Hydrolysis of *N*-Succinyl-L,L-diaminopimelic Acid by the *Haemophilus influenzae dapE*-Encoded Desuccinylase: Metal Activation, Solvent Isotope Effects, and Kinetic Mechanism†

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ABSTRACT: Hydrolysis of *N*-succinyl-L,L-diaminopimelic acid by the *dapE*-encoded desuccinylase is required for the bacterial synthesis of lysine and *meso*-diaminopimelic acid. We have investigated the catalytic mechanism of the recombinant enzyme from *Haemophilus influenzae*. The desuccinylase was overexpressed in *Escherichia coli* and purified to homogeneity. Steady-state kinetic experiments verified that the enzyme is metal-dependent, with a K_m for *N*-succinyl-L,L-diaminopimelic acid of 1.3 mM and a turnover number of 200 s^{-1} in the presence of zinc. The maximal velocity was independent of pH above 7 but decreased with a slope of 1 below pH 7. The pH dependence of *V*/*K* was bell-shaped with apparent p*K*s of 6.5 and 8.3. Both L,L- and D,L-diaminopimelic acid were competitive inhibitors of the substrate, but D,D-diaminopimelic acid was not. Solvent kinetic isotope effect studies yielded inverse isotope effects, with values for $^{D_2O}V/K$ of 0.62 and ^{D_2O}V of 0.78. Determination of metal stoichiometry by ICP-AES indicated one tightly bound metal ion, while sequence homologies suggest the presence of two metal binding sites. On the basis of these observations, we propose a chemical mechanism for this metalloenzyme, which has a number of important structurally defined homologues.

Lysine is one of the 10 essential amino acids that mammals are unable to synthesize and must therefore acquire in their diet. The pathway of lysine biosynthesis, one of four amino acids derived from aspartic acid, has been elucidated in a number of organisms (*1*−*4*). In addition to lysine, the pathway produces *meso*-diaminopimelic acid (*meso*-DAP[1]), an essential component of bacterial cell walls. Disruption of *meso*-DAP biosynthesis results in cell death, presumably due to the instability of the peptidoglycan (*5*). Blocking this pathway prevents the synthesis of two compounds required for cell viability, and therefore, enzymes in this pathway have become attractive targets for the development of novel antibiotics. Determination of the detailed reaction mechanisms of these enzymes will be required for the rational design of potent and specific inhibitors.

Bacteria, most algae, and higher plants synthesize lysine and *meso*-DAP via three related, albeit slightly different, pathways. The initial steps in the pathway involve the four-step conversion of aspartic acid to L-tetrahydrodipicolinate (Figure 1). This compound either can be directly converted to *meso*-DAP by the dehydrogenase pathway (*6*−*8*), can proceed through a series of acetylated intermediates resulting in the production of *meso*-DAP (*9*, *10*), or can proceed through a series of succinylated intermediates leading to *meso*-DAP formation (*10*−*12*). The presence of multiple biosynthetic pathways for the production of *meso*-DAP and lysine is most likely a result of their importance in bacterial survival. An alternative pathway of lysine biosynthesis, the α-aminoadipic acid pathway, is utilized by yeast and *Euglena* (*13*, *14*). These organisms have no requirement for *meso*-DAP as it is not a component of their cell walls.

The *dapE*-encoded *N*-succinyl-L,L-diaminopimelic acid desuccinylase catalyzes the hydrolysis of *N*-succinyl-L,L-diaminopimelic acid (SDAP), forming L,L-diaminopimelic acid and succinate, in the succinylase pathway (Figure 1). Previous studies on the *Escherichia coli* enzyme suggested that the desuccinylase exists as a homodimer and demonstrated that the addition of either zinc or cobalt resulted in increased enzyme activity (*11*, *15*). The same studies also indicated that the desuccinylase could discriminate between substrate stereoisomers. The amino acid sequences of bacterial desuccinylases have been reported from *E. coli* (*16*), *Haemophilus influenzae* (*17*), *Corynebacterium glutamicum* (*18*), *Helicobacter pylori* (*19*), and *Mycobacterium tuberculosis* (*20*). Alignment of these sequences revealed a number of highly conserved residues also identified in other metal-dependent enzymes (*21*), including *E. coli argE*-encoded acetylornithinase, *Pseudomonas cpg2*-encoded carboxypeptidase G2, *Lactobacillus pepV*-encoded carnosinase, bacterial *pepT*-encoded tripeptidase, and both the yeast *ysc*-encoded carboxypeptidase and the mammalian aminoacylase. Recently, the three-dimensional structure of carboxypeptidase

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[1] Abbreviations: BSA, bovine serum albumin; CPG2, carboxypeptidase G2; DAP, diaminopimelic acid; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; ICP-AES, inductively coupled plasma atomic emission spectroscopy; IPTG, isopropyl thio-β-D-galactoside; *meso*-DAP, D,L-diaminopimelic acid; SDAP, *N*-succinyldiaminopimelic acid; TEA, triethanolamine.

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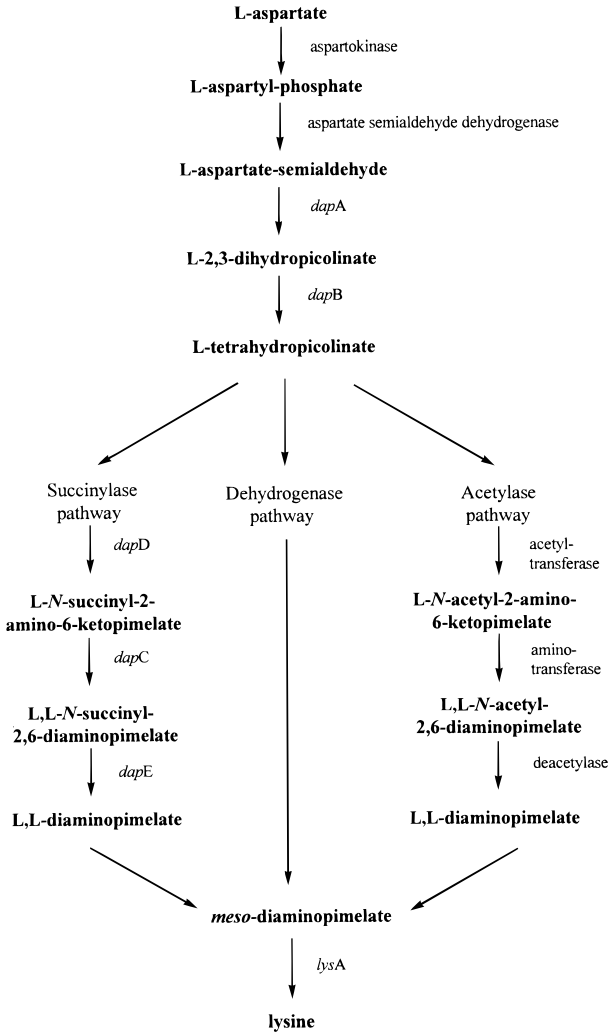


FIGURE 1: Biosynthetic pathways of *meso*-diaminopimelic acid and lysine in bacteria.

G2 from *Pseudomonas* was determined (*22*), revealing a dinuclear zinc center at the active site.

The chemistry catalyzed by the desuccinylase, amide bond hydrolysis, is a common enzymological reaction. Other enzymes catalyzing the same chemistry include aminopeptidases, carboxypeptidases, endopeptidases, β-lactamases, urease, and penicillin amidase, as well as those listed above. One group of these amidases utilizes an active site amino acid residue such as serine, cysteine, or aspartic acid as an attacking nucleophile, forming an acyl−enzyme intermediate prior to hydrolysis by water, while a second group catalyzes the direct hydrolysis of substrate by water with the help of one or two metal ions located at the active site.

In this paper, we report the cloning, overexpression, and purification of the *dapE*-encoded desuccinylase from *H. influenzae*, as well as the determination of k_{cat} and K_m for SDAP hydrolysis using a newly developed direct assay. The stereospecificity of the enzyme is further delineated by product inhibition studies. The pH dependence of the enzyme activity has allowed us to identify two ionizable groups that are important for binding and catalysis. Finally, ICP-AES and solvent isotope effects have been used to show that the desuccinylase binds 1 mol of metal per mole of protein tightly, and this metal is directly involved in enzyme catalysis.

MATERIALS AND METHODS

Materials. Diaminopimelic acid was purchased from Aldrich (Milwaukee, WI). 2-Naphthalenesulfonic acid, 1-hydrate was from Acros (Pittsburgh, PA). Oligonucleotide primers used for PCR amplification were synthesized by Life Technologies (Gaithersburg, MD). *Nde*I and *Bam*HI restriction enzymes were purchased from New England BioLabs (Beverly, MA), and pET23a(+) vector DNA and *E. coli* BL21(DE3) cells were from Novagen (Madison, WI). *H. influenzae* RD was from ATCC (Rockville, MD). Chromatographic supports for protein purification were obtained from Pharmacia (Piscataway, NJ). D_2O was from Cambridge Isotope Laboratories (Andover, MA). Water used was freshly deionized in a Milli-Q plus system (Millipore).

Synthesis of SDAP. *N*-Succinyl-L,L-diaminopimelic acid was synthesized essentially as described (*15*). Briefly, the D,D- and L,L-isoforms of diaminopimelic acid were separated from the D,L-isoform by recrystallization in the presence of 2-naphthalenesulfonic acid, 1-hydrate (*23*). Commercial diaminopimelic acid (50 mmol) and naphthalene (135 mmol) were combined and dissolved in 1 N HCl (180 mL) while being heated, and the naphthalene salt of the D,D- and L,L-DAP isoforms was crystallized from solution upon cooling to room temperature. Six recrystallizations were required to completely remove D,L-DAP, as determined by enzymatic assay with *meso*-diaminopimelate dehydrogenase (*24*). Removal of the naphthalenesulfonic acid was accomplished by suspension of the recrystallized solid in 5 volumes of 15% pyridine in absolute ethanol (v/v) for 2 days at room temperature and was confirmed by NMR. SDAP was then prepared by codissolving DAP (10 mmol) and $KHCO_3$ (20 mmol) in 100 mL of water at 50 °C. After the compounds had completely dissolved, the solution was cooled to room temperature and succinic anhydride (10 mmol) was added while the mixture was stirred. The reaction was allowed to proceed overnight before SDAP was purified as described (*15*). The identity and purity of the final product were confirmed by mass spectrometry and NMR.

To obtain pure L,L-SDAP, a mixture of the D,D- and L,L-isoforms was separated on a chirobiotic T column (Alltec), eluting the compounds in an isocratic gradient of 10% methanol using a Hewlett-Packard series 1100 HPLC apparatus. The two isoforms were present in an approximately 1:1 ratio, with L,L-SDAP eluting first. The identity of the two compounds was determined by mass spectrometry and enzymatic assays.

PCR and Expression of H. influenzae N-Succinyl-L,L-diaminopimelic Acid Desuccinylase. The sequence of the *H. influenzae dapE* gene has been reported (*17*). Two oligonucleotides (5′-TACATATGAAAGAAAAAGTGGTTTCG-3′ and 5′-GCGGATCCGTTAGCTATCCAATAAATTCACTA-3′) which were complementary to the amino-terminal coding and carboxyl-terminal noncoding strands were synthesized containing *Nde*I and *Bam*HI restriction sites, respectively, in noncomplementary overhangs. These primers were used to amplify the *H. influenzae dapE* gene from genomic *H. influenzae* RD using standard PCR conditions (Perkin-Elmer). The PCR product was purified by electrophoresis on low-melting agarose, digested with *Nde*I and *Bam*HI, and ligated into a pET23a(+) expression vector which had previously been digested with the same

restriction enzymes. The recombinant plasmid was transformed into competent *E. coli* BL21(DE3) cells. The transformed cells were grown at 37 °C in LB media containing 50 μg/mL carbenicillin until OD_{600} equaled 0.8, induced by addition of 0.6 mM IPTG, and grown for an additional 3 h at 37 °C. Analysis by SDS−PAGE with Coomassie blue staining indicated that the cell extracts contained a significant amount of a 43 kDa protein.

*Purification of N-Succinyl-*L,L*-diaminopimelic Acid Desuccinylase.* Six liters of LB media containing 50 μg/mL carbenicillin was innoculated with an overnight culture, grown to an OD_{600} of 0.5−0.8, and induced with 1 mM IPTG. After 3 h, approximately 12 g of cells was recovered by centrifugation. All subsequent steps were performed at 4 °C. The cells were resuspended in 50 mL of 25 mM triethanolamine (TEA, pH 7.8) containing protease inhibitors (Complete protease inhibitor cocktail tablets, Boehringer Mannheim) and 10 mg of lysozyme, and the mixture was stirred for 30 min. The cells were broken by sonicating four times for 1 min intervals with a Sonifier cell disruptor 350 (Branson), and the cell debris was removed by centrifugation at 12000*g* for 45 min. Nucleic acids were precipitated by the addition of streptomycin sulfate (1% w/v final) to the supernatant, and the solution was stirred for 30 min before pelleting the nucleic acids at 17000*g* for 30 min. The nucleic acid-free supernatant was dialyzed against 25 mM TEA (pH 7.8) for 6 h. The precipitate which formed during dialysis was removed by centrifugation for 30 min at 17000*g*. The clear supernatant was loaded onto a 400 mL fast-flow Q-Sepharose anion-exchange column which had been equilibrated with 25 mM TEA (pH 7.8). The protein was eluted at 2 mL/min with a 1500 mL linear 0 to 1 M NaCl gradient. The enzyme activity eluted between 0.5 and 0.6 M NaCl. The active fractions were pooled and concentrated (YM-10 membrane, Amicon) to 18 mL, and ammonium sulfate was added to a final concentration of 0.8 M. After the mixture was stirred for 30 min, the precipitate was pelleted at 17000*g* for 30 min and the supernatant applied to a 2.4 cm × 16 cm phenyl-Sepharose column equilibrated in 25 mM TEA (pH 7.8) and 0.8 M $(NH_4)_2SO_4$. The protein was eluted at 1 mL/min in a 600 mL linear 0.8 to 0 M $(NH_4)_2SO_4$ gradient, and the active fractions, which exhibited a single band on SDS−PAGE with Coomasie blue staining, were pooled.

Measurement of Enzyme Activity. Reaction rates were determined by monitoring the decrease in amide bond absorbance due to SDAP desuccinylation at 215, 220, or 225 nm (ϵ = 698, 344, and 157 M^{-1} cm^{-1}, respectively) in a UVIKON 9310 or 943 spectrophotometer equipped with thermospacers and connected to a constant-temperature circulating water bath. Unless otherwise noted, the substrate used for assays was a mixture of D,D- and L,L-SDAP. Assays were performed in 50 mM K_2HPO_4 (pH 7.6) containing 100 mM $ZnSO_4$ (or $CoSO_4$ where noted) at a temperature of 25 °C. Assays were initiated by the addition of enzyme unless otherwise noted. Initial velocity kinetic data were analyzed by Lineweaver−Burk analysis and fitted to eq 1

$$v = VA/(K + A) \quad (1)$$

using the programs of Cleland (*25*), where V is the maximal velocity, A is the substrate concentration, and K is the Michaelis constant (K_m).

pH Profiles. Enzyme activity was measured over the pH range of 5.5−8.9 using either phosphate (5.5−8.0) or Tris (7.7−8.9) as buffers to avoid spectrophotometric interference. Assays were performed at 25 °C in 50 mM buffer containing 25 mM $ZnSO_4$ or $CoSO_4$. Assays were initiated by the addition of substrate, and pH values were determined at the end of each assay using an Accumet model 20 pH meter equipped with a microelectrode. The kinetic parameters V and V/K were determined using six different substrate concentrations at each pH value and fitted to eqs 2 and 3, respectively, using the Sigma Plot program.

$$\log V = \log[C/(1 + H/K_a)] \quad (2)$$

$$\log V/K = \log[C/(1 + H/K_a + K_b/H)] \quad (3)$$

where C is the pH-independent plateau value, K_a is the ionization constant for the acidic group, K_b is the ionization constant for the basic group, and H is the hydrogen ion concentration.

Product Inhibition Studies. The three stereoisomers of DAP were separated on a chirobiotic T column (Alltec), eluting the compounds with an isocratic gradient of 50% methanol using a Hewlett-Packard series 1100 HPLC apparatus. The three stereoisomers eluted in the order L,L-, D,L-, and D,D-DAP in an approximately 1:2:1 ratio. Inhibition assays were performed at 25 °C using five concentrations of each DAP isomer in an assay containing 50 mM K_2HPO_4 (pH 7.6) and 100 mM $ZnSO_4$. For each concentration of inhibitor, the enzyme was assayed at six concentrations of substrate and maximal velocities were determined using eq 1. Data for competitive inhibition were fitted to eq 4 using the programs of Cleland (*25*), where K_{is} is the dissociation constant of the enzyme−inhibitor complex and I is the inhibitor concentration.

$$v = VA/[K(1 + I/K_{is}) + A] \quad (4)$$

Solvent Kinetic Isotope Effects. Solvent kinetic isotope effects on V and V/K were determined by measuring the initial velocity of SDAP desuccinylation at various substrate concentrations in H_2O and 80% D_2O (v/v). Each assay was conducted at 25 °C in buffer containing 50 mM K_2HPO_4 (pH 7.6) and 100 mM $ZnSO_4$. Solvent deuterium kinetic isotope effects were calculated from eq 5 using the programs of Cleland (*25*).

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

where V, A, and K are equivalent to those same parameters in eq 1, F_i is the fraction of isotopic label, and $E_{V/K}$ and E_V are the isotope effects on V/K and V, respectively.

Proton inventories were determined by varying the atom fraction of D_2O from 0 to 0.8, in increments of 0.1, in the same buffer as above at a saturating substrate concentration (5 mM).

ICP-AES Experiments. Metal stoichiometries were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES). Glassware was soaked overnight in 6 M nitric acid, washed extensively in deionized water purified with a Milli-Q plus system (Millipore), soaked overnight in 1 mM EDTA, and again extensively washed with Milli-Q water. Enzyme samples, prepared so that the

metal concentrations would be in the range of 1 ppm, were incubated with metal ($ZnSO_4$ or $CoSO_4$) followed by extensive dialysis against 25 mM HEPES (pH 7.5). Sample blanks were taken from the final dialysis buffer for each sample. The metal concentrations were measured using a Perkin-Elmer Optima 3000SC ICP-spectrophotometer, calibrating the instrument at the beginning and end of the experiments with a standard known to contain 1 ppm cobalt and zinc. Emission wavelengths chosen to monitor cobalt and zinc were 228.616 and 213.856 nm, respectively, and sample was introduced at a pump speed of 1 mL/min. Protein concentrations were determined by the Bio-Rad protein assay using BSA as the standard.

RESULTS

*Expression and Purification of N-Succinyl-*L,L*-diaminopimelic Acid Desuccinylase.* The *H. influenzae N*-succinyl-L,L-diaminopimelic acid desuccinylase was overexpressed in *E. coli* by cloning the corresponding gene into the pET23a-(+) vector. Upon IPTG induction, BL21(DE3) cells containing this vector overproduced a soluble protein of the expected monomer molecular mass, with the desuccinylase comprising greater than 50% of the total soluble protein as determined by SDS−PAGE. Following cell lysis, the desuccinylase was purified by anion exchange over a Fast-Q Sepharose column, and hydrophobic interaction on a phenyl-Sepharose column. Approximately 0.5 g of homogeneous desuccinylase were purified 1.4-fold in 60% overall yield from 12 g of cells using this purification procedure. Automated Edman amino-terminal sequencing confirmed that the first 10 residues were identical to the derived amino acid sequence of the *H. influenzae* protein. The monomer molecular mass was determined to be 41 350 Da by electrospray mass spectrometry, consistent with the predicted molecular mass of 41 353 Da. The *E. coli* enzyme is purported to exist as a homodimer or tetramer in solution (*15*).

Determination of Steady-State Parameters. To assay the desuccinylase, previous studies either took advantage of the fact that DAP reacts more quickly with ninhydrin than SDAP does (*11*) or else coupled the production of succinate to succinate thiokinase, pyruvate kinase, and lactate dehydrogenase to generate a discontinuous spectrophotometric assay (*15*). The ninhydrin-based assay suffers from high background and a lack of reproducibility, while the coupled assay suffers from its discontinuous nature. In this study, we have developed a method in which we directly monitor desuccinylase activity by spectrophotometrically following the hydrolysis of the amide bond of SDAP at wavelengths from 215 to 225 nm. Since the change in absorbance is monitored at wavelengths of <230 nm, buffers that do not interfere with the assay must be chosen. Using this assay, initial velocities were linear and proportional to the amount of added enzyme (data not shown).

Initial velocities were measured using as substrate both pure L,L-SDAP and a mixture of D,D- and L,L-SDAP, with either Zn^{2+} or Co^{2+} as the metal activator. The SDAP mixture contained approximately equimolar ratios of the two SDAP isomers. The results of these experiments are summarized in Table 1. In the presence of a saturating zinc concentration, the values obtained for k_{cat} are essentially identical for both pure L,L-SDAP and the mixture, while the K_m value calculated for L,L-SDAP was ca. 40% of the value for the mixture. In the presence of a saturating cobalt concentration, the value of k_{cat} with L,L-SDAP is ca. 80% of that of the mixture, and the value of K_m is ca. 35% of that of the mixture. Lin et al. (*15*) reported values of 0.4 mM and 267 s^{-1} for K_m and k_{cat}, respectively, for the zinc-activated *E. coli* desuccinylase using their coupled assay.

Table 1: Kinetic Parameters for the Hydrolysis of *N*-Succinyl-L,L-diaminopimelic Acid Using Zinc and Cobalt as Metal Activators

sample	metal	K_m (mM)	k_{cat} (s^{-1})
SDAP mixture	Zn^{2+}	3.2 ± 0.4	230 ± 20
L,L-SDAP	Zn^{2+}	1.3 ± 0.2	200 ± 10
SDAP mixture	Co^{2+}	4.7 ± 0.5	470 ± 30
L,L-SDAP	Co^{2+}	1.6 ± 0.2	370 ± 20

pH Dependence of the Kinetic Parameters. The pH dependence of the desuccinylase reaction was measured over the pH range of 5.5−8.9, using either zinc or cobalt as the enzyme activator. Since many buffers absorb strongly in the spectral region in which the reaction is monitored, pH studies were performed using two buffers with low absorbance in this region, phosphate and Tris, and the pH of the reaction mixture was measured at the end of each assay. As can be seen in Figure 2A, the maximal velocity was independent of pH above pH 7 when zinc was used to activate the enzyme. Below pH 7, the maximum velocity decreased with an apparent slope of 1, indicating that protonation of a single ionizable group, exhibiting a p*K* value of 6.6 ± 0.2, resulted in a loss of activity. The pH dependence of the *V*/*K* value of SDAP (Figure 2B) is a bell-shaped curve, with *V*/*K* decreasing with an apparent slope of 1 at lower pH values and a slope of −1 at higher pH values. The calculated p*K* values of these two groups were 6.5 ± 0.2 and 8.3 ± 0.4. The decrease in the *V*/*K* value of SDAP at high pH values is due to a steadily increasing K_m value for SDAP, resulting in our inability to reach K_m values of SDAP above pH 8.9.

The pH dependence of the kinetic parameters of the cobalt form of the desuccinylase was qualitatively identical to that of the zinc form. The calculated p*K* values for the maximum velocity differed slightly from the values calculated in the presence of zinc, with the p*K* value determined in the presence of cobalt shifted to a lower pH value (5.9 ± 0.3). The pH dependence of *V*/*K* for the cobalt enzyme was also bell-shaped, decreasing with a slope of 1 at lower pH values and a slope of −1 at higher pH values. The calculated p*K* values for these two groups were 6.2 ± 0.2 and 8.3 ± 0.3. The p*K* of the ionizable group whose deprotonation decreases the *V*/*K* of SDAP was essentially indistinguishable between the zinc and cobalt forms of the enzyme.

Inhibition by Product. Both of the products of the desuccinylase reaction, succinate and DAP, were tested as inhibitors of the enzyme. Succinate was a poor inhibitor of the desuccinylase, and although at 100 mM concentrations of succinate there was evidence of enzyme inhibition, this inhibition could be mimicked by a similar change in ionic strength (data not shown).

A mixture of the three DAP isomers was separated via HPLC on a chiral column, and each compound was tested as an inhibitor of the desuccinylase. Both L,L-DAP and D,L-DAP are competitive inhibitors of the desuccinylase, exhibit-

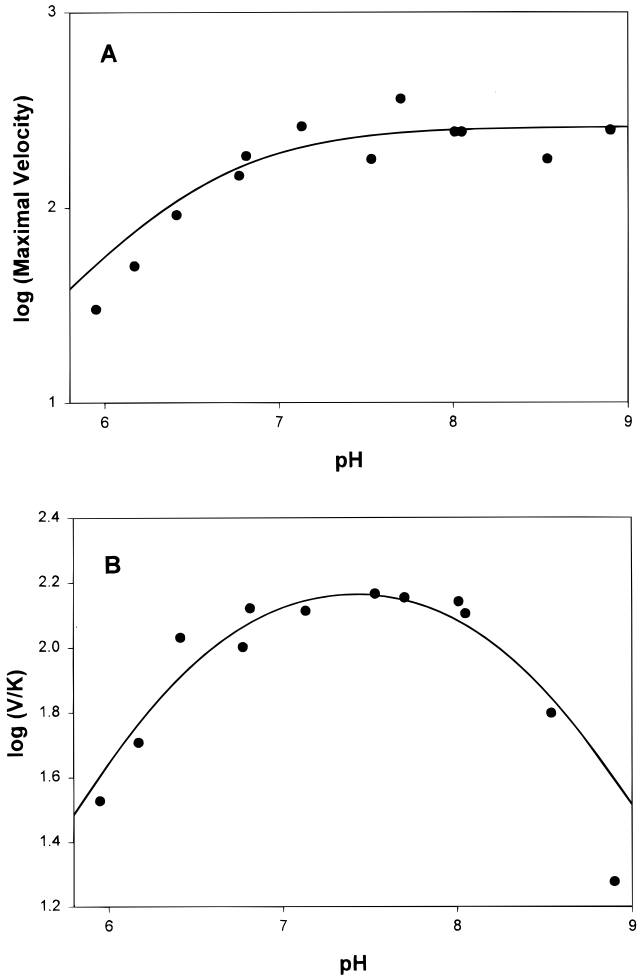
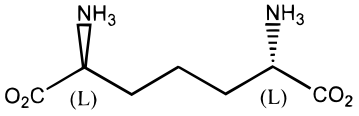
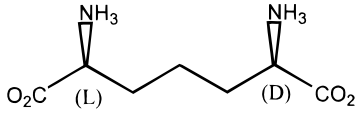
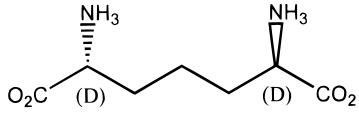


FIGURE 2: Dependence of the kinetic parameters of the desuccinylation of SDAP by the zinc form of the *dapE*-encoded desuccinylase on pH. Experiments were conducted as described in Materials and Methods. (A) Dependence of the maximal velocity on pH. The symbols represent the experimentally determined values, while the curves are fits to values calculated from eq 2. The maximal velocity is measured in units of micromoles per minute per milligram. (B) Dependence of *V*/*K* on pH. The symbols represent the experimentally determined values, while the curves are fits to values calculated from eq 3. The values of *V*/*K* are measured in units of micromoles per minute per milligram per millimolar.

Table 2: Inhibition of the Desuccinylase by the Three Stereoisomers of Diaminopimelic Acid

inhibitor	structure	K_{is} (mM)
L,L-DAP		8 ± 2
D,L-DAP		12 ± 3
D,D-DAP		90 ± 60

ing K_{is} values of 8 and 12 mM, respectively (Table 2). D,D-DAP was an extremely poor inhibitor of SDAP desuccinylation, exhibiting a K_{is} value of 90 mM.

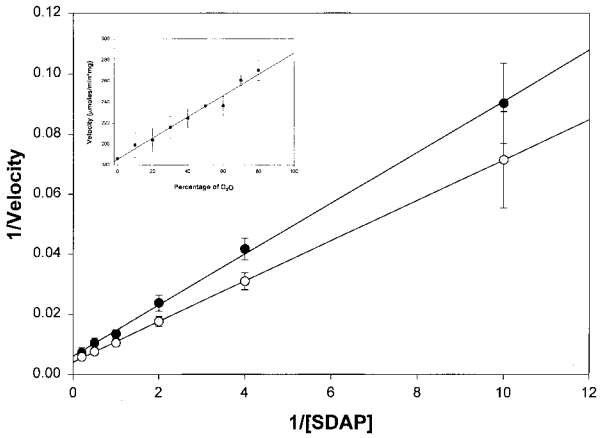


FIGURE 3: Double-reciprocal plot of the desuccinylation of SDAP by the desuccinylase in H_2O and D_2O. Experiments were performed as described in Materials and Methods: (○) experiments performed in 80% D_2O and (●) experiments performed in H_2O. The data plotted are the averages of three experiments ± the standard error. The units of substrate concentration are millimolar, while the units of maximal velocity are micromoles per minute per milligram. (Inset) Proton inventory. Maximal velocities were measured at incrementally increasing percentages of D_2O as described in Materials and Methods. The data plotted are the averages of three experiments ± the standard error.

Solvent Kinetic Isotope Effects. Solvent kinetic isotope effects were determined by measuring initial velocities in both H_2O and 80% D_2O at six different SDAP concentrations using zinc as the metal activator. These experiments were performed at pH 7.6, where both *V* and *V*/*K* are maximal and independent of pH. As shown in Figure 3, the observed isotope effects are inverse. Fitting the data to eq 5 yielded values for the solvent isotope effect on *V*/*K* of 0.62 ± 0.06 and on *V* of 0.78 ± 0.05, assuming a linear dependence of the isotope effect on fractional deuterium abundance.

A proton inventory experiment was performed by varying the atom fraction of D_2O at saturating concentrations of SDAP. Each data point was determined in triplicate, and yielded a linear relation between the rate and mole fraction of deuterium (Figure 3, inset).

Determination of Metal Stoichiometry. Metal stoichiometries have not been previously determined for the desuccinylase due to an inability to obtain the required amounts of enzyme. Our expression system produced sufficient quantities of enzyme, allowing us to measure metal stoichiometries by ICP-AES. Enzyme samples (~25 mM) were incubated with 100 mM $ZnSO_4$ or $CoSO_4$ for 30 min (set I) or 2 h (set II) to fill all of the metal binding sites. The samples were then dialyzed against 25 mM HEPES (pH 7.5) for either 2 days (set I) or 1 day (set II). Metal concentrations for all the samples were determined by ICP-AES, and after the appropriate blanks were subtracted, the stoichiometries were calculated on the basis of the protein concentration of each sample. These results are summarized in Table 3. From the results, it is evident that each protein sample contains 1 mol of metal per mole of protein. In the first sample set, the enzyme incubated with cobalt contained only zinc, while in the second sample set, the cobalt stoichiometry was 0.4.

DISCUSSION

Deacylation of *N*-succinyl-L,L-diaminopimelic acid by the desuccinylase was first demonstrated by Gilvarg in 1960 after

Table 3: ICP-AES Determination of Metal Bound by *N*-Succinyl-L,L-diaminopimelic Acid Desuccinylase

sample[a]	ppm zinc	ppm cobalt	moles of zinc/ mole of protein	moles of cobalt/ mole of protein
zinc, set I	1.532	nd[b]	1.160	–
cobalt, set I	1.196	0.039	0.934	0.0338
zinc, set II	1.982	nd	1.072	–
cobalt, set II	0.992	0.636	0.565	0.403
as purified	0.842	0.0016	0.838	0.0022

[a] Samples in set I were incubated with metal for 30 min followed by a 2 day dialysis against several changes of metal-free buffer, while samples in set II were incubated with metal for 120 min prior to a 1 day dialysis against several changes of metal-free buffer. [b] nd, not detectable.

it was observed that SDAP was an intermediate in the biosynthesis of diaminopimelic acid. Since that time, limited enzymological information has been reported for the desuccinylase, primarily due to the fact that it has been difficult to obtain sufficient quantities of the enzyme. In their original study (*11*), Kindler and Gilvarg demonstrated that crude lysates from *E. coli* hydrolyzed SDAP with a Michaelis constant of 1.3 mM and showed that enzyme activity was stimulated by the addition of metal ions. Cobalt was identified as the most effective metal in stimulating activity, approximately 4 times more effective than zinc, manganese, or iron.

In 1988, the native *E. coli* enzyme was purified 7100-fold to homogeneity by Lin et al. (*15*). They reported a K_m of 0.4 mM and a turnover number of 16 000 min^{-1} with SDAP as the substrate for the zinc form of the desuccinylase. The cobalt form of the enzyme was found to be twice as active as the zinc form, and concentrations of metal in the millimolar range were inhibitory. From substrate specificity studies, it was shown that the desuccinylase catalyzed desuccinylation of the L-stereocenter but not the D-stereocenter. In addition, the enzyme demonstrated a strong preference for an L-amino acid center at the distal end of SDAP.

Bouvier et al. (*16*) reported the first *dapE* gene sequence, from *E. coli*, in 1992. Since then, *dapE* sequences have been reported from *H. influenzae*, *C. glutamicum*, *He. pylori*, and *M. tuberculosis*. We have cloned the *dapE* gene from *H. influenzae*, overexpressed it to high levels in *E. coli*, and purified the protein to homogeneity. The purification of large quantities of enzyme, combined with the development of a direct spectrophotometric assay for desuccinylase activity, has enabled us to conduct a detailed investigation of the kinetic mechanism of the desuccinylase.

We have confirmed that maximal desuccinylase activity requires the presence of either zinc or cobalt, with the cobalt form of the enzyme exhibiting twice the activity of the zinc form. Enzyme activity can be completely inhibited by the addition of EDTA. The kinetic parameters determined for the *H. influenzae* enzyme are similar to those reported earlier for the *E. coli* enzyme. The measured K_m of 1.3 mM is similar to that determined by Gilvarg and 3 times higher than that determined by Lin et al., and the turnover number of 200 s^{-1} is only slightly lower than the previously reported value (*11*, *15*). The close agreement between these data demonstrates that the enzymes from the two different sources have very similar kinetic properties.

Previous work had indicated that the desuccinylase is intolerant of most changes in substrate structure (*11*, *15*). The ability of the isomeric DAPs to inhibit the desuccinylase was investigated as a means of further identifying structural features important for substrate binding. Succinate was a poor product inhibitor, with the inhibitory effects observed at 100 mM succinate being mimicked by a corresponding increase in ionic strength with NaCl. While this may suggest that the identity of the acyl group is relatively unimportant, *N*-acetyl-L,L-diaminopimelic acid has been reported to not be a substrate for the *E. coli* desuccinylase (*11*).

All three isomers of DAP were tested as inhibitors of the desuccinylase, with both L,L- and D,L-DAP demonstrating linear competitive inhibition versus L,L-SDAP. The K_{is} values for both of these compounds are approximately 10 mM, which is 5−10-fold higher than the K_m values for SDAP. D,D-DAP exhibited no inhibition versus SDAP at the concentrations tested (up to 10 mM). These data, in combination with results reported by Lin et al., indicate that the desuccinylase requires an L-configuration at the acylated amino acid center where hydrolysis occurs, and prefers an L-configuration at the distal amino acid center but will tolerate a D-configuration.

Experiments aimed at determining the effect of pH on desuccinylase activity were complicated by the necessity of using buffers possessing a low absorbance at wavelengths of <230 nm. Both phosphate and Tris buffers satisfy this requirement, and experiments at overlapping pH values produced equivalent kinetic parameters. The pH of each assay was determined by directly measuring the reaction pH, since some assays were conducted at the extremes of the buffering capacity of these buffers.

The pH dependencies of the maximum velocity of both the zinc and cobalt forms of the desuccinylase are qualitatively identical; the maximal velocities are independent of pH in the alkaline region but decrease as a single ionizable group is protonated. We have observed a loss of enzyme stability below pH 6, accounting for the deviation of the experimental points from the predicted values for a single ionizable group at the acidic extremes of the pH curves. The calculated p*K* values for this group are similar for the two enzymes (Zn, 6.6; and Co, 5.9), with the p*K* value observed for the cobalt form being slightly lower. A similar observation has been reported for carbonic anhydrase (*26*). It is known that coordination of a water molecule to a metal center significantly lowers its p*K*, with reported values as low as 5−6 (*27*−*29*). The dependence of the p*K* value observed in the *V* profile on metal ion identity suggests that the pH-sensitive group is possibly a metal-coordinated water, an observation we have incorporated into our mechanistic proposal.

Plots of *V*/*K* versus pH for both metal forms of the desuccinylase are bell-shaped curves. We suggest that the ionizable group whose protonation decreases *V*/*K* is the same metal-coordinated water molecule observed in the *V* profile based on the similarity of the p*K* values determined. *V*/*K* includes kinetic contributions of substrate binding and all subsequent steps through the first irreversible step, which is likely to be attack of the metal-coordinated hydroxide on the amide carbonyl. The coupling of substrate binding and metal-bound water hydrolysis has been observed in other metal-dependent enzymes, and is one explanation for our

results. The ionizable group whose deprotonation affects *V*/*K* for SDAP which is not observed in the *V* profile is likely to be a group on the free enzyme which interacts with the substrate. SDAP contains three carboxylate moieties, and it is likely that positively charged amino acid side chains, in particular, those of lysine residues, are present in the substrate binding site to electrostatically interact with the substrate. Alternatively, the ionizable group may be on the substrate, and the most likely candidate would be the α-amino group of the distal L-amino acid center. The p*K* values determined from the *V*/*K* profiles of both the Zn and Co enzymes are identical and reasonably close to the expected values for that group.

These experiments suggest that the desuccinylase is a metalloenzyme, as are a number of enzymes which catalyze amide bond hydrolysis. Leucine and methionine aminopeptidases, which hydrolyze the N-terminal amino acid from polypeptides, contain two zinc and two cobalt ions, respectively, per monomer, as determined by X-ray crystallography (*30*, *31*). Members of the thermolysin and stromelysin family of matrix metalloproteinases contain a single zinc ion at their active sites (*32*). Interestingly, the class B β-lactamases are also metal-dependent amidases. Although all of the known members of this β-lactamase family contain two metal binding sites, some require occupancy of both sites for full activity (*33*, *34*), while others are fully active in the monozinc form and are inactivated upon binding of the second metal ion (*35*).

An alignment of the known *dapE* sequences identified a number of residues that are highly conserved in a variety of metal-dependent enzymes (*21*). Furthermore, when these sequences are aligned with that of *Pseudomonas* carboxypeptidase G2, an amidase known from its three-dimensional structure to contain a dinuclear metal center (*22*), all of the CPG2 metal binding residues are absolutely conserved in the desuccinylases (Figure 4). To determine whether the desuccinylase contained one or two metal ions per monomer, metal stoichiometries were determined using ICP-AES to simultaneously detect the presence of zinc and cobalt. Somewhat surprisingly, it was found that 1 mol of metal was tightly bound per mole of protein, in contrast to the 2 mol of metal expected from sequence comparisons with carboxypeptidase G2. The sample preparation procedure involved extensive dialysis for removing adventitious metal nonspecifically bound to the protein. We suggest that the desuccinylase contains two metal binding sites, one of high affinity and one of low affinity, and upon extensive dialysis, the metal occupying the low-affinity site is lost. In support of this suggestion, we note that the enzyme as purified contains 1 mol of metal, but activity can be stimulated 2−7-fold by the addition of exogenous metal. This suggests that the single-metal form of the enzyme is able to catalyze desuccinylation, but maximal activity is reached only upon metal binding to both metal sites.

Attempts to load cobalt into the high-affinity site of the enzyme were only partially successful. In the first set of samples, greater than 93% of the high-affinity site was occupied by zinc, while only 3% was occupied by cobalt. When the desuccinylase was incubated with cobalt for a longer period, the cobalt occupancy increased to 40%, suggesting that even longer incubation periods would be required for complete substitution of cobalt for zinc. It is interesting to note that, under the experimental assay conditions, exogenous metal is added immediately prior to assaying the enzyme, suggesting that cobalt binding to the low-affinity site stimulates the desuccinylase twice as effectively as zinc binding to the low-affinity site. Thus, the differences seen in the values of K_m and k_{cat}, as well as the differences in ionization constants, between the zinc and cobalt forms of the desuccinylase must be due to binding of metal at the low-affinity site.

Solvent kinetic isotope effect studies revealed inverse, and approximately equivalent, effects on *V* and *V*/*K* of 0.78 (±0.05) and 0.62 (±0.06). Similar inverse solvent kinetic isotope effects on *V*/*K* have been observed for the metalloproteases thermolysin and stromelysin, both of which contain a single active site zinc atom. Mechanisms have been proposed for these latter enzymes which account for the inverse solvent kinetic isotope effects (*37*−*40*). On the other hand, we are unaware of any similar isotopic studies on metalloproteases, such as leucine or methionine aminopeptidases, which have been shown to contain bimetallic centers (*30*, *31*). On the basis of the strong sequence homology between the *dapE*-encoded desuccinylases and carboxypeptidase G2, whose three-dimensional structure revealed the presence of two zinc atoms at the active site, we propose a model invoking two metal ions, one of which is very tightly bound. The fractionation factors of water or hydroxide metal ligands are considerably inverse, and for carbonic anhydrase isozymes have been measured as 0.72−0.77 (*41*). These reactant-state fractionation factors can account for the observed effects that we measure for the desuccinylase. Two criteria must further be met for this explanation to be plausible: that the transition-state fractionation factor be unity and that both *V* and *V*/*K* be reporting on the same reactant-state to transition-state event. As described earlier, *V*/*K* includes kinetic contributions between the binding of the free substrate to the free enzyme and the first irreversible step, while *V* includes kinetic contributions from the conversion of the enzyme−substrate complex through the dissociation of the last product. There is thus a single step common to both rate expressions, the conversion of the enzyme−SDAP Michaelis complex to the tetrahedral intermediate. We thus suggest that this is the step that is sensitive to solvent isotopic composition and that the inverse fractionation factor of the single hydroxide proton and the unitary value of the fractionation factor of the tetrahedral intermediate formed by hydroxide ion attack on the amide carbonyl can account for the solvent kinetic isotope effects.

We propose a mechanism for the desuccinylase which is similar to those proposed for other metal-dependent amidases (Figure 5). Prior to substrate binding, a water molecule occupies the active site, coordinated to both metal ions, based on the CPG2 crystal structure (*22*). We postulate that this water molecule is hydrogen bonded to an active site base in what is essentially a [A^-−H−O(H)−Zn] active site pairing that combines to lower the p*K* of H_2O to the observed value of ca. 6.6 for the zinc enzyme and ca. 5.9 for the cobalt enzyme. The active site base is likely to be Glu134 (*H. influenzae* sequence), which is conserved in all of the *dapE* sequences, as well as in CPG2 (Figure 4). Glutamic acid residues have been proposed to fulfill this role in other metalloproteases (*22*, *37*, *38*, *42*, *43*).

```
                    101                                                 150
H. influenzae       .D.TLNLWA. .KHG.TS.EP V.IAFAGHTD VVPTGDENQW SSPPFSAEII
E. coli             .D.TQNFWA. .WRG.QG.E. T.LAFAGHTD VVPPGDADRW INPPFEPTIR
H. pylori           KEHAEKEHAK EKHAKENVKP LHFSFAGHID VVPPGD.N.W QSDPFKPIIK
M. tuberculosis     L..A.R..T. K.LNRSS.R. V.L.LAGHLD TVPVAG.NL. ...PSR..RE
C. glutamicum       L..A.R..T. N.RGLAS.R. V.M.LAGHID TVPIAD.NL. ...PSR..VE
Pseudomonas CPG2    V..GDNI.VG KIKGRGG.KN L.L.LMSHMD TVYLKG.IL. AKAPFR..VE
Consensus           ---------- ---------- -------H-D -V-------- ---P------
                    151                                                *200
H. influenzae       DGMLYGRGAA DMKGSLAAMI .VAAEEYVKA NPNHK.GTIA LLITSDEE.A
E. coli             DGMLFGRGAA DMKGSLAAMV .VAAERFVAQ HPNHT.GRLA FLITSDEE.A
H. pylori           EGFLYGRGAQ DMKGGVGAFL .SASLNF... NPKTP.FLLS ILLTSDEE.G
M. tuberculosis     NDQLHGCGAA DMKSGDAVFL HLAATLAEPT ...HDL.TL. VFYDC.EEI.
C. glutamicum       DGIMYGCGTV DMKSGLAVYL HTFATLATST ELKHDL.TL. IAYEC.EEVA
Pseudomonas CPG2    GDKAYGPGIA DDKGGNAVIL HTLKLLKE.Y GVR.DYGTIT VLFNTDEEKG
Consensus           -----G-G-- D-K------- ---------- ---------- ------EE--
                    201                                                 250
H. influenzae       T.AKDGTIHV VETLMARDEK ITY.CMVGEP SSAKNLGDV. VKNGRRGSIT
E. coli             S.AHNGTVKV VEALMARNER LDY.CLVGEP SSIEVVGDV. VKNGRRGSLT
H. pylori           P.GIFGTKLM LEKLKEKD.L LPHMAIVAEP TCEKVLGDS. IKIGRRGSIN
M. tuberculosis     DSAANGLGRI QREL.P.DWL SADVAILGEP T.A...G..C IEAGCQGTLR
C. glutamicum       DH.LNGLGHI RDEH.P.EWL AADLALLGEP T.G...G..W IEAGCQGNLR
Pseudomonas CPG2    SFGSRDL..I QEE..A.K.L .ADYVLSFEP TSA...GDEK LSLGTSGIAY
Consensus           ---------- ---------- --------EP ------G--- ---G--G---
                    251                                                 300
H. influenzae       GNLYIQGIQG H.VAYPHLAE NPIHKAALFL QEL.T..T.. YQWDKGNEFF
E. coli             CNLTIHGVQG H.VAYPHLAD NPVHRAAPFL NEL.V..A.. IEWDQGNEFF
H. pylori           GRLILKGVQG H.VAYPQKCQ NPIDTLASVL PSI.S..G.. VHLDDGDEYF
M. tuberculosis     VVLSVTGTRA HS.ARSWLGD NAIHKLGAVL DRLAVYRARS VDID.GCTY.
C. glutamicum       IKVTAHGVRA HS.ARSWLGD NAMHKLSPII SKVAAYKAAE VNID.GLTY.
Pseudomonas CPG2    VQVNITGKAS HAGAAPELGV NAL..VEA.S D.L.VLRTMN ID.DKAKNL.
Consensus           ------G--- H--A------ N--------- ---------- ---D------
                    301                                                 350
H. influenzae       PPTSLQIANI HAGTGSNNVI PAELYIQFNL RYC.TEVTDE IIKQKVAEML
E. coli             PATSMQIANI QAGTGSNNVI PGELFVQFNF RFS.TELTDE MIKAQVLALL
H. pylori           DPSKLVVTNL HAGLGANNVT PGSVEITFNA RHS.LKTTKE SLKEYLEKVL
M. tuberculosis     .REGLSAVRV AGGV.AGNVI PDAASVTINY RFAPDRSVAA A....LQH.V
C. glutamicum       .REGLNIVFC ESGV.ANNVI PDLAWMNLNF RFAPNRDLNE A....IEHVV
Pseudomonas CPG2    .RFNWTIAK. AGNV.S.NII PASATLNADV RYARNEDFDA AMKT.LEERA
Consensus           ---------- -------N-- P--------- R--------- ----------
                    351                                                 400
H. influenzae       EKHNLK.Y.R IEWNLS.GKP FLTK.PG..K .LLD.SITSA IEETIGITPK
E. coli             EKHQLR.Y.T VDWWLS.GQP FLTA.RG..K .LVD.AVVNA VEHYNEIKPQ
H. pylori           .K.DLP.H.T LELESS.SSP FITASHS..K .LTS.VLKEN ILKTCRTTPL
M. tuberculosis     HDV.FDGLD. VQIEQTDA.. AAGALPGLSE P.AAKALV.. .EAAGGQ.VR
C. glutamicum       ETLELDGQDG IEWAVEDG.. AGGALPGLGQ Q.VTSGLI.. .DAVGREKIR
Pseudomonas CPG2    QQKKLPEAD. VKVIVTRGRP AFNAGEG.GK KLVDKAVAYY KEAGGTLGVE
Consensus           ---------- ---------- ---------- ---------- ----------
                    401                                                 450
H. influenzae       AETGGGTSD. GRFIALMGAE VVEFGP.L.N STIHKVNECV ~~~~~~~~~~
E. coli             LLTTGGTSD. GRFIARMGAQ VVELGP.V.N ATIHKINECV NAADLQLLAR
H. pylori           LNTKGGTSD. ARFFSAHGIE VVEFGV.I.N DRIHAIDERV SLKE~~~~~~
M. tuberculosis     AKY.GWT.DV SRFAAL.GIP AVNYGPGDPN LA.HCRDERV PVGNITAAVD
C. glutamicum       AKF.GWT.DV SRFSAM.GIP ALNFGAGDPS FA.HKRDEQC PVEQITDVAA
Pseudomonas CPG2    ERTGGGT.DA A.YAALSGKP VIE.SLGLPG FGYHS.D.K. .AEYV.DISA
Consensus           ----G-T-D- -------G-- ---------- ---H------ ----------
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FIGURE 4: Sequence alignment of the five known *dapE* sequences with the *Pseudomonas* CPG2 sequence. Consensus residues are those conserved between all six sequences. The residues in bold are the metal binding residues determined from the CPG2 structure (*22*). The starred residue is postulated to be an active site general acid.

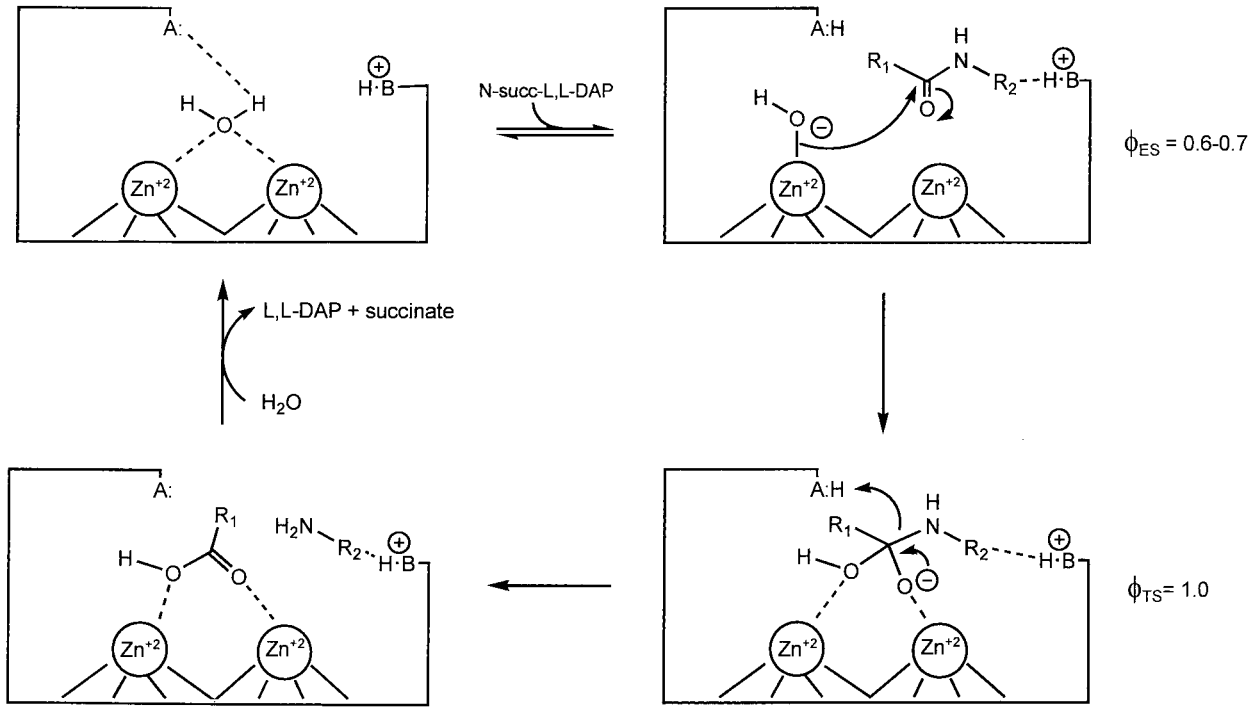

FIGURE 5: Proposed enzymatic mechanism for the desuccinylation of SDAP by the *dapE*-encoded desuccinylase.

Substrate binding is accompanied by coordination to the loosely bound zinc ion through the amide carbonyl with displacement of the water molecule from its bridging position, shifting it closer to the active site base. This promotes hydrolysis of the water molecule for generating the reactive hydroxyl nucleophile in the precatalytic complex. Substrate binding is further stabilized by interaction with a positively charged group, possibly a lysine residue.

From this precatalytic complex, the hydroxide attacks the carbonyl to form a tetrahedral intermediate. The tetrahedral intermediate then collapses to form products, presumably assisted by protonation of the amide nitrogen. It is tempting to postulate that the proton donor is the same active site base which earlier abstracted a proton from the nucleophilic water molecule, although we have no experimental evidence to support this second role. Because both products are poor inhibitors of the enzyme, their dissociation constants are expected to be high and dissociation rates are likely to be fast and not kinetically significant for the overall reaction.

Our mechanism suggests a number of functions for the zinc ions. First, they are involved in coordinating the nucleophilic water molecule at the active site, correctly positioning it for attack at the substrate carbonyl. They are also involved in lowering the p*K* of the water nucleophile, probably in conjunction with an active site base, thus activating it for attack on substrate. We also postulate that the substrate is coordinated to the zinc center upon binding. While we have no direct evidence to support this contention, it is reasonable, and precedented in other metalloproteases, that such coordination would provide both favorable polarization of the carbonyl and orientation for nucleophilic attack by hydroxide. There is both structural and enzymatic precedence for proposing such a function for the metal center (*27*, *44*). Formation of the tetrahedral intermediate results in a significant buildup of negative charge on both of the oxygens, which can be stabilized through coordination to the two metals.

The data presented in this paper firmly place the *dapE*-encoded desuccinylases in the family of metal-dependent amidases, a family that includes the pharmaceutically important enzymes thermolysin, stromelysin, and the class B β-lactamases. The members of this family appear to catalyze amide bond hydrolysis via a highly similar mechanism, suggesting that information determined for one family member will be applicable to other family members as well. Structural studies of the desuccinylase, however, will be required if the results presented here are to be placed in a framework that will make them applicable to other family members.

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