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Formation of β-Hydroxy Histidine in the Biosynthesis of Nikkomycin Antibiotics

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Summary

Nikkomycins, a group of peptidyl nucleoside antibiotics produced *Streptomyces tendae* Tü901, are potent competitive inhibitors of chitin synthase. In this study, three nikkomycin biosynthetic enzymes, NikP1, NikQ, and NikP2, were overexpressed, purified, and characterized. The NikP1 activated L-His and transferred it to the carrier protein domain to form L-His-S-NikP1, which served as the β-hydroxylation substrate of NikQ. The β-OH-His was then hydrolytically released from NikP1 by NikP2. The results reported here substantiate our earlier proposal that the covalent tethering of an amino acid onto a carrier protein domain prior to downstream modification is a general strategy for diverting a fraction of the amino acid into secondary metabolism.

Introduction

Nikkomycins, a group of peptidyl nucleoside antibiotics produced by *Streptomyces tendae* Tü901, are potent competitive inhibitors of chitin synthase [1–3]. These antibiotics are structurally similar to the chitin synthase substrate UDP-*N*-acetylglucosamine and show high fungicidal, insecticidal, and acaricidal activity [1–3]. Nikkomycin X, a dipeptidyl nucleoside natural product isolated as a major component from the culture filtrate of *S. tendae* Tü901, contains two unusual amino acids, hydroxypyridylhomothreonine (HPHT) and aminohexuronic acid with an *N*-glycosidically linked 4-formyl-4-imidazolin-2-one (imidazolone) base (Figure 1). The other major component, nikkomycin Z, has a similar molecular structure to nikkomycin X, but the imidazolone base is replaced with uracil (Figure 1). Minor components of the culture filtrate are nikkomycins I/J, tripeptide antibiotics containing an additional C-terminal glutamate to the nikkomycin X/Z, respectively (Figure 1). The aminohexuronic acid derivatives isolated from some mutant strains are designated as nikkomycins C_X/C_Z (Figure 1).

Early radiolabeled precursor feeding experiments established that the pyridyl residue in HPHT originates from L-lysine and that L-His is the precursor of the imidazolone base [4]. The biogenesis of the aminohexuronic acid moiety is believed to be similar to that of the polyoxins, in which a ribose sugar is condensed with phosphoenolpyruvate (PEP) [5, 6]. The entire *nik* cluster has been isolated and heterologously expressed in *Streptomyces lividans* to produce all of the nikkomycins [7]. The ongoing sequencing of the *nik* cluster and systematic analysis of the disrupted mutants, as well as some initial biochemical characterizations, have led to the proposal of a biosynthetic pathway for the first amino acid residue HPHT [8, 9].

Although L-His was shown to be the precursor of the imidazolone base, insight into the formation of the base from histidine was only obtained recently when a gene encoding a heme-dependent protein (NikQ) was disrupted and shown to be involved in imidazolone biosynthesis, probably by β-hydroxylating L-His [10]. NikQ is similar to a distinct subgroup of the heme-dependent enzyme family that includes ORF20 of the chloroeremomycin cluster [11], NovI of the novobiocin cluster [12], CumD of the coumermycin A_1 cluster [13], and SimI of the simocyclinone cluster (direct deposit to the databank by L. Heide, accession number AF321122). NovI has been established to be a β-hydroxylase specific for L-Tyr covalently tethered to the phosphopantetheine (Ppant) prosthetic group of the peptidyl carrier protein (PCP) domain of the partner protein NovH [14]. NovH resembles a typical nonribosomal peptide synthetase (NRPS) with two domains, an L-Tyr specific adenylation (A) domain and a PCP domain [15, 16]. Free L-tyrosine and the small molecule mimic of aminoacyl-*S*-enzyme, L-Tyr-*S*-(*N*-acetyl)cysteamine thioester (L-Tyr-*S*-NAC), failed to be recognized and processed by NovI [14]. All of these factors prompted us to look for a potential didomain NRPS partner protein that may function with NikQ in the β-hydroxylation of L-His. Indeed, examination of the *nik* cluster in a surrounding region of *nikQ* quickly revealed the *nikP1* gene (Figure 2A), which encodes an A-PCP NRPS didomain [17]. Interestingly, there is a thioesterase (TE) gene, *nikP2*, flanked by the *nikP1* and *nikQ* genes, that encodes a protein which is similar to the free-standing type II TEs found in polyketide synthase (PKS) and NRPS clusters. We surmised that, analogous to the NovH and NovI pair, NikP1 and NikQ may function in concert to carry out L-His β-hydroxylation to give the β-OH-His-*S*-NikP1 intermediate. Subsequent hydrolysis by NikP2 would release free β-OH-His (Figure 2B). Further oxygenation on the imidazole ring and cleavage of the two-carbon unit (glycine) will lead to the desired imidazolone base found in nikkomycins X/I. Here we report the biochemical characterizations of three purified enzymes, NikP1, NikQ, and NikP2, for their role in the biosynthesis of β-OH-His. The results presented here reinforce our recent proposal that the A domain of a didomain NRPS can be used to divert a fraction of the amino acid pool for β-hydroxylation and further processing to a secondary metabolite [14].

Results

Expression, Purification, and Initial Characterization of NikP1, NikQ, and NikP2

All three genes were individually amplified from the genomic DNA of *S. tendae* Tü901 by PCR and cloned into

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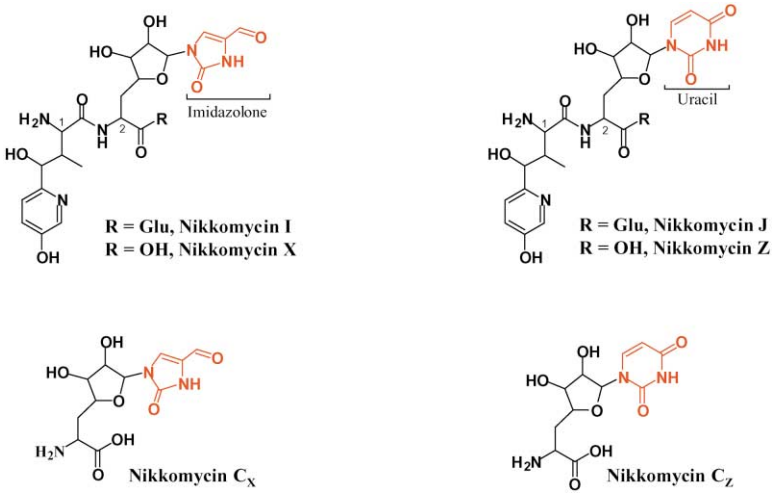

Figure 1. Chemical Structures of the Nikkomycin Antibiotics with Bases Highlighted in Red

appropriate pET vectors for protein expression in *E. coli*. Improved soluble protein yield (70 mg/L) of NikP1 was obtained by lowering the expression temperature to 24°C after induction with isopropyl β-D-thiogalactoside (IPTG). NikP1 was purified to near homogeneity in a single step by nickel-NTA affinity chromatography (Figure 3A). NikP1 was also coexpressed with Sfp, a phosphopantetheinyl-transferase (PPTase) from *Bacillus subtilis* [18], and purified similarly. NikQ was isolated fully loaded with heme cofactor when grown at 15°C for 72 hr without the addition of IPTG. Including $FeCl_3$ and δ-aminolevulinic acid in the culture medium was essen-

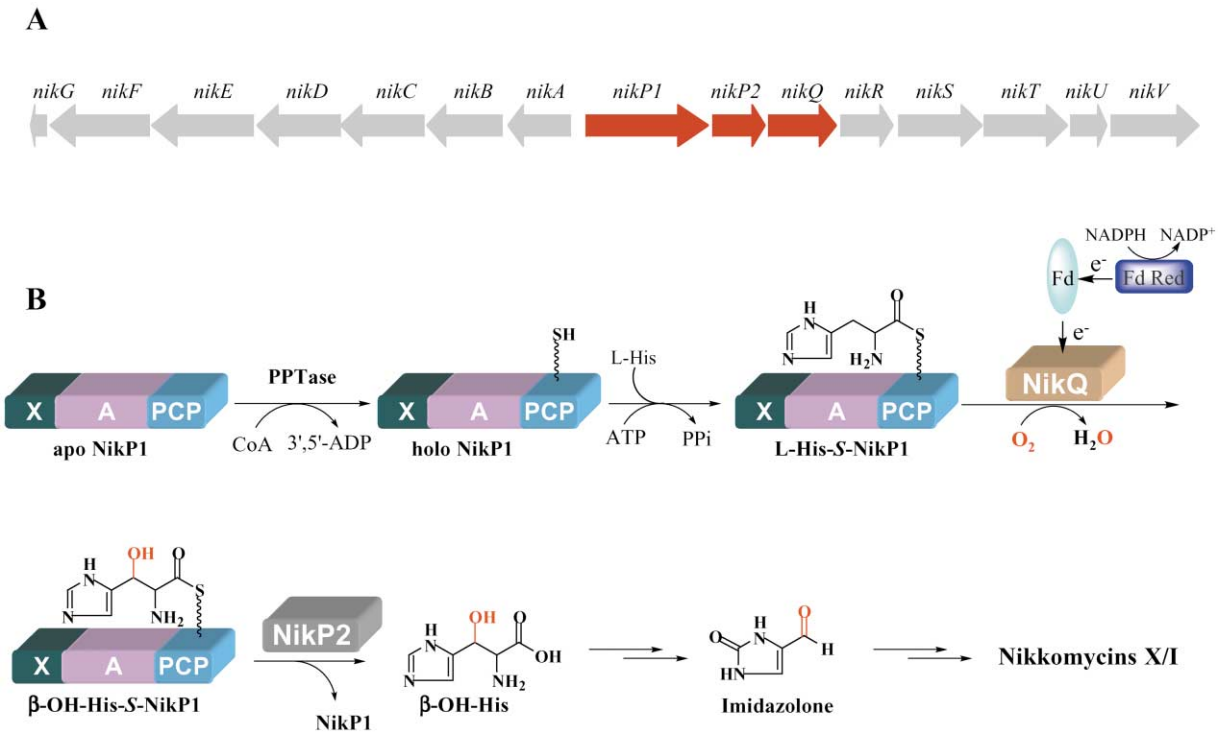

Figure 2. Biosynthesis of β-OH-His Found in Nikkomycins

(A) The partial nikkomycin biosynthetic gene cluster and three genes that are involved in β-OH-His biogenesis are highlighted in red.

(B) Proposed functions of NikP1, NikQ, and NikP2 in the biosynthesis of β-OH-His. The function of the N-terminal MbtH-like domain (designated as X) is not clear. The PCP domain in NikP1 can be primed by a PPTase to install the 4′-phosphopantetheine (Ppant) prosthetic group with free CoA. The A domain activates and loads L-His onto the free thiol of the prosthetic arm to generate L-His-*S*-NikP1 aminoacyl-*S*-enzyme. The β-hydroxylation reaction is effected by the heme-associated monooxygenase NikQ when molecular oxygen and external electrons are provided. Finally, β-OH-His is released from the PCP domain by a type II TE NikP2. Further transformations will afford the imidazolone base found in nikkomycins X/I.

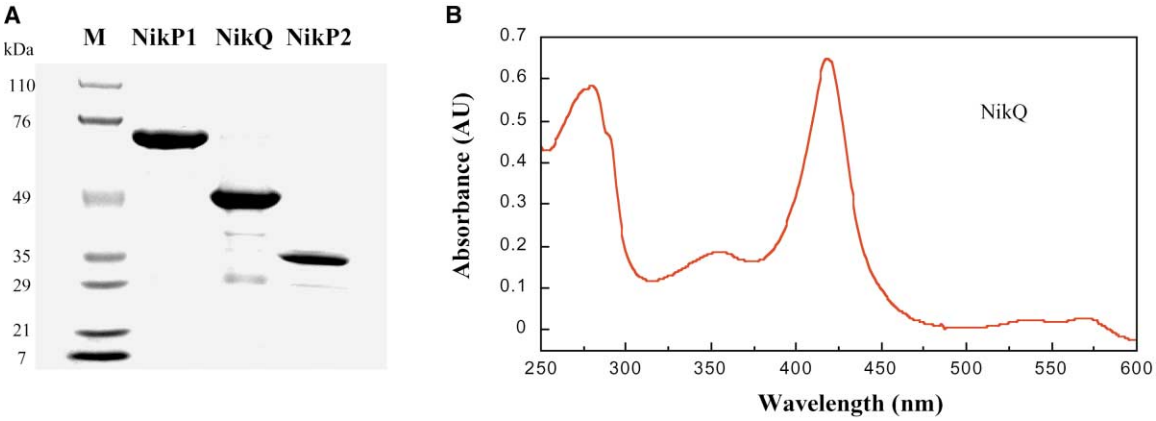

Figure 3. Purification of Nik Proteins

(A) Coomassie brilliant blue-stained 5%–15% SDS-PAGE gradient gel showing isolated NikP1, NikQ, and NikP2 proteins from *E. coli*.
(B) The UV-visible spectrum of the purified NikQ protein containing one equivalent of heme cofactor.

tial to obtain holo heme protein (Figure 3B). Unlike the cases of NikP1 and NikQ, initial attempts to produce soluble NikP2 protein failed when the *nikP2* gene was expressed from the pET16b vector alone. Inspection of the *nik* cluster revealed that the *nikP2* and *nikQ* are cotranscribed genes overlapping by 11 bases. The solubility of NikP2 protein in *E. coli* was much improved when *nikQ* and *nikP2* genes were expressed together from the pET16b vector. Production of both proteins was observed by SDS-PAGE, and NikP2 was readily isolated by nickel affinity chromatography when a His10 tag was specifically installed on the NikP2 N terminus.

Characterization of NikP1 as an L-His-Specific Didomain NRPS for L-Histidinyl-*S*-NikP1 Aminoacyl Enzyme Formation

Translated *nikP1* has 667 amino acid residues and contains three discernible domains by sequence analysis [17]. The N-terminal domain (1–80 amino acids) is similar to MbtH of the mycobactin cluster from *Mycobacterium tuberculosis*, a protein with unknown function. The remainder of NikP1 (90-667 amino acids) displays significant sequence similarity to an A-PCP didomain NRPS module. The C-terminal PCP domain has a conserved Ser626 surrounded by a canonical sequence (^{617}DFFQVGGHSLLAA^{630}) for posttranslational attachment of 4′-phosphopantetheine (Ppant) group to become a holo protein. We used a versatile PPTase, Sfp, for phosphopantetheinylation of the NikP1 apo PCP domain [18]. Time-dependent covalent attachment of [^{3}H]-Ppant to form holo NikP1 was observed by trichloroacetic acid (TCA) precipitation assays using [^{3}H]CoA as substrate (data not shown). The stoichiometry of Ppant incorporation was estimated to be ~50%. NikP1 protein coexpressed with Sfp was presumably modified in vivo and was used directly without in vitro modification.

The A domains of nonribosomal peptide synthetases are bifunctional catalysts for both aminoacyl-AMP formation and subsequent loading of the activated amino acid to the free thiol of the Ppant group on the cognate PCP domain. Detailed analysis of the amino acid sequence of the NikP1 A domain revealed that it contained all ten signature sequences (A1–A10) [16], but the potential substrate remained unclear even after a careful comparison with A domains of known specificity in the data bank. The amino acid code of the NikP1 A domain (DAESIAVITK) is significantly different from the only known L-His-specific A domain in BacC (DSEATAEVCK) involved in bacitracin biosynthesis [19, 20]. The other putative L-His-specific A domain (DSAAIAEVWK) lies in NRPS8 of the bleomycin cluster [21], but it is possible that β-OH-His is the native substrate for that A domain. To determine the substrate specificity of the NikP1 A domain and see if the NikP1 A domain specifically activates L-His using ATP as a cosubstrate, the classical ATP-pyrophosphate (PPi) exchange assay was used to monitor the reversible incorporation of [^{32}P]PPi into ATP in a substrate-dependent fashion [22]. Of all the common amino acids screened, L-His showed the highest activity, while the others (including D-His) possessed near background activity. The steady state kinetic parameters of the PPi-ATP exchange assay were determined to be $K_m = 100 \pm 7$ μM, $k_{cat} = 5.8 \pm 0.5$ min^{-1} for L-His, and $K_m = 490 \pm 40$ μM, $k_{cat} = 0.48 \pm 0.06$ min^{-1} for (2*S*,3*R*)β-OH-His. The NikP1 A domain exhibited a 60-fold specificity (k_{cat}/K_m) for L-His compared with β-OH-His.

The substrate-dependent PPi-ATP assays validated that the A domain of NikP1 selectively activates L-His as L-His-AMP. The subsequent thioesterification activity of the A domain was investigated by incubations where [^{3}H]-L-His and ATP were incubated with holo-NikP1 enzyme. The covalent transfer of L-His to the PCP domain is evidenced by the autoradiogram shown in Figure 4B, while Figure 4A shows the corresponding SDS-PAGE stained by Coomassie brilliant blue. Without pretreatment with Sfp, a much weaker radioactive band was observed, indicating that *E. coli*-produced NikP1 was largely in apo form when not coexpressed in vivo with Sfp. This result also implies that L-His loading was specific to the Ppant arm in the PCP domain. The time dependence of NikP1 aminoacylation was measured by TCA precipitation assays. The aminoacyl-*S*-NikP1 species accumulated up to a stoichiometry of 45% within 30 min under the conditions used in this report (data not shown).

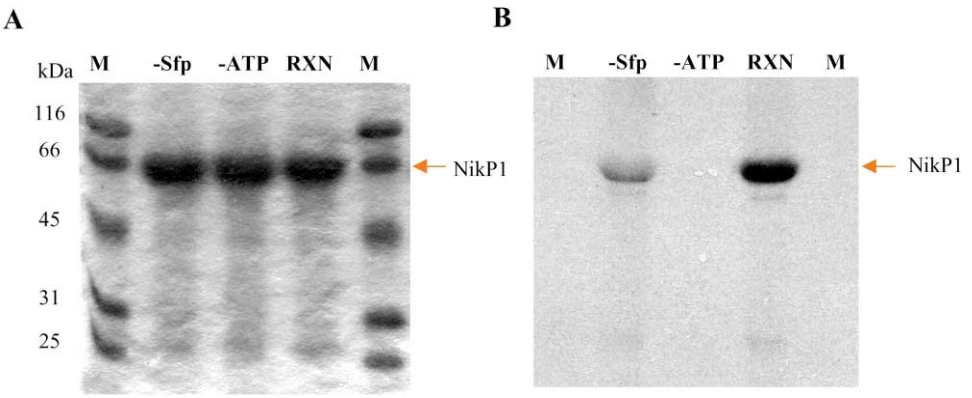

Figure 4. Self-Aminoacylation of NikP1 with [^{3}H]L-His

(A) Coomassie brilliant blue-stained 10% SDS-PAGE gel of various NikP1 samples. Lane 1, molecular weight marker; lane 2, control reaction without pretreatment with the PPTase Sfp; lane 3, control reaction without adding ATP; lane 4, complete reaction containing holo NikP1, ATP, [^{3}H]L-His, and Mg^{2+}.

(B) Autoradiogram of the gel in (A).

Characterization of NikQ as a Heme Hydroxylase for the Formation of β-OH-L-Histidinyl-*S*-NikP1

NikQ was isolated as a red heme protein when the culture medium was supplemented with δ-aminolevulinic acid and ferric iron ($FeCl_3$). NikQ was incubated with L-His-*S*-NikP1 to determine if it can function as a heme hydroxylase in the formation of β-OH-L-His-*S*-NikP1. For all heme monooxygenases, the organic substrate donates two electrons; two additional electrons are provided by the cosubstrate NADPH, mediated by two dedicated electron transfer proteins, ferredoxin and ferredoxin reductase. The cognate electron transferring proteins of *S. tendae* Tü901 have yet to be identified, so the commercially available spinach ferredoxin and ferredoxin reductase were used instead. By analogy to the NovH/NovI system, the hydroxylation product would be tethered to the PCP domain after the incubation complicating product analysis. The small molecule product was released from the PCP domain by KOH treatment and analyzed by strong cation exchange (SCX) HPLC. The retention properties of β-OH-L-His and L-His on a SCX analytical column were established using authentic standards. When [^{3}H]L-His-*S*-NikP1 was incubated with NikQ and other necessary components, HPLC analysis of the KOH eluant revealed a new radioactive peak possessing an identical retention time to that of the β-OH-His standard (Figure 5, trace A versus C). Coinjection experiments with various elution profiles confirmed the consistent coelution of the new radioactive peak with the β-OH-His standard. Parallel control experiments, omitting either NikQ or any component of the electron transfer chain, failed to give a detectable product signal (Figure 5, trace B). It should be noted that free L-His was not a substrate for NikQ (data not shown).

Characterization of NikP2 as a Thioesterase That Releases β-OH-Histidine from NikP1

The NikP2 protein exhibits sequence homology to type II TEs found in PKS and NRPS clusters. The exact role these TEs play in natural product biosynthesis has yet to be established. NikP2 contains the conserved active site Ser residue in the GxSxG motif and also the critical His residue essential for catalytic activity [23, 24]. In the NikP1/Q/P2 incubation, β-OH-His was detected in the supernatant of the reaction mixture (Figure 6, trace C), while there was no detectable product signal in the supernatant of the control reactions where NikP2 (Figure 6, trace B) or ferredoxin reductase was omitted (Figure 6, trace A). Prolonged incubation generated no detectable β-OH-His in the supernatant of a control reaction lacking NikP2, implying that the β-OH-His-*S*-NikP1 thioester linkage is stable and the release of the product was effected by NikP2 and not by adventitious hydrolysis. A time course of NikP2-catalyzed release of β-OH-His was recorded, showing that the β-OH-His peak grew linearly with time in the first 100 min, with over 30 turnovers observed for NikP2 during the incubation (Figure

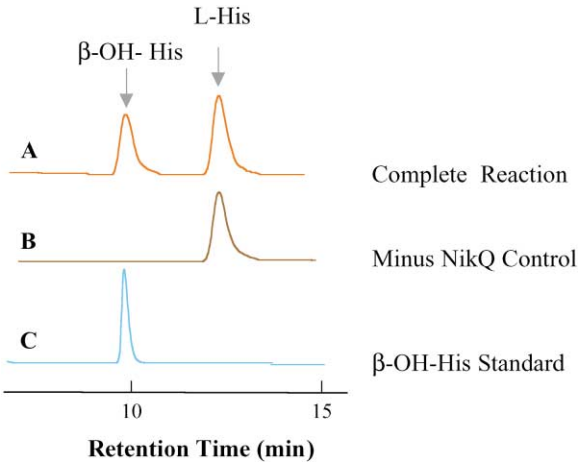

Figure 5. SCX-HPLC Analysis of the KOH-Released Small Molecule Product from the PCP Domain of NikP1 in a NikQ Catalyzed Reaction

Trace A, KOH-released materials from the L-His-*S*-NikP1/NikQ incubation; trace B, control incubation lacking NikQ; trace C, chemically prepared (2*S*,3*R*)β-OH-His. See "Incubation of NikQ with L-His-*S*-NikP1" for detailed information.

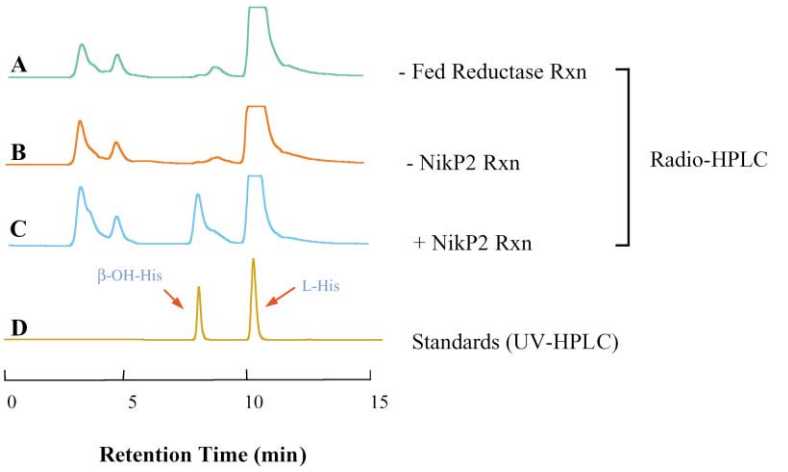

Figure 6. SCX-HPLC Analysis of the Supernatant of a NikP1, NikQ, and NikP2 Catalyzed Reaction

Trace A, control incubation omitting ferredoxin reductase; trace B, control incubation omitting NikP2; trace C, full NikP1/NikQ/NikP2 reaction; trace D, standards of L-His and (2*S*,3*R*)β-OH-His. See "NikP2 Incubation and Hydrolysis Time Course" for detailed information.

7B). A k_{obs} of 0.14 min^{-1} was calculated from the linear part of the time course (Figure 7A).

In Vitro Selectivity of NikP2

To assess the selectivity of NikP2 toward different aminoacyl groups presented by NikP1, time courses of the NikP2-catalyzed hydrolysis of [^{3}H]His-*S*-NikP1 and [^{3}H]β-OH-His-*S*-NikP1 were recorded (Figure 8). ATPase was added prior to the addition of NikP2 to remove excess ATP and prevent the reloading of L-His by the A domain of NikP1. NikP2 effectively hydrolyzed both L-His and β-OH-His from the PCP domain of NikP1 in a time-dependent manner, although NikP2 exhibited a modest (~2 fold) selectivity for β-OH-His-*S*-NikP1 (Figure 8, trace C versus trace D). The hydrolytic process for both substrates was almost complete within 10 min. Both [^{3}H]His-*S*-NikP1 and [^{3}H]β-OH-His-*S*-NikP1 species were kinetically stable under the experimental conditions in the absence of NikP2 (Figure 8, traces A and B). Without ATP, loading of [^{3}H]L-His was not observed (Figure 8, trace E).

Characterization of the NikP1/Q/P2 Reaction Product

A large-scale incubation of NikP1/Q/P2 allowed isolation of enough β-OH-His product for mass characterization. The product was separated by an isocratic elution (25 mM ammonium formate [pH 3.5]) on SCX-HPLC, lyophilized, and subjected to MALDI-TOF MS analysis. The measured mass of 172.0 ($M + H^+$ mode) matched well with the predicted value of β-OH-His ($C_6H_9N_3O_3$, calculated 171.1).

Although the newly generated chiral β-hydroxy center will be lost later in imidazolone biosynthesis (Figure 2B), it is still of interest to examine if the hydroxylation reaction effected by NikQ is stereospecific and to establish

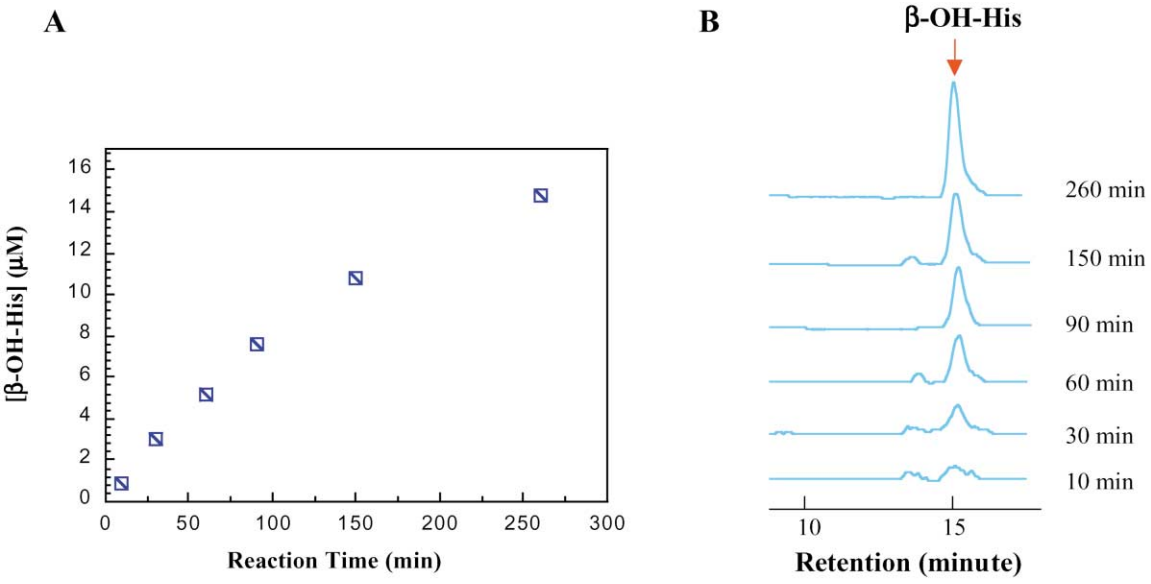

Figure 7. Time Course of NikP2-Catalyzed (2*S*,3*R*)β-OH-His Released from NikP1

(A) Plot of the product released by NikP2 over time calculated from the HPLC analysis.
(B) Stack of SCX-HPLC traces of each time points.

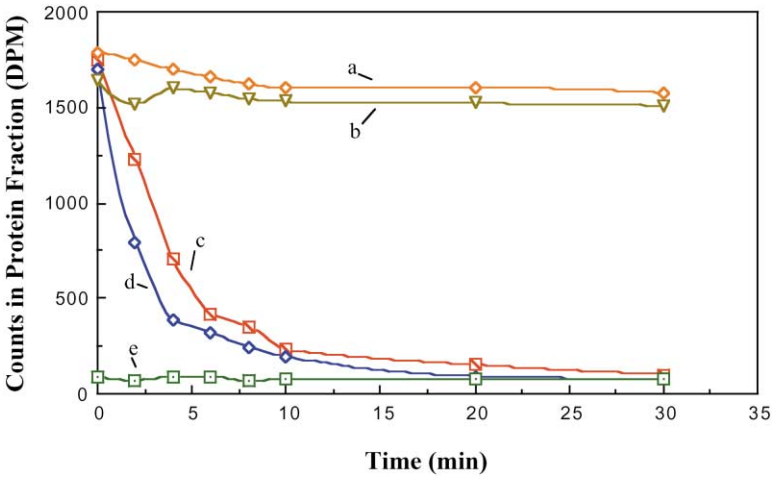

Figure 8. Time Course of NikP2-Catalyzed Aminoacyl-*S*-NikP1 Hydrolysis Analyzed by TCA Precipitation Assay

ATPase was added to the reaction mixture prior to the addition of NikP2 to remove the excess ATP and thus prevent L-His reloading by the A domain of NikP1. Trace A, control reaction not including NikP2; trace B, control reaction omitting NikQ and NikP2; trace C, release of L-His from NikP1 in the incubation omitting NikQ; trace D, release of β-OH-His from NikP1 by NikP2 in the full incubation; trace E, control incubation omitting ATP.

the absolute stereochemistry of the product. As shown in Figure 9, the SXC-HPLC-purified [^{3}H]β-OH-His had an identical retention time and peak shape as the chemically prepared (2*S*,3*R*)-3-OH-His (trace A versus trace B) when analyzed by chiral HPLC (Daicel ChiralPAK WH). Thus, NikQ is, in fact, stereospecific and generates an *R* chiral center of the β-carbon. Although the (2*S*,3*S*)-3-OH-His standard is not available for chiral HPLC analysis, it was unlikely that the 3*R*- and the 3*S*-diastereomer of β-OH-His would coelute, as the coelution of the enzymatic product with (2*S*,3*R*)-3-OH-His was always maintained under various HPLC conditions when control compounds D/L His and D/L Tyr were adequately resolved (data not shown). In conjunction with the fact that β-*pro*-*S* proton of L-His was stereospecifically retained in the radiolabeled precursor feeding experiments [4], β-hydroxylation of L-His catalyzed by NikQ proceeds with retention of configuration to produce (2*S*,3*R*)-3-OH-His.

Discussion

Nikkomycins X/I, peptidyl nucleoside antibiotics produced by *S. tendae* Tü901, are potent competitive inhibitors of chitin synthase [1–3]. These natural products contain an aminohexuronic acid residue with an *N*-glycosidically linked 4-formyl-4-imidazolin-2-one (imidazolone) base (Figure 1). Early radiolabeled precursor feeding experiments established that the imidazolone base was derived from L-His, presumably via a β-OH-His intermediate. In this report, an enzymatic route for the formation of β-OH-His used in the biosynthesis of imidazolone in nikkomycins was proven to involve a free-standing didomain NRPS module (NikP1), a heme hydroxylase (NikQ), and a type II thioesterase (NikP2). NikP1 specifically activates L-His as L-His-AMP and then thioesterifies it to the terminal thiol of the Ppant prosthetic group of the holo PCP domain (Figure 2B). The apo-PCP domain of NikP1 can be primed in vitro with the Ppant group by the versatile PPTase Sfp or in vivo by coexpression with an *sfp* gene-containing plasmid [18]. The transfer of the L-His-AMP to the holo-PCP domain was detected by SDS-PAGE/autoradiography and by TCA protein precipitation assays that detect and quantify the stoichiometry of L-His loading.

NikQ recognized L-His presented by NikP1 as an aminoacyl-*S*-enzyme and functioned as a β-hydroxylase with regio- and stereospecificity. NikQ could not hydroxylate free L-His, demonstrating that the A-PCP protein scaffold is critical for substrate recognition. NikQ functioned catalytically on the L-His-*S*-NikP1 substrate (data not shown) and the β-hydroxylated product remained covalently linked to the PCP domain of NikP1. KOH was used to cleave the thioester linkage, releasing the hydroxylated product whose identity was established to be (2*S*,3*R*)-3-OH-His by HPLC and by MALDI-TOF

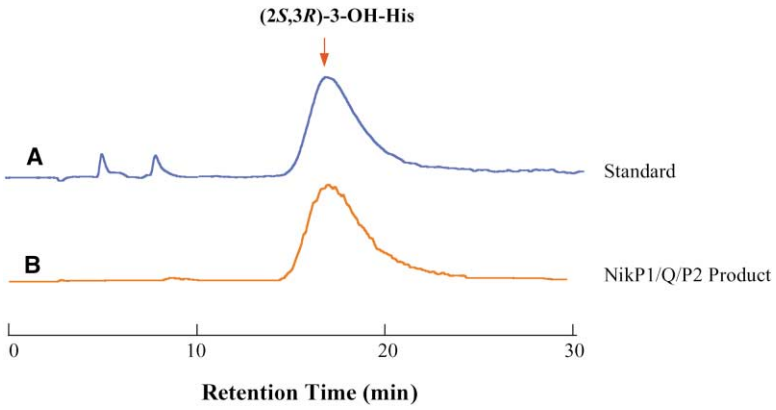

Figure 9. Determination of the Stereochemistry of the Newly Generated β-Center Introduced by NikQ Using Chiral HPLC with a ChiralPAK WH Analytical Column

(A) UV-HPLC trace of the (2*S*,3*R*)β-OH-His standard.

(B) Radio-HPLC trace of the NikP1/Q/P2 enzymatic product.

analysis. While further C-2′ hydroxylation on the imidazole ring and removal of a two-carbon unit are required to afford the imidazolone base (Figure 2B), we did not observe a second hydroxylation reaction catalyzed by NikQ under the assay conditions specified in this report. It remains to be seen whether an additional hydroxylase exists for the C-2′ oxygenation.

Many PKSs and NRPSs contain a terminal TE domain (type I) for the release of the fully processed polyketide or polypeptide chain, either in linear or cyclized form depending on the nature of the TE [25, 26]. While the role of the integrated type I TE has been clearly established as a chain releasing agent, the function of the type II TE, which is presented in many PKS and NRPS clusters as a free-standing identity, remains controversial. Deletion of type II TEs from various clusters adversely affects the in vivo level of metabolite production, and it has been proposed that type II TEs may play an editing or gate-keeping role during PKS/NRPS assembly by removing aberrant intermediates, thus keeping the assembly line unblocked [27, 28]. When NikP2, which is similar to type II TE proteins, was disrupted in *S. tendae* Tü901, the mutant strain failed to produce nikkomycins X/I (which contain the β-OH-His-derived imidazolone base). Notably, the NikP2 disruption did not affect production of the uracil base containing nikkomycins Z/J [17], suggesting a role for NikP2 in imidazolone biosynthesis. In this report, we clearly establish that NikP2 functions as a hydrolytic thioesterase to release the (2*S*,3*R*)β-OH-His from the PCP domain of NikP1. In a metabolic economic sense, it would be beneficial for the host bacteria to have a highly selective NikP2, but NikP2 failed to exhibit substantial selectivity for β-OH-His over His in the in vitro assay. Whether NikP2 exhibits higher selectivity in vivo or how such selectivity can be achieved remains to be elucidated. Potentially, selectivity can be achieved by controlling the relative concentrations of these three proteins, even though it was shown that *nikP1*, *nikP2*, and *nikQ* are cotranscribed genes [17]. Other possible means to achieve in vivo selectivity, e.g., by having a highly efficient NikQ (to keep L-His-NikP1 at a very low concentration) or by sequestering the L-His-NikP1 species with a coordinated interaction of NikP1 and NikQ proteins, could be envisioned. Nonetheless, NikP2, unlike other free-standing type II TEs, clearly has an essential function as a hydrolytic thioesterase in β-OH-His biosynthesis.

Previous studies have indicated that *nikP2* and *nikQ* disruption mutants produced nikkomycins Z/J, which contain a uracil base, at the same level as the wild-type strain [17]. While the *nikQ* disruption mutant failed to produce any detectable nikkomycins X/I (which have the L-His-derived imidazolone base), the *nikP2* mutant produced about 2% of the wild-type level [17], indicating that adventitious hydrolysis of the β-OH-His from the PCP domain of NikP1 occurred in the absence of the thioesterase NikP2. Unlike the *nikP2* and *nikQ* disruption mutants, the *nikP1* mutant could not produce the uracil containing nikkomycins Z/J, yielding only the uncoupled intermediate nikkomycin C_z (Figure 1) [17]. Since the consecutive *nikP1*, *nikP2*, and *nikQ* genes in the β-OH-His pathway are polycistronic (Figure 2A), single gene disruption mutants should give a similar phenotype. The abnormal disruption results strongly suggest the involvement of NikP1 in nikkomycin peptide bond formation in addition to its role in imidazolone biosynthesis. The presence of a small domain (1–80 amino acids) on the N terminus of NikP1 is intriguing. Similar MbtH homology can be found in many other NRPS systems, such as ORF6 in the chloroeremomycin cluster from *Amycolatopsis orientalis* [11], CumB in the novobiocin cluster from *S. tendae* Tü90 [13], ORFX in the calcium-dependent antibiotic (CDA) cluster of *Streptomyces coelicolor* and ORF13 in the bleomycin cluster [21]. The function of these small proteins in nonribosomal peptide biosynthesis is not clear, but this region of NikP1 may be important for coordinating all the necessary components for the conversion of nikkomycins Cz/C_x to nikkomycins Z/X (Figure 1), presumably through protein–protein interactions.

The adenylation, condensation (C), and carrier protein domains reconstitute the core elements in modules of the nonribosomal peptide synthetase assembly line [15, 16, 26]. For each amino acid monomer incorporated into a nonribosomal peptide, there is a corresponding module in the NPRS gene cluster. The order and number of the modules dictate the sequence and the length of the polypeptide. The initiation modules are typically A-PCP didomains, which are sufficient to activate and load the starter unit, while all other modules have an additional condensation domain to reconstitute the C-A-PCP minimal assemblage for extension and termination. The C domain is an amide bond formation catalyst that adds a new amino acid monomer to the growing polypeptide chain. In our studies of the novobiocin biosynthetic pathway, we recently discovered that the A-PCP didomain can be utilized for amino acid sequestration in the biosynthesis of β-hydroxy amino acid, and the results obtained in this report generalize this proposition [14]. In vancomycin group antibiotics, a similar set of proteins, ORF19 (A-PCP), ORF20 (heme protein), and ORF19 (thioesterase), exist for the biosynthesis of β-OH-Tyr for incorporation into positions 2 and 6 of the linear peptide [11]. Other β-hydroxy amino acids, found in various antibiotics, may also be synthesized using this molecular logic [29]. Cytochrome P450-type heme protein monooxygenases, and possibly nonheme iron oxygenases, have coevolved with NRPSs to recognize the aminoacyl thioester presented by the specific carrier protein domain scaffold, resulting in the β-hydroxylation of a specific fraction of the proteinogenic amino acid pool [29]. The β-hydroxy amino acid moiety can be subjected to further transformations and eventual incorporation into the natural product or can be hydrolyzed and utilized as a free acid by a downstream NRPS assembly line [29]. The strategy of covalent docking of a natural amino acid in a kinetically stable thioester linkage on a carrier protein could be a general practice for tailoring the particular amino acid by hydroxylation [14], epimerization [30, 31], desaturation [32], or some other modification prior to incorporation as a building block for the assembly of a secondary metabolite.

Significance

Nikkomycins X/Z are potent antifungal antibiotics containing an unusual 4-formyl-4-imidazolin-2-one (imi-

dazolone) base derived from L-His. This report establishes the roles played by three enzymes in the biosynthetic pathway to (2*S*,3*R*)3-OH-His, a key intermediate for imidazolone formation. The first step involves NikP1, an A-PCP didomain NRPS that selects, activates, and tethers L-His to the PCP domain. The heme-dependent hydroxylase NikQ can then recognize the sequestered L-His and perform a β-hydroxylation reaction in a regio- and stereospecific manner in the presence of molecular oxygen and two external electrons. In the third step, β-OH-His is hydrolytically released from NikP1 by the type II thioesterase NikP2. This molecular logic, in which an A-PCP NRPS is utilized to tether a proteinogenic amino acid as aminoacyl-*S*-enzyme for modification by tailoring enzymes, could represent a general strategy to sequester, and thus divert, a fraction of proteinogenic amino acid to provide building blocks for the construction of secondary metabolites.

Experimental Procedures

Cloning, Expression, and Purification of NikP1, NikQ, and NikP2

The *nikP1*, *nikQ*, and *nikP2* genes of nikkomycin cluster were individually amplified from *S. tendae* Tü901 (ATCC31160) genomic DNA by polymerase chain reaction (PCR). Primers NikP1-Nterm*Nde*I (5′-CCAAA CAT ATG GTC AAC CCG ATT CAT GAC GAC AAC-3′) and NikP1-CtermXhoI (5′-GGTTT CTC GAG GCT ACG TCC AGC GCG GGC CGC CGC-3′) were used to amplify the *nikP1* gene. The *nikQ* gene was amplified with primers NikQ-CtermNdeI (5′-CCC TTT CAT ATG CGC GTT GAC CTG TCC GAC CC-3′) and NikQ-NtermXhoI (5′-GGG TTA CTC GAG TCA GGC CCT CGG GAC GAA TTT-3′), while the *nikP2* gene was amplified together with *nikQ* gene using primers NikP2-NtermNdeI (5′-GGG TTT CAT ATG GCA CCG CGT CAG GCT GCC GCC-3′) and NikQ-CtermXhoI. The introduced restriction site on each primer is underscored. The PCR products were purified, digested with NdeI and XhoI restriction enzymes, and ligated to pET22b, pET28b, and pET16b vector to give C-terminally His6-tagged NikP1, N-terminally His6-tagged NikP2, and N-terminally His10-tagged NikQ constructs, respectively. The resulting plasmids were transformed into *E. coli* BL21(DE3) for protein overexpression.

Production and Purification of NikP1, NikP2, and NikQ Proteins

Cells harboring the NikP1 plasmid were grown in LB medium supplemented with 100 μg/mL ampicillin. Culture was grown at 37°C until OD_{600} reached 0.5, at which point temperature was lowered to 25°C, 50 μM of IPTG was added to induce protein production, and cell culturing was continued for 4 hr before harvesting. To generate phophopantetheinylated NikP1 protein, BL21(DE3) competent cells were cotransformed with *nikP1* construct and a plasmid containing the *sfp* gene. Cells were grown in LB medium in the presence of ampicillin and chloramphenicol. Cells were grown at 25°C to an OD_{600} of 0.6, at which time the temperature was lowered to 15°C, the cells induced with 60 μM IPTG and allowed to grow for an additional 18 hr. An overnight culture of NikQ (10 mL) was used to inoculate 1L LB medium (100 μg/ml ampicillin, 50 mg/L $FeCl_3$, 50 mg/L δ-aminolevulinic acid) and the culture was allowed to grow at 15°C for 72 hr without adding IPTG for induction. Cells containing NikP2 plasmid were cultured under same conditions as that of NikP1 overexpressed alone. A general procedure of Ni-NTA affinity chromatography was used to purify all three proteins. Cells were harvested by centrifugation, resuspended in binding buffer (25 mM Tris [pH 8.0], 400 mM NaCl, and 5 mM imidazole), and lysed by French Press (two passages at 15000 psi); cellular debris was removed by centrifugation (30 min at 15,000 g). Supernatant was incubated with 3 ml nickel NTA resin (Pharmacia) and allowed to bind in batch for 2 hr. The resin was decanted into a column and washed with 15 bed volumes of binding buffer. Protein elution was achieved by a step gradient washes with increasing imidazole concentration (10, 20, 40, 60 mM imidazole) and eluted with 200 mM imidazole. Fractions from the washes and elution were analyzed by SDS-PAGE, and those containing the target protein with desired purity were pooled, dialyzed against a buffer (25 mM Tris [pH 7.5], 50 mM NaCl, 1 mM DTT, and 10% glycerol), concentrated, flash frozen in liquid nitrogen, and stored at −80°C. The purity of NikP2 was not satisfactory and it was further purified with a Resource Q (Pharmacia) anion exchange column and proteins was eluted with a linear gradient of 0-1 M NaCl in 25 mM Tris (pH 8.0). Fractions containing protein were pooled, concentrated, flash frozen in liquid nitrogen, and stored at –80°C. The concentration of the purified protein was determined by Bradford assay.

Priming of the PCP Domain of NikP1 with Sfp

NikP1 was isolated as an apo protein from *E. coli* when overexpressed in the absence of the *sfp* plasmid. The PCP domain was modified posttranslationally with the phosphopantetheinyl group using purified Sfp. A TCA precipitation assay was adopted to measure the time course and the stoichiometry of the apo to holo conversion of NikP1 using free [^{3}H]CoA. Reactions (200 μl) contained 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5 mM tris-(2-carboxyethylphosphine (TCEP, Sigma), 50 μM [^{3}H]CoA (74 Ci/mol, DuPont NEN), 4.2 μM NikP1, and 0.3 μM of Sfp. Samples of 15 μl were withdrawn at various time points and quenched with 0.5 ml of 10% TCA. The precipitated proteins were pelleted, washed with 10% TCA, redissolved in 0.5 ml of 88% formic acid, and submitted for liquid scintillation counting. Percent modification of NikP1 was calculated from the specific activity of the [^{3}H]CoA and the protein concentration. Cold CoA was used for preparative priming of the NikP1 required by other studies. When coexpressed with Sfp, NikP1 was isolated as holo protein from *E. coli*, so in vitro priming with CoA by Sfp could be eliminated.

ATP-[^{32}P]PP_i Exchange Assay for A Domain Activity

Reactions (300 μL) contained 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5 mM TCEP, 2 mM ATP, 1 μM NikP1, and 1 mM [^{32}P]-pyrophosphate (3.2 Ci/mol). Reactions were initiated by addition of NikP1 and allowed to proceed at 24°C. Aliquots (50 μL) were withdrawn at various time points and quenched by the addition of a charcoal suspension (0.5 ml of 1.6% [w/v] activated charcoal, 4.5% [w/v] tetrasodium pyrophosphate, and 3.5% perchloric acid in water). The charcoal was pelleted by centrifugation, washed twice with quenching buffer lacking charcoal, resuspended in water (1 mL), and subjected to liquid scintillation counting. The amount of radioactivity was converted to reaction rate by using the specific activity of the pyrophosphate. Kinetic parameters of L-His were determined by carrying out reactions under the conditions described above, with the exception that the concentration of L-His was varied. Kinetic parameters of (2*S*,3*R*)-3-OH-His were determined from reactions containing 2 μM NikP1.

Aminoacylation of L-His on the PCP Domain of NikP1

To examine the second activity of the A domain, e.g., the covalent transfer of activated L-His-AMP to the terminal free thiol of pantetheine arm on the PCP domain of NikP1, a radio autographic study was performed. Loading of NikP1 with [^{3}H]L-His was established by analyzing the reaction by electrophoresis on a 10% SDS-PAGE gel. Control reactions, in which either Sfp or ATP was removed, were carried out and analyzed on the same gel. After electrophoresis, the gel was stained with Coomassie brilliant blue, destained, and soaked in Amplify (Amersham) for 15 min. The dried gel was exposed to a Biomar blue sensitive film (Marsh) for 36 hr at −80°C before developing. TCA precipitation assays were used to quantify the stoichiometry of auto aminoacylation of L-His by NikP1. Reactions were performed at 24°C in 100 μl volumes containing 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5 mM TCEP, 3.3 μM NikP1, 5 mM [^{3}H]L-His (5.3 Ci/mol), and 3 mM ATP. Aliquots of 10 μl were withdrawn at various time points and quenched with 0.5 ml of 10% TCA in the presence of 10 μl of the carrier protein BSA (10 mg/mL). The precipitated proteins were pelleted, washed, and counted for radioactivity. Percentage of loading was calculated from the specific activity of [^{3}H]L-His and the concentration of NikP1.

Incubation of NikQ with L-His-*S*-NikP1

A reaction mixture (250 μl) containing 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.2 mM [^{3}H]L-His (72 Ci/mol), 3 mM ATP, 2 mM TCEP, and 30 μM holo NikP1 was incubated at 24°C to load L-His onto the PCP domain of NikP1. After 2 hr of incubation, NADPH (2mM), spinach ferredoxin (5 μM), ferredoxin reductase (0.1 unit), and NikQ (2.5 μM) were added to the above incubation mixture to a final volume of 300 μl. The reaction was quenched with 0.5 ml 10% TCA after two additional hours of incubation. The precipitated proteins were pelleted by centrifugation, washed twice with 0.5 ml 10% TCA, and redissolved in 100 μl of 0.1 N KOH solution. The enzymatic product was released from the carrier protein by incubating for 10 min at 60°C. Routinely, a small amount of authentic (2*S*,3*R*)-β-OH-L-His was added in the workup as a nonlabeled carrier compound. The proteins were precipitated by acidifying the mixture with 5 μl of 50% TFA and removed by centrifugation. New product formation was analyzed by HPLC using a Zorbax 300-SCX strong cation exchange column (5 μm, 4.6 × 250 mm, Agilent) at 220 nm with a 1 mL/min flow rate. The elution profile was a linear gradient of 0%–20% B over 20 min (A = 50 mM potassium phosphate [pH 3.0]; B = 500 potassium phosphate [pH 3.0]). Radioactivity was monitored by an online liquid scintillation counter. Two control incubations were performed in parallel by removing NikQ or ferredoxin.

NikP2 Incubation and Hydrolysis Time Course

An incubation mixture (200 μl) contained 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 2 mM TCEP, 20 μM holo NikP1, 3.0 μM NikQ, 6.0 μM ferredoxin, 0.15 units ferredoxin reductase, 0.2 mM [^{3}H]L-His (72 Ci/mol), 2 mM NADPH, and 0.4 μM NikP2. The reaction was quenched by the addition of 100 μl 10% TCA after a 2 hr incubation. The supernatant was analyzed by SCX-HPLC following the procedure described above for β-OH-His analysis but a different elution gradient was used (0%–20% B over 15 min). To obtain a time course, an incubation mixture (400 μl) containing 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 2 mM TCEP, 20 μM holo NikP1, 3.0 μM NikQ, 0.5 μM NikP2, 6.0 μM ferredoxin, 0.15 units ferredoxin reductase, 0.2 mM [^{3}H]L-His (72 Ci/mol), and 2 mM NADPH was carried out at 24°C. NikP2 was added last to initiate the hydrolytic reaction and aliquots (20 μl) were withdrawn and quenched at 10, 30, 60, 90, 150, and 260 min with 10% TCA. After removal of precipitated proteins, the supernatants of the aliquots were analyzed by SCX-HPLC with a linear elution gradient of 20–40 mM potassium phosphate (pH 3.0) over 20 min. The integral of the product peak was converted to the amount of product formation using the specific radioactivity of [^{3}H]L-His.

Substrate Specificity of the NikP2 Catalyzed Reaction In Vitro

An experiment was designed to investigate the in vitro specificity of NikP2-catalyzed hydrolysis of L-His and β-OH-His covalently linked to the PCP domain of NikP1. A reaction mixture (175 μL) containing 85 mM Tris (pH 7.5), 10 mM $MgCl_2$, 6 mM TCEP, 5.3 mM [^{3}H]L-His (25 Ci/mol), 0.6 mM ATP, and 18.9 μM NikP1 was incubated for 1 hr at 24°C to load L-His onto NikP1. Then 30 μl ferredoxin (150 μM), 30 μl ferredoxin reductase (0.05 units/μl), 80 μl NikQ (50 μM), and 5 μl NADPH (100 mM) were added, and the resultant mixture was incubated for another hour. ATPase (10 μL, 0.1 unit) was then added to hydrolyze the remaining ATP, and the reaction was allowed to continue for a further 30 min. Finally, 20 μl of NikP2 (6 μM) was added to start the thioesterase reaction, aliquots (40 μl) were withdrawn at specific time points, and the proteins were coprecipitated with 100 μg of BSA using 10% TCA (500 μl). After centrifugation, the protein pellet was washed twice with 300 μl 10% TCA, redissolved with 300 μl 85% formic acid, and measured for radioactivity by a liquid scintillation counter. Four parallel incubations omitting NikQ, NikP2, NikP2 and NikQ, or ATP were also performed, and a time course was recorded for each.

Determination of the Stereochemistry and Mass Analysis of Enzymatic Product of NikP1/Q/P2

A large-scale reaction (2 mL) contained 75 mM Tris (pH 7.5), 10 mM $MgCl_2$, 3 mM TCEP, 0.4 mM L-His, 3 mM ATP, 4 mM NADPH, 15 μM NikP1, 6 μM NikQ, 1 μM NikP2, 15 μM ferredoxin, and 0.2 units ferredoxin reductase. Incubation was carried out at 24°C for 6 hr. After proteins were removed by filtration with a Centricon 10 (Amicon), the β-OH-His product was isolated by SCX-HPLC using an isocratic elution with 50 mM ammonium formate (pH 3.5). Repeated cycles of lyophilization removed the majority of the buffer salt, and the solid residue was subjected to MALDI-TOF analysis. Similarly, [^{3}H]-β-OH-His was enzymatically synthesized using the procedure described in the previous section and isolated by ammonium formate elution on SCX-HPLC. After lyophilization, the cold reference compound (2*S*,3*R*)-3-OH-His was mixed with the purified [^{3}H]-β-OH-His and the stereochemistry of the β center was established by chiral HPLC analysis with a ChiralPAK WH column (Daicel). Isocratic elution of 4.0 mM $CuSO_4$ was used at a flow rate of 1 mL/min. The UV absorbance (at 220 nm) and radioactivity detectors were used to monitor the retention times of the cold reference compound and the hot NikP1Q/P2 enzymatic product, respectively.

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