Mechanistic Analysis of the *argE*-Encoded *N*-Acetylornithine Deacetylase†

Farah Javid-Majd and John S. Blanchard*

Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

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ABSTRACT: The *E. coli argE*-encoded *N*-acetyl-L-ornithine deacetylase has been cloned, expressed, and purified in high yield. The substrate specificity of the enzyme is relatively broad, with a number of α-*N*-acyl-L-amino acids exhibiting activity, including both α-*N*-acetyl- and α-*N*-formylmethionine that exhibit higher activity than α-*N*-acetyl-L-ornithine. Sequence homolgy suggests that the enzyme is a member of the metal-dependent aminoacylase family, and the purified enzyme contains a single atom of zinc per monomer. The activity of this enzyme can be increased greater than 2-fold by the addition of zinc, or 8-fold by the addition of cobalt. This suggests that the enzyme can accommodate two metal ions at the active site. The pH dependence of the kinetic parameters has been determined and revealed the presence of two enzymic groups, one functioning as a general base and one functioning as a general acid. Solvent kinetic isotope effects on the hydrolysis of *N*-acetylornithine have been determined, and a linear proton inventory suggests that a single proton transfer occurs in a partially rate-limiting step. A chemical mechanism is proposed and compared with other mechanisms determined for other members of the aminoacylase family.

Arginine has been a focus of biochemical interest for over 40 years because of its complex multistep biosynthesis, the intricate genetic regulation of its biosynthesis and degradation, and the role of biosynthetic intermediates in several catabolic pathways (*1*−*4*). Intermediates in arginine biosynthesis serve as precursors for other pathways such as polyamine and pyrimidine biosynthesis. Arginine itself is directly utilized in four different catabolic pathways (*1*): the deiminase pathway, the succinyltransferase pathway, the dehydrogenase pathway, and the decarboxylase pathway. In microorganisms, arginine can serve as a source of both carbon and nitrogen (*1*).

Arginine biosynthesis in bacteria occurs in eight enzymatic steps, shown in Figure 1 (*2*−*6*). Biosynthesis is initiated by the formation of *N*-acetylglutamate from glutamate and acetyl-CoA in a reaction catalyzed by *N*-acetylglutamate synthase. *N*-Acetylglutamate is then phosphorylated, reduced to *N*-acetylglutamate semialdehyde, and aminated to generate *N*-acetylornithine. In procarayotes, the acetyl group is removed from *N*-acetylornithine by a hydrolytic enzyme, *N*-acetylornithine deacetylase, to form ornithine, an obligatory intermediate in the arginine biosynthetic pathway, and a branchpoint in the synthesis of polyamines.

The gene encoding *N*-acetylornithine deacetylase, *argE*, has been cloned and sequenced from *Escherichia coli* (*7*). The bacterial enzyme is a homodimer of monomers of subunit molecular mass 42 350 Da, and has been reported as a metalloenzyme which is activated by cobalt and inorganic phosphate (*8, 9*). *N*-Acetylornithine deacetylase shares significant sequence homology and biochemical features with three other enzymes known to have amino-acylase activity, including the *dapE*-encoded *E. coli* succinyl-diaminopimelate desuccinylase, the *cpg2*-encoded *Pseudomonas* carboxypeptidase G2, and the *aacI*-encoded pig aminoacylase I (*6, 8, 9*). These hydrolytic enzymes are Zn-dependent metalloproteins with subunits of very similar molecular mass (∼43 kDa), and all hydrolyze an amide bond of α-*N*-acylated-L-amino acids. The amino acid sequence alignment of these hydrolytic enzymes (Figure 2) shows a high degree of identity and similarity among their sequences, supporting the hypothesis that these enzymes share a common origin (*7, 8*). Despite their similar sequence and properties, *N*-acetylornithine deacetylase displays some differences in substrate specificity from carboxypeptidase G2 and aminoacylase I (*8*−*10*). The recent report of the three-dimensional structure of *Pseudomonas* CPG2 clearly indicated the presence of two Zn(II) ions in the active site with each zinc ion coordinated by a histidine residue, a glutamate residue, a bridging aspartate residue, and a water molecule (*11*). These amino acid ligands are present in all of the aligned amino acid sequences of the *argE*-, *dapE*, *cpg2*-, and *accI*-encoded aminoacyl deacylases (boldface residues in Figure 2).

In this report, we describe the subcloning, overexpression, and purification of *N*-acetylornithine deacetylase from *E. coli*. We have developed a continuous, direct assay method to determine the steady-state kinetic parameters for *N*-acylamino acids. The stoichiometry and specificity of metal binding have been determined directly using inductively coupled plasma atomic emission spectrometry. Solvent kinetic isotope effect studies and the analysis of the pH dependence of the kinetic parameters provide the basis for a proposed chemical mechanism of *N*-acetylornihine deacetylase. These studies have allowed us to compare this enzyme to other metal-dependent *N*-acyl-L-amino acid hydrolyases.

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* To whom correspondence should be addressed. Phone: (718) 430-3096. Fax: (718) 430-8565. E-mail: blanchar@aecom.yu.edu.

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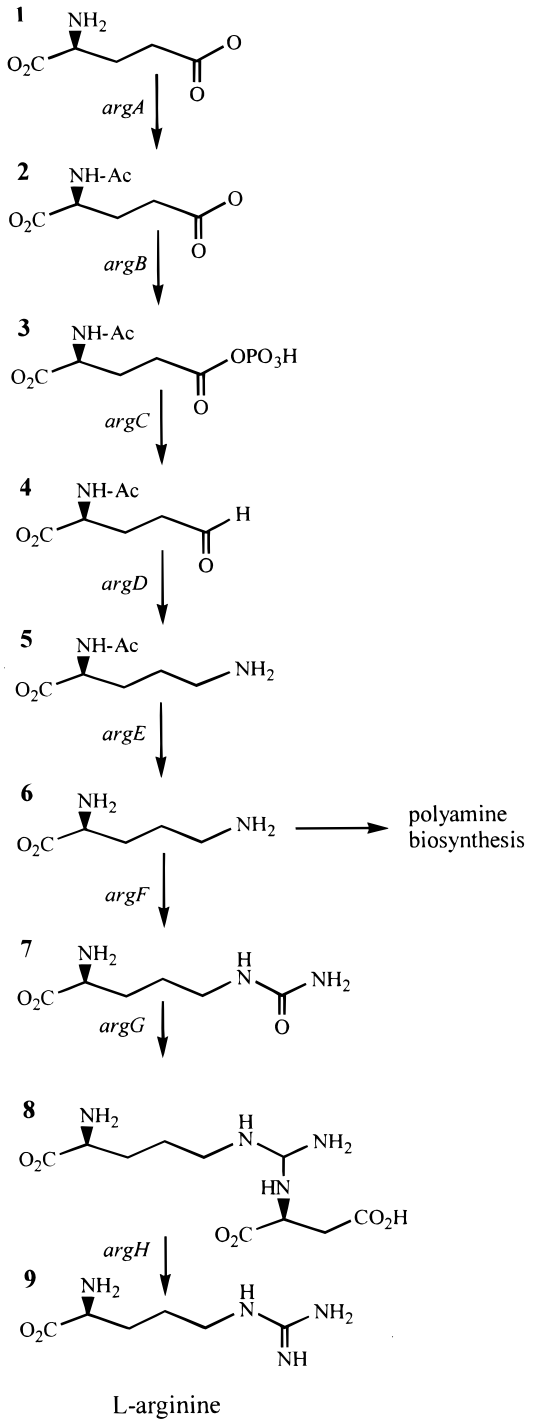

FIGURE 1: Arginine biosynthesis in *E. coli*.

MATERIALS AND METHODS

Materials. All chemicals used were purchased from Sigma Chemical Co., Aldrich Chemical Co., or Fisher Scientific. Enzymes used in molecular biology were supplied by Stratagene, New England Biolabs, or Promega. DNA purification kits and plasmid pET-27a(+) were purchased from Novagen. Oligonucleotides used in this work were synthesized by the Albert Einstein Oligonucleotide Synthesis Facilities. *E. coli* strains XL10-Gold and BL21(DE3) were obtained from Stratagene.

Construction of the Overexpression Plasmid for E. coli N-Acetylornithine Deacetylase. Two oligonucleotide primers, A (containing a *Nco*I restriction site) and D (containing an *Eco*RI restriction site), were designed to amplify the *argE* gene from *E. coli* genomic DNA. A pair of mutagenic primers (B&C) was designed to remove the preexisting *Nco*I site at nucleotide position 989 by changing a T to a C in the wild-type sequence. The method of overlap extension PCR[1] (*12*) was used which consisted of performing the reactions with two separate sets of primers. The first set of primers, primer A and primer B, was used to make an AB fragment. The second set of primers, consisting of the corresponding C primer and D primer, was used to make a CD fragment. The resulting AB and CD fragments were purified by agarose gel electrophoresis and further purified using a Qiagen DNA purification kit. Fragments AB and CD were then combined and used in the third round of PCR in the presence of A and D primers for further amplification of the AD fragment. The AD fragment was then purified by agarose gel electrophoresis, digested with *Nco*I and *Eco*RI, and ligated into the *Nco*I/*Eco*RI site of the pET-27a(+) plasmid. The ligation product, pET-27a(+):*argE*, was then transformed into XL10-Gold. Colonies were screened for those vectors containing the insert by digestion with *Nde*I. The sequence of the PCR-amplified *argE* was verified by nucleic acid sequencing of the PCR product. The primers used in this work are indicated in Table 1.

Overexpression and Purification of N-Acetylornithine Deacetylase. The recombinant expression plasmid was first transformed into *E. coli* BL21(DE3) competent cells. A single colony from the transformed BL21(DE3) pET-27a(+):*argE* was then used to inoculate 50 mL of LB medium containing 50 μg/mL ampicillin. After the cells grew overnight at room temperature, 1 mL of this culture was used to inoculate 12 L of fresh LB medium containing 50 μg/mL ampicillin. The cell cultures were grown at 37 °C to a density of approximately 1 OD at 600 nm and were cooled to 20 °C for 15 min. The cells were induced with 0.2 mM isopropyl 1-thio-β-D-galactopyranoside, and cultures were grown for 8−10 h at 20 °C.

All procedures were carried out at 4 °C for the purification of *N*-acetylornithine deacetylase. Cells were collected by centrifugation at 12000*g*. The cells were resuspended in 50 mM KP_i, pH 7.6, buffer containing protease inhibitors (Boehringer Mannheim), lysozyme (0.5 μg/mL), and DNase I (1 μg/mL) and stirred for 20 min. The cells were then lysed by passage through a French Press at 12 000 psi. The cell debris was removed by centrifugation at 12000*g* for 40 min. The supernatant was then loaded onto a fast flow Q-Sepharose column (Amersham Pharmacia Biotech) that had been equilibrated with 50 mM KP_i buffer, pH 7.6. The protein was eluted with a 2 L linear gradient of 0−1 M potassium chloride in 50 mM KP_i buffer, pH 7.6, at a flow rate of 2 mL/min. The fractions containing the highest *N*-acetylornithine deacetylase activity were combined and concentrated. Solid ammonium sulfate was then added to the sample to a final concentration of 1.0 M. The clear

[1] Abbreviations: Bis-Tris-propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; EDTA, ethylenediaminetetraacetic acid; ICP-AES, inductively coupled plasma atomic emission spetrometery; IPTG; isopropyl thio-β-D-galactosidase; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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Eco NAO                 MKNKLPPFIEIYRALIATPSISATEEALDQSNADLITLLADWFKDLGFN     49
Eco SDAP                      MSCPVIELTQQLIRRPSLSPDDAGCQALLIERLQAIGFT     39
P. CPG2          ALAQKRDNVLFQAATDEQPAVIKTLEKLVNIETGTGDAEGIAAAGNFLEAELKNLGFT     58
Pig ACYI               MASKGREGEHPSVTLFRQYLRIRTVQPEPDYGAAVAFLEERARQLGLG     48
Hu ACYI                MTSKGPEEEHPSVTLFRQYLRIRTVQPKPDYGAAVAFFEETARQLGLG     48

Eco NAO          VEVQPVPGTRNKFNMLASI-GQGAGGLLLAGHTDTVPFDDG-RWTRDPFTLTEHDGKL   105
Eco SDAP         VERMDFADTQN-F--WAW-RGQG-ETLAFAGHTDVVPPGDADRWINPPFEPTIRDGML    92
P. sp. CPG2      VTRSKSAGLVVGDNIVGKIKGRGGKNLLLMSHMDTV-YLK-GILAKAPFRV--EGDKA   112
Pig ACYI         CQKVEVVPGHVVTVLTWPGTNPTLSSILLNSHTDVVPVFKEHWSHDPFEGFKDADGYI   106
Hu ACYI          CQKVEVAPGYVVTVLTWPGTNPTLSSILLNSHTDVVPVFKEHWSHDPFEAFKDSEGYI   106

Eco NAO          YGLGTADMKGFF-AFILDALRDVDVTKLK--KPLYILATADEETSMAGARYFAETTAL   160
Eco SDAP         FGRGAADMKGSLAAMVVAAERFVAQHPNHT-GRLAFLITSDEEASAHNGTVKVVEALM   149
P. sp. CPG2      YGPGIADDKGGN-AVILHTLKLLKEYGVRDYGTITVLFNTDEEKGSFGSRDLIQEEAK   169
Pig ACYI         YGRGAQDMKCVSIQYLEAVRR-LKVEGHHFPRTIHMTFVPDEEVGGHQGMELFVKRPE   164
Hu ACYI          YARGAQDMKCVSIQYLEAVRR-LKVEGHRFPRTIHMTFVPDEEVGGHQGMELFVKQRP   164
                                                    *
Eco NAO          ----RPDCAIIGEEPTSLQPV-----RAHKGHISNA----IRIQGQSGH-SSDPARGVN   204
Eco SDAP         ARNERLDYCLVGEEPSSIEVVGDVVKNGRRGSLTCN----LTIHGVQGH-VAYPHLADN   202
P. sp. CPG2      ----LADYVLSFEEPTSAGDE-----KLSLGTSGIA-YVQVNITGKASHAGAAPELGVN   217
Pig ACYI         FQALRAGFALD-EGLASPTDA----FTVFYSERSPWWLRVTSTGKPGHGSRFIEDTAA   217
Hu ACYI          FHALRAGFALD-EGIANPTDA----FTVFYSERSPWWVRVTSTGRPGHASRFMEDTAA   217

Eco NAO          AIELMHDAIGHILQLRDNLKE--RYHYEAFTVPYPTLNLGHIHGGDASNRI-CACCEL   259
Eco SDAP         PVHRAAPFLNELVAIE-------WDQGNEF-FPATSMQIANIQAGTGSNNVIPG--EL   250
P. sp. CPG2      ALVEASDLVLRTMNIDDKAKNL-RFNWTIAKAGNV------------SNII-PASATL   261
Pig ACYI         EKLHKVINSILAFREKEKQRLQSN-QLKPGAVT-SVNLTMLEGGVA-YNVVPATMSAC   272
Hu ACYI          EKLHKVVNSILAFREKEWQRLQSNPHLKEGSVT-SVNLTKLEGGVA-YNVIPATMSAS   273

Eco NAO          HMDIRPLPGMTLNELN---GLLNDALAPVSERWPGRLTVDELHPPIPGYECPPNH---   311
Eco SDAP         FVQFNFRFSTELTD-----EMIKAQVLALLEKHQLRYTVDWWLSGQPFLTARGKLVD-   302
P. sp. CPG2      NADVRYARNEDFDAAMKTLEERAQQKKLPEADVKVIVTR-GRPAFNAGEGGKKLVD--   316
Pig ACYI         FDFRVAPDVDLKAFEEQLQSWC---QAAGEGVTFEFVQK-WMETQVTSTDDSDPWWAA   326
Hu ACYI          FDFRVAPDVDFKAFEEQLQSWC---QAAGEGVTLEFAQK-WMHPQVTPTDDSNPWWAA   327

Eco NAO          -QLVEVVEKLLGAKTEVVNY--CTEAPFIQTLCPTLV-LGPGSINQAHQPDEYLETRF   365
Eco SDAP         -AVVNAVEHYNEIKPQLLTTGGTSDGRFIARMGAQVVELGPVN-ATIHKINECVNAAD   358
P. sp. CPG2      KAVAYYKEAGGTLGVEERTGGGTDAAYAALSGKPVIESLGLPG-FGYHSDKAEYVDIS   373
Pig ACYI         FSGVFKDMKLALELEI-CPASTDARYIRAAGVPALGFSPMNHTPVLLHDHDERLHEAV   383
Hu ACYI          FSRVCKDMNLTLEPEI-MPAATDNRYIRAVGVPALGFSPMNRTPVLLHDHDERLHEAV   384

Eco NAO          IKPTRELITQVIHHFCWH                                          383
Eco SDAP         LQLLARMYQRIMEQLVA                                           375
P. sp. CPG2      AIPRRLYMAARLIMDLGAGK                                        393
Pig ACYI         FLRGVDIYTQLLSALASVPALPSES                                   408
Hu ACYI          FLRGVDIYTRLLPALASVPALPSDS                                   409
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FIGURE 2: Comparison of *E. coli N*-acetylornithine deacetylase with *E. coli* DapE, *Pseudomonas sp.* Cpg2, pig AcyI, and *H. sapiens* AcyI. The sequences have been aligned by introducing gaps indicated by dashes. In this alignment, the 22 amino acid leader sequence of the Cpg2 sequence (*9*) has not been included. The residues in boldface are the metal binding residues determined from the Cpg2 structure (*8*). The asterisked residue is postulated to act as a general acid in the active site of *N*-acetylornithine deacetylase.

Table 1: List of Mutagenic and Flanking Primers (5′ → 3′)

primer A	ATT CCA TAT GAA AAA CAA ATT ACC GCC
primer B	GTG AGT TGC ACA TGG ATA TTC G
primer C	CGA ATA TCC ATG TGC AAC TCA C
primer D	CGA ATA TCC TAA TGC CAG CAA AAA TGG TG

supernatant was then loaded onto a phenyl-Sepharose (Amersham Pharmacia Biotech) column that had been equilibrated with 50 mM KP_i buffer, pH 7.6, containing 1 M $(NH_4)_2SO_4$. The protein was eluted with a 1 L linear 1.0−0.0 M ammonium sulfate gradient at a flow rate of 1 mL/min. The active fractions, that were >95% homogeneous as determined by SDS−gel electrophoresis, were pooled and stored at −80 °C. Electrospray ionization/mass spectrometery confirmed that no unexpected mutations were introduced during the amplification of *argE* using PCR.

Apoenzyme and Reconstituted Enzyme Preparation. The metal-free enzyme was prepared by dialyzing the *N*-acetylornithine deacetylase against a 200-fold volume excess of 50 mM KP_i containing 50 mM EDTA at 4 °C for 48 h. EDTA was removed by extensive dialysis against four changes of a 200-fold volume excess of metal-free, 50 mM KP_i, pH 7.6, buffer at 4 °C for 8 h. Metal-free buffers were prepared by passing the buffers through Chelex-100 resin according to the manufacturer's instructions (Bio-Rad). Plasticware was successively washed with 20% nitric acid and metal-free water before use. Metal-free dialysis tubing was prepared as described by Auld (*13*). A stoichiometric excess of Zn(II) or Co(II) was added directly into apoenzyme solution at 4 °C overnight. Each sample was dialyzed against metal-free 50 mM KP_i buffer, pH 7.6, to remove unbound metal ion.

Determination of Metal Ion Content of N-Acetylornithine Deacetylase. The metal content of chromatographically purified apo- and metal-reconstituted *N*-acetylornithine deacetylase was determined using atomic absorption spectrophotometry and inductively coupled plasma atomic emission spectrometry.

Spectrophotometric Enzyme Activity Assays. All assays were conducted at 25 °C in 50 mM KP_i, pH 5.3−8.7, in a final reaction volume of 1 mL. Enzyme activity was determined by measuring the change of amide bond absorbance at 214 nm accompanying the hydrolysis of the *N*-acetylornithine and using the molar absorption coefficient of 0.103 mM^{-1} mm^{-1}. Initial velocity data were obtained by nonlinear fitting to eq 1 using a program of Cleland (*14*):

$$v = V\,[A]/(K + [A]) \qquad (1)$$

where V is the maximal velocity, [A] is the substrate concentration, and K is the Michaelis constant (K_m). Values of the kinetic parameters are reported with their associated standard errors.

Product Inhibition. The inhibitory nature of ornithine and acetate, the two products of the reaction catalyzed by *N*-acetylornithine deacetylase, was determined. The assays were performed at 25 °C using five concentrations of each inhibitor in 50 mM KP_i buffer, pH 7.0, containing 100 μM Co(II). For each concentration of inhibitor, the enzyme was assayed at seven concentrations of *N*-acetylornithine, and velocities were fit to eq 2 using the programs of Cleland (*14*), where I is the inhibitor concentration and K_{is} is the inhibition constant for I.

$$v = VA[K(1 + I/K_{is}) + A] \qquad (2)$$

Inhibition of N-Acetylornithine Deacetylase by Fluoride. To determine if a metal-liganded water molecule or hydroxide participates as the reactive nucleophile in the hydrolysis of *N*-acetylornithine, we investigated the inhibition of fluoride on *N*-acetylornithine deacetylase. The initial rates of hydrolysis of *N*-acetylornithine were monitored as a function of NaF concentration in 50 mM KP_i buffer, pH 7.0, containing 100 μM Co(II). The activity assay was determined at five concentrations of NaF (0−16 mM). For each concentration of fluoride, the enzyme activity was determined at seven concentrations of substrate. The experimental data were fit to eq 3 describing uncompetitive inhibition, where I is the inhibitor concentration and K_{ii} is the inhibition constant for I binding to the ES complex.

$$v = VA[K + A(1 + I/K_{ii})] \qquad (3)$$

pH−Rate Profiles. The effect of pH on deacetylase activity was assessed using either Bis-Tris (pH 5.3−6.0) or Bis-Tris-propane (pH 6.5−8.7). The enzyme was inhibited by most organic buffers, and the highest activity was observed in phosphate buffer. The enzyme displays a pH optimum near neutral pH (pH 7.0). Assays were conducted at 25 °C in 50 mM buffer containing 100 μM Co(II). At all pH values employed, the concentration of the substrate was varied between 1 and 40 mM, and all velocities were determined in triplicate. The kinetic parameters V and V/K were determined at each pH value by fitting the data to eq 1, and the pK values of the groups which caused V and V/K to vary with pH were determined using eq 4 (*14*).

$$\log v = \log [C/1 + [H^+]/K_a + K_b/[H^+])] \qquad (4)$$

In eq 4, C is the pH-independent plateau value, $[H^+]$ is the hydrogen ion concentration, and K_a and K_b are the acid and base dissociation constants, respectively.

Solvent Kinetic Isotope Effects. Solvent isotope effects were obtained by performing the reaction in D_2O and H_2O. For reactions in D_2O, the buffer and substrate stock solutions were prepared in 99.8% D_2O. pH measurements were corrected for the isotope effect on the glass electrode by addition of 0.4 to the observed reading. The buffer was titrated to pD using 10% DCl in D_2O. Deuterium solvent kinetic isotope effects were fit to eq 5 (14).

$$v = VA/[K(1 + F_iE_{V/K}) + A(1 + F_iE_V)] \qquad (5)$$

In eq 5, V, A, and K are the same parameters as those in eq 1, F_i is the fraction of isotopic label, and $E_{V/K}$ and E_V are the isotopic effects minus 1 on V/K and V, respectively.

Proton inventories were measured by mixing the appropriate volume of H_2O and D_2O buffers to the reported atom % excess of deuterium at saturating substrate concentration.

RESULTS

Overexpression and Purification of N-Acetylornithine Deacetylase. The *argE* gene from *E. coli* was subcloned into the *E. coli* expression system. Very little soluble expressed protein was found in transformed cells grown at 37 °C and induced with 1.0 mM IPTG at midlog phase ($OD_{600\ nm}$ = 0.6). The solubility of the expressed protein was optimal when the cultures were induced at 18 °C with 0.2 mM IPTG concentration. The enzyme was greater than 95% homogeneous after standard chromatographic purification as judged by SDS−PAGE gel. Electrospray ionization mass spectrometry revealed a monomer molecular mass of 42 345 ± 5 Da, in agreement with the 42 346.9 Da molecular mass deduced from the nucleotide sequence of the *argE* gene.

Assay for N-Acetylornithine Deacetylase. The activity of *N*-acetylornithine deacetylase was determined spectrophotometrically, following the hydrolysis of the amide bond of *N*-acetylornithine at 214 nm and using potassium phosphate buffer. The concentration of *N*-acetylornithine was kept below 50 mM in kinetic experiments because of the observation of substrate inhibition at higher concentrations. The steady-state kinetic parameters of *N*-acetylornithine deacetylase were determined in the absence of any added metal or in the presence of 100 μM Cd(II), Co(II), Mg(II), Ni(II), or Zn(II). An 8-fold enhancement in activity was observed in the presence of 100 μM cobalt, whereas inhibition of activity was observed in the presence of 100 μM Cd(II). Although the activity of the enzyme is stimulated 2-fold in the presence of 100 μM Zn(II), a further increase in the concentration of Zn(II) resulted in inhibition of the deacetylase activity (Table 2).

Substrate Specificity of N-Acetylornithine Deacetylase. *N*-Acetylornithine deacetylase is unable to hydrolyze any of the tested *N*-acyl-D-amino acids (Table 3). The length and degree of branching of *N*-acyl-L-amino acid side chains affect the enzyme-catalyzed deacylation reaction. While *N*-acetyl-L-lysine exhibits a k_{cat}/K_m value that is 10% that of *N*-

Table 2: Kinetic Parameters for the Hydrolysis of *N*-Acetylornithine Deacetylase in the Absence and Presence of Metals

M^{2+} (100 μM)	k_{cat}, s^{-1}	K_m, mM[a]	k_{cat}/K_m, $M^{-1}\,s^{-1}$
none[b]	130 ± 6	2.5 ± 0.3	5.2×10^4
Co	1100 ± 20	1.3 ± 0.1	8.5×10^5
Cd	92 ± 6	3.8 ± 0.5	2.4×10^4
Mg	110 ± 7	2.2 ± 0.3	5.0×10^4
Ni	110 ± 4	1.3 ± 0.1	8.5×10^4
Zn	290 ± 20	7.2 ± 0.9	4.0×10^4

[a] K_m value of *N*-acetylornithine. [b] This enzyme preparation contains 1 mol of Zn/mol of enzyme.

Table 3: Substrate Specificity of Purified *E. coli N*-Acetylorntihine Deacetylase

substrate[a]	k_{cat}, s^{-1}	K_m, mM	k_{cat}/K_m, $M^{-1}\,s^{-1}$
*N*α-acetyl-L-ornithine	1100 ± 20	1.3 ± 0.1	8.5×10^5
*N*α-acetyl-L-alanine	120 ± 3	1.0 ± 0.1	1.2×10^5
*N*α-acetyl-L-asparagine	92 ± 6	8.3 ± 0.8	1.1×10^4
*N*α-acetyl-L-cysteine	100 ± 10	13 ± 2	8×10^3
*N*α-acetyl-L-glutamine	260 ± 7	2.4 ± 0.2	1.1×10^5
*N*α-chloroacetylglycine	130 ± 10	7.0 ± 1	1.9×10^4
*N*α-acetyl-L-leucine	420 ± 40	7.0 ± 1	6.0×10^4
*N*α-acetyl-L-lysine	370 ± 10	4.1 ± 0.2	9.0×10^4
*N*α-acetyl-L-methionine	1700 ± 40	0.81 ± 0.1	2.1×10^6
*N*α-formyl-L-methionine	1800 ± 40	2.0 ± 0.2	9.0×10^5
*N*α-acetyl-DL-serine	80 ± 7	6.7 ± 1	1.2×10^4

[a] No detectable activity was observed with the following compounds at 50 mM concentrations: *N*α-acetyl-D-alanine, *N*α-acetyl-L-arginine, *N*α-acetyl-L-aspartate, *N*α-acetyl-L-glutamate, *N*α-acetylglycine, *N*α-acetyl-L-histidine, *N*ε-acetyl-L-lysine, *N*ε-formyl-L-lysine, *N*α-acetyl-D-methionine, *N*α-acetyl-L-phenylalanine, *N*α-acetyl-L-proline, *O*-acetyl-L-serine, *N*α-acetyl-L-tyrosine, *N*α-acetyl-L-valine, acetylcholine iodide, *N*-succinyldiaminopimelatic acid, *N*α-chloroacetyl-L-leucine, *N*α-acetyl-L-phenylalanine, *N*α-acetyl-L-phenylalanine, *N*α-acetyltryptophan, *N*α-chloroacetyltryptophan, *N*α-chloroacetyl-L-valine. One unit of activity is defined as the hydrolysis of 1.0 μmol of substrate min^{-1} (mg of protein)$^{-1}$.

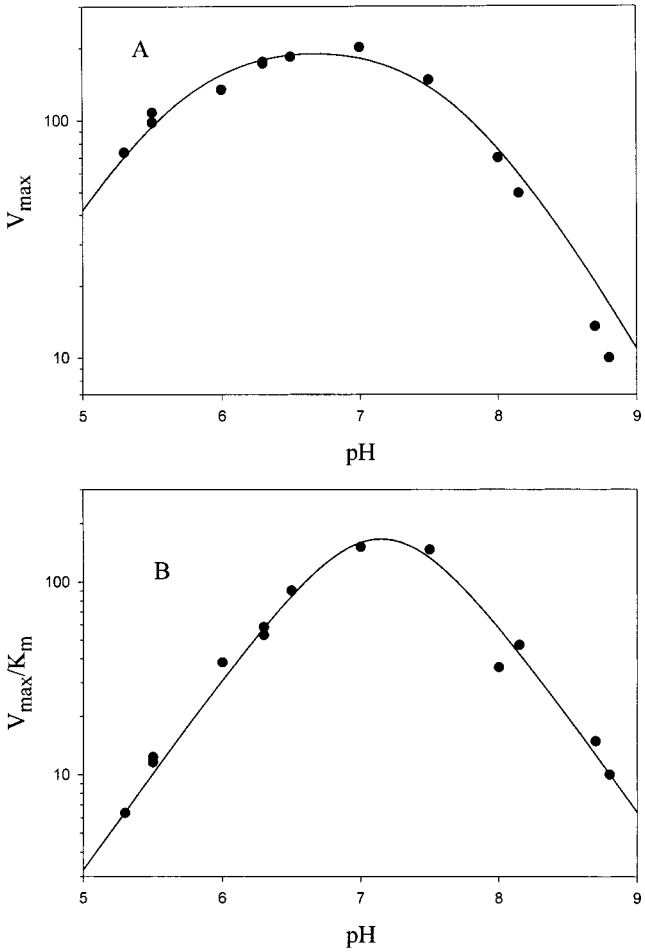

FIGURE 3: pH dependence of the maximum velocities (A) and *V*/*K* values (B) of the deacetylation of *N*-acetylornithine by *N*-acetylornithine deacetylase in the presence of 100 μM Co(II). The points in the plots are experimentally determined, while the smooth lines are fits of the data to eq 4.

acetylornithine, no detectable activity was observed using *N*-acetyl-L-arginine. The α-acetyl group is exclusively deacetylated by the enzyme as evidenced by a lack of reactivity with ε-acetyllysine, and the enzyme is unable to hydrolyze the *N*-acetylated derivatives of aromatic amino acids. The enzyme also exhibits a k_{cat}/K_m value for chloro-*N*-acetylglycine that is 22% that of *N*-acetylornithine. Remarkably, the enzyme hydrolyzes both the *N*-acetylated and *N*-formylated forms of L-methionine with higher catalytic efficiency than *N*-acetylornithine. These compounds are not intermediates in the biosynthesis of methionine, nor are they likely to be found in vivo under normal physiological conditions.

Product Inhibition Studies. The inhibitory effects of ornithine and acetate, the two products of the deacetylation of *N*-acetylornithine, were determined. Ornithine and acetate are linear competitive inhibitors vs *N*-acetylornithine, exhibiting K_{is} values of 0.67 and 13 mM, respectively.

Inhibition of N-Acetylornithine Deacetylase by Fluoride. In metalloproteases, monovalent anions such as fluoride have been used for probing water or hydroxide binding to active site metal centers (*15*−*17*). To determine if a metal-centered water or hydroxide participates as the reactive nucleophile in the hydrolysis of *N*-acetylornithine, we investigated the inhibition by fluoride on the *N*-acetylornithine deacetylase reaction (K_{ii} = 3.4 ± 0.1 mM). Sodium fluoride exhibits linear uncompetitive inhibition versus *N*-acetylornithine, suggesting that this inhibitor binds exclusively to the ES complex, resulting in the formation of an inactive ESI complex.

pH Studies. The pH dependences of *V* and *V*/*K* for *N*-acetylornithine were determined using a variable concentration of *N*-acetylornithine in the presence of 100 μM cobalt. The pH dependence of V_{max}, shown in Figure 3A, is bell-shaped, with apparent p*K* values of 5.6 ± 0.2 for the group that must be unprotonated for activity, and 7.7 ± 0.2 for the group that must be protonated. The pH dependence of *V*/*K* shows a similar bell-shaped dependence, and yielded p*K* values of 7.1 ± 0.2 and 7.2 ± 0.2 (Figure 3B). The kinetic parameters for *N*-acetylornithine could not be precisely determined at pH values <5.3 and >8.7 due to the steadily increasing K_m values for the substrate.

Solvent Isotope Effects. We examined the effect of solvent deuterium substitution on the kinetic parameters of the deacetylase. We observed a large solvent kinetic isotope effect on V_{max} of 2.1 ± 0.1 (Figure 4A) and a smaller solvent kinetic isotope effect on *V*/*K* of 1.3 ± 0.1 at pH 7.0. A proton inventory of the reaction of *N*-acetylornithine deacetylase was performed at pH 7.0 at various mole fractions of D_2O and at saturating *N*-acetylornithine concentrations. Plotting the value of k_{cat} against the mole fraction of D_2O yielded a

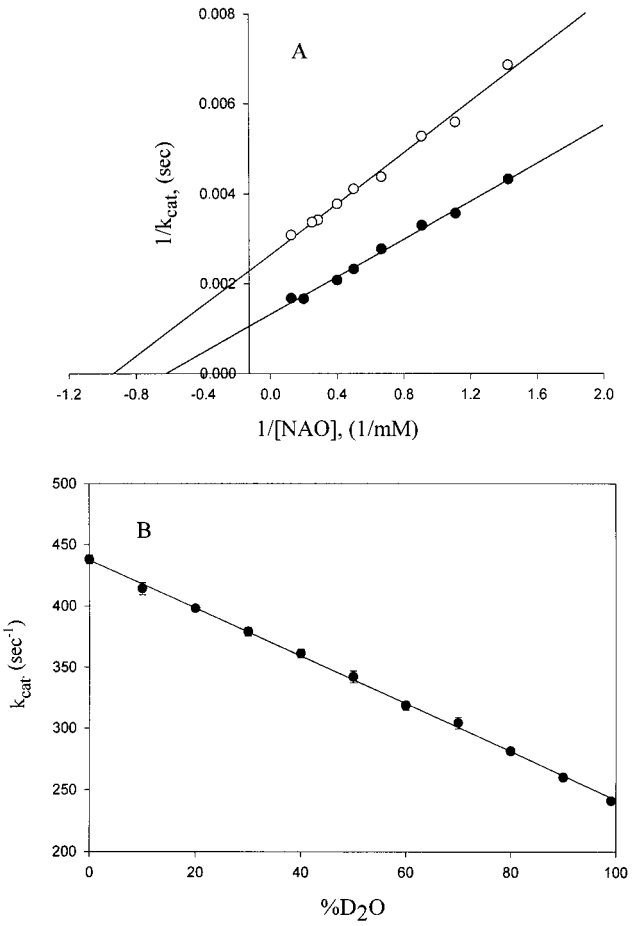

FIGURE 4: (A) Double-reciprocal plot of deacetylation of *N*-acetylornithine by *N*-acetylornithine deacetylase in (●) H_2O and (○) D_2O. (B) Proton inventory of the *N*-acetylornithine deacetylase reaction.

Table 4: Determination of Metal Content of *N*-Acetylornithine Deacetylase by ICP-AES[a]

sample	ICP, ppm	M^{2+}/E
(1) native (1)	Zn = 0.78	1.0 ± 0.002
(2) native (2)	Zn = 0.53	0.9 ± 0.01
(3) apo (1)	Zn = 0.12	0.3 ± 0.01
(4) apo (2)	Zn = 0.15	0.3 ± 0.01
(5) recons. w/ Zn (1)	Zn = 0.70	1.1 ± 0.01
(6) recons. w/ Zn (2)	Zn=0.65	1.3 ± 0.01
(7) recons. w/ Co (1)	Zn = 0.072	0.25 ± 0.01
	Co = 0.067	0.25 ± 0.01
(8) recons. w/ Co (2)	Zn = 0.093	0.26 ± 0.01
	Co = 0.090	0.28 ± 0.01

[a] Apoenzyme was prepared by incubation with 20 mM EDTA as described under Materials and Methods. The reconstituted deacetylase with either Zn(II) or Co(II) was prepared from apoenzyme as described under Materials and Methods.

linear relationship between rate and solvent isotopic composition (Figure 4B), suggesting the involvement of a single proton in the rate-limiting step.

Metal Analysis. The metal analyses were determined by two independent methods, ICP-AES and atomic absorption spectrometry (Table 4). The results indicate that the chromatographically purified protein from three preparations in metal-free buffer contains a single (1.0 ± 0.002) mole of Zn(II) per mole of protein and no cobalt was detected in the protein sample by either ICP-AES or atomic absorption spectrometry. The concentration of enzyme in these samples was estimated from measurements of A_{280} assuming an extinction coefficient of 49 412 M^{-1} cm^{-1}.

The metal content of apoenzyme prepared by extensive dialysis of the protein against 20 mM EDTA was determined. The Zn content of the apoenzyme was determined to be 0.27 ± 0.003 mol/mol of enzyme by ICP-AES or atomic absorption spectrometry. The apoenzyme was incubated at room temperature with 10 equiv of $ZnSO_4$ or $CoSO_4$ for 12 h. The metal-reconstituted enzymes were then dialyzed extensively against metal-free buffer before being analyzed by ICP-AES. The Zn-reconstituted enzyme contained 1.3 equiv of Zn(II) bound per monomer of the enzyme. The Co(II)-reconstituted protein, however, showed equivalent, but nonstoichiometric amounts of Co(II) and Zn(II) bound per monomer. This suggests that zinc can bind stoichiometrically to the tight binding metal site but that cobalt binds poorly to this site. The preparation of apoenzyme by dialysis versus solutions containing 5 mM 1,10-phenanthroline resulted in nearly complete removal of metal (0.07 mol of Zn/mol of enzyme) and (99%) loss of activity. Maximal deacetylase activity of the apoenzyme was obtained in the presence of a higher concentration of cobalt (100 μM).

DISCUSSION

The activity of the enzyme *N*-acetylornithine deacetylase (EC 3.5.1.16) was demonstrated more than 3 decades ago (*3*). The *argE* gene encoding the deacetylase was cloned and sequenced from *E. coli* in 1992 (*7*), and has more recently been included as a member of a large family of bacterial and lower eucaryotic metallopeptidases that cleave *N*-acylated amino acids and short peptides. Other members of this family include the following: the *dapE*-encoded *N*-succinyl-L,L-diaminopimelate desuccinylase (EC 3.5.1.8), an enzyme involved in the biosynthesis of L-lysine; the yeast *cpg2*-encoded carboxypeptidase (EC 3.4.17.11), an enzyme involved in the hydrolysis of the polyglutamyl moiety of folate derivatives; the *aacI*-encoded porcine aminoacylase; and several other bacterial di- and tripeptidases (*6*−*10*). The three-dimensional structure of the yeast carboxypeptidase has recently been reported (*11*), and two metals were observed at the active site, which included a bridging water molecule. Although the overall sequence homology among family members is approximately 70%, residues that have been identified by this structural study as responsible for metal binding are completely conserved in the sequences of all members of this metallopeptidase family.

The recombinant *E. coli N*-acetylornithine deacetylase was expressed at levels that allowed the purification of >20 mg of homogeneous enzyme per liter of culture. This represents a significantly higher level of expression than previously reported (*7*). The purified enzyme exhibited a mass that matched within experimental error that calculated from the amino acid sequence, suggesting that during PCR amplification of the gene no mutations were introduced into the gene product.

The substrate specificity of the deacetylase has been briefly described (*6*, *8*), but we have reexamined the specificity of the enzyme for a wide variety of *N*-acyl-L-amino acids and derivatives using the direct assay that we have developed. The enzyme exhibits absolute stereospecificity for acylated L-amino acids, being unable to hydrolyze *N*-acyl-D-amino

acids. It similarly is specific for α-*N*-acylated amino acids, showing no activity with ϵ-acetyl-L-lysine. The rate of deacylation of α-*N*-acylated amino acid substrates is sensitive to the nature of the amino acid, with amino acids containing either aromatic or acidic side chains showing no activity. The corresponding amide-containing side chains are reasonable substrates, and those containing cationic side chains are good substrates. Perhaps surprisingly, the best substrates for the deacetylase, as evaluated by k_{cat}/K_m values, are α-*N*-acetyl- and α-*N*-formyl-L-methionine. Since α-*N*-acylmethionine derivatives or precursors are not involved in bacterial methionine biosynthesis, it is unclear whether this observation has any physiological significance. However, it is clear that *N*-acetylornithine deacetylase exhibits a much broader range of substrate specificity than either *N*-succinyl-L,L-diaminopimelate desuccinylase or the yeast *cpg2*-encoded carboxypeptidase.

The pH dependence of the kinetic parameters *V* and *V*/*K* was determined in the presence of a saturating concentration of Co(II). The *V*/*K* pH profile is sharply bell-shaped, suggesting the presence of two ionizable groups: one exhibiting a p*K* value of 7.1 that must be unprotonated, and a second exhibiting a p*K* value of 7.2 that must be protonated. The kinetic expression for *V*/*K* for this simple unimolecular hydrolysis includes rate constants for steps between the initial binding of the free substrate to the free enzyme through the first irreversible step, which in this reaction is likely the hydrolysis of the substrate. The groups whose ionization may influence the *V*/*K* value for the substrate are thus ionizable groups either on the free substrate or on free enzyme. *N*-Acetylornithine has two ionizable groups that may potentially be assigned as one of these groups: the α-carboxylate and the δ-amino groups, whose p*K*s of ∼1.71 and 10.76 would be far below and above the experimentally accessible region, respectively (*18*). Furthermore, the finding that α-*N*-acyl-L-methionines are excellent substrates for the enzyme makes it clear that a specific interaction between the protonated δ-amino group and the enzyme is not required. This argues that both groups that are observed in the *V*/*K* pH profile are ionizable groups on the enzyme involved in catalysis, and both groups are observed in the *V* pH profile. The group that must be protonated for activity exhibits a p*K* value of 7.2 and 7.7 in both the *V*/*K* and *V* profiles, suggesting that its macroscopic p*K* is perturbed upon substrate binding. Given the highly precedented peptidase chemistry that the enzyme catalyzes, we suggest that this group is a general acid that functions to protonate the leaving amino acid product (*23*−*25*). Interestingly, no such general acid is observed in the reaction catalyzed by *N*-succinyl-L,L-diaminopimelate desuccinylase (*10*). The group that must be unprotonated for catalysis is likely to be either the metal-bound hydroxide or a general base responsible for deprotonating a metal-bound water. The p*K* value of this group is highly perturbed upon substrate binding, and we favor an assignment for this group as a general base. In the related *N*-succinyl-L,L-diaminopimelate desuccinylase, the group whose protonation abolishes catalytic activity has been assigned to a conserved glutamate residue homologous to one observed near the metal ion center in carboxypeptidase (*10*). A conserved glutamate residue in a homologous position is also present in the *E. coli N*-acetylornithine deacetylase (Glu144). Given likely modes of substrate binding to the metal center, the perturbation of the p*K* value of this general base in the free enzyme and enzyme−substrate complex is expected.

Solvent kinetic isotope effects were performed at pH 7, a region where neither kinetic parameter is sensitive to modest changes in the pH value. Solvent kinetic isotope effects were observed on both *V*/*K* ($^{D20}V/K = 1.3$) and *V* ($^{D20}V = 2.1$). To determine whether these effects were due to a single, or multiple, proton transfer, we performed a proton inventory experiment under *V* conditions. The linear dependence of the observed rate on solvent isotopic composition argues that the *V*, and by analogy the *V*/*K*, solvent kinetic isotope effects are due to a single proton-transfer step. The magnitude of the *V* effect suggests that we are looking at a partially rate-limiting proton transfer, and the step that we are probing is the general base-assisted deprotonation of the metal-bound water to transiently generate a nucleophilic metal-bound hydroxide anion equivalent. Such general base catalysis generates normal solvent kinetic isotope effects of 2−4, as has been observed in a number of metalloproteases (*19*−*22*). These data also point out a second difference between *N*-acetylornithine deacetylase and *N*-succinyl-L,L-diaminopimelate desuccinylase, since in this latter enzyme inverse solvent kinetic isotope effects are observed on *V* and *V*/*K* (*10*). This suggests that the rate-limiting steps are different for the two enzymes, a feature that we have included in our mechanistic analysis.

Metallopeptidases can use either one or two metal ions to promote substrate carbonyl polarization and assist in water hydrolysis (*15*−*17*). *N*-Acetylornithine deacetylase is predicted to use a binuclear metal center for this purpose, based on the significant homology to the yeast carboxypeptidase that has been shown to bind two metal ions at the active site. Qualitatively, the deacetylase exhibits features of a typical metalloenzyme, including inhibition of activity by metal chelators, including EDTA and the zinc-specific chelator 1,10-phenanthroline. To probe the stoichiometry of metal binding to the deacetylase, we measured the metal content using both ICP-AES and atomic absorption spectrometry. The enzyme as isolated, or extensively dialyzed versus solutions containing no metal chelators, contains a single mole of zinc per mole of enzyme, and is active in the absence of exogenously added metal salts. The activity of this enzyme can be reduced by ∼99% by dialysis of enzyme solutions versus buffered solutions of 1,10-phenanthroline. The activity of the metal-free enzyme can be restored by the addition of either Zn(II) or Co(II). The activity of the isolated enzyme containing a single mole of zinc is substantially, and specifically, increased by the addition of Co(II), whereas addition of Zn(II) to these preparations is slightly activating at low concentrations, but inhibitory at high concentrations. All of these data suggest that the enzyme contains two metal binding sites: a high-affinity site which shows a preference for Zn(II); and a second lower affinity site that shows a strong preference for Co(II). Although similar to results reported for *N*-succinyl-L,L-diaminopimelate desuccinylase, the preference for Co(II) at the lower affinity metal ion site is a third difference between these homologous enzymes.

On the basis of sequence alignments, the structure of the metal binding sites in the yeast carboxypeptidase, and our

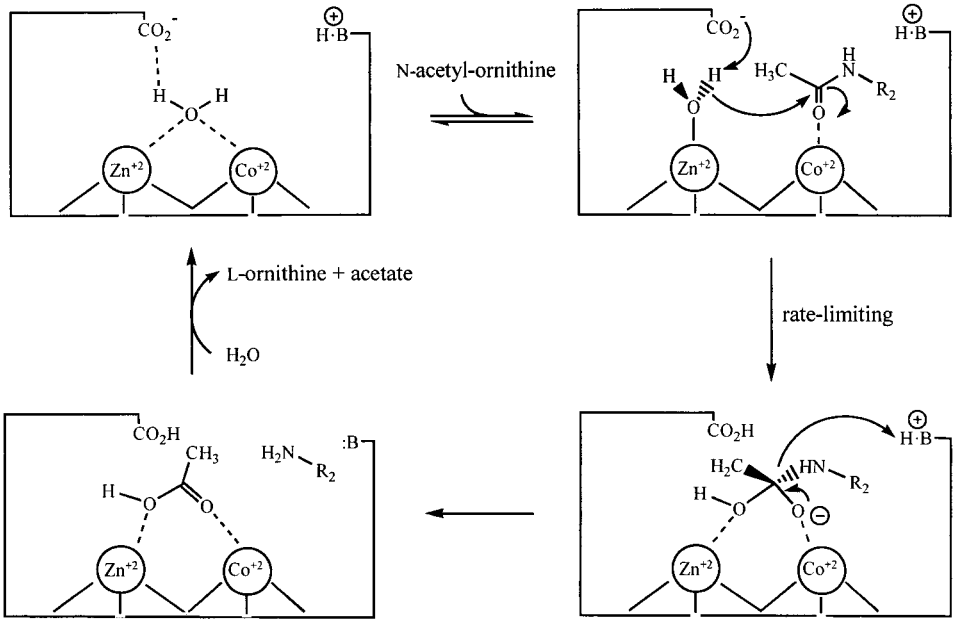

FIGURE 5: Proposed chemical mechanism for *argE*-encoded *N*-acetylornithine deacetylase.

kinetic and analytical results, we propose the chemical mechanism shown in Figure 5. The active site includes two metal ions, shown as Zn and Co, and a single bridging water molecule that is hydrogen bonded to glutamate 144, the residue that we have assigned as the general base. Substrate binding occurs with the carbonyl group of the acyl moiety interacting with one of the metal ions, resulting in the polarization of the amide bond. Substrate binding also perturbs the p*K* value of the general base, either as a result of simple exclusion of water from the active site or due to reorganization of the metal−water−base hydrogen bond interactions. The finding that fluoride ion exhibits linear uncompetitive inhibition versus *N*-acetylornithine is generally supportive of the latter interpretation, since it binds only to the enzyme−*N*-acetylornithine binary complex and not the free enzyme. The abstraction of a proton from water by glutamate 144 is partially rate-limiting, as reported by the solvent kinetic isotope effect on maximal velocity, and the metal-bound hydroxide ion attacks the polarized carbonyl to generate the tetrahedral intermediate. Collapse of this intermediate requires the additional involvement of a general acid to donate a proton to the leaving amino acid, ornithine. In the related *N*-succinyl-L,L-diaminopimelate desuccinylase, this function appears to be provided by the now protonated glutamate residue, while in the deacetylase, the involvement of a second residue functioning as a general acid is supported by the appearance of such a residue in the *V* pH profile. The mechanism proposed here appears to account for all of our experimental evidence, and points out the subtle mechanistic differences that may be apparent between even two functionally and structurally homologous enzymes.

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