CHARACTERIZATION OF A NOVEL COBALTOENZYME AND ITS METALLOCHAPERONE IN RHODOCOCCUS

by

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ABSTRACT

Rhodococcus jostii RHA1 grows on various nitriles. Proteomic studies revealed that RHA1 utilizes an *nth* pathway, which includes an Fe$^{3+}$-dependent nitrile hydratase (NHase), to catabolize phenylacetonitrile. By contrast, RHA1 utilizes the *anh* pathway, which includes a novel acetonitrile hydratase (ANHase), a αβ-heterodimeric metalloenzyme, to catabolize acetonitrile.

To better characterize ANHase, a *Rhodococcus*-E. coli shuttle vector was constructed using a benzoate-inducible promoter. ANHase transformed nitriles containing up to four carbons, with highest specificity for acetonitrile and propionitrile. The enzyme contained cobalt, copper and zinc, and lacked sequence identity with known NHases. Accordingly, ANHase is proposed to belong to a previously unknown class of NHases.

The α subunit of ANHase possesses two potential metal-binding sequences: an N-terminal sequence rich in His and acidic residues; and a Cys-rich motif (CLLGCAC). The latter is reminiscent of the conserved catalytic motif in Co$^{3+}$-dependent NHases, suggesting that it coordinates Co$^{3+}$ in ANHase. The N-terminal sequence is similar to that found in some other cobaltoenzymes. Spectrophotometric analysis of a synthetic N-terminal peptide (MPDHGHDHGHNDACDSE) demonstrated that it formed a 2:1 complex with Co$^{2+}$ and that Cys is a probable ligand. Isothermal titration calorimetry (ITC) studies demonstrated that the peptide binds Co$^{2+}$, Zn$^{2+}$ and Ni$^{2+}$ with similar affinities ($K_d = 3.2–4.3$ µM).

Finally, AnhE, an 11-kDa protein whose gene occurs between the ANHase structural genes, *anhA* and *anhB*, was characterized. A Δ*anhE* mutant grew on acetonitrile only if *anhE* was provided in trans. Co-expression of *anhE* with *anhA* enabled reconstitution of ANHase in vitro.
in the presence of low concentrations of Co$^{2+}$. Co$^{2+}$ and other divalent metal ions stabilized a dimeric form of AnhE. ITC studies demonstrated that the dimer binds two Co$^{2+}$ ions with different affinities ($K_{d1} = 0.12$ nM, $K_{d2} = 110$ nM) but only one Zn$^{2+}$ ($K_d = 11$ nM) and one Ni$^{2+}$ ($K_d = 49$ nM). Together, these data suggest that AnhE acts as a dimeric metallochaperone to deliver cobalt to ANHase.

Overall, these studies provide insight into a novel NHase and its maturation. The findings have potential applications in the bacterial transformation of nitriles as well as implications for metal-trafficking in biological systems.
PREFACE

Parts of this thesis have been published in peer-reviewed journals. Characterization of ANHase appeared in Molecular Microbiology (Okamoto, S. and Eltis, L. D. “Purification and characterization of a novel nitrile hydratase from Rhodococcus sp. RHA1” *Mol. Micro.* 2007, 65, 828-838.). In this study, I was responsible for every aspect of the work.

Characterization of AnhE will be published in the Journal of Biological Chemistry (Okamoto, S., van Petegem, F., Patrauchan, M. A. and Eltis, L. D. “AnhE, a metallochaperone involved in the maturation of a cobalt-dependent nitrile hydratase” *J. Biol. Chem.* In Press.). In this study, I was responsible for every aspect of the work, except for the construction of *anhE* deletion mutant (provided by Dr. Marianna Patrauchan). Dr. Filip Petegem assisted with ITC data analysis and interpretation.
# TABLE OF CONTENTS

ABSTRACT................................................................................................................................... ii  

PREFACE..................................................................................................................................... iv  

TABLE OF CONTENTS .............................................................................................................. v  

LIST OF TABLES ..................................................................................................................... viii  

LIST OF FIGURES ..................................................................................................................... ix  

LIST OF ABBREVIATIONS ..................................................................................................... xi  

ACKNOWLEDGEMENTS ....................................................................................................... xii  

CHAPTER ONE: INTRODUCTION .............................................................................................1  
1.1 Nitrile compounds......................................................................................................................1  
1.2 Microbial nitrile catabolism ........................................................................................................2  
  1.2.1 Microbial catabolism of nitriles .....................................................................................2  
  1.2.2 Nitrile-hydrolyzing enzymes ......................................................................................3  
  1.2.3 Other nitrile catabolic genes .......................................................................................5  
  1.2.4 Regulation of nitrile catabolic genes ...........................................................................7  
1.3 Rhodococci ................................................................................................................................8  
  1.3.1 Biotransformation of nitriles by rhodococci ...............................................................8  
  1.3.2 *Rhodococcus jostii* RHA1 ........................................................................................8  
1.4 Nitrile hydratase .........................................................................................................................9  
  1.4.1 Structure of NHase ....................................................................................................9  
  1.4.2 Mechanism of NHase ...............................................................................................11  
1.5 Shuttle vector ...........................................................................................................................12  
1.6 Assembly of metalloenzymes ..................................................................................................14  
  1.6.1 Metal homeostasis .................................................................................................14  
  1.6.2 Cobalt in biology .....................................................................................................14  
  1.6.3 Metallochaperones ....................................................................................................16  
    1.6.3.1 Transfer of metal ions from the metallochaperone to their targets ...............18  
    1.6.3.2 ATP/GTPase activity for metal incorporation ...............................................19  
  1.6.4 Terminal metal-binding motifs in proteins .............................................................20  
  1.6.5 NHase activator proteins - Putative metallochaperones .......................................... 22  
1.7 Aim of this study......................................................................................................................23  

CHAPTER TWO: MATERIALS AND METHODS ........................................................................26  
2.1 Chemicals and reagents ...........................................................................................................26  
2.2 Bacterial strains, plasmids and growth conditions ...............................................................26  
2.3 Proteomics ..............................................................................................................................27
2.4 DNA manipulation ...................................................................................................................28
  2.4.1 Cloning of anh genes ........................................................................................................29
  2.4.2 Construction of shuttle vector ..........................................................................................30
2.5 Protein purification ..................................................................................................................30
  2.5.1 Purification of Ht-AnhC .....................................................................................................31
  2.5.2 Purification of ANHase ........................................................................................................31
  2.5.3 Purification of AnhA ...........................................................................................................32
  2.5.4 Purification of AnhB ..........................................................................................................32
  2.5.5 Purification of AnhE ..........................................................................................................33
  2.5.6 Purification of the reconstituted ANHase ..........................................................................33
2.6 In vitro reconstitution of ANHase ..............................................................................................34
2.7 Protein analysis ........................................................................................................................34
2.8 Molecular weight determination .............................................................................................35
2.9 Amino acid sequence determination .......................................................................................36
2.10 Metal analysis ........................................................................................................................36
2.11 Steady-state kinetics ..............................................................................................................37
  2.11.1 Phenol-hypochlorite colorimetric assay ........................................................................37
  2.11.2 HPLC-based assay .......................................................................................................37
  2.11.3 Analysis of steady-state kinetic data and evaluation of reaction conditions ...............38
2.12 Electronic absorption spectroscopy .........................................................................................38
2.13 Isothermal titration calorimetry (ITC) ..................................................................................39

CHAPTER THREE: RESULTS ........................................................................................................40
3.1 Nitrile catabolic pathway of RHA1 .........................................................................................40
  3.1.1 Growth study of RHA1 on a variety of nitriles .................................................................41
  3.1.2 Identification of nitrile catabolic enzymes .....................................................................41
    3.1.2.1 Identification of acetonitrile catabolic enzymes ...................................................41
    3.1.2.2 Identification of an acetonitrile catabolic gene cluster ...........................................42
    3.1.2.3 Proteomic analysis of other nitrile grown RHA1 ..................................................43
  3.1.3 Production and characterization of AnhC .........................................................................51
  3.1.4 Characterization of ANHase .............................................................................................53
    3.1.4.1 Purification of ANHase from RHA1 ......................................................................53
    3.1.4.2 Biophysical characterization of ANHase ..............................................................56
    3.1.4.3 Metal content of ANHase .....................................................................................57
    3.1.4.4 Kinetic properties and substrate specificity of ANHase ..........................................57
    3.1.4.5 Construction of *Rhodococcus*-E.coli shuttle vector ............................................61
3.2 Metal-binding site in AnhA ....................................................................................................65
  3.2.1 Sequence analysis ..........................................................................................................65
  3.2.2 IMAC of untagged AnhA ...............................................................................................66
  3.2.3 Spectrophotometric analysis Co$^{2+}$-binding to the N-terminal peptide .......................67
  3.2.4 Calorimetric analysis of metal ion binding by N-terminal peptide ..................................69
3.3 Characterization of AnhE .....................................................................................................72
  3.3.1 Growth phenotypes of RHA1 and mutant .....................................................................72
  3.3.2 Reconstitution of ANHase ..............................................................................................73
  3.3.3 AnhE cloning, expression and purification .....................................................................76
  3.3.4 Metal-dependent dimerization .......................................................................................76
3.3.5 Binding of Co^{2+} to AnhE by Electronic absorption spectra ...........................................78
3.3.6 Isothermal titration calorimetry .....................................................................................79
3.3.7 The role of His41 in cobalt binding .............................................................................84

CHAPTER FOUR: DISCUSSION .............................................................................................85
4.1 Discovery of anh gene cluster .........................................................................................85
4.2 Characterization of AnhC ...............................................................................................86
4.3 Characterization of ANHase .........................................................................................86
4.4 Other genes encoded on anh gene cluster ......................................................................89
4.5 Construction of the a shuttle vector ...............................................................................90
4.6 N-terminal metal binding motif in ANHase .....................................................................91
4.7 Characterization of AnhE – metallochaperone ...............................................................95
4.8 Concluding remarks .......................................................................................................101

BIBLIOGRAPHY ......................................................................................................................103
LIST OF TABLES

Table 1. Oligonucleotides used in this study........................................................................................................29

Table 2. Growth parameters of RHA1 on a variety of nitriles.................................................................41

Table 3. Products of the acetonitrile catabolic gene cluster (anh) of RHA1 and their proteomic identification.............................................................................................................................44

Table 4. Propionitrile and phenylacetonitrile catabolic gene products in RHA1 and their proteomic identification..............................................................................................................................49

Table 5. Purification of AnhE from E. coli GJ1158......................................................................................51

Table 6. Purification of ANHase from RHA1.................................................................................................54

Table 7. Steady state kinetic parameters of the ANHase for selected nitriles.............................................59

Table 8. Purification of ANHase using a Rhodococcus-E. coli shuttle vector............................................64

Table 9. Thermodynamic parameters for the binding of divalent metals by the AnhA N-terminal peptide.........................................................................................................................................71

Table 10. Thermodynamic parameters for the binding of divalent metals by AnhE...............................83
LIST OF FIGURES

Figure 1. Microbial nitrile metabolic pathways ................................................................. 2

Figure 2. Microbial nitrile hydrolysis pathways ................................................................. 3

Figure 3. The structure of heterodimeric Co-NHase from *Pseudonocardia thermophila* ...... 4

Figure 4. The Nitrile catabolic pathway of *P. chlororaphis* B23 ...................................... 6

Figure 5. The active site of Co-NHase from *P. thermophila* .............................................. 10

Figure 6. Proposed mechanisms of nitrile hydrolysis by NHase ........................................ 11

Figure 7. Theoretical mechanism for Cu$^{1+}$ transfer between ATX1 and CCC2 ............. 18

Figure 8. Transformation of the modified tetrapyrrole intermediate sirohydrochlorin into siroheme and cobalamin (vitamin B12) ........................................................................ 21

Figure 9. Multiple sequence alignment of C-terminal region of CbiX ............................ 22

Figure 10. The occurrence of genes encoding activator proteins in representative nitrile catabolic gene clusters .................................................................................. 22

Figure 11. The nitriles used in this study ........................................................................ 41

Figure 12. Proteomics analysis of RHA1 grown on acetate and ammonia and acetonitrile .... 43

Figure 13. Deduced acetonitrile catabolic pathway of RHA1 .......................................... 45

Figure 14. Proteomics analysis of RHA1 grown on propionic acid and ammonia and propionitrile ............................................................................................................. 47

Figure 15. Proteomics analysis of RHA1 grown on phenylacetic acid and ammonia and phenylacetonitrile .................................................................................................. 48

Figure 16. Nitrile catabolic gene clusters in RHA1 .......................................................... 50

Figure 17. SDS-PAGE of the purified AnhC from *E. coli* GJ1158 .................................... 51

Figure 18. Steady-state kinetic analyses of acetamide hydrolysis by AnhC ...................... 52

Figure 19. Relative activity of AnhC against a range of amides ....................................... 53

Figure 20. SDS-PAGE of the ANHase purified from RHA1 ............................................. 54
Figure 21. Mass spectrum obtained by MALDI-ToF analysis of ANHase ..................................55

Figure 22. Electronic absorption spectrum of the ANHase from RHA1 ..................................56

Figure 23. The ANHase-catalyzed transformation of acrylonitrile to acrylamide .......................58

Figure 24. The pH-dependence of ANHase activity ..................................................................60

Figure 25. The ben gene cluster located on chromosome of RHA1 and nucleotide sequence of the upstream region of benA .................................................................62

Figure 26. Construction of the Rhodococcus-E. coli shuttle vector .............................................62

Figure 27. Optical density at 600 nm and specific activity of RHA1 cell extract .......................64

Figure 28. Alignment of the N-terminal amino acid sequences of Co-NHase α subunits with that of AnhA from RHA1 ...................................................................................................66

Figure 29. Purification of AnhA using Ni-NTA chromatography ................................................67

Figure 30. The formation of peptide-cobalt complex ..................................................................68

Figure 31. Binding of N-terminal peptide to cobalt ion .............................................................68

Figure 32. ITC analysis of the binding of Co²⁺, Zn²⁺, Ni²⁺ and Cu²⁺ to N-terminal peptide ......70

Figure 33. The anh gene cluster located on pRHL2, the deduced acetonitrile catabolic pathway of RHA1 and the growth of RHA1 wild type strain, anhE deletion mutant and anhE deletion mutant complemented in trans on acetonitrile and acetamide ..................72

Figure 34. The reconstitution of ANHase ..................................................................................74

Figure 35. Electronic absorption spectra of recombinant ANHase from RHA1 and purified reconstituted enzyme ........................................................................................................75

Figure 36. Molecular weight determination of apo-AnhE and metal-bound AnhE by size exclusion chromatography ........................................................................................................77

Figure 37. Electronic absorption spectrum of cobalt-bound AnhE ..............................................79

Figure 38. ITC analysis of the binding of Co²⁺, Zn²⁺, Ni²⁺ and Cu²⁺ to AnhE .........................82

Figure 39. Proposed mechanisms of the influence of Co²⁺–binding on the activity of AnhE.....98

Figure 40. Proposed ANHase maturation mechanism .............................................................100
LIST OF ABBREVIATIONS

ANHase acetonitrile hydratase
EDTA ethylenediaminetetraacetic acid
ESI-MS electrospray ionization mass spectrometry
ESI-MS/MS electrospray ionization source tandem mass spectrometry
HPLC high-performance liquid chromatograph
ICP-MS inductively coupled plasma-atomic emission mass spectrometer
IEF isoelectric focusing
IMAC immobilized metal affinity chromatography
IPTG isopropyl-β-D-thio-galactoside
ITC isothermal titration calorimetry
LB lysogeny broth
MALDI-ToF matrix-assisted laser desorption ionization time of flight
NHase nitrile hydratase (EC 3.5.5.1)
Ni-NTA nickel-nitrilotriacetic acid
PCBs polychlorinated biphenyls
PMF peptide mass fingerprint
PMSF phenylmethanesulfonylfluoride
RT qPCR reverse transcriptase quantitative
SOD superoxide dismutase
tBuNC tert-butylisonitrile
2DGE 2D gel electrophoresis
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CHAPTER ONE: INTRODUCTION

1.1 Nitrile compounds

Nitriles are organic compounds that contain a cyanide (-C≡N) functional group. These compounds are widespread in the environment as a result of biological and industrial activity. Naturally occurring nitriles mainly comprise cyanoglycosides, cyanolipids and phenylacetonitrile (1) that are produced as defensive metabolites in plants (2). Cyanogenic glycosides and cyanolipids also serve as storage compounds for reduced nitrogen (3). In the chemical industry, nitriles extensively used as feedstocks, extractants, solvents, polymers, pharmaceuticals, pesticides and drug intermediates. For example, acrylonitrile and adiponitrile are frequently used for the production of polyacrylonitrile and nylon 66 polymers. Nitriles are also important intermediates in the organic synthesis of amides, amines, carboxylic acids, esters and ketones (4).

Most nitriles are highly toxic, mutagenic and carcinogenic in nature (5). For example, chronic exposure of rats to acrylonitrile induces neoplasias in their brain and stomach (6) and the International Agency for Research on Cancer (IARC) has classified acrylonitrile as a 'possible carcinogen for humans' (7). While the mechanisms of carcinogenesis for acrylonitrile in rats remain uncertain, oxidative stress is a likely cause of tumor development (8). In humans, the general toxicities of nitriles are expressed as gastric problems, bronchial irritation, respiratory distress and coma. Nitriles inactivate the respiratory system by tightly binding to cytochrome c oxidase (9).
1.2 Microbial nitrile catabolism

1.2.1 Microbial catabolism of nitriles

In the environment, nitriles are mostly degraded by bacteria. Bacteria from many genera, including *Acinetobacter*, *Corynebacterium*, *Arthrobacter*, *Pseudomonas*, *Klebsiella*, *Nocardia*, and *Rhodococcus*, utilize nitriles as a growth substrate and/or nitrogen source. These microbial activities have been studied for a variety of applications in bioremediation and green chemistry. For example, the production of acrylamide from acrylonitrile using *Rhodococcus rhodochrous* J1 is one of the most commercially successful microbial biotransformations (10). Similarly, the biotransformation of 3-cyanopuridine to nicotinamide and nicotinic acid is commercially viable (11). Finally, an efficient process for removing toxic acetonitrile using *Rhodococcus pyridinivorans* S85-2 from wastewater has been demonstrated (12).

**Figure 1.** Microbial nitrile metabolic pathways. Modified from (4).
Bacteria transform nitriles using at least three different chemical strategies (4) summarized in Figure 1: hydrolysis, oxidation (oxygenase) (13), and reduction (nitrogenase) (14). Of these strategies, that involving a two-step hydrolysis of the nitrile to the corresponding carboxylic acid via an amide appears to be the most prevalent and widespread. The two steps are catalyzed by either a nitrilase or by the successive actions of a NHase and an amidase (Fig. 2).

**Figure 2.** Microbial nitrile hydrolysis pathways

### 1.2.2 Nitrile-hydrolyzing enzymes

Nitrilase (EC 3.5.5.1), the first nitrile-metabolizing enzyme to be discovered (15), catalyzes the hydrolysis of a nitrile to the corresponding carboxylic acid and ammonia using two equivalents of water. A nitrilase superfamily, members of which occur in prokaryotes and eukaryotes, consisting of 13 classes of enzymes has been identified based on amino acid sequence identities (4,16). Despite the moniker of this superfamily, only one of the 13 classes is known to possess nitrilase activity: the other classes appear to act on amides or are involved in amide-condensation (17). However, all possess a Glu-Lys-Cys catalytic triad that catalyzes the hydrolysis of the non-peptide carbon-nitrogen bond via a thiol acyl-enzyme intermediate (18). In nitrile hydrolysis, the reaction is initiated by nucleophilic attack of the carbon atom of the nitrile
by the thiol of the catalytic triad and subsequence protonation of the nitrile nitrogen atom to form a thioimidate intermediate. Addition of a water molecule leads to a further protonation of the nitrile nitrogen which results in a release of ammonia. Attack of a second water molecule produces the acid and regenerates the enzyme (19,20).

NHases (EC 4.2.1.84) are αβ-heterodimeric metalloproteins that have been well characterized in Gram-negative (21) and Gram-positive bacteria, (22,23) (Fig. 3). The catalytically essential metal of these enzymes is either a non-heme Fe$^{3+}$ or non-corrinoid Co$^{3+}$ (4), the ligands of which occur in a Cys-rich motif, C(T/S)LCS(L/Y/T), located in the middle of the α subunit.

**Figure 3.** The structure of the αβ protomer of the heterodimeric Co-NHase from *Pseudonocardia thermophila* (PDB ID: 1IRE). The α and β subunits are coloured orange and gray, respectively. The cobalt ion is coloured purple. Modified from (22).

In Co-NHase, the second and seventh residues of this motif are Thr and Tyr, whereas in Fe-NHases they are Ser and Thr. The motif provides five metal ligands: three Cys residues and two
backbone amides (23). In addition to catalyzing R-CN hydration, the metal ions are considered to play a critical role in polypeptide folding. The structure and catalytic mechanism of these enzymes is discussed in more detail in sections 1.4 and 1.5.

The amides produced by NHases are further hydrolyzed by amidases (EC 3.5.1.4) to generate the corresponding carboxylic acids and ammonia. The amidases involved in bacterial nitrile catabolism typically belong to the amidase signature (AS) family and are encoded in the same gene cluster as the NHase subunits (24). The AS family is characterized by a conserved C-terminal region of approximately 130 amino acids known as the AS sequence which is rich in Ser and Gly residues (25). Amide hydrolysis is proposed to be catalyzed by a highly conserved Ser-Ser-Lys catalytic triad via a covalent acyl-enzyme intermediate (26).

1.2.3 Other nitrile catabolic genes

In general, the genes encoding NHases in bacteria are clustered in putative operons with genes encoding catabolic enzymes and other functions related to NHase function, such as amidases. For example, aldoxime dehydratase (OxdA), a heme-containing lyase, was isolated from \textit{P. chlororaphis} B23 (Fig. 4) (27-29). This strain utilizes butyraldoxime as a growth substrate and sole nitrogen source, and the purified OxdA catalyzes the dehydration of butyraldoxime to butyronitrile. A similar aldoxime dehydratase, phenylacetaldoxime dehydratase (PAOx), was found in \textit{Bacillus} sp. strain OxB (30). Although the production of phenylacetonitrile from \textit{z}-phenylacetaldoxime by PAOx resembles that of OxdA, a gene encoding a nitrilase instead of an NHase occurs upstream of PAOx.
The nitrile catabolic cluster in *Pseudomonas chlororaphis* B23 also contains genes encoding an amidase (21,31) and a short-chain acyl-CoA synthetase (AcsA), (Fig. 4) (32). The amidase exhibited activity toward a broad range of substrates, including aliphatic, unsaturated and aromatic amides. It had highest activity against isobutyramide, followed by propioamide and butyramide (31). The acyl-CoA synthetase produced butyryl-CoA from butyric acid and CoA as substrates, indicating the essential role of AcsA in acid utilization in the nitrile-degradating pathway. Together, the aldoxime dehydratase, amidase and acyl-CoA synthetase constitute an entire nitrile catabolic pathway.

![Figure 4](image)

**Figure 4.** The nitrile catabolic pathway of *P. chlororaphis* B23. **A.** Organization of nitrile catabolic gene cluster: *nhpR*, transcriptional activator; *oxdA*, aldoxime dehydratase; *amiA*, amidase; *nhpA*, NHase α subunit; *nhpB*, NHase β subunit; *nhpC*, P47K; *nhpS*, hypothetical protein; *acsA*, acyl-CoA synthetase (27,32). **B.** Deduced catabolic pathway based on **A** and biochemical studies summarized in the text.

Other functions that are often encoded in nitrile catabolic gene clusters are those involved in the uptake and trafficking of the metal ions required for NHase activity. Metal-trafficking proteins, exemplified by P47K encoded by the nitrile catabolic cluster of *P. chlororaphis* B23, are discussed in section 1.6.3. Metal ion transporters are exemplified by that encoded by the
**nhlF** gene in *R. rhodochrous* J1 which occurs downstream of the genes encoding the cobalt type L-NHase (33). NhlF is an integral membrane protein with eight membrane-spanning helices and possesses significant amino acid sequence similarity with a variety of nickel transporters such as HoxN from *Alcaligenes eutrophus* (34), HupN from *Bradyrhizobium japonicum* (35), and NixA from *Helicobacter pylori* (36). NhlF uses the proton gradient to drive the uptake of radiolabelled Co^{2+}. This activity was inhibited in the presence of Ni^{2+}, but not other divalent cations. NhlF activity was not detected in the absence of the growth substrate crotonamide, suggesting that *nhlF* is co-transcribed with the other nitrile catabolic genes on the cluster. The presence of a potent ρ-independent transcriptional terminator immediately downstream of the *nhlF*-containing operon (44) further supports this hypothesis.

### 1.2.4 Regulation of nitrile catabolic genes

Nitrile catabolic genes are transcriptionally regulated by at least two distinct systems: one comprising an amide-sensing system involving two components and a second comprising a XylS/AraC-type regulator. The amide-binding system was first investigated in *R. rhodochrous* J1 (37,38) where two genes encoding regulatory proteins were found upstream of genes encoding the H-NHase subunits. The encoded regulators, NhhC and NhhD, are homologues of AmiC and AmiA, respectively, which regulate the transcription of *amiE*, an aliphatic amidase gene from *Pseudomonas aeruginosa* (39). AmiA is a DNA-binding protein that represses the expression of *amiE*. AmiC lifts this repression when it binds acetamide.

The second transcriptional regulatory system is exemplified by NhpR(40). Recently found in *P. chlororaphis* B23 (Fig. 4), this is the first demonstrated transcriptional regulator of NHase-encoding genes that belongs to the XylS/AraC family. A homologue, NitR, also
regulates a nitrilase-encoding gene cluster in R. rhodochrous J1 (41). NhpR is a transcriptional activator that strongly induces transcription of the NHase-encoding genes. Indeed, when this strain is cultured in the presence of methacrylamide, the amount of NHase produced comprises more than 50% of the total soluble protein in P. chlororaphis B23 (42).

1.3 Rhodococci

1.3.1 Biotransformation of nitriles by rhodococci

Rhodococci are aerobic, high G+C content actinomycetes. They are most closely related to Nocardia, corynebacteria, Gordonia and mycobacteria, all of which contain mycolic acids in their outer membrane (43) and change morphology (cocci or filaments) in response to the environment. Rhodococci are considered to be environmentally and biotechnologically important due to their broad catabolic diversity (44). Indeed, they have been extensively studied for their ability to degrade hydrophobic pollutants, including polychlorinated biphenyls (PCBs). Rhodococci are also well known in nitrile transformation (45). Indeed, as mentioned previously, the production of acrylamide from acrylonitrile using a R. rhodochrous J1 is one of the most commercially successful examples in biotransformations used by the chemical industry (46). Nitrile degradation by rhodococci has been intensively studied because this organism is well suited for bioremediation, due to their ability to survive in nutrient poor environments (44).

1.3.2 Rhodococcus jostii RHA1

Understanding the basis of the catabolic versatility of rhodococci was greatly facilitated by the genome sequence of Rhodococcus jostii RHA1 (47). As of October 2009, three rhodococcal genomes have been completely sequenced and four others are partially completed.
RHA1 was originally characterized for its potent ability to degrade PCBs (48). However, a number of other catabolic pathways have now been elucidated in this organism, including those responsible for the catabolism of cholesterol, phenylacetate, and benzoate (49-51).

1.4 Nitrile hydratase

As noted above, NHases are αβ-heterodimeric metalloproteins that utilize Fe\(^{3+}\) or Co\(^{3+}\) to transform nitriles to amides.

1.4.1 Structure of NHase

The quaternary structure of NHases is \((αβ)_x\) where \(x\) is 9-10. The subunits range in size from 26 to 35 kDa. (4). The first crystal structure of a NHase was that of the Fe-NHase from *Rhodococcus* sp. R312 (52). This structure revealed the catalytic iron ion to be coordinated in a distorted octahedral geometry (Fig. 5) by the three Cys residues, two main-chain amide nitrogen atoms and a hydroxide ion (52). It was subsequently discovered in Fe-NHase of *Rhodococcus* sp. N-771 that two of Cys ligands are post-translationally modified to Cys-sulfenic acid (Cys-SOH) and Cys-sulfinic acid (Cys-SO\(_2\)H), respectively (53,54). Interestingly, although all the ligands to the iron ion are provided by the α subunit, in a holo-enzyme structure, both Cys-SOH and Cys-SO\(_2\)H are deprotonated and hydrogen-bonded with the guanidinium groups of two conserved Arg residues of the β subunit (βArg141 and βArg56) (55), which appear to stabilize the active site. The subsequent structure of a Co-NHase from *Pseudonocardia thermophila* JCM 3095 revealed that the metallocentres in Co- and Fe-NHases share many features, including the two oxidized Cys residues (22).
**Figure 5.** The active site of Co-NHase from *P. thermophila*. The Co$^{3+}$ ion is coordinated by three Cys sulfurs, two amide nitrogens, and one solvent species in a distorted octahedral geometry. Cys111 and Cys113 are post-translationally oxidized to sulfinic and sulfenic acids, respectively. Adapted from (56).

In general, Fe-NHases preferentially hydrolyze aliphatic nitriles whereas Co-NHases prefer aromatic ones (22,34,35,50-52). Substrate preference appears to be determined by the hydrophobic pocket which accommodates either the alkyl chain or aromatic ring of the nitrile. In *P. thermophila* Co-NHase, three residues from the β subunit line the substrate-binding pocket: βLeu48, βPhe51 and βTrp72. In Fe-NHase from *Rhodococcus* sp. N-771, these are replaced by βVal52, βVal56 and βTyr76, respectively, (22,52,54). These three residues are conserved in Fe-NHase from *P. chlororaphis* B23 and both Fe-NHases form narrower substrate-binding pockets which contribute to the recognition of aliphatic nitriles (22). By contrast, βLeu48 and βPhe51 of Co-NHase force βTrp72 (βTyr76 in Fe-NHase) away from the substrate-binding pocket, which contributes to the formation of a larger substrate binding pocket consistent with this enzyme’s higher substrate specificity for aromatic nitriles.
1.4.2 Mechanism of NHase

The mechanism of nitrile hydrolysis by NHase has been subject to some debate, with three mechanisms having been proposed over the past decade: (A) attack of the nitrile by the metal-bound hydroxide of the resting state enzyme; (B) activation of a water molecule by the metal-bound hydroxide, with the former attacking the nitrile (57,58); and (C) binding of the nitrile to metal ion, activating the former for attack by a water molecule (Fig. 6).

![Figure 6. Proposed mechanisms of nitrile hydrolysis by NHase. (A) Direct nucleophilic attack by metal bound hydroxide ion. (B) Metal-bound hydroxide activates water molecule. (C) Nitrile binds to metal ion directly and becomes susceptible to water attack.](image)

The last of these represents the most widely accepted mechanism whereby the substrate binds to the metal ion and displaces the solvent species (59). Nitriles are extremely resistant to hydrolysis as the limited electrophilicity of nitrile carbon makes it resistant to direct attack by water (60). The metal ion is thought to act as a Lewis acid, increasing the electrophilicity of the nitrile carbon and thus activating it for nucleophilic attack. The crystal structure of Co-NHase from *P. thermophila* JCM3095 bound with *n*-butyric acid, a weak competitive inhibitor, supports this mechanism. In this structure, a carboxylate oxygen atom is bound to the metal ion, while the metal-coordinated solvent species that occurs in the resting state enzyme is displaced (61).
Additional evidence is provided by time-resolved X-ray crystallography using Fe-NHase from *R. erythropolis* N-771 and tert-butylinonitrile (tBuNC), a slow substrate, which enabled visualization of a metal-bound nitrile species (62).

Although the structural data support the binding of nitrile to the metal ion, other details of the enzymatic mechanism remain unclear. Recently, two potential mechanisms were postulated. A recent study investigating the pH and temperature dependence of the kinetic parameters of Co-NHase from *P. thermophila* JCM 3095 (56) highlighted the role of a potential catalytic triad, Ser-Tyr-Trp. Of these residues, αSer112 is strictly conserved and occurs between two Cys of the active site motif, C(T/S)YCSC(Y/T), while βTyr68 and βTrp72 are conserved among the β subunits of Fe- and Co-NHases and occur in the motif YYE(H/K)(W/Y) (residues 68–72 in Co-NHase from *P. thermophila* JCM 3095). Based on the pH dependence of the $k_{cat}$ value as well as the solvent isotope effect, Mitra and Holz determined that an ionizing group occurs in the active site and proposed that αSer112 functions as a general base to deprotonate βTyr68 which in turn deprotonates a water molecule for nucleophilic attack of the metal-bound nitrile. The above-mentioned time-resolved X-ray crystallography of Fe-NHase from *R. erythropolis* N-771 with the slow substrate, tert-butylinonitrile, resulted in a different proposed mechanism (58). In this study, the appearance of electron density near the sulfenate oxygen of αCys114 as the reaction progressed led the authors to propose that this sulfenate oxygen acts as a general base to activate water for nitrile hydrolysis.

The occurrence of Co$^{3+}$ in NHases might be surprising since this metal ion exchanges ligands relatively slowly (63). However, Co-NHase demonstrates equivalent activity to Fe-NHase (57). Studies using model compounds indicate that the incorporation of anionic sulfur
into the coordination sphere dramatically increases the ligand exchange rate, especially in the presence of *trans*-thiolate sulfur (64).

### 1.5 Shuttle vectors

Recombinant DNA technology has greatly facilitated the study of low-abundance proteins, particularly with respect to the generation of the relatively large amounts of material required for biophysical methods. Nevertheless, many heterologously produced proteins do not possess their native activity. This can be due to the misfolding of the protein caused by the absence of proper folding system, such as chaperone proteins, or the lack of post-translational modification essential for the activity. Indeed, heterologous expression of NHases in *E. coli* usually results in the accumulation of insoluble protein lacking activity (65). Such problems have been overcome by co-expressing NHases with their “activator proteins” (see section 1.6.7) (66,67) or with GroES and GroEL, universal bacterial heat shock proteins (68).

An alternate strategy for producing rhodococcal NHases has been to produce them in a rhodococcal strain using an *Rhodococcus – E. coli* shuttle vector (69). Shuttle vectors are designed to propagate in multiple host species (70). The major advantage of this vector is it may be manipulated in species such as *E. coli*, for which molecular genetic methodologies have been well established. Once introduced into the vector, the target gene may be transferred into the appropriate species for further study. In general, the shuttle vector contains multiple origins of replication and selection markers for manipulation and propagation of the DNA, as well as inducible promoters to control gene expression. Examples of shuttle vectors include yeast-*E. coli* vectors, which are commonly used to produce eukaryotic proteins in *Saccharomyces*
cerevisiae (71), and actinomycete-\textit{E. coli} systems used (72) to facilitate the study and production of secondary metabolites (73).

1.6 Assembly of metalloenzymes

1.6.1 Metal homeostasis

Transition metal ions are commonly found as a natural component of proteins. Indeed, more than 30% of structurally characterized proteins possess metal ions (74). The latter play critical roles in electron transfer, oxygen transport, gene regulation and structure stabilization. Nevertheless, cells must strictly regulate cytosolic metal concentrations since transition metals are toxic (75). Such toxicity can arise from their binding to inappropriate sites in proteins, preventing the latter’s proper function, as well as the production of highly reactive oxygen-based free radicals. To balance the essential and highly toxic properties of metal ions, nature has developed intracellular trafficking systems to capture trace metal ions in their free forms and maintain metal homeostasis. The most studied, almost universally distributed metal-storage proteins are ferritin and metallothionein, which are specific for iron and copper/zinc ions, respectively. These proteins play essential roles in metal-storage and detoxification. As a result of such metal trafficking systems, free metal ions occur at extremely low intracellular concentration in the cell (76). For example, the concentration of free zinc ion in the cytosol of \textit{E. coli} is less than one per cell (77).

1.6.2 Cobalt in biology

Cobalt is a first-row transition metal located between iron and nickel in the periodic table. Although this metal occurs less frequently in metalloproteins than other first-row transition
metals such as iron, copper or zinc, it is an essential trace element for a variety of metabolic functions in both prokaryotes and eukaryotes. Cobalt exhibits oxidation states from Co\textsuperscript{1+} to Co\textsuperscript{4+}, although Co\textsuperscript{4+} is rare in nature (78). In proteins, cobalt is often found as a corrinoid prosthetic group, where it functions in electron transfer, transmethylation and rearrangement reactions. In non-corrinoid form, the catalytic role of the metal ion is often to activate water molecules for hydrolytic reactions.

Corrinoids are complex organometallic cofactors consisting of a tetrapyrrole-derived framework (corrin macrocycle) in which the cobalt atom is coordinated equatorially to four pyrrolic nitrogens (79). The most well known corrinoid is vitamin B\textsubscript{12}, also known as cobalamin. Related cofactors include chlorophyll and heme which contain magnesium and iron, respectively, instead of cobalt. Most prokaryotes and eukaryotes possess vitamin B\textsubscript{12}-dependent enzymes, with the notable exception of plants and fungi. However, only some of the bacterial and archaeal species are known to be able to synthesize cobalamin (80). To date, cobalamins are known to serve as cofactors in three different classes of enzymes: isomerases, methyltransferases, and dehalogenases (79). They are synthesized by two pathways: aerobic and anaerobic, each of which requires up to 30 enzymes (81). Within these pathways, a cobaltochelatase catalyzes the insertion of cobalt ion into the tetrapyrrole. In the aerobic pathway, the cobaltochelatase is a CobNST complex, whereas anaerobic pathways use either CbiX or CbiK (82).

Cobalt is also found in metalloenzymes in a form other than corrinoid cofactor. To date, eight non-corrin cobalt-containing enzymes have been isolated and characterized (78): methionine aminopeptidase, prolidase, NHase, glucose isomerase, methylmalonyl-CoA carboxytransferase, aldehyde decarboxylase, lysine-2,3-aminomutase, and bromoperoxidase.
Among these enzymes, His and Asp/Glu are the prevalent metal ligands; Cys is used as a ligand only in Co-NHase. Methionine aminopeptidase, which cleaves the N-terminal methionine from newly translated polypeptide chains in both prokaryotes and eukaryotes, contains a dinuclear Co\(^{2+}\) centre, coordinated by two Glu, two Asp, and a His residue (83). Methylmalonyl-CoA carboxytransferase, which mediates the transfer of CO\(_2^-\) from methylmalonyl-CoA to pyruvate and yield propionyl-CoA and oxaloacetate, contains a cobalt ion octahedrally coordinated by two His, one Asp, a water molecule, and the CO\(_2^-\) from the carbamylated Lys residue (84).

The presence of free cobalt ion is highly toxic to cells, as is the case with other redox-active metal ions. Cobalt toxicity is associated with various human diseases such as contact dermatitis, pneumonia, allergic asthma, and lung cancer (85). Recently, the regulation of the internal metal ions levels by various metal trafficking proteins have been discovered, especially in copper and iron homeostasis. However, because of the rare occurrence of cobalt in metalloproteins, very little is known about the storage and trafficking of this metal ion.

1.6.3 Metallochaperones

The tight regulation of cytosolic metal ion concentrations mentioned in section 1.6.1 prevents the existence of a “free metal pool” which is readily available for the synthesis of various metalloproteins. This raises the question of metal acquisition mechanisms, since simple diffusion and collision of metal ions and apo-enzymes are unlikely to occur (86). Accessory proteins are required for the maturation of many metalloenzymes whose physiological roles rely on transition metal ions in their active sites. These accessory proteins, called metallochaperones, have high affinity for specific metal ions.
Metallochaperones are cytosolic proteins that play essential roles in intracellular metal trafficking (72,78). While these proteins have only recently been discovered, they are now known to be widely distributed in prokaryotes and eukaryotes. Metallochaperones differ from universal metal-storage proteins whose purposes are mainly to store and detoxify transition elements. Instead, metallochaperones deliver the physiologically relevant metal ions to specific apo-enzymes and actively facilitate the maturation of the latter to the active metalloenzyme. Several metal-specific metallochaperones have been isolated to date, of which the best characterized are arguably those involved in the intracellular trafficking of copper. In eukaryotes, CCS, the copper chaperone for superoxide dismutase (SOD), binds a copper ion via a GMXCXXC motif and inserts the metal ion into Cu,Zn-superoxide dismutase (SOD) (87,88). The latter catalyzes the disproportionation of the superoxide anion to oxygen and hydrogen peroxide to help protect the cell against reactive oxygen species (89).

Other characterized metallochaperones include IscA, UreE and HypB. IscA, an ~12 kDa protein found in bacteria and eukaryotes, is essential for the assembly of iron-sulfur clusters. IscA exists as a tetramer and binds iron ion with sub-picomolar affinity (90). The probable iron binding site is located in a central channel formed at the interface of the monomers (91,92) and consists of Cys and Gln residues. UreE (~20 kDa) and HypB (~15 kDa) are prokaryotic metallochaperones involved in the maturation of the nickel-containing enzymes urease and [NiFe]-hydrogenase, respectively. These metallochaperones have $K_d$’s for nickel ion in the sub-picomolar range (85-87), and deliver this metal to the active sites of their cognate enzymes.
1.6.3.1 Transfer of metal ions from the metallochaperone to their targets

Metallochaperones have extremely high affinity for metal ions to compete with other metal storage proteins. However, their coordination environment must also allow for easy access for the target proteins so that the metal transfer occurs. It has been discovered that metallochaperones have evolved atypical coordination chemistries for this purpose. Indeed, many of these coordination chemistry are quite novel in biology.

The atypical coordination chemistry of metallochaperones is illustrated by the copper metallochaperones in yeast. ATX1, the first copper chaperone to be discovered in *S. cerevisiae* (88), sequesters copper and delivers it to specific protein targets in the cytoplasm (87,93) known as P-type copper transporters. One such copper-transporting ATPase is Ccc2, also known as Wilson disease protein (94), which transports Cu$^{1+}$ into a trans-Golgi compartment where it is incorporated into copper oxidases (91). Both ATX1 and Ccc2 bind Cu$^{1+}$ in a low-coordination-number environment by means of two Cys residues in a CXXC motif. When ATX1 docks with the cytosolic domain of Ccc2, the Cu$^{1+}$ is delivered from one CXXC motif to the other (Fig. 7). The formation of the intermediate, a metal-bridged ATX1-Ccc2 complex, as well as their binding interface has been characterized in solution by NMR (95).

![Figure 7](image.png)

**Figure 7.** Theoretical mechanism for Cu$^{1+}$ transfer between ATX1 and CCC2. The formation of a metal-bridged intermediate is supported by the crystal structure of Atox1. Adapted from (86).
In all systems studied to date, the docking of the loaded metallochaperone to its target protein is an essential step for metal ion transfer. However, the metal ion is not the only factor that contributes to complex formation. A copper chaperone for Cu,Zn-SOD, CCS, activates the SOD by directly inserting the copper ion into the apo-SOD (87,96). A yeast two-hybrid system provided evidence of the formation of CCS-SOD1 heterodimer (97). Fluorescence anisotropy measurements subsequently established that the binding affinity between CCS and SOD increases by an order of magnitude in the presence of copper ion (98). These results clearly demonstrate that there is a docking site at the interface of the heterodimer complex which is further stabilized by copper ion.

1.6.3.2 ATP/GTPase activity for metal incorporation

Metallochaperones often possess ATP/GTPase activity. Although the exact role of this activity remains uncertain, it has been speculated that the hydrolysis is essential for the release of metal ion to the apo-enzyme or dissociation of metallochaperone from the target protein following metal transfer (99). Recently, the role of GTP hydrolysis in nickel ion delivery has been investigated in UreE and HypB, the above-mentioned metallochaperones for urease and hydrogenase, respectively. The two metallochaperones share ~30% amino acid sequence similarity to each other and contain a G-nucleotide-binding domain. GTPase activity is required for nickel ion insertion into both hydrogenase (100) and urease (101). Structural studies of HypB revealed that HypB undergoes GTP-dependent dimerization and that the GTP is found at the homodimer interface (102). The GTP hydrolysis induced a conformational change at the dimer interface, and the metal-binding site was found in close proximately to the G-nucleotide-binding domain, leading to the hypothesis that GTP hydrolysis functions as a molecular switch that induces the delivery of the nickel ion to hydrogenase (102). Indeed, mutations introduced
into the G-nucleotide-binding domain resulted in reduced hydrogenase activity \textit{in vivo} due to a loss of nickel ion incorporation into the large subunit of the hydrogenase. Overall, these data indicate that GTP hydrolysis is critical for nickel ion incorporation into hydrogenases.

### 1.6.4 Terminal metal-binding motifs in proteins

Many metallochaperones and other metalloproteins possess an N- or C-terminal metal-binding motif. The major role of these motifs appears to be to sequester metal ions and to either transport or transfer them to the required site. Accordingly, these motifs usually have high affinity for metal ions, and are mobile and accessible to the surroundings. Such motifs are found in UreE, HypB and CooJ, a metallochaperone that delivers nickel ions to CO dehydrogenase (103). The \textit{ureE} gene occurs downstream of the urease structural genes and contains a C-terminal His-rich motif that binds up to 6 nickel ions per UreE dimer (104). HypB (100) and CooJ also deliver nickel ions to apo-enzymes from an N-terminal Cys-rich motif or C-terminal His-rich motif, respectively.

Among metalloenzymes, some of the Cu,Zn-SODs from Gram-negative bacteria possess an N-terminal metal-binding motif. Such an enzyme was isolated from \textit{Haemophilus ducreyi}, a pathogen responsible of a genital ulcerative disease known as chancroid (102,103). This sequence contains a His-rich motif followed by Met-rich motif and binds divalent copper and zinc ions with picomolar and nanomolar affinities, respectively. The bound divalent metal ions are eventually inserted into the metallocentre of the apo-enzyme (105).

As mentioned in section 1.6.4, CbiX is essential for the anaerobic biosynthesis of vitamin B$_{12}$ (cobalamin). A homologue of CbiX, SirB, plays key role in the insertion of Fe$^{2+}$ into
sirohydrochlorin as part of the biosynthesis of siroheme (106) (Fig. 8). Interestingly, the C-terminal sequence of some CbiXs, particularly those from bacillus and archaea, contain a region that is unusually rich in His and acidic residues (107)(Fig. 9). This sequence does not occur in any known SirB. Since the truncation of the C-terminus of CbiX did not abolish the latter’s cobaltocheatase activity, the C-terminus has been proposed to play a role in cobalt trafficking/storage. Nevertheless, no direct evidence for cobalt ion binding by this sequence has been provided to date.

Figure 8. Transformation of the modified tetrapyrrole intermediate sirohydrochlorin into siroheme and cobalamin (vitamin B$_{12}$). A, acetic acid side chain; P, propionic acid side chain. (Adapted from (108))
3.4

Figure 9. Multiple sequence alignment of C-terminal sequences of CbiX homologs. Of 41 CbiX sequences in the Pfam database, 13 contain the C-terminal His/Asp/Glu-rich region. These 13 sequences were aligned using ClustalX. His, Asp and Glu residues are indicated in blue, red, and pink, respectively. The consensus displayed below the alignment is the percentage of the modal residue per column.

1.6.5 NHase activator proteins - Putative metallochaperones

Fe- and Co-NHases require “activator proteins” for their maturation (Fig. 10). The type of activator protein depends on the identity of the metal ion of the NHase. P47K of

Figure 10. The occurrence of genes encoding activator proteins in representative nitrile catabolic gene clusters. (1) *Rhodococcus* sp. N-771 and (2) *R. rhodochrous* J1. nha1: Fe-NHase α subunit, nha2: Fe-NHase β subunit, nha3: Fe-NHase activator P47K, nhlB: Co-NHase β subunit, nhlA: Co-NHase α subunit, nhlE: Co-NHase activator P14K, nhlF: cobalt transporter. Adapted from (66) and (109).
Rhodococcus sp. N-771 represents the class of activator proteins involved in the maturation of Fe-NHases (110). Similarly, P14K (NhlE) of *R. rhodochrous* J1 represents the class involved in the maturation of Co-NHase (109).

Although it appears likely that NHase activator proteins act as metallochaperones, their direct association with metals has not been reported. For example, P47K interacts directly with the NHase and contains a conserved Cys-rich motif, CXCC, which potentially plays a key role in iron binding. However, no direct evidence of the interaction with iron has been reported (109). Similarly, a complex comprising two P14K (NhlE), the α-subunit of NHase (NhlA) and one cobalt ion was isolated. P14K was demonstrated to mediate cobalt ion insertion and oxidation of the NHase cysteine residues (109), processes that are essential for NHase activity (111). At some point during NHase maturation, Co$^{2+}$ is oxidized to Co$^{3+}$ (109). Nevertheless, it has yet to be established whether P14K binds cobalt. Overall, activator proteins have been shown to be essential for the maturation of both Fe- and Co-NHases.

1.7 Aim of this study

The overall objective of the current study was to characterize the function and assembly of ANHase, a nitrile hydratase from RHA1. The enzyme was initially identified as part of the nitrile catabolic pathways of RHA1 using bioinformatics, proteomics, and targeted gene deletion. First, RHA1 was grown on a variety of aliphatic and aromatic nitriles to determine the suitable growth substrate. Then, the cytosolic proteome of acetonitrile-grown RHA1 was analyzed by 2D gel electrophoresis (2DGE) and proteins specific to this proteome were identified using matrix-assisted laser desorption ionization time of flight (MALDI-ToF) mass spectrometry. An acetonitrile catabolic gene cluster encoding a novel NHase was discovered and compared with
other nitrile catabolic systems. The identified ANHase shares no significant amino acid sequence similarity with any protein in the databases.

To characterize the novel ANHase from RHA1, the enzyme was homologously produced and purified to apparent homogeneity. Homologous production was necessary as ANHase could not be produced in *E. coli* strains. A *Rhodococcus*-*E. coli* shuttle vector containing the ANHase genes was constructed and culture condition were optimized to improve the total yield of ANHase. The purified enzyme was characterized with respect to molecular mass and metal content using gel filtration chromatography and inductively coupled plasma-atomic emission mass spectrometer (ICP-MS), respectively. To characterize the enzyme kinetically, colorimetric and HPLC assays were developed. The substrate specificity of the enzyme was investigated. The characteristics of ANHase are discussed with respect to those of other NHases.

The third goal of this study was to examine a potential metal-binding motif in ANHase. The N-terminus of AnhA, the ANHase α subunit, contained a His-rich sequence (MPDHGHDHGHNH) which is hypothesized to play role in metal-binding. In order to probe this, a peptide with this amino acid sequence was synthesized and its metal-binding properties were studied using isothermal titration calorimetry (ITC) and electronic absorption spectrophotometry. These results were discussed in comparison to other potential metal-binding motifs found in Co-NHase and cobaltochelatases.

The final goal of this study was to elucidate the role of AnhE, an 11.1 kDa protein of unknown function whose gene occurs between *anhA* and *anhB*, the structural genes of ANHase. To elucidate the physiological role of this protein, *anhE* was deleted in-frame and the resulting mutant was evaluated for growth on acetonitrile. AnhE was also produced heterologously,
purified, and its metal-binding properties were investigated using ITC and electronic absorption spectrophotometry. The role of AnhE in ANHase maturation was studied using gel-filtration chromatography and *in vitro* reconstitution experiments. A model in which AnhE functions as a metallochaperone was developed.
CHAPTER TWO: MATERIALS AND METHODS

2.1 Chemicals and reagents

Restriction enzymes and Taq DNA ligase were purchased from New England Biolabs (Pickering, ON). T4 DNA ligase was from Fermentas (Burlington, ON), Expand high fidelity PCR system was from Roche Applied Science (Laval, Quebec) and Taq DNA polymerase was from Promega (Madison, WI). The peptide (MPDHGDHGHNH-DACDSE) was purchased from Genscript (Piscataway, NJ). Buffers for protein purification and characterization were prepared using water purified on a Barnstead NANOpure UV apparatus to a resistivity greater than 17 MΩ·cm. All the other chemicals were of analytical grade and used without further purification.

2.2 Bacterial strains, plasmids and growth conditions

For the growth study, RHA1 was grown in W minimal medium (112) supplemented with 20 mM nitrile as sole carbon- and nitrogen-containing compound. Acrylonitrile, benzonitrile and phenylacetonitrile were also provided to cultures in vapor form. For proteomics analysis, RHA1 was grown in W minimal medium supplemented with either 0.1 M acetonitrile, 20 mM propionitrile, phenylacetonitrile (vapor) or their corresponding carboxylic acids with ammonium sulfate, in 2 l shake flasks containing 0.5 l medium. For ANHase purification, RHA1 was grown in 2 l shake flasks containing 1 l medium. Cultures were shaken at 200 rpm, 30 °C and harvested at mid-log (OD$_{600}$ ~ 1.5). For ANHase purification using constructed *Rhodococcus* - *E. coli* shuttle vector, RHA1 was grown in Lysogeny broth (LB) supplemented with 100 µg ml$^{-1}$ of ampicillin. Overnight cultures were inoculated in 2 l shake flasks containing 1 l 1/5 diluted LB.
supplemented with trace elements (112) and 0.1 mM CoCl₂. At an OD₆₀₀ = 2.5, ANHase was induced by adding benzoate to a final concentration of 10 mM. Cultures were shaken at 200 rpm, 30 °C and harvested at OD₆₀₀ ~4.0.

*E. coli* DH5α was used to propagate DNA. Ht-AnhC was produced in *E. coli* GJ1158 (113) using a derivative of pT7HP20 (114). AnhA, AnhB and AnhE were produced in *E. coli* BL21 using derivatives of pET41-b. *E. coli* strains were grown at 37 °C in LB supplemented with either 100 µg ml⁻¹ ampicillin or 25 µg ml⁻¹ kanamycin. Ht-AnhC production was induced in cells that had attained an OD₆₀₀ = 0.5 by adding sodium chloride to a final concentration of 0.3 M and cells were harvested after 3 hours of further incubation. Production of AnhA, AnhB and AnhE was induced in cells that had attained an OD₆₀₀ = 0.5 by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM; cells were harvested after 16 hours of further incubation at 20 °C. All the cell pellets for protein purification were frozen in liquid nitrogen and stored at -80 °C until use. In the cobalt supplementation study, *E. coli* BL21 carrying either pET41AnhA or pET41AnhAE were grown in LB supplemented with up to 50 µM CoCl₂. Expression was induced in cultures at OD₆₀₀ = 0.5 by adding IPTG to a final concentration of 0.5 mM. Cells were harvested after 16 hours of further incubation at 20 °C.

### 2.3 Proteomics

The cytosolic proteome was resolved using 2DGE and analyzed essentially as described previously (51). Briefly, the cells were disrupted using 0.7 g of 0.1 mm zirconia/silica beads (Biospec Products, Bartlesville, OK) and a Fast Prep Bio 101 Thermo Savant bead beater operated at speed 6.0 for 5 cycles of 20 s each. Between each cycle, the sample was cooled on ice for 2 min and the chamber of the bead beater was immersed in ice water. Cell debris was
removed by centrifugation and the resultant cell extracts were stored at -80 °C. Aliquots containing 100 µg protein were separated in the first dimension by isoelectric focusing (IEF) using 24-cm, non-linear IPG strips and a pH gradient of 3 to 7. Proteins were separated in the second dimension using 12% SDS-PA gels. Gels were stained using Sypro Ruby. Gel images were digitized using a Typhoon 9400 (GE Healthcare). Spot detection and pattern matching were performed using Progenesis Workstation Software (Nonlinear Dynamics, Durham, NC).

The relative abundance of each protein was determined from the integrated signal intensity of spots after subtraction of background values. The signal intensity of each spot was normalized against the total signal intensity of the gel. Patterns, including spot intensities, of each growth condition were based on gels from three independent cultures. Protein spots of interest were identified using a Voyager DESTR MALDI-ToF Mass spectrometer (Applied Biosystems, Foster City, CA) based on peptide mass fingerprint (PMF) analysis combined with a MASCOT search engine (www.matrixscience.com) at the Proteomics Centre, University of Victoria. Identified proteins fulfilled three criteria: the MASCOT search score was above 55 for the RHA1 database, a minimum of 4 peptides were matched and the protein sequence coverage was at least 20%.

2.4 DNA manipulation

DNA was purified, digested, ligated and amplified using standard protocols (115). PCR was performed using a RoboCycler GRADIENT 96 (Stratagene, La Jolla, CA). The nucleotide sequence of cloned amplicons was verified at the Nucleic Acid Protein Service Unit (NAPS), University of British Columbia.
Table 1. Oligonucleotides used in this study.

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<td>Reverse primer for <em>anhC</em>, PstI</td>
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<td>Forward primer for <em>anhA</em>, NdeI</td>
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<td>Reverse primer for <em>anhA</em>, HindIII</td>
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<td>Forward primer for <em>anhB</em>, NdeI</td>
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<td>Reverse primer for <em>anhB</em>, HindIII</td>
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<tr>
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<td>Forward primer for <em>anhE</em>, NdeI</td>
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<td>Reverse primer for <em>benA</em> promoter region, NdeI</td>
</tr>
</tbody>
</table>

<sup>a</sup>The recognition sequences for the indicated restriction sites are underlined.

2.4.1 Cloning of *anh* genes

The *anh* genes were amplified using RF00111aA02 selected from the RHA1 fosmid library (116) and primers based on the RHA1 genome sequence (47)(Table 1). The *anhC* gene (1035 bp) was cloned immediately downstream of, and in-frame with, the sequence encoding a six-histidine tag in pT7HP20, yielding pT7HC. PCR amplicons containing each of *anhA* (1545 bp), *anhAE* (1839 bp), *anhB* (1704 bp), and *anhE* (300 bp) were cloned into pET41-b yielding
pET41AnhA, pET41AnhAE, pET41AnhB and pET41AnhE, respectively. The anhE gene was also cloned into pTipQC2, yielding pTipAnhE.

2.4.2 Construction of shuttle vector

A fragment containing anhAEB was amplified from fosmid RF00111A02 using anhAF and anhBR as primers. The resulting 3543 bp amplicon was cloned in pT7-7 using the NdeI and HindIII sites, yielding pT7AEB. A 104 bp fragment containing the promoter and ribosome binding site (rbs) of the benA gene of RHA1, Pben, was amplified from fosmid RF00125A18 using primers benAF and benAR (Table 1). The rbs of pT7AEB was replaced with this fragment using XbaI and NdeI to yield pT7PbenAEB. The benAF oligonucleotide contained an additional HindIII site for subsequent cloning. To construct a Rhodococcus-E. coli shuttle vector carrying anhAEB, pNC9501 (117) was first digested with EcoRI and HindIII. The fragment containing the thiostrepton resistance gene and the rhodococcal origin of replication was cloned into pUC18, yielding pNU. Finally, the HindIII fragment from pT7 PbenAEB carrying Pben and the anhAEB genes was cloned into pNU to yield pNUPbenAEB.

2.5 Protein purification

Unless otherwise specified, E. coli cell pellets were obtained from 2 l cultures and were resuspended in 80 to 100 ml of 20 mM HEPES, pH 7.5 containing 1 mM phenylmethanesulfonylfluoride (PMSF) at 4 °C. The cells were disrupted by passing the suspension through an Emulsiflex-05 homogenizer (Avestin, Ottawa, Canada) operated at 10,000 p.s.i. The cell suspension was centrifuged (36,000 g x 40 min, 4 °C) and the resultant supernatant was passed through a 0.45 µm filter immediately prior to chromatography.
Immobilized metal ion chromatography (IMAC) was performed using nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen, Mississauga, Ontario, Canada) according to the instructions of the manufacturer unless otherwise specified. Other chromatography was performed using an ÄKTA Explorer (GE Healthcare). During protein purification, samples were concentrated and/or buffer-exchanged using concentrated using an ultrafiltration cell (Amicon, Beverly, MA) equipped with a filter of the appropriate molecular weight cut-off (Millipore, Nepean, Ontario, Canada). Purified proteins were flash frozen as beads in liquid nitrogen and stored at -80 °C.

2.5.1 Purification of Ht-AnhC

Ht-AnhC was produced *E. coli* GJ1158 containing pT7HAC1, and was purified from the filtered supernatant using 5 ml Ni-NTA resin. Activity-containing fractions were pooled and concentrated to 16 mg/ml using ultrafiltration.

2.5.2 Purification of ANHase

The RHA1 cell pellet obtained from 70 l of culture (~116 g wet weight) was thawed and resuspended in 200 ml of 10 mM HEPES, pH 7.5 containing 1 mM PMSF at 4 °C. The cells were disrupted using 10 g of beads and 5 × 1 min cycles bead beating as described above. The cell suspension was centrifuged (40,000 g x 40 min, 4 °C) and the resultant supernatant was passed through a 0.45-μm filter immediately prior to chromatography. The filtered cellular extract was applied to a 2 x 9 cm column of Source Q anion-exchange resin (GE Healthcare) which had been pre-equilibrated with 10 mM HEPES, pH 7.5. The column was washed at a flow rate of 10 ml min⁻¹ with a 10-column-volume linear gradient of 0 to 30% of 10 mM HEPES, pH 7.5 containing 1 M NaCl. The ANHase activity was eluted using a 10-column-volume linear
gradient of 0.3 to 0.5 M NaCl in 10 mM HEPES, pH 7.5 (flow rate of 5 ml min⁻¹). The activity-containing fractions from three runs were pooled and concentrated to 3.7 mg/ml by ultrafiltration. The enzyme solution was brought to 40% saturation sodium sulfate by adding a 100% saturated solution, incubated at room temperature for 15 min, and then centrifuged (16,000 g x 15 min at 4 °C). The supernatant was applied to a 0.5 x 5 cm column of Source Phenyl resin (GE Healthcare) equilibrated with 67% of 10 mM HEPES, pH 7.5 containing 1.3 M of sodium sulfate. The column was washed with 30-column volumes of the equilibration buffer at a flow rate of 1.0 ml min⁻¹. The protein was eluted using a linear gradient of 0.84 to 0 M sodium sulfate (80 column volumes). Activity-containing fractions were pooled, dialyzed against 10 mM HEPES, pH 7.5, concentrated to 7 mg ml⁻¹ by the ultrafiltration.

2.5.3 Purification of AnhA

AnhA was produced using *E. coli* BL21 containing pET41AnhA or pET41AnhAE and was purified from the filtered supernatant using 5 ml Ni-NTA resin. AnhA-bound resin was washed with 100 ml 20 mM HEPES, pH 8.0 containing 10 mM imidazole. AnhA was eluted using 10 ml 20 mM HEPES, pH 8.0 containing 250 mM imidazole. The eluate was concentrated to 10 ml by ultrafiltration and applied to a 2 x 9 cm column of Source Q anion-exchange resin which had been equilibrated with 20 mM HEPES, pH 8.0. The proteins were eluted at 5 ml min⁻¹ using a 14-column-volume linear gradient of 0 to 0.7 M NaCl in 20 mM HEPES, pH 8.0. The AnhA-containing fractions were concentrated to 40 - 50 mg/ml by ultrafiltration.
2.5.4 Purification of AnhB

AnhB was produced using *E. coli* BL21 containing pET41AnhB. The filtered cellular extract was applied to a 2 x 9 cm column of Source Q anion-exchange resin which had been pre-equilibrated with 20 mM HEPES, pH 7.5. The proteins were eluted at 5 ml min\(^{-1}\) using a 6-column-volume linear gradient of 0 to 0.3 M NaCl in 20 mM HEPES, pH 7.5. Fractions containing AnhB were pooled and concentrated to 4.0 ml by ultrafiltration and applied to a HiLoad 26/60 Superdex 200 column equilibrated with 20 mM HEPES, pH 7.5 containing 50 mM NaCl, and operated at 3 ml min\(^{-1}\). Purified AnhB was concentrated to 10 mg ml\(^{-1}\) by the ultrafiltration.

2.5.5 Purification of AnhE

AnhE was produced using *E. coli* BL21 containing pET41AnhE. The filtered cellular extract was applied to a 2 x 9 cm column of Source Q anion-exchange resin which had been equilibrated with 20 mM HEPES, pH 7.5. The proteins were eluted at 5 ml min\(^{-1}\) using a 7-column-volume linear gradient of 0 to 0.2 M NaCl in 20 mM HEPES, pH 7.5. Fractions containing AnhE were pooled and concentrated to 4.0 ml by ultrafiltration using Centricon YM10 and applied to a HiLoad 26/60 Superdex 75 column equilibrated with 20 mM HEPES, pH 7.5 containing 50 mM NaCl, and operated at 3 ml min\(^{-1}\). Purified AnhE was concentrated to 21 mg ml\(^{-1}\) by the ultrafiltration and dialyzed overnight at 4 °C against a 20 mM HEPES, pH 7.5 buffer containing 5 mM ethylenediaminetetraacetic acid (EDTA) using molecularporous membrane tubing (Spectra/Por) followed by further dialysis with 20 mM HEPES buffer to remove EDTA.
2.5.6 Purification of the reconstituted ANHase

*In vitro*-assembled ANHase was purified by passing an incubated mixture of AnhA and AnhB through a 0.45-µm filter and applying this to a 0.5 x 5 cm column of Source Q anion-exchange resin which had been equilibrated with 20 mM HEPES, pH 7.5. The proteins were eluted at 1 ml min\(^{-1}\) using a 10-column-volume linear gradient of 0.2 to 0.5 M NaCl in 20 mM HEPES, pH 7.5. Activity-containing fractions were pooled and concentrated to 4.0 ml and applied to a HiLoad 26/60 Superdex 200 column operated at 3 ml min\(^{-1}\) and equilibrated with 20 mM HEPES, pH 7.5 containing 50 mM NaCl. Activity-containing fractions were pooled, dialyzed against 20 mM HEPES, pH 7.5, and concentrated to 21 mg ml\(^{-1}\).

2.6 *In vitro* assembly of ANHase

ANHase was activated using three different methods. In the first, 400 µg of AnhA-containing cell extract of *E. coli* grown in the presence of various concentrations of CoCl\(_2\) was incubated with 600 µg of purified AnhB in a total volume of 100 µl for 16 hours at 4 ºC. In the second, 300 µg AnhA was used instead of cell extract. In the third, 5 mg AnhA (purified from *E. coli* BL21 carrying pET41AnhAE, grown on LB media supplemented with 5 µM CoCl\(_2\)) and 10 mg AnhB were incubated for 16 h at 4 ºC. ANHase was purified from this mixture as described above in Protein Purification. ANHase activity was assayed discontinuously by quantifying ammonia release using a phenol-hypochlorite colorimetric assay adapted to 96-well plates (118).

2.7 Protein analysis

Protein concentration was determined using the micro BCA protein assay (Pierce). The concentration of purified AnhE was determined using the Edelhoch method and an extinction
coefficient of 16,960 M$^{-1}$cm$^{-1}$ at 280 nm, as estimated from the deduced amino acid sequence of protein using ProtParam software (www.expasy.org/tools/protparam.html) (119). SDS-PAGE was performed using separating gels containing 12% acrylamide (115). Tricine-SDS-PAGE (120) was performed using separating gels containing 15% acrylamide. Gels were stained using Coomassie Brilliant Blue.

2.8 Molecular weight determination

Subunit molecular weights of ANHase and AnhE were determined using 40 µg of purified protein and an Applied Biosystems VOYAGER-DE STR workstation, MALDI-ToF MS. The apparent molecular size, M_r, of ANHase was determined using a HiLoad 26/60 Superdex 200 column (GE Healthcare) equilibrated with 10 mM HEPES, pH 7.5 containing 100 mM NaCl, and operated at 2 ml min$^{-1}$. The column was calibrated using the HMW Gel Filtration Calibration Kit (GE Healthcare), comprising aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), thyroglobulin (669 kDa). The calibration curve was constructed by plotting $K_{av}$ versus log M_r where $K_{av} = (V_e - V_0)/(V_t - V_0)$. In this equation, $V_e$ is the measured elution volume of each standard; $V_0$, the void volume of the column determined by $V_e$ of blue dextran; and $V_t$, the total column volume as specified by the manufacturer.

The ratio of $\alpha$ and $\beta$ subunits in the native enzyme was calculated from HPLC traces or digitized SDS-PAGE gels. For HPLC, 10 µg of ANHase was denatured by incubating it for 15 min at 95 °C in 10 mM HEPES, pH 7.5 containing 1 mM DTT. The protein sample was loaded onto a Waters HPLC system equipped with a 250 x 4.6 mm C18 Aqua column (Phenomenex, Torrance, CA) operated at 1.0 ml min$^{-1}$. The mobile phase was deionized water containing 0.5% phosphoric acid: methanol (9:1). The absorbance of the column eluate was monitored at 280 nm.
SDS-PA gels were stained with either Coomassie Brilliant Blue or Sypro Ruby. Absorption spectra were recorded using a Cary 5000 UV-Visible spectrophotometer (Varian). The size of native AnhE, as well as AnhE-metal complex, was determined using a Tricorn™ Superdex™ 75 (GE Healthcare) equilibrated with 20 mM HEPES, pH 7.5 containing 50 mM NaCl, and operated at 0.4 ml min⁻¹. For AnhE-metal complex, AnhE was incubated with equivalent divalent metals for 1 minutes 25 ºC before loaded on the column. The column was calibrated using low molecular mass standards comprising lysozyme (15 kDa), chymotrypsinogen (25 kDa), carbonic anhydrase (32 kDa), β-lactoglobulin (36 kDa) and bovine serum albumin (68 kDa).

2.9 Amino acid sequence determination

To determine the amino acid sequence of each ANHase subunit, 30 µg of ANHase was denatured by incubation at 95 ºC for 15 min in a solution of 50 mM Tris-Cl, pH 8.0, 1 mM DTT and 5.8 M urea. The denatured protein was washed twice with 50 mM Tris-Cl, pH 8.0 using a 30 K NMWL membrane microcon (Millipore) to lower the urea concentration to below 1 M. Acetonitrile was added to the sample (10% v/v) which was then digested overnight at 37 ºC using 0.75 µg of trypsin. The peptides were separated by HPLC and analyzed by an API QSTAR Pulsar system equipped with an electrospray ionization mass spectrometry (ESI-MS). Selected peptides were sequenced by MS/MS using the same instrument.

2.10 Metal analysis

Fifty-five µg of ANHase in 27 µl 10 mM HEPES, pH 7.5 was dialyzed against the same buffer, dried in a SpeedVac concentrator (model SC110A, Thermo Savant, Milford, MA), and decomposed by heating with nitric acid. Control samples containing no ANHase consisted of the
same volume of buffer. The metal content was analyzed using a Thermo Finnigan ELEMENT2 High Resolution inductively coupled plasma-atomic emission mass spectrometer (ICP-MS) at the Pacific Centre for Isotopic and Geochemical Research, University of British Columbia. Solutions of AnhA were dialyzed extensively against 20 mM HEPES, pH 7.5, dried in a SpeedVac concentrator (model SC110A, Thermo Savant, Milford, MA), and decomposed by heating with nitric acid. Controls consisted of the same volume of buffer. The metal content was analyzed using inductively coupled plasma-atomic emission mass spectrometer (ICP-MS) at Exova (Santa Fe Springs, Ca).

2.11 Steady-state kinetics

2.11.1 Phenol-hypochlorite colorimetric assay

Amidase (AnhC) and ANHase (AnhAB) activities were routinely assayed discontinuously by quantifying ammonia release using a phenol-hypochlorite colorimetric assay (121) adapted to 96-well plates. Reactions were performed in a total volume of 20 µl containing 10 mM HEPES, pH 7.5. In the amidase assay, the standard reaction contained 50 mM acetamide and was initiated by the addition of ~2.9 mU of AnhC. In the ANHase assay, the standard reaction contained 50 mM acetonitrile and 6 mU of AnhC to couple amide and ammonia production. Reaction mixtures were incubated at either 25 °C or 37 °C for 6-16 min and were stopped by adding 100 µl each of solutions A (10 g l⁻¹ phenol; 0.4 g l⁻¹ sodium nitroprusside) and B (5 g l⁻¹ NaOH; 7.0 ml/l sodium hypochlorite). The colorimetric reaction was incubated for 3 min at 100 °C, after which the absorbance was determined at 650 nm using a V_max Microplate Reader (Molecular Devices, Sunnyvale, CA). Absorbance was converted to ammonia
concentration using a standard curve established using 0.01-1.0 mM NH₄Cl. One unit of the enzyme activity was defined as the amount of enzyme which produces 1 µmol of NH₃ per min.

2.11.2 HPLC-based assay

ANHase activity was also assayed using an high-performance liquid chromatography (HPLC) to detect the amide reaction product. Reactions were performed in 100 µl 10 mM HEPES, pH 7.5 containing 25 mM nitrile. Reaction mixtures were incubated at 37 °C for 15 min and were stopped using 100 µl methanol containing 0.5% phosphoric acid. Stopped reactions were analyzed using an HPLC as described in Protein analyses. The absorbance of the column eluate was monitored at 210 nm and amide concentrations were determined from peak areas using standard curves established with the corresponding amides.

2.11.3 Analysis of steady-state kinetic data and evaluation of reaction conditions

Steady-state kinetic parameters and the effect of reaction conditions were evaluated using the phenol-hypochlorite colorimetric assay. In evaluating the former, initial rates were determined at substrate concentrations from 0.1 to 25 mM. Steady-state rate equations were fit to data using the least squares and dynamic weighting options of LEONORA (122). The pH-dependence of the ANHase activity was evaluated using 20 mM acrylonitrile and the following buffers: 10 mM sodium acetate (pH 4.0-5.5), 10 mM MES (pH 5.5-7.0), 10 mM HEPES (pH 7.0-8.0), 10 mM BICINE (pH 8.0-9.0), 10 mM ethanolamine (pH 9.0-10.0), 10 mM CHES (pH 9.0-10.0), and 10 mM CAPS (pH 10.0-11.0). To study the effect of metal ions on ANHase activity, the purified enzyme was dialyzed three times against 10 mM phosphate, pH 7.5 containing 5 mM EDTA. The sample mixture was further dialyzed against 10 mM phosphate.
buffer to reduce the concentration of EDTA to below 0.1 mM. Prior to initiating the reaction, the assay mixture containing the enzyme was incubated for 10 min at 37 ºC with 1 mM of one of the following salts: CoCl$_2$, CuCl$_2$, ZnCl$_2$, FeCl$_2$, MgCl$_2$ or NiCl$_2$. Product inhibition was studied using 6.4 mM acrylonitrile and either 0.1, 1.0 or 10 mM of either acetamide or propioamide.

2.12 Electronic absorption spectroscopy

Electronic absorption spectra were recorded and analyzed on a Cary 4000 UV-visible spectrophotometer (Varian Canada, Mississauga, ON). All the samples were prepared in 20 mM HEPES, pH 7.5 and the spectra were recorded at 25 ºC. For the time-dependent binding of N-terminal peptide to cobalt ion, 100 µM of CoCl$_2$ was mixed with 40 µM of peptide and spectra were recorded at 3 minute intervals. For the titration experiment, 75 µM of peptide was mixed with 10-100 µM of CoCl$_2$ and incubated at 25 ºC for 2 hours. Then the absorbance was measured at 300 and 500 nm. To investigate the binding of AnhE to cobalt ion, 500 µM AnhE homodimer was mixed with varied concentration of CoCl$_2$. Background corrections for the cuvettes and buffers were applied as appropriate.

2.13 Isothermal titration calorimetry (ITC)

The binding of metal ions by AnhE and the N-terminal peptide of AnhA, respectively, was investigated at 25 ºC using iTC$_{200}$ system (Microcal, Northampton, MA). Stock solutions of metal ions (CoCl$_2$, ZnCl$_2$, NiCl$_2$ and CuCl$_2$) were prepared to 100 mM in water then further diluted to 0.5 – 2.0 mM using 20 mM HEPES, pH 7.5. ITC measurements were performed by injecting 0.5 – 1.0 µl of metal solution into 250 µl of 100 µM peptide or 50 – 100 µM AnhE. In order to allow the system to reach the equilibrium, samples were incubated for 120 s between
each injection. Experiments were performed in triplicate. Two types of control experiments were performed: each metal ion solution was titrated into the buffer alone; and solutions of peptide and protein were titrated with buffer. Integrated heat release from each metal injection was fit using the Microcal Origin 7.0 which uses non-linear least squares algorithm and the best fits were achieved using one-site model or two-site model.
CHAPTER THREE: RESULTS

3.1 Nitrile catabolic pathways of RHA1

3.1.1 Growth study of RHA1 on a variety of nitriles

The ability of RHA1 to utilize nitriles as organic growth substrate and sole source of nitrogen was investigated. Accordingly, W minimal media was supplemented with various nitriles (Fig. 11) as sole carbon- and nitrogen-containing compound. RHA1 was able to grow on most of the short-chain aliphatic nitriles except for acrylonitrile. By contrast, no growth was observed on aromatic nitriles when they were added directly to the medium at 20 mM. Finally, benzonitrile and phenylacetonitrile, but not acrylonitrile, supported the growth of RHA1 when provided to cultures in the vapor form. Growth parameters are summarized in Table 2.

<table>
<thead>
<tr>
<th>CH$_3$-CN</th>
<th>CH$_2$=CH-CN</th>
<th>C$_2$H$_5$-CN</th>
<th>C$_3$H$_7$-CN</th>
<th>C$_4$H$_9$-CN</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetonitrile</td>
<td>acrylonitrile</td>
<td>propionitrile</td>
<td>butyronitrile</td>
<td>valeronitrile</td>
</tr>
</tbody>
</table>

Figure 11. The nitriles used in this study.
Table 2. Growth parameters of RHA1 on a variety of nitriles.

<table>
<thead>
<tr>
<th>Nitrile</th>
<th>Growth rate (h⁻¹)</th>
<th>Lag phase (h)</th>
<th>Maximum OD₆₀₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetonitrile</td>
<td>0.021 (0.002)</td>
<td>26 (6)</td>
<td>0.42 (0.07)</td>
</tr>
<tr>
<td>acrylonitrile</td>
<td>ND (ND)</td>
<td>ND (ND)</td>
<td>ND (ND)</td>
</tr>
<tr>
<td>propionitrile</td>
<td>0.023 (0.002)</td>
<td>35 (10)</td>
<td>0.83 (0.07)</td>
</tr>
<tr>
<td>butyronitrile</td>
<td>0.028 (0.001)</td>
<td>42 (2)</td>
<td>1.12 (0.05)</td>
</tr>
<tr>
<td>valeronitrile</td>
<td>0.0097 (0.0003)</td>
<td>78.1 (0.2)</td>
<td>0.46 (0.02)</td>
</tr>
<tr>
<td>benzonitrile</td>
<td>ND (ND)</td>
<td>ND (ND)</td>
<td>ND (ND)</td>
</tr>
<tr>
<td>phenylacetonitrile</td>
<td>ND (ND)</td>
<td>ND (ND)</td>
<td>ND (ND)</td>
</tr>
<tr>
<td>2-methyl-2-butenenitrile</td>
<td>0.0052 (0.0004)</td>
<td>74 (3)</td>
<td>0.22 (0.01)</td>
</tr>
<tr>
<td>acrylonitrile (v)</td>
<td>ND (ND)</td>
<td>ND (ND)</td>
<td>ND (ND)</td>
</tr>
<tr>
<td>benzonitrile (v)</td>
<td>0.056 (0.009)</td>
<td>31.4 (0.3)</td>
<td>1.71 (0.09)</td>
</tr>
<tr>
<td>phenylacetonitrile (v)</td>
<td>0.0028 (0.0001)</td>
<td>67.2 (0.2)</td>
<td>1.41 (0.04)</td>
</tr>
</tbody>
</table>

*RHA1 was grown at 30 °C in W minimal medium (112) supplemented with 20 mM nitrile.

*b Acrylonitrile, benzonitrile and phenylacetonitrile were provided as vapor.

*c The values in parentheses represent standard errors calculated from two batches.

*d No growth detected.

3.1.2 Identification of nitrile catabolic enzymes

3.1.2.1 Identification of acetonitrile catabolic enzymes

To identify enzymes involved in the catabolism of acetonitrile in RHA1, the cytosolic proteome of cells growing exponentially on 0.1 M acetonitrile as sole organic growth substrate and nitrogen source was compared to that of cells growing on acetic acid and ammonia. Quantitative 2D gel electrophoresis (2DGE) analyses revealed that these two proteomes were very similar to each other in the range of pI 3 to 7: of ~1500 spots detected under each growth
condition, only 19 differed by more than two-fold in intensity. Of these 19, three were unique to acetonitrile-grown cells (Fig. 12), 11 were up-regulated at least two-fold in these cells, and five were at least two-fold down-regulated. Fifteen of the 19 spots were identified by MALDI-ToF MS, corresponding to 14 different proteins, and among them, three of them were encoded on the same gene cluster (Table 3).

The identified proteins include two that are predicted to be involved in acetonitrile catabolism. The first possesses 75% amino acid sequence identity with the broad-spectrum aliphatic amidase of *Brevibacterium* sp., and may catalyze hydrolysis of the amide produced from acetonitrile to yield acetic acid. The second possesses 75% amino acid sequence identity with an acetyl-CoA hydrolase of *Burkholderia pseudomallei* K96243 (123) that is known to be involved in acetic acid utilization. The predicted amidase was not detected in acetate-grown cells. The other two spots that were only detected in acetonitrile-grown cells corresponded to the same protein: one possessing no identifiable homologue in the databases. The occurrence of two spots may reflect carbamylation, as observed in other proteomic analyses of RHA1 (51).
Figure 12. Proteomic analysis of RHA1 grown on acetate and ammonia (1) and acetonitrile (2). (3) Sections of the gels shown in (1) and (2) containing anh-encoded catabolic proteins: AnhC (A, D); AnhB (B, E); and AnhD (C, F). Proteins were visualized by staining with Sypro Ruby. The proteins spots of interest are indicated by arrows and were identified by MALDI-ToF MS as summarized in Table 2.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene product</th>
<th>Length (aa)</th>
<th>Closest Homologue</th>
<th>Identity (%)</th>
<th>No. peptides matched</th>
<th>Sequence coverage (%)</th>
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<td>anhP</td>
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<td>AmiA, M.</td>
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<td>smegmatis</td>
<td>(DAA01132)</td>
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<td>hypothetical protein</td>
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<td>NitR, Rhodococcus</td>
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<td>globerulus A-4</td>
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<td>anhT</td>
<td>Probable cobalt transporter</td>
<td>364</td>
<td>(BAA11038)</td>
<td>72</td>
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<td>Acu-8, Neurospora</td>
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<td>anhD</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>opacus (AAC38799)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AnhB and AnhC were picked from a gel of acetonitrile-grown cells. AnhD was picked from a gel of acetate-grown cells.

\(^a\)Gene ID

\(^b\)Closest homologue of experimentally verified function (gene product name, species, accession number).

\(^c\)Percent amino acid sequence identity was calculated over the entire length of the proteins using alignments generated with BlastP.

\(^d\)Number of peptides from RHA1 gene product matched to MASCOT database.
3.1.2.2 Identification of an acetonitrile catabolic gene cluster

Analysis of the RHA1 genome ((47); www.rhodococcus.ca) revealed that the protein of unknown function, the amidase, and the acetyl-CoA hydrolase are encoded by genes that are clustered on pRHL2, a 450 kb linear plasmid of RHA1 (Fig. 13, Table 3). In addition to these genes, annotated here as anhB, anhC and anhD, respectively, the cluster contains genes encoding two other proteins possessing no significant sequence identity with known proteins: anhA and anhE. The proteins encoded by anhA and anhB share 32% amino acid sequence identity to each other. Finally, the cluster also encodes a possible cobalt transporter, and three regulatory genes (anhP, anhQ, and anhR). AnhP and AnhQ share 41% and 48% amino acid sequence identity, respectively, with the negative and positive transcriptional regulators of amiE in Mycobacterium smegmatis, AmiA and AmiC (124). The anhP gene is divergently orientated with respect to the other genes, which are organized in an operon-like structure. The content of the cluster together with the increased abundance of three of the encoded proteins during growth on acetonitrile strongly suggests that the cluster encodes acetonitrile catabolic enzymes.

Figure 13. Deduced acetonitrile catabolic pathway of RHA1. (A) The enzymes and metabolites. (B) The anh gene cluster located on pRHL2. Genes encoding catabolic enzymes are filled.
3.1.2.3 Proteomic analysis of RHA1 grown on other nitriles

To investigate other nitrile catabolism pathways in RHA1, the cytosolic proteomes of propionitrile- and phenylacetonitrile-grown cells were analyzed. Cells grown on propionitrile or phenylacetonitrile as sole organic growth substrate and nitrogen source were compared to cells grown on propionic acid or phenylacetic acid, respectively, and ammonia. 2DGE resolved approximately 1,500 protein spots in the cytosolic proteomes of RHA1 cells grown on each substrate. In contrast to acetonitrile- versus acetate- and ammonia-grown cells, the proteomes of propionitrile and phenylacetonitrile grown cells differed significantly from the corresponding carboxylic acid-grown cells (Fig. 14, 15). To identify nitrile catabolic proteins, ~40 protein spots were picked from gels of propionitrile- and phenylacetonitrile-grown cells and analyzed by MALDI-ToF MS. The identified proteins from propionitrile-grown cells included AnhB and AnhC, identified in acetonitrile-grown cells. In addition, the gene product of anhA was present in propionitrile-cells. This protein has no homologue in the database and its gene occurs immediately downstream of anhC. By contrast, three of the identified proteins from phenylacetonitrile-grown cells are encoded by a cluster of genes that occur on the RHA1 chromosome. The proteins, annotated here as NthA, NthB and NthC, share high sequence identity (79 ~ 91%) with the α and β subunits of Fe-NHase and an amidase (AmiD) from Rhodococcus erythropolis. The deduced catabolic pathways for propionitrile and phenylacetonitrile, respectively, are shown in Fig. 16.
Figure 14. Proteomic analysis of RHA1 grown on propionic acid and ammonia (1) and propionitrile (2). (3) Sections of the 2D gels from (1) and (2) containing anh-encoded catabolic proteins: AnhA (A, D); AnhC (B, E); and AnhB (C, F). Proteins were visualized using Sypro Ruby dye. The proteins spots of interest are indicated by arrows and were identified by MALDI-ToF MS as summarized in Table 3.
Figure 15. Proteomic analysis of RHA1 grown on phenylacetic acid and ammonia (1) and phenylacetonitrile (2). (3) Parts of the 2D gels from (1) and (2) containing nth-encoded catabolic proteins: NthC (A, C); NthA and NthB (B, D). Proteins were visualized by staining with Sypro Ruby. The proteins spots of interest are indicated by arrows and were identified by MALDI-ToF MS as summarized in Table 3.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene product</th>
<th>Length (aa)</th>
<th>Closest Homologue(^b)</th>
<th>Identity(^c) (%)</th>
<th>No. peptides match(^d)</th>
<th>Sequence coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Propionitrile</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>anhC</em> (ro10169)(^a)</td>
<td>aliphatic amidase</td>
<td>345</td>
<td>AmiE, <em>Brevibacterium</em> sp. R312 (Q01360)</td>
<td>75</td>
<td>6</td>
<td>25</td>
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<tr>
<td><em>anhA</em> (ro10170)</td>
<td>hypothetical protein</td>
<td>514</td>
<td></td>
<td></td>
<td>9</td>
<td>29</td>
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<tr>
<td><em>anhB</em> (ro10172)</td>
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<td></td>
<td></td>
<td>16</td>
<td>32</td>
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<tr>
<td><strong>Phenyl acetonitrile</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>nthD</em> (ro00358)</td>
<td>aldoxime dehydratase</td>
<td>357</td>
<td><em>R. erythropolis</em> (BAD17969)</td>
<td>82</td>
<td></td>
<td></td>
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<tr>
<td><em>nthC</em> (ro00359)</td>
<td>amidase</td>
<td>519</td>
<td>AmiD, <em>R. erythropolis</em> (CAQ16887)</td>
<td>79</td>
<td>15</td>
<td>41</td>
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<tr>
<td><em>nthA</em> (ro00360)</td>
<td>NHase α subunit</td>
<td>207</td>
<td><em>R. erythropolis</em> (AAP57664)</td>
<td>91</td>
<td>7</td>
<td>37</td>
</tr>
<tr>
<td><em>nthB</em> (ro00361)</td>
<td>NHase β subunit</td>
<td>212</td>
<td><em>R. erythropolis</em> (AAP57638)</td>
<td>89</td>
<td>4</td>
<td>24</td>
</tr>
<tr>
<td><em>nthE</em> (ro00362)</td>
<td>P47K</td>
<td>418</td>
<td><em>Rhodococcus erythropolis</em> (CAD36563)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Gene ID
\(^b\)Closest homologue of experimentally verified function (gene product name, species, accession number).
\(^c\)Percent amino acid sequence identity was calculated over the entire length of the proteins using alignments generated with BlastP.
\(^d\)Number of peptides from RHA1 gene product matched to MASCOT database.
Figure 16. Nitrile catabolic gene clusters in RHA1 and the deduced catabolic pathway. (1) The anh gene cluster identified in propionitrile-grown cells and the deduced propionitrile catabolic pathway. (2) The nth gene cluster identified in phenylacetoneitrile-grown cells and the deduced phenylacetoneitrile catabolic pathway. Genes encoding products that were identified by 2DGE and MALDI-ToF analysis are filled.
3.1.3 Production and characterization of AnhC

To investigate enzyme function and to establish a coupled assay for the characterization of ANHase, the anhC-encoded amidase was heterologously produced as a His-tagged amidase (Ht-AnhC). An amplicon containing anhC was inserted downstream of the T7 promoter in pT7HP20 as described in Material and Methods, and its nucleotide sequence was verified. Purification of Ht-AnhC using Ni-NTA resin from cell extracts of E. coli GJ1158 containing pT7HAC1 yielded ~4.2 mg of protein per a liter of cell culture. SDS-PAGE analysis indicated that the protein in the preparation was >95% AnhC (Fig. 17, Table 5).

![Figure 17. SDS-PAGE of the purified AnhC from E. coli GJ1158. Lanes: 1, molecular weight markers; 2, crude extract of E.coli. (30 µg of protein); 3, Purified AnhE using Ni-NTA (5 µg).](image)

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Sp. activity (U mg⁻¹)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>31.8</td>
<td>44.5</td>
<td>1.4</td>
<td>100</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>4.2</td>
<td>12.6</td>
<td>3.0</td>
<td>28.3</td>
</tr>
</tbody>
</table>

aEnzyme activity was measured at pH 7.5, 37 °C using acetonitrile as a substrate. One unit of enzyme activity was defined as the amount of enzyme which produces 1 µmol of NH₃ per min.
The transformation of acetamide by Ht-AnhC was investigated using a phenol-hypochlorite colorimetric assay (121) adapted to 96-well plates. Using 2.9 mU Ht-AnhC, the production of ammonia was linear for at least 10 min at an initial concentration of 0.25 mM acetamide, and for 13 min at 50 mM acetamide. At 50 mM acetamide, the specific activity of Ht-AnhC was 2.94 U mg\(^{-1}\), which was 4.6-fold higher than in the cell extract. At concentrations of acetamide ranging from 0.25 to 50 mM (10 mM HEPES, pH 7.5, 37 °C), Ht-AnhC displayed Michaelis-Menten behavior (Fig. 18). Under these conditions, the steady-state kinetic parameters for acetamide were \(K_m = 1.05 \pm 0.09\) mM, \(k_{cat} = 3.00 \pm 0.05\) s\(^{-1}\), and \(k_{cat}/K_m = 2.9 \pm 0.1\) mM\(^{-1}\) s\(^{-1}\).

Figure 18. Steady-state kinetic analyses of acetamide hydrolysis by AnhC. The dependence of the production of ammonia on acetamide concentration is shown. The line represents a best fit of the Michaelis-Menten equation to the data. Steady-state rate equations were fit to data using the least squares and dynamic weighting options of LEONORA, yielding parameters of \(K_m = 1.05 \pm 0.09\) mM, \(k_{cat} = 3.00 \pm 0.05\) s\(^{-1}\) and \(k_{cat}/K_m = 2.9 \pm 0.1\) mM\(^{-1}\) s\(^{-1}\). All the experiments were performed at 37 °C in 10 mM HEPES pH 7.5.
The relative activity of Ht-AnhC against various amides was examined (Fig. 19). In the presence of 50 mM substrate, the enzyme had specific activities for propioamide and acrylamide that were 2.2- and 1.2-fold higher, respectively, than for acetamide. Butyramide and phenylacetamide were hydrolyzed at 42% and 20% the rate of acetamide, respectively. Finally, Ht-AnhC did not detectably transform acetonitrile, indicating that the enzyme has no significant nitrilase activity.

![Relative activity of AnhC against amides](image)

**Figure 19.** Relative activity of AnhC against a range of amides. Experiments were performed at 37 °C in 10 mM HEPES pH 7.5, in the presence of 50 mM amide.

### 3.1.4 Characterization of ANHase

#### 3.1.4.1 Purification of ANHase from RHA1

As a NHase was not identified from either proteomic studies or sequence analysis of the *anh* genes, the enzyme was purified from acetonitrile-grown cells. As summarized in Table 6, ANHase was purified 22-fold to apparent homogeneity using anion exchange and hydrophobic
interaction chromatographies with a yield of 2%. SDS-PAGE analysis indicated that the enzyme was apparently homogeneous and that it consisted of two subunits (Fig. 20). MALDI-ToF MS analyses on purified ANHase extracted from SDS-PA gel and subjected to trypsin-digest revealed that the ANHase subunits are encoded by \textit{anhA} and \textit{anhB}, respectively (AnhA: 17 peptide peptides matches; 43 % overall sequence coverage, AnhB: 25 peptides matches; 47 % overall sequence coverage).

![Figure 20. SDS-PAGE of the ANHase purified from RHA1. Lanes: 1, molecular weight markers; 2, crude extract of RHA1 (20 µg of protein); 3, Source Q fraction (8 µg); 4, Source Phenyl fraction (3 µg). Arrows indicate the α and β subunits of ANHase.](image)

\textbf{Table 6.} Purification of ANHase from RHA1.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity ((U))</th>
<th>Sp. activity ((U\text{mg}^{-1}))</th>
<th>Yield (%)</th>
</tr>
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<tr>
<td>Cell extract</td>
<td>2550</td>
<td>684</td>
<td>0.269</td>
<td>100</td>
</tr>
<tr>
<td>Source Q</td>
<td>29.6</td>
<td>82.6</td>
<td>2.79</td>
<td>12</td>
</tr>
<tr>
<td>Source Phenyl</td>
<td>2.2</td>
<td>13</td>
<td>5.9</td>
<td>1.9</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Enzyme activity was measured at pH 7.5, 37 °C using acetonitrile as a substrate. One unit of enzyme activity was defined as the amount of enzyme which produces 1 µmol of NH\(_3\) per min.
Mass spectrometric studies confirmed the identity of the gene products and that the coding sequences had been correctly identified. First, MALDI-ToF mass spectrometric analyses indicated that the molecular masses of the ANHase α and β subunits, 55745.90 Da and 63000.83 Da, respectively, correspond closely to the predicted masses of AnhA (55757.57 Da) and AnhB (63018.53 Da) (Fig. 21).

![Mass spectrum](image)

**Figure 21.** Mass spectrum obtained by MALDI-ToF analysis of 40 µg of ANHase. The masses of each subunit deduced from the genomic sequence were 55757.57 Da and 63018.53 Da. Additional details are provided in Materials and Methods.

Second, electrospray ionization source tandem mass spectrometry (ESI-MS/MS) analyses of trypsin-digested ANHase identified peptides whose molecular masses (1128.5472 Da and 2368.2865 Da) and amino acid sequences (GELELTETTH and FPTAVQLTAAAMEAPVQTVVPTP) corresponded to the respective C-terminal peptides of AnhA and AnhB.
3.1.4.2 Biophysical characterization of ANHase

Analysis of ANHase using a calibrated gel filtration column yielded a single peak that eluted at a volume corresponding to a molecular mass of 430 ± 13 kDa. The ratio of α and β subunits was estimated to be 1:1.9 by HPLC (absorbance at 280 nm), 1:1.9 from Coomassie Blue-stained gels and 1:2 from Sypro Ruby-stained gels. From these values, we provisionally concluded that the protomer of the native enzyme is $\alpha\beta_2$. Two protomers would have an overall subunit composition of $\alpha_2\beta_4$ and a molecular mass of 364 kDa. The absorption spectrum of ANHase in 10 mM HEPES, pH 7.5 (25 °C) had maxima at 414 nm, 525 nm, and 560 nm, in order of decreasing intensity (Fig. 22). The ratio of $A_{280}/A_{414}$ in the purified enzyme was 5.88.

Figure 22. Electronic absorption spectrum of the ANHase from RHA1. (A) The sample contained 1.0 mg ml$^{-1}$ purified ANHase in 10 mM HEPES, pH 7.5, 25 °C. (B) Expanding view of 300 nm to 800 nm region of spectrum shown in A.
3.1.4.3 Metal content of ANHase

Metal analyses using inductively coupled plasma-atomic emission mass spectrometer (ICP-MS) revealed that 1 µg ANHase (~5.6 ± 0.2 pmol) contained 5.8 ± 1 pmol cobalt ion, 12 ± 5 pmol copper ion, and 6 ± 2 pmol zinc ion. Metal ions that were not detected at significant levels include iron, manganese, nickel and molybdenum. We therefore concluded that the holoenzyme contains 1 cobalt ion, 2 copper ions, and 1 zinc ion. Calculated as a function of cobalt ion content, the molar extinction coefficient of native ANHase was $\varepsilon_{414} = 17,857 \text{ M}^{-1}\text{cm}^{-1}$. This value was used to calculate $k_{\text{cat}}$ in subsequent analyses. By comparison to the absorbance at 414 nm, $\varepsilon_{525} = 388 \text{ M}^{-1}\text{cm}^{-1}$.

3.1.4.4 Kinetic properties and substrate specificity of ANHase

To confirm the activity of the ANHase, the reaction product was investigated using high-performance liquid chromatography (HPLC) and acrylonitrile, to facilitate product detection. A reaction mixture containing 21 mU ANHase, 25 mM acrylonitrile, 10 mM HEPES, pH 7.5 was incubated for 10 min at 37 °C then stopped with methanol containing 0.5% phosphoric acid. HPLC analysis of the reaction mixture revealed peaks at 6.0 and 4.5 min which corresponded to authentic samples of acrylonitrile and acrylamide, respectively (Fig. 23). Acrylamide was only detected in reaction mixtures containing ANHase, and the amount of acrylamide detected was linearly dependent on the incubation time up to 15 min.
Figure 23. The ANHase-catalyzed transformation of acrylonitrile to acrylamide. Assays contained 21 mU ANHase, 25 mM acrylonitrile, 10 mM HEPES, pH 7.5 (37 °C). Assays were analyzed by HPLC as described in Materials and Methods after 0 and 10 min of incubation. Reactions were stopped by adding methanol with 0.5% phosphoric acid.

Due to the metal content of the enzyme, we investigated the dependence of ANHase activity on exogenous metal ions using the HPLC-based assay. After repeated dialysis against 5 mM EDTA (three times against 50 sample-volumes), the enzyme retained 88% of its specific activity. The dialyzed NHase was then incubated for 10 min at 37 °C with 1 mM of various metal salts. Under these conditions, CuCl$_2$ strongly inhibited ANHase activity: compared with the EDTA-dialyzed sample, 19% of the activity remained. Zinc and ferrous ions also inhibited the activity (56% and 38% activity remained, respectively). By contrast, cobalt and magnesium ions slightly enhanced the activity of the enzyme (120% and 130% activity, respectively). Based on these results, no metals were added to the standard assay.

ANHase was subject to relatively little product inhibition. Thus, in the presence of 10 mM acetamide and propanoamide, the acrylonitrile-degrading activity of ANHase was reduced from 1.2 U mg$^{-1}$ to 1.0 and 0.91 U mg$^{-1}$, respectively (i.e., ~80% of the activity remained).
To facilitate the steady-state kinetic characterization of ANHase, a coupled assay was established using 5.9 mU Ht-AnhC and the phenol-hypochlorite colorimetric assay described above. Using 4.1 mU ANHase, the production of ammonia was linear for at least 12 min at an initial concentration of 0.1 mM acetonitrile, and for 20 min at 50 mM acetonitrile. The specific activity of ANHase was 5.9 ± 0.4 and 2.6 ± 0.2 U mg\(^{-1}\) for each of acetonitrile and acrylonitrile (50 mM), respectively. Similar results were obtained using the HPLC-based assay (5.7 ± 0.2 and 2.8 ± 0.2 U mg\(^{-1}\), respectively), indicating that Ht-AnhC was not limiting in the coupled assay. At concentrations of acetonitrile from 0.1 to 50 mM, ANHase displayed Michaelis-Menten behavior.

The substrate specificity of ANHase was investigated using the coupled assay. ANHase detectably transformed nitriles containing up to 4 carbon atoms, with specificity being highest for C\(_2\) and C\(_3\) saturated nitriles (acetonitrile ~ propionitrile > acrylonitrile >> butyronitrile (Table 7)).

<table>
<thead>
<tr>
<th>substrate</th>
<th>(K_m) (mM)</th>
<th>(k_{cat}) (s(^{-1}))</th>
<th>(k_{cat}/K_m) (mM(^{-1})s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetonitrile</td>
<td>5.4 (0.3)</td>
<td>41 (0.9)</td>
<td>7.6 (0.3)</td>
</tr>
<tr>
<td>acrylonitrile</td>
<td>6.4 (0.3)</td>
<td>28 (0.5)</td>
<td>4.4 (0.2)</td>
</tr>
<tr>
<td>propionitrile</td>
<td>0.38 (0.10)</td>
<td>2.6 (0.2)</td>
<td>6.8 (1.7)</td>
</tr>
<tr>
<td>butyronitrile</td>
<td>1.9 (0.2)</td>
<td>0.99 (0.04)</td>
<td>0.52 (0.06)</td>
</tr>
</tbody>
</table>

\(^a\)Assays were performed using 10 mM HEPES, pH 7.5 at 37 °C. The values in parentheses represent standard errors.

Although the \(k_{cat}/K_m\) of the enzyme for acetonitrile and propionitrile were similar, the \(k_{cat}\) and \(K_m\) for acetonitrile were an order of magnitude larger than for the latter. Larger nitriles that were not detectably transformed by ANHase include valeronitrile (C\(_5\)), 2-methyl-2-butene-nitrile,
benzonitrile and phenylacetonitrile. Similar results were obtained using the HPLC-based assay, indicating that Ht-AnhC was not limiting in these experiments.

The pH dependence of ANHase activity was investigated using the HPLC-based assay to avoid any effect from the amidase used in the coupled assay. As shown in Fig. 24, ANHase possessed a broad pH maximum between 5.5 and 10.0, with a maximum around pH 9. The activity dropped sharply below pH 5.5 and above pH 10.0. ANHase activity was markedly lower in the presence of ethanolamine, suggesting that this compound inhibits the enzyme.

![Figure 24](image_url)

**Figure 24.** The pH-dependence of ANHase activity. Assay buffers contained one of the following components; 10 mM sodium acetate (•), 10 mM MES (□), 10 mM HEPES (△), 10 mM BICINE (⊗), 10 mM ethanolamine (★), 10 mM CAPS (+), or 10 mM CHES (◦). Activities were normalized to the average value at pH 9.0.
3.1.4.5 Construction of *Rhodococcus*-*E.coli* shuttle vector

To heterologously produce ANHase, an amplicon containing *anhAEB* (α and β subunits of ANHase with an intervening gene described below) was inserted downstream of the T7 promoter in pT7-7. However, the ANHase produced in *E. coli* GJ1158 had <60% the activity of native ANHase. Moreover, a significant amount of free α subunit was produced (data not shown). The poor production of functional ANHase suggests that the enzyme may not fold and/or mature properly in *E. coli*.

To investigate whether ANHase produced in a rhodococcal strain would have a higher specific activity, a *Rhodococcus*-*E. coli* shuttle vector was constructed. The vector was based on five elements: (i) the thiostrepton resistance gene from pNC9501 (117); (ii) the origin of replication of pNC903, which was originally isolated from *R. ruber* P-II-123-1 (125); (iii) the colE1 origin of replication from pUC18; (iv) the ampicillin resistance gene from pUC18; and (v) the benzoate promoter from the RHA1 chromosome (126) (Fig. 24). Previous work had established that the promoter is induced 10,000-fold in the presence of benzoate (126), and primer extension analysis had elucidated minor and major transcription initiation sites 58 bp and 66 bp upstream of *benA* (127). As part of the latter, putative -10 and -35 σ70-type consensus regions were identified. This promoter region was cloned upstream of *anhAEB* in the shuttle vector to regulate the expression of these genes by adding benzoate to the growth medium (Fig. 25)
**Figure 25.** The *ben* gene cluster located on the RHA1 chromosome (A) and nucleotide sequence of the upstream region of *benA* (B). The vertical arrows indicate estimated major and minor transcriptional start points. The putative σ70-type promoter sequence and the deduced ribosome-binding site (RBS) for *benA* are enclosed in boxes. The start codon of *benA* is underlined. Adapted from (127).

**Figure 26.** Construction of the *Rhodococcus-E. coli* shuttle vector. Restriction enzyme sites used are indicated. Ap, ampicillin resistance gene; Thio, thiostrepton resistance gene; Km, kanamycin resistance gene.
The constructed shuttle-vector was introduced into RHA1 using electroporation conditions established in *Rhodococcus opacus* PD630 (117). Expression of the target protein was first tested in W minimal media (125) supplemented with 20 mM acetic acid. At an OD$_{600} = 0.6$, ANHase expression was induced by adding 20 mM benzoate. The expression of the target protein was evaluated by specific activity of ANHase using phenol-hypochlorite colorimetric assay on cell extracts and OD$_{600}$ was monitored to ensure the sufficient biomass for the further protein purification. To overcome the slow growth of RHA1 on W minimal media, expression was also tested in LB. As summarized in Fig. 27, when RHA1: pNUP$_{ben}$AEB was grown in W minimal media, relatively high levels of the target protein were produced 24 and 34 hours after addition of benzoate. However, the culture required more than 70 hours to reach OD$_{600} = 0.6$ before induction. On the other hand, in LB, cells reached OD$_{600} = 0.6$ within 24 hours. However, the level of ANHase activity was approximately 20% that produced in the W minimal medium-grown cells. The lower expression in LB may be due to catabolite repression caused by some component of the medium. To mitigate such an effect, expression was tested using 1/5 diluted LB. As on full strength LB, the culture reached OD$_{600} = 0.6$ within 24 hours. However, within 24 hours of adding benzoate to 20 mM, the specific activity of the ANHase was with 80% that of cells grown on W minimal media. Considering that much more biomass was obtained using 1/5 LB, this medium was used for protein production. Interestingly, no expression of ANHase was observed in 1/5 LB until the OD$_{600}$ reached 2.5, even in the presence of benzoate. For this reason, benzoate was not added until the culture reached OD$_{600} = 2.5$. Using this system, 1.7 mg l$^{-1}$ of ANHase was obtained which is over 30-fold more compared to wild-type RHA1 grown on acetonitrile (Table 8).
Figure 27. Optical density at 600 nm and specific activity of RHA1 cell extract grown on W minimal (W), LB, and 1-5 diluted LB (1/5 LB) media. Grey, optical density at OD$_{600}$; white, specific activity.

Table 8. Purification of ANHase using a Rhodococcus-E. coli shuttle vector.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Sp. activity (U mg$^{-1}$)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>211</td>
<td>106</td>
<td>0.5</td>
<td>100</td>
</tr>
<tr>
<td>Source Q</td>
<td>10.5</td>
<td>34</td>
<td>3.2</td>
<td>32</td>
</tr>
<tr>
<td>Source Phenyl</td>
<td>1.7</td>
<td>11</td>
<td>6.3</td>
<td>10</td>
</tr>
</tbody>
</table>

$^a$Cells were grown on W minimal media supplemented with 100 mM acetonitrile.
3.2 Metal-binding sites of AnhA

3.2.1 Sequence analysis

As mentioned in section 1.2.1, characterized NHases contain a Cys-rich motif C(T/S)LSC(Y/T) in the α subunit that binds the catalytic Co^{3+} or Fe^{3+} ion. Inspection of the ANHase amino acid sequence revealed no exact match of this motif. However, the ANHase α subunit, AnhA, contains a Cys-rich region in its C-terminus: CLLGCACW which potentially serves as the active site for the nitrile hydrolysis.

It was further noted that the N-terminal sequence of AnhA contains a relatively high number of histidinyl (5 in the first 12 residues), and acidic residues (5 Asp and Glu in the first 18) and thus could potentially bind metal ions. A multiple sequence alignment revealed that many of these His and acidic residues are conserved in the N-terminal sequences of Co-NHase α subunits (Fig. 28) despite the fact the AnhA and the Co-NHase α subunits share no other significant amino acid sequence similarity. Interestingly, this N-terminal sequence was not found in any Fe-NHase α subunit. This analysis suggests that the N-terminal sequence may play a role in cobalt ion binding.
3.2.2 IMAC of untagged AnhA

As a preliminary investigation of the metal-binding capacity of the N-terminal sequence of AnhA, the protein was heterologously produced and purified. An amplicon containing anhA was inserted downstream of the T7 promoter in pET41-b as described in Material and Methods, yielding pET41AnhA. The protein was heterologously produced in E. coli BL21 containing pET41AnhA and purified from cell extracts using Ni-NTA resin. This purification yielded 3.2 mg of protein per liter of cell culture, and is consistent with the N-terminal His-rich motif possessing high affinity for divalent metal ions. SDS-PAGE analysis indicated that the preparation was of >95% purity and that the molecular weight of the enzyme is about 65 kDa.
(Fig. 29), consistent with the deduced amino acid sequence of the \textit{anhA} gene product. This preparation was further purified using HiLoad 26/60 Superdex 200 column.

![Figure 29. Purification of AnhA using NiNTA chromatography. Lanes: 1, molecular weight markers; 2, crude extract of \textit{E. coli}. (20 µg of protein); 3, fraction eluted from NiNTA using 20mM HEPES buffer pH 8.0, containing 250 mM imidazole. (5 µg).](image)

3.2.3 Spectrophotometric analysis Co\textsuperscript{2+}-binding to the N-terminal peptide

To investigate the ability of the N-terminus of AnhA to bind cobalt, a synthetic peptide comprising the first 18 amino acid residues of AnhA, MPDHGHGDHGHNHDACDSE, was incubated with a 2.5 molar excess CoCl\textsubscript{2} and the change in the absorption spectrum was monitored. The electronic absorption spectrum changed in a time-dependent fashion, consistent with the binding of cobalt by the peptide (Fig. 30). The electronic absorption spectrum indicates the blue shift of the d-d electronic transition band of Co\textsuperscript{2+} and also the appearance of intense band at 300 nm.
Figure 30. The formation of peptide-cobalt complex. (A) 40 µM of N-terminal peptide was mixed with 100 µM of CoCl₂ 25 °C in 20 mM HEPES pH 7.5 and spectroscopic change was monitored every 3 minutes. (B) 300 nm to 800 nm region of spectra (A).

Figure 31. Binding of the AnhA N-terminal peptide to cobalt ion. Seventy-five µM of peptide was mixed with 10 – 100 µM of CoCl₂ and incubated at 25 °C for 2 hours (20 mM HEPES, pH 7.5). Then the absorbance was measured at 300 nm.

To investigate the stoichiometry of the binding, the N-terminal peptide was titrated with CoCl₂ and the change of the absorption at 300 nm was monitored as a function of the Co²⁺
concentration (Fig. 31). The molar extinction coefficient, calculated on the basis of the total cobalt ion, were $\varepsilon_{300} = 3.2 \pm 0.3 \times 10^3 \text{M}^{-1}\text{cm}^{-1}$ and $\varepsilon_{512} = 198 \pm 3 \text{M}^{-1}\text{cm}^{-1}$.

3.2.4 Calorimetric analysis of metal ion binding by N-terminal peptide

The binding of metal ions to the AnhA N-terminal peptide was further characterized through a series of ITC experiments. Thermograms shown in Fig. 31 were obtained by titrating 100 µM peptide with 0.5 – 1.0 µL aliquots of 0.5 – 1.0 mM divalent metal ions (20 mM HEPES, pH 7.5, 25 °C). The addition of either Co$^{2+}$, Zn$^{2+}$, Ni$^{2+}$ or Cu$^{2+}$ resulted in exothermic reactions. In titrations with each of these divalent metal ions, a one-site model fit to the integrated heat released upon each addition. The fits revealed that the peptide bound Co$^{2+}$, Zn$^{2+}$ and Ni$^{2+}$ with similar, low micromolar affinities and with almost identical stoichiometries (Table 9) indicating formation of a 2:1 peptide:metal ion complex. By contrast, the affinity of the peptide for Cu$^{2+}$ was an order of magnitude lesser and the stoichiometry was slightly different.
Figure 32. ITC analysis of the binding of (A) Co$^{2+}$, (B) Zn$^{2+}$, (C) Ni$^{2+}$ and (D) Cu$^{2+}$ to the AnhA N-terminal peptide. Experiments were performed at 25 ºC in 20 mM HEPES pH 7.5. Upper panel indicates calorimetric titrations (0.5 – 1 µl) of each divalent metal into 100 µM N-terminal peptide. Lower panel displays the integrated heats from the upper panel as a function of the metal/protein molar ratio.
Table 9. Thermodynamic parameters for the binding of divalent metals by the AnhA N-terminal peptide$^a$.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$K_d$ (µM)</th>
<th>$\Delta H$ (kcal mol$^{-1}$)</th>
<th>$\Delta S$ (cal mol$^{-1}$ K$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co$^{2+}$</td>
<td>0.45 (0.09)</td>
<td>2.9 (0.8) x 10$^5$</td>
<td>3.6 (0.8)</td>
<td>- 4.6 (0.7)</td>
<td>9.6 (2.1)</td>
</tr>
<tr>
<td>Ni$^{2+}$</td>
<td>0.51 (0.06)</td>
<td>3.2 x 10$^5$ (0.3 x 10$^5$)</td>
<td>3.2 (0.3)</td>
<td>- 15.8 (0.07)</td>
<td>27.8 (0.4)</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>0.45 (0.03)</td>
<td>2.3 x 10$^5$ (0.02 x 10$^5$)</td>
<td>4.3 (0.1)</td>
<td>- 6.3 (0.4)</td>
<td>3.3 (1.3)</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>0.78 (0.1)</td>
<td>1.8 x 10$^4$ (0.4 x 10$^5$)</td>
<td>56.1 (13.4)</td>
<td>- 12.9 (0.2)</td>
<td>23.7 (7.2)</td>
</tr>
</tbody>
</table>

$^a$0.5 mM divalent metal solution was titrated into 100 µM peptide (20 mM HEPES, pH 7.5 at 25 °C). The values in parentheses represent standard errors.
3.3 Characterization of AnhE

3.3.1 Growth phenotypes of RHA1 and mutant

The *anhE* gene (Locus ID RHA1_ro10171, accession number YP_708522) occurs between the two structural genes of ANHase and is predicted to encode an 11.1 kDa protein. To investigate the role of AnhE in the catabolism of nitriles by RHA1, *anhE* was subject to in-frame deletion (Fig. 33A) and the growth phenotype of the resultant mutant, RHA023, was investigated.

![Figure 33](image)

**Figure 33.** (A) The *anh* gene cluster located on pRHL2. Genes encoding AnhE (*anhE*) and ANHase subunits (*anhA* and *anhB*) are shaded in dark and light grey and a probable cobalt transporter (*anhT*) is shown in stripe. (B) Deduced acetonitrile catabolic pathway of RHA1. (C) The growth of RHA1 wild type strain (WT), *anhE* deletion mutant (*ΔanhE*) and *anhE* deletion mutant complemented in trans (*ΔanhEC*) on acetonitrile and acetamide.

Unlike wild-type RHA1, RHA023 was unable to utilize acetonitrile as a growth substrate. In contrast, the mutant strain grew on acetamide at the same rate as the parent strain and to the
same growth yield (Fig. 33C). Complementation of \( \Delta anhE \) in trans using a construct based on the pTip-QC2 shuttle vector restored growth on acetonitrile. Since the in-frame deletion of \( anhE \) affected growth on acetonitrile but not on acetamide, the hydration product of acetonitrile (Fig 33B), these results strongly indicate that AnhE plays some role in the transformation of acetonitrile. As AnhE was not detected in preparations of purified ANHase, we hypothesized that the protein is involved in the maturation of ANHase.

3.3.2 Assembly of ANHase in vitro

AnhA, the \( \alpha \) subunit of ANHase, contains the sequence CLLGCAC which is reminiscent of the CTSLCSC sequence of Co-NHases that binds the catalytically essential cobalt ion. To investigate the role of AnhE in ANHase maturation, \( anhA \) was expressed in \( E. coli \) either alone or with \( anhE \) (Fig 34A). The strains were grown in LB supplemented with different amounts of \( \text{CoCl}_2 \). Extracts of these cells were then incubated with purified AnhB and the ANHase activity was measured. As shown in Fig 34B, activation of ANHase required the \( \alpha \) and \( \beta \) subunits. Moreover, activity was only obtained when AnhA-containing extracts were prepared from cells grown in the presence of supplemental \( \text{CoCl}_2 \). When AnhA was produced in cells without AnhE, 50 \( \mu \text{M} \) \( \text{CoCl}_2 \) was required in the growth medium of AnhA-containing cells to observe ANHase activity. By contrast, when AnhA was co-produced in cells with AnhE, 0.5 to 5 \( \mu \text{M} \) of \( \text{CoCl}_2 \) was sufficient.
Figure 34. The assembly of ANHase in vitro. (A) The portion of the anh gene cluster used to construct pETAnhA and pETAnhAE. (B) The specific activity of ANHase reconstituted using cell extracts containing AnhA or AnhAE. Cells were grown in media supplemented with the indicated concentrations of CoCl$_2$. (C) The specific activity of ANHase reconstituted using purified AnhA. AnhA was purified from cells grown in media supplemented with the indicated concentrations of CoCl$_2$. In the assembly experiments, purified AnhB was incubated with either cell extracts or purified AnhA. The concentration of CoCl$_2$ supplemented in the growth media is indicated in (B) and (C).

To further investigate the role of AnhE in the biosynthesis of ANHase, AnhA was purified from the various cell extracts and its cobalt content was analyzed using ICP-MS. Samples of AnhA were also incubated with purified AnhB, and ANHase activity was determined using the colorimetric assay. When purified from cells containing no AnhE grown in LB supplemented with 5 µM CoCl$_2$, AnhA contained 0.06 ± 0.02 equivalents of cobalt (Fig 34B) and the specific activity of the incubation mixture was 0.08 ± 0.01 mg ml$^{-1}$ (Fig 34C). When the cells were then grown in LB supplemented with 50 µM CoCl$_2$, the obtained AnhA contained 0.49 ± 0.02 equivalents of cobalt. Moreover, the specific activity of the assembly mixture was 0.86 ± 0.07 mg ml$^{-1}$. Finally, the cobalt content of purified AnhA was even higher (0.56 ± 0.04 mol/mol) when the protein was purified from cells containing AnhE, even when the latter were grown in the presence of less CoCl$_2$ (5 µM). Similarly, this preparation of AnhA yielded unpurified ANHase of the highest specific activity: 0.97 ± 0.07 mg ml$^{-1}$. Attempts to assemble
ANHase *in vitro* by incubating apo-AnhA, AnhE, AnhB and CoCl$_2$ yielded no detectable ANHase activity.

To better characterize the ANHase assembled *in vitro*, the activity was purified from the incubation mixture. In this experiment, 5 mg of AnhA (purified from AnhE-containing cells) was incubated with 10 mg AnhB at 4 ºC for 16 h and further purified using anion exchange and gel filtration chromatographies. The specific activity of purified, in vitro assembled ANHase was 2.1 ± 0.1 U mg$^{-1}$ (20 mM HEPES, pH 7.5, 25 ºC). This compares favorably with the wild-type enzyme (118) under these conditions: 2.3 ± 0.1 U mg$^{-1}$. Overall, these results indicate that AnhE plays a role in delivering cobalt to AnhA in the maturation of ANHase.

Interestingly, the absorption spectrum of ANHase from RHA1 (20 mM HEPES, pH 7.5, 25 ºC) had maxima at 414 nm, 525 nm, and 560 nm with the shoulder around 300 – 350 nm, whereas the reconstituted ANHase from *E. coli* BL21 had a slight shoulder around 300 – 350 nm with no characteristic peak, in contrast to the wild-type enzyme (Fig. 35).

**Figure 35.** Electronic absorption spectra of recombinant ANHase from RHA1 and purified reconstituted enzyme. The concentration of protein in both samples was 2.0 mg ml$^{-1}$ and the buffer was 20 mM HEPES, pH 7.5, 25 ºC.
3.3.3 AnhE cloning, expression and purification

To further characterize the function of AnhE, the protein was heterologously produced and purified. The \textit{anhE} gene was cloned downstream of the T7 promoter in pET41-b. Purification of AnhE using anion exchange and gel-filtration chromatographies from cell extracts of \textit{E. coli} BL21 containing pETAnhE yielded over 53 mg of protein per litre of cell culture. SDS-PAGE analysis indicated that purified AnhE was apparently homogeneous. The molecular mass of AnhE, 11,071.13 Da, determined by MALDI-ToF mass spectrometry was within 0.2% of the predicted value of 11,093.51 Da. As outlined below, the behavior of AnhE was strongly dependent on the presence of divalent metal ions. For this reason, AnhE preparations were dialyzed extensively against 5 mM EDTA and then EDTA-free buffer prior to experiments.

3.3.4 Metal-dependent dimerization

The oligomeric state of AnhE was investigated using gel-filtration chromatography. As shown in Fig. 36, AnhE existed as an equilibrium between two forms (20 mM HEPES, pH 7.5) with apparent molecular weights of 16.2 kDa and 29.0 kDa, respectively. These were taken to represent monomeric and dimeric forms of AnhE, respectively. Integration of the peak areas indicated that the equilibrium was ~2:3 at 0.1 mM AnhE and shifted to ~1:4 at 0.5 mM AnhE. Addition of 5 mM EDTA in the equilibration buffer did not prevent the dimerization of AnhE. Incubation of 0.1 mM AnhE with 0.1 mM CoCl$_2$, ZnCl$_2$, or NiCl$_2$ shifted the equilibrium to essentially 100% of the dimeric form, consistent with metal ion-dependent dimerization of AnhE (Fig 36C). By contrast, incubation of AnhE with 0.1 mM CuCl$_2$ had no detectable effect on the dimerization property of AnhE. Higher order complexes were not detected in any of these experiments.
Figure 36. Molecular weight determination of apo-AnhE and metal-bound AnhE by size exclusion chromatography. Elution profile of (A) 0.1 mM AnhE, (B) 0.5 mM AnhE in the absence/presence of equivalent of CoCl$_2$. 100 µl of 0.1 mM or 0.5 mM protein solution were applied onto Superdex 75 10/300 GL column (Amersham Biosciences) pre-equilibrated with the buffer containing 20 mM HEPES (pH 7.5), 50 mM NaCl. (C) Elution profile of 0.1 mM AnhE in the presence of 0.1 mM ZnCl$_2$, NiCl$_2$ and CuCl$_2$. (D) Standard curve for the molecular weight determination was calculated using lysozyme (15 kDa), chymotrypsinogen (25 kDa), carbonic anhydrase (32 kDa), β-lactoglobulin (36 kDa) and bovine serum albumin (68 kDa). AnhE monomer and dimer are also indicated.
3.3.5 Spectrophotometric analysis of Co$^{2+}$-binding to AnhE

The d-d transitions of Co$^{2+}$ give rise to weak bands between 450 and 700 nm in the electronic absorption spectrum and can be used to investigate the coordination geometry of the bound metal ion in proteins (128). The addition of 500 µM AnhE to a solution of 500 µM CoCl$_2$ resulted in a blue-shifted, more intense absorption band of the latter (Fig. 37). This is consistent with a change in the coordination sphere of the cobalt ion and thus its binding to AnhE.

![Figure 37](image.png)

**Figure 37.** Electronic absorption spectrum of cobalt-bound AnhE. The spectrum of 500 µM CoCl$_2$ and the difference spectrum for AnhE titrated with 1 equiv of CoCl$_2$ are shown. The difference spectrum was generated by subtracting the spectrum of apo-AnhE. (20 mM HEPES pH 7.5 at 25 °C).

The molar extinction coefficient of the AnhE-Co$^{2+}$ complex at 490 nm, calculated on the basis of the cobalt concentration, was $\varepsilon_{490} = 9.7 \pm 0.1$ M$^{-1}$cm$^{-1}$. The spectrum of the AnhE-Co$^{2+}$ complex prepared using 500 µM AnhE and 250 µM CoCl$_2$ was very similar, but half as intense (results not shown).
3.3.6 Isothermal titration calorimetry

The electronic absorption spectra and gel-filtration chromatography studies indicate that AnhE interacts directly with divalent metal ions. To characterize the binding of metal ions by AnhE, a series of ITC experiments was performed. Inspection of the thermogram in Figure 5A reveals that titration of a 50 µM solution of AnhE with 0.5 µL aliquots of 0.5 mM CoCl$_2$ (20 mM HEPES, pH 7.5, 25 °C) resulted in exothermic reactions (Fig. 38A). As titration continued, a rapid transition to endothermic reactions was observed. These reactions were dependent on both the metal ion and the protein, and strongly suggest that Co$^{2+}$ is involved in two distinct binding events. Indeed, a two-site model better fit the integrated heat release from each Co$^{2+}$ addition. In the best fit, each site possessed a stoichiometry of N = 0.5 and nanomolar dissociation constants differing by three orders of magnitude. Based on replicate titrations, these constants were $K_{d1} = 0.12 \pm 0.06$ nM and $K_{d2} = 110 \pm 35$ nM. Although the value for $K_{d1}$ is relatively low for the ITC$_{200}$ system, the quality of the data (Fig. 37A) indicates that the value is accurate. Together with the gel filtration studies, these results suggest the existence of two distinct metal-binding sites in AnhE homodimer.

Titration of AnhE with Zn$^{2+}$ or Ni$^{2+}$ also resulted in exothermic reactions (Fig. 38BC), similar to those observed during the initial titration of the protein with Co$^{2+}$. Unlike Co$^{2+}$ and Ni$^{2+}$, when Zn$^{2+}$ ion was titrated into buffer alone, a consistent endothermic reaction was observed, similar to that observed in the titration with AnhE after the exothermic reactions were completed. This pattern indicates that the endothermic reaction with Zn$^{2+}$ was not due to the binding of the metal ion to AnhE. Titration of AnhE with neither Zn$^{2+}$ nor Ni$^{2+}$ at concentrations up to 2.5 mM yielded the second phase of endothermic events observed with Co$^{2+}$. Accordingly,
a one-site model fit the binding of Zn$^{2+}$ and Ni$^{2+}$ to AnhE with $N = 0.5$ and $K_d = 11 \pm 2$ nM and $49 \pm 17$ nM, respectively. It is possible that these metal ions bound to a second site but that this binding was either too weak or was driven entirely by entropy. Finally, AnhE did not detectably bind Cu$^{2+}$ at concentrations up to 2.5 mM.

The calculated thermodynamic parameters for the above-described binding events, based on three to five replicates, are summarized in Table 10. The binding of Zn$^{2+}$ and Ni$^{2+}$, as well as the initial binding of Co$^{2+}$ were driven by both favorable enthalpy and entropy reflecting the strength of the protein:metal ion interaction (129,130). Moreover, enthalpies of Ni$^{2+}$ and Zn$^{2+}$ binding were more favorable than for Co$^{2+}$: the higher affinity of AnhE for Co$^{2+}$ ($\Delta \Delta G = -2.7$ kcal mol$^{-1}$ and -3.6 kcal mol$^{-1}$, respectively) was due to the more favorable entropy. By contrast, the second binding transition for Co$^{2+}$ was driven by favorable entropy, which offset the positive enthalpy. This is consistent with increased conformational freedom, such as desolvation, accompanying the occupation of the second binding site.
Figure 38. ITC analysis of the binding of (A) Co$^{2+}$, (B) Zn$^{2+}$, (C) Ni$^{2+}$ and (D) Cu$^{2+}$ to AnhE. All the experiments were performed at 25 °C in 20 mM HEPES pH 7.5. Upper panel indicates calorimetric titrations (0.5 – 1 µl) of each divalent metal into 50 µM AnhE. Lower panel displays the integrated heats from the upper panel as a function of the metal/protein molar ratio.
Table 10. Thermodynamic parameters for the binding of divalent metals by AnhE.

<table>
<thead>
<tr>
<th>First-binding</th>
<th>$N$</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$K_d$ (nM)</th>
<th>$\Delta H$ (kcal mol$^{-1}$)</th>
<th>$\Delta S$ (cal mol$^{-1}$ K$^{-1}$)</th>
<th>$\Delta G^a$ (kcal mol$^{-1}$)</th>
<th>$K_a/K_{a(co)}$</th>
<th>$\Delta \Delta G$ (kcal mol$^{-1}$)</th>
<th>$n^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co$^{2+}$</td>
<td>0.48 (0.03)</td>
<td>$10 \times 10^9 (5 \times 10^9)$</td>
<td>0.12 (0.06)</td>
<td>-3.7 (0.2)</td>
<td>32 (3)</td>
<td>-13.6 (0.9)</td>
<td>1</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>0.478 (0.002)</td>
<td>$10 \times 10^7 (2 \times 10^7)$</td>
<td>11 (2)</td>
<td>-6.8 (0.2)</td>
<td>13.7 (0.9)</td>
<td>-10.9 (0.1)</td>
<td>0.01</td>
<td>-2.7</td>
<td>3</td>
</tr>
<tr>
<td>Ni$^{2+}$</td>
<td>0.49 (0.06)</td>
<td>$2.2 \times 10^7 (0.7 \times 10^7)$</td>
<td>49 (17)</td>
<td>-7.9 (0.4)</td>
<td>7 (2)</td>
<td>-10.0 (0.2)</td>
<td>0.0022</td>
<td>-3.6</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Second-binding</th>
<th>$N$</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$K_d$ (nM)</th>
<th>$\Delta H$ (kcal mol$^{-1}$)</th>
<th>$\Delta S$ (cal mol$^{-1}$ K$^{-1}$)</th>
<th>$\Delta G^a$ (kcal mol$^{-1}$)</th>
<th>$K_a/K_{a(co)}$</th>
<th>$\Delta \Delta G$ (kcal mol$^{-1}$)</th>
<th>$n^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co$^{2+}$</td>
<td>0.42 (0.04)</td>
<td>$10 \times 10^8 (4 \times 10^8)$</td>
<td>110 (35)</td>
<td>1.6 (0.4)</td>
<td>36 (2)</td>
<td>-9.0 (0.8)</td>
<td>1</td>
<td>-</td>
<td>5</td>
</tr>
</tbody>
</table>

0.5 mM divalent metal solution was titrated into 50 µM AnhE in 20 mM HEPES, pH 7.5 at 25 °C.
The values in parentheses represent standard errors.

$^a$Δ$G = RT \ln K_a$, $T = 298$ K

$^b$Δ$\Delta G = RT \ln (K_d/K_{a(co)})$

$^c$n = number of observations
3.3.7 The role of His41 in cobalt binding

AnhE contains no cysteine residues and only a single histidine residue, His41. To investigate the role of this residue as a ligand, it was substituted with alanine and the metal-binding properties of the resultant H41A variant were investigated. ITC data obtained using Co$^{2+}$ fit a two-site model with $K_{d1}$ of $0.13 \pm 0.08$ nM and $K_{d2} = 140 \pm 60$ nM, and each site possessing a stoichiometry of $N = 0.5$. These results are remarkably similar to what was observed in WT AnhE, indicating that His41 is not involved in metal ion binding.
CHAPTER FOUR: DISCUSSION

4.1 Discovery of nitrile catabolic gene clusters

Proteomics, genome analysis, biochemical studies and molecular genetics were used to identify the enzymes responsible for the catabolism of nitriles in *Rhodococcus jostii* RHA1. Growth studies indicated that RHA1 utilizes a variety of nitriles with varied efficiency. Proteomic and genomic approaches further revealed that this bacterium possesses at least two distinct nitrile catabolic pathways, encoded by the *anh* and *nth* gene clusters, respectively. The *nth* genes are chromosomally located and the encoded proteins were more abundant in phenylacetonitrile-grown cells (Fig. 15). By contrast, the *anh* genes are located on pRHL2, one of RHA1’s three linear plasmids. The *anh*-encoded enzymes include an amidase (AnhC) and an acetyl-CoA hydrolase (AnhD), which are predicted to transform acetamide to acetyl-CoA and ammonia (Fig.12). Other encoded proteins include three predicted transcriptional regulators (AnhP, AnhQ, and AnhR), a probable cobalt transporter (AnhT), and three proteins whose respective amino acid sequences shared no significant sequence identity with anything in the databases. As discussed below, these three proteins comprised ANHase (AnhAB) and a cobalt chaperone (AnhE).

Bioinformatic analyses strongly suggest that the *nth* genes encode an Fe-NHase that is also involved in aldoxime catabolism. First, the encoded NHase contains the sequence motif CSLCSCT, which ligates the Fe$^{3+}$ and is the signature of Fe-NHases (52). Second, *nthE* encodes a protein that shares 86% amino acid sequence identity with P47K. The latter is involved in the maturation of Fe-NHases, and likely is an iron chaperone (110). Finally, the other *nth*-encoded
enzymes are predicted to be an aldoxime dehydratase and an amidase. The former transforms aldoximes to nitriles.

4.2 Characterization of AnhC

AnhC, the aliphatic amidase encoded by the *anh* gene cluster, belongs to the nitrilase superfamily. This is in contrast to the majority of amidases characterized from nitrile-degrading bacteria to date, which belong to the AS family. Aliphatic amidases contain the Glu-Lys-Cys catalytic triad of the nitrilase superfamily, but hydrolyze amides instead of nitriles. Aliphatic amidases have been characterized in a variety of bacteria including *Pseudomonas*, *Bacillus*, *Brevibacteria* and *Helicobacteria* strains (16). In addition to their role in amide catabolism, aliphatic amidases can also play critical roles in pathogenesis. For example, the human pathogen *H. pylori* uses an aliphatic amidase to produce ammonia which serves as a nitrogen source (131). The production of ammonia in the gastric environment of *H. pylori* is also essential for the acid resistance of this pathogen (132).

AnhC of RHA1 shares 75% amino acid sequence identity with AmiE, an aliphatic amidase, in *Brevibacterium* sp. R312 (133,134), and its catalytic triad is predicted to be Glu59, Lys134 and Cys166. AmiE acts on aliphatic amides with highest specific activity against propioamide (16.7 µmol min⁻¹mg⁻¹) followed by acetamide (9.0 mol min⁻¹mg⁻¹) and benzamide (0.8 µmol min⁻¹mg⁻¹). As shown in Fig. 17 and 18, the relative activities of AnhC indicate that this enzyme has a similar substrate preference to that of AmiE. Nevertheless, the gene clusters containing *anhC* and *amiE* share no similarity other than the amidase-encoding genes, indicating independent recruitment events during the evolution of these clusters.
4.3 Characterization of ANHase

ANHase appears to be the first recognized member of a novel class of NHases. While NHases have been identified in diverse bacteria (23,135), these studies have used approaches such as Southern hybridization and PCR that select for enzymes sharing significant sequence similarity with known NHases. Although ANHase is similar to other NHases in that it is an αβ-heterodimeric metalloenzyme, it shares no significant amino acid sequence similarity with any protein in the databases, including NHases. Moreover, the sequence similarity of the α and β subunits of ANHase is unusual: in characterized NHases, the subunits share no significant sequence similarity. The previously characterized NHases are classified based on the identity of their catalytically essential metal ion: non-heme iron or non-corrinoid cobalt (1). These enzymes share a common structural fold and a seven-residue motif in the middle of their α subunits:, C(T/S)LCSC(Y/T), which contains the three Cys residues that ligate the metal ion, (23). Neither ANHase subunit contains this motif. Interestingly, the ANHase α subunit contains another cysteine-rich motif in its C-terminus: CLLGCACW.

In addition to its sequence, ANHase of RHA1 differs from characterized NHases in at least three additional respects: size, metal content and substrate specificity. The α and β subunits of known NHases range in molecular weight from 26 to 35 kDa. By contrast, the subunits of ANHase are 63 kDa and 56 kDa, respectively. It is currently unclear why the subunits of ANHase are so much larger than that of known NHases. With respect to substrate specificity, ANHase appears to be unique in that it has a relatively high specificity for acetonitrile (Table 6). By contrast, the characterized NHases that preferentially transform aliphatic nitriles over aromatic nitriles have greatest specificity for longer aliphatic ones such as propionitrile,
butyronitrile or valeronitrile (136-138). Nevertheless, the specificity of ANHase towards its preferred substrates \( k_{\text{cat}}/K_m = 7.6 \text{ mM}^{-1}\text{s}^{-1} \) for acetonitrile appears to be similar to that of other NHases for their preferred substrates. For example, the \( k_{\text{cat}}/K_m \) of propionitrile NHase from \( P. \) \textit{chlororaphis} B23 for propionitrile is 1.0 mM\(^{-1}\)s\(^{-1}\) (136). ANHase from \textit{Rhodococcus jostii} RHA1 also has unique feature in pH dependence. It shows a relatively broad range of optimum pH which is between pH 5.5-10.0 with sharp drops of activity beyond this range (Fig. 23). This range is significantly wider than other NHases from \textit{R. rhodochrous} J1 (H-NHase, pH 6.0-8.5; (139), \textit{P. chlororaphis} B23 (pH 6.0-7.5;(137)) and \textit{Rhodococcus sp}. N774 (pH 7.0-8.5;(140)), which showed a decrease in activity above pH 7.5-8.5. This suggests that the active site of ANHase might differ with respect to key amino acid residues or metal ions that comprise it.

The roles of the metal ions in ANHase are unclear. However, the metal analyses following treatment with EDTA indicate that all four ions (the cobalt, the two copper and the zinc) are tightly bound. The similarity of the absorption maximum of ANHase at 414 nm to that at \( \approx 410 \text{ nm} \) in Co-NHases (58) suggests that cobalt is also responsible for the peak in ANHase and that this ion might be ligated by three cysteine residues as in Co\(^{3+}\)-type NHases (141). The occurrence of a gene encoding a probable cobalt transporter in the \textit{anh} cluster (Fig. 2) further suggests the importance of the cobalt ion in ANHase. A similar gene is located downstream of the genes encoding the cobalt-containing L-NHase in \textit{R. rhodochrous} J1 (33). This transporter contains putative membrane-spanning domains and facilitates the cellular uptake of cobalt. The stoichiometry of 1 cobalt ion per \( \alpha \beta_2 \) protomer suggests that this ion occurs in the \( \alpha \) subunit. Moreover, the C-terminal CLLGCAC sequence of this subunit contains a number of residues that may ligate the ion.
4.4 Other genes encoded on *anh* gene cluster

An *anh* gene cluster encodes three regulatory proteins that share common features with other regulatory proteins that control the expression of genes by detection nitrile or its metabolites. The probable transcriptional regulators AnhPQ share sequence similarities with well characterized Ami proteins (AmiAC) of *M. smegmatis*, which implies a similar mechanism of action. AmiE is an amidase that enables *M. smegmatis* to utilize short chain amides, including acetamide, as sole organic growth substrate and nitrogen source (142). Expression of *amiE* is strongly induced in the presence of acetamide due to the actions of the two-protein regulatory system encoded by *amiA* and *amiC* (143,144). AmiA, the homologue of AnhP, is a DNA-binding protein that represses the expression of *amiE*. This repression is lifted by AmiC, the homologue of AnhQ, when the latter binds acetamide. The crystal structure of an AmiC:acetamide binary complex (145) indicates that the amide of the bound effector forms four hydrogen bonds with the protein: two to Ser85 and one with each of Tyr150 and Tyr104. These three residues are conserved in AnhQ of RHA1, suggesting that acetamide is also the effector of the AnhPQ system. Finally, homologues of the Anh regulatory proteins also are involved in regulating nitrile catabolic genes in *R. rhodochrous* J1 (38,41). Thus, NhhD, a homologue of AnhP, is encoded by the operon that encodes high molecular mass-nitrile hydratase (H-NHase), and NhlC and NhhC, homologues of AnhQ, are encoded by the operons that encode low molecular mass-nitrile hydratase (L-NHase) and H-NHase, respectively.

The third regulatory protein of the *anh* cluster, AnhR, shares sequence identity with the XylS/AraC-family that are involved in regulating the transcription of nitrile catabolic genes in rhodococci. The best characterized of these, NitR, is a 319 residue protein that shares 42%
amino acid sequence identity with AnhR and regulates nitrilase in R. rhodochrous J1. Upon activation by isovaleronitrile, NitR binds to the promoter region of nitA, which encodes a nitrilase, inducing transcription of the latter (41). However, AnhR is predicted to be much shorter than NitR and related proteins, consisting only of a carboxyl terminal helix-turn-helix (HTH) DNA-binding motif and a 20-residue N-terminal region possessing no recognizable sequence motifs. The mechanism of AnhR is therefore unclear. Nevertheless, the presence of multiple transcriptional regulators in the anh cluster suggests that this operon has multiple promoters.

Another gene of the anh cluster, anhT, encodes a probable cobalt transporter. AnhT shares 72% amino acid sequence identity with NhlF, a characterized cobalt transporter in R. rhodochrous J1. As mentioned in section 1.2.2, NhlF is a membrane protein that takes up Co\(^{2+}\) in a proton-dependent manner (33). Homologues of this gene occur in clusters that encode Co-NHases but not Fe-NHases. These cobalt transporters contain a conserved motif, GX\(_2\)HAXDADH, in a transmembrane domain. The His residues of this motif are proposed to be cobalt ligands during the metal ions transport. In the motif that occurs in AnhT, the His residues correspond to His65 and His71. Overall, the presence of an nhlF homologue in the anh cluster strongly suggests that ANHase is a cobalt-dependent enzyme.

4.5 Construction of a Rhodococcus-E. coli shuttle vector

The Rhodococcus-E. coli shuttle vector constructed for the homologous production of ANHase should be useful for the production of other proteins using rhodococci as hosts. A number of bacterial proteins have been produced to higher levels in rhodococci than in E. coli (146,147) using vectors such as pTip, in which gene expression is driven by the thiostrepton-
inducible tipA promoter (P_{tipA}) (148,149). Another vector, pMind, has been used to express genes in mycobacteria (148,149), a related actinomycete. A major advantage of the vector constructed in this study is the utilization of the P_{ben} promoter of RHA1. Transcriptional analysis using reverse transcriptase quantitative PCR (RT qPCR) has established that the benzoate catabolic gene cluster, benABCDK, is up-regulated 10,000-fold in the presence of benzoate (126). In comparison, thiostrepton induces P_{tipA} up to 200-fold (148,149) while tetracycline induces the tetA promoter (P_{tetA}) of pMind up to 70-fold (148,149). In addition, thiostrepton is much more expensive than benzoate. Finally, catabolite repression was observed when cells were grown on a mixture of benzoate and phthalate in Rhodococcus sp. strain DK17 (150). In their study, the consumption of phthalate only started after benzoate had been completely depleted, indicating the preferred metabolism of benzoate over phthalate. Overall, the extremely high expression of benzoate catabolic genes, the low cost of benzoate and the preference of RHA1 for benzoate as a carbon source makes P_{ben} a good promoter for the expression of target genes in Rhodococcus.

### 4.6 N-terminal metal binding sequence in ANHase

As part of this thesis, a metal-binding sequence was identified in the N-terminus of the ANHase α subunit that is potentially involved in the maturation of this enzyme. Analyses of the sequence, which is rich in histidinyl and acidic residues, revealed that it is conserved in the N-terminus of the α subunit of some characterized and predicted Co-NHases but not in any of the characterized or predicted Fe-NHases. IMAC experiments on heterologously produced AnhA suggested that the sequence has an affinity for divalent metal ions. Spectrophotometric and ITC studies of a 21-residue synthetic peptide based on the N-terminus of AnhA established that the sequence has low micromolar affinity for several divalent metals including Co^{2+}, Zn^{2+} and Ni^{2+}. 

91
The occurrence of the sequence in the N-terminus of some cobalt-containing NHases, but not in any iron-containing homologues, is consistent with its role in cobalt binding and NHase maturation.

The occurrence of the potential Co$^{2+}$-binding sequence in the N-terminus of the $\alpha$ subunit of ANHase and some Co-NHases is particularly striking given the otherwise lack of amino acid sequence similarity in these proteins. Moreover, the lack of this N-terminal sequence in all known and predicted Fe-NHases suggests that this sequence has a role in Co-trafficking. The possible binding capacity of these residues has been implied in other cobalt-binding proteins discussed later in this section. However, this is the first demonstration of Co$^{2+}$-binding to any of these sequences.

The high affinity of the peptide for divalent metal ions is consistent with the ability of AnhA to be purified using IMAC (Fig 28). The affinity of the His/Met-rich N-terminal sequence in Cu, Zn-SOD, mentioned in section 1.6.3, for divalent metals was also first demonstrated using IMAC (151). Subsequent studies established the physiological role of this sequence in copper and zinc trafficking (105). Nevertheless, further experiments are required to establish the role of the AnhA N-terminal sequence in resin-binding.

The current studies help establish the utility of “peptide maquettes”, or synthetic peptides, to investigate the role of sequence motifs in proteins. Peptide maquettes have been used to characterize the metal-binding sites of HypB from E. coli (152) and Cu,Zn-SOD from Haemophilus ducreyi (153), respectively. In the former case, a 10-residue peptide, MCTTCGCGEG, was used to characterize the Ni$^{2+}$-binding site of HypB. The peptide had a dissociation constant of $2.0 \times 10^{-11}$ M for this metal ion. Moreover, XAS studies established that
the Ni\(^{2+}\) is coordinated by three Cys thiolates and the N-terminal amine in a square-planar geometry (152). This agreed with the coordination environment revealed by EXAFS and near-edge spectra using intact HypB (154). As described in section 1.6.4, Cu,Zn-SOD from *H. ducreyi* possesses an N-terminal metal-binding sequence which is distinct from the active metallocentre and plays a role in metal ion uptake. The N-terminal peptide of SOD, HGDGMGNGDTK, was also used to establish its high affinity for Cu\(^{2+}\) \((K_d = 5.0 \times 10^{-12} \text{M})\) and Zn\(^{2+}\) \((K_d = 1.6 \times 10^{-9} \text{M})\) (105). The metal-binding properties of the N-terminal peptide of AnhA thus provides good evidence that this motif has a physiological role in the metal-binding properties of ANHase.

The ITC studies indicate that the N-terminal peptide has similar, low micromolar affinity for Co\(^{2+}\), Zn\(^{2+}\) and Ni\(^{2+}\) \((K_d = 3.2 – 4.3 \mu\text{M})\). Although this result suggests that the N-terminal sequence plays no role in determining the specificity of ANHase for cobalt, it is possible that determinants located elsewhere in AnhA contribute to this specificity. Alternatively, it is possible that only Co\(^{2+}\) is delivered to the N-terminal sequence through the actions of the AnhT cobalt transporter and/or (AnhE, both of which are encoded by the same gene cluster as ANHase.

The electronic absorption spectrum of the Co\(^{2+}\)-bound peptide suggests that the metal is 5-coordinate and that two of the ligands are cysteine thiolates. Involvement of the cysteine is indicated by the absorbance of the complex at 300 nm (Fig. 29), which corresponds to the typical sulfur-to-cobalt charge transfer resulting from thiolate coordination (155). More specifically, the extinction coefficients of ligand-to-metal charge transfer (LMCT) bands are normally 900 – 1300 M\(^{-1}\)cm\(^{-1}\) per cobalt-thiolate bond (156). The obtained extinction coefficient, \(\varepsilon_{300} = 3.2 (\pm 0.3) \times 10^3 \text{M}^{-1}\text{cm}^{-1}\), suggests that two or three of the Co\(^{2+}\) ligands are Cys residues. Considering the
Evidence for a 5-coordinate Co\(^{2+}\) site is derived from the intensity of the band at 512 nm. Co\(^{2+}\) complexes absorb between 450 and 700 nm due to d-d transitions. The intensity of the band is characteristic of the coordination geometry: four-coordinate sites, typically have extinction coefficients greater than 300 M\(^{-1}\)cm\(^{-1}\); five-coordinate, between 50 and 300 M\(^{-1}\)cm\(^{-1}\); and six-coordinate sites are below 50 M\(^{-1}\)cm\(^{-1}\). Thus, the relatively strong d-d transitions (\(\epsilon_{512} = 198 \pm 3\) M\(^{-1}\)cm\(^{-1}\)) obtained from the binding of N-terminal peptide to cobalt ion suggest a five-coordinate geometry. While it is unclear how a dimeric scaffold would provide five ligands, it is possible that one is a solvent species. In any case, further studies are warranted as the type of ligands and the symmetry of the coordination sphere also influence the intensity of absorption.

Both the electronic absorption spectroscopic and ITC titrations indicate that the stoichiometry of peptide:metal in the complex was 2:1. This stoichiometry might be physiological relevant as there are two AnhA subunits in the holoANHase. Thus, a dimer of AnhA may bind Co\(^{2+}\) through their N-terminal regions. It is also possible that the stoichiometry of the peptide does not reflect that of AnhA. Further study is required to elucidate the coordination chemistry of the Co\(^{2+}\)-binding site in N-terminus of ANHase.

The N-terminal sequence of AnhA is reminiscent of the potential Co\(^{2+}\)-binding sequence at the C-terminus of some CbiX-type cobaltochelatases, mentioned in section 1.6.2. Significant numbers of histidinyl and acidic residues occur in both sequences. As discussed above, these kinds of residues are often involved in coordinating cobalt. The N-terminal sequence of AnhA differs from that of other Co-NHases and the cobaltochelatases in that it also contains a Cys residue that is likely serves as a cobalt ligand. Further studies may reveal whether the
involvement of this Cys residue reflects the unique nature of ANHase and its maturation. Regardless, the presented data provide important new insights into Co^{2+}-trafficking, which has been less well characterized than that of other metal ions such as copper, nickel and iron.

4.7 Characterization of AnhE – a cobalt metallochaperone

The current study establishes that AnhE is required for the \textit{in vivo} production of functional ANHase. It was further demonstrated that this small protein binds a number of divalent metal ions with nanomolar affinity and that these ions promote the dimerization of AnhE. However, AnhE bound Co^{2+} with the highest affinity, and bound twice as many equivalents of Co^{2+} as other tested metal ions. As discussed below, the requirement of AnhE for the production of active ANHase, the high affinity of AnhE for its physiologically relevant metal ion and its metal-ion induced conformational changes are characteristic of metallochaperones and strongly suggest that the physiological role of AnhE is to deliver cobalt ion to ANHase.

The high affinity of AnhE for Co^{2+} is consistent with the high affinity of other metallochaperones for their physiologically relevant metal ions. More specifically, the value for AnhE ($K_{d} = 0.12 \pm 0.06$ nM) is intermediate between those of UreE ($K_{d} = 1.6$ nM (158)) and HypB, a Ni^{2+}-specific metallochaperone for [NiFe]-hydrogenase, ($K_{d} = 0.13$ to 0.44 pM (159)). Similarly, IscA, a metallochaperone involved in the assembly of iron-sulfur clusters, binds to Fe^{2+} with sub-picomolar affinity (160). The much higher affinity of AnhE for Co^{2+} versus Zn^{2+} ($K_{d} = 11$ nM) is remarkable given that these two metal ions often utilize similar donor ligands and can accommodate the same coordination geometry (161,162). Nevertheless, the higher affinity of AnhE for Co^{2+} versus Ni^{2+} or Zn^{2+} is consistent with the higher affinity of UreE for Ni^{2+} versus Zn^{2+} (158),
The metal ion binding sites in metallochaperones are often quite unusual as they must not only tightly bind the metal ion so as to avoid cytotoxic effects, but also release it to the target protein. The presented data indicate that the Co\textsuperscript{2+} binding sites of AnhE are hexacoordinate and that the lone histidinyl residue in the protein does not contribute directly to them. More particularly, the low extinction coefficient of AnhE:Co\textsuperscript{2+} (\( \varepsilon_{490} = 9.7 \text{ M}^{-1}\text{cm}^{-1} \)) is within the range of that observed for six-coordinate sites. By contrast, four- and five-coordinate sites have extinction coefficients greater than 50 and 300 M\(^{-1}\)cm\(^{-1} \), respectively (157). Further study is required to identify the metal ligands of AnhE and to elucidate the protein’s metal ion specificity.

The Co\textsuperscript{2+}–induced dimerization of AnhE is similar to metal ion-induced conformational changes in metallochaperones and other proteins. For example, the metal-binding sites of UreE, HypB and IscA occur at their respective subunit interfaces and appear to stabilize the functional oligomeric forms of these proteins (91,92,102,104,163). Similarly, the binding of Zn\textsuperscript{2+} to the zinc finger induces a conformational change from an extended \( \beta \)-sheet to the \( \beta\beta\alpha \) hairpin structure required for recognition of target DNA sequences (164). Moreover, the binding of Ni\textsuperscript{2+} to NikR, a prokaryotic transcription factor, triggers a conformational change which results in the regulation of the expression of nickel-containing enzymes and transporters. Intriguingly, this rearrangement was not induced by Zn\textsuperscript{2+}-binding (165). This specificity of the conformational change in NikR for Ni\textsuperscript{2+} is similar to the Co\textsuperscript{2+}-specific changes in AnhE activity.

The current data suggest a model in which a functional homodimer of AnhE binds two equivalents of Co\textsuperscript{2+}. While the thermodynamic analyses clearly revealed that AnhE binds two half-equivalents of Co\textsuperscript{2+} with different affinities, specific metal-binding sites cannot be proposed. Nevertheless, the current data indicate that binding could involve one of two scenarios.
**Figure 39.** Proposed mechanisms of the influence of Co\(^{2+}\)-binding on the activity of AnhE. (A) Co\(^{2+}\) binds to high- and low-affinity sites at the AnhE homodimer interface. (B) Co\(^{2+}\) binds away from the dimer interface but drives dimerization through conformational changes. Circular and square surfaces represent high and low-affinity sites, respectively. The implied equivalence of the two sites in the AnhE\(_2\)(Co\(^{2+}\))\(_2\) species is unintended.

In the first case (Fig. 39A), the ions bind at the AnhE dimer interface. In this case, there are two such sites for Co\(^{2+}\), and either one or two for Zn\(^{2+}\) or Ni\(^{2+}\) (with the second site being too weak to detect). In a second scenario (Fig. 39B), the binding site does not involve residues at the dimer interface, but drives dimerization through conformational changes and, as a result, alters the affinity of the ion for the second molecule in the dimer. The first scenario is reminiscent of UreE, a Ni\(^{2+}\)-specific metallochaperone involved in the maturation of urease. The UreE homo-dimer binds two nickel ions at non-identical sites present at the dimer interface (166). These sites also accommodate Cu\(^{2+}\) and Co\(^{2+}\). However, a variety of spectroscopic methods indicate that the coordination environment is different for Ni\(^{2+}\), facilitating its selection for urease activation (166,167). It is possible that Co\(^{2+}\) interacts with AnhE in a similar manner.

The lack of significant sequence identity between AnhE and two other potential Co\(^{2+}\)-specific metallochaperones, P14K and CobW, limits comparison of their respective modes of
action. Nevertheless, the model for AnhE action shares some similarities with that proposed for P14K in the self-subunit swapping model of Co-NHase maturation (109). These include the action of the chaperone as a dimer and the preferred binding of a single Co$^{2+}$ by the dimer. For its part, CobW occurs in the aerobic biosynthesis of cobalamin pathway (82). In the latter, Co$^{2+}$ is inserted into the corrin ring by cobaltochelatase, an enzyme consisting of three subunits: CobN, CobS and CobT (168). CobW, whose gene is always located immediately upstream of cobN (82), is essential for cobalamin biosynthesis (169) and has been proposed to deliver cobalt to the chelatase complex. As in P14K, no direct evidence of cobalt-binding to CobW has been reported. Interestingly, CobW shares 20–30% amino acid sequence identity with HypB and UreG, the GTP-utilizing, Ni$^{2+}$-specific metallochaperones discussed above, as well as ~30% identity and a possible metal-binding motif with P47K, the potential metallochaperone of Fe-NHase. Neither ATP/GTPase activity nor metal-binding has been reported in either P47K or P14K.

While this study establishes that AnhE binds Co$^{2+}$ and facilitates its incorporation into AnhA, the oxidation state of the cobalt in ANHase is unclear. In other Co-NHases, the cobalt is trivalent. The hydrolysis of nitrile is mediated by the inert Co$^{3+}$, whose ligand exchange rate is significantly increased in the presence of thiolate ligands (64). In addition, the Lewis acidity of the Co$^{3+}$ is increased by the oxidation of cysteiny1 ligands to sulfenic and sulfinic acids (60). Co-NHase has been reconstituted aerobically using either Co$^{2+}$ or Co$^{3+}$ (111). However, the exact mechanism of Co$^{2+}$ oxidation remains uncertain. AnhA contains a Cys-rich motif that may bind cobalt. Moreover, mass spectrometry data indicate that one of these residues is a sulfinic acid (unpublished data). Thus, ANHase may utilize Co$^{3+}$ in a similar mechanism as other Co-NHases. Nevertheless, ITC experiments performed using Co(NH$_3)_6$Cl$_3$ indicated that AnhE does not bind Co$^{3+}$ (data not shown).
Based on the findings in this study, a potential ANHase maturation mechanism in RHA1 may be proposed (Fig. 40). In rhodococcus, metal ions are likely to transported passively into the cytoplasm by outer membrane porins (170). In response to the short-aliphatic nitriles or its amide intermediate, a probable cobalt transporter AnhT encoded on anh gene cluster is transcribed. AnhT, located in the cytoplasmic membrane facilitates the uptake of cobalt ion into the cytoplasm. The form of cobalt is unclear: it may exist as a complex with a metallophore as observed in nickel uptake (171). AnhE either docks with AnhT and receives AnhT-bound cobalt or senses the increased concentration of cobalt ion by its subnanomolar affinity against Co$^{2+}$, then undergoes dimer formation followed by the conformational change, which is potentially critical for the recognition of apo-AnhA. Cobalt ion, is then transferred to another probable cobalt binding site, N-terminal domain of AnhA, releasing metal-free AnhE and forming mature ANHase. The oxidation state of cobalt in ANHase, as well as potential oxidation of Co$^{2+}$ by AnhE, remains unclear.

**Figure 40.** Proposed ANHase maturation mechanism.
4.8 Concluding remarks

This thesis describes the characterization of nitrile catabolic genes and enzymes in RHA1, a soil saprophyte. Two pathways were identified, \textit{anh} and \textit{nth}, responsible for the catabolism of acetonitrile and phenylacetonitrile, respectively. The discovery of the \textit{anh} gene cluster using a proteomic approach led to the biochemical and molecular genetic characterization of ANHase, a novel Co-dependent nitrile hydratase and AnhE, a cobalt-specific metallochaperone. Characterization of this system also provided insights into the biological trafficking of cobalt.

The characterization of ANHase revealed that this novel enzyme contains a catalytically essential cobalt ion and preferentially transforms short-chain aliphatic nitriles. Nitrile hydrolysis by the relatively inert Co$^{3+}$ has been the subject of considerable study. Kovacs (60,64) has provided evidence that the combination of cysteine thiolates and their oxidations are essential for the catalytic activity of the metallocentre. Characterization of the unique Cys-rich motif of ANHase and its role in binding Co$^{3+}$ may provide important new insight into the modulation of cobalt reactivity in biological systems.

The novel ANHase reported herein is also of biotechnological significance in light of the use of non-homologous rhodococcal NHases in green chemistry and bioremediation applications (12,46). The relative lack of product inhibition in ANHase may be useful for the efficient transformation of nitriles in such applications. Although product inhibition has not been widely studied in NHases, propioamide was reported to inhibit NHase of \textit{Rhodococcus} sp. with a $K_i$ of 0.6 mM (172). Under the conditions used here (\textit{i.e.}, 10 mM propioamide, concentration of nitrile $= K_m$), a $K_{ic}$ of this magnitude would reduce the activity of ANHase by 62%, much more that the 20% observed.
The N-terminal sequence of ANHase and AnhE shed light on the trafficking of cobalt ions which normally exist in trace amounts. The subnanomolar affinity of AnhE for the physiological metal ion, and the protein’s apparent conformational change, are particularly intriguing with respect to target recognition and metal ion transfer. The relative lack of known Co$^{2+}$ ligands in AnhE also implies a unique metal coordination environment in this protein. Additional studies of the structure and the metal-binding sites of AnhE as well as the mechanism of metal-transfer to apo-ANHase should provide further insights into the maturation of NHases as well as the metal specificity and mechanism of metallochaperones.
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107