Crystallography Using a Novel Substrate *tert*-Butylisonitrile. *J. Biol. Chem.* 283, 36617–36623.
- (19) Martinez, S., Wu, R., Sanishvili, R., Liu, D., and Holz, C. H. (2014) The Active Site Sulfenic Acid Ligand in Nitrile Hydratases Can Function as a Nucleophile. *J. Am. Chem. Soc.* 136, 1186–1189.
- (20) Miyanaga, A., Fushinobu, S., Ito, K., and Wakagi, T. (2001) Crystal Structure of Cobalt-Containing Nitrile Hydratase. *Biochem. Biophys. Res. Commun.* 288, 1169–1174.