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A New Subfamily of Agmatinases Present in Methanogenic *Archaea* Is Fe(II) Dependent

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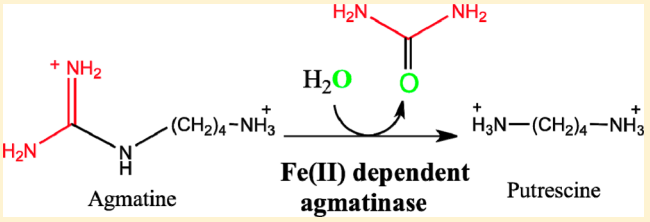
ABSTRACT: Here we report that the *Methanocaldococcus jannaschii* enzyme derived from the MJ0309 gene is an Fe(II) dependent agmatinase (SpeB). This is the first report of an iron-dependent agmatinase. We demonstrate that aerobically isolated recombinant enzyme contains two disulfide bonds and only a trace amount of any metal and requires the presence of both dithiothreitol (DTT) and 4 equiv of Fe(II) for maximum activity. The DTT activation could be indicative of the presence of a redox system, which would regulate the activity of this as well as other enzymes in the methanogens. Site-directed mutagenesis of the four conserved cysteines C71, C136, C151, and C229 to alanine or serine showed that only the C71 and C151 mutants showed a significant drop in activity indicating that the disulfide bond responsible for regulating activity was likely between C136 and C229. We propose that the C71 and C151 cysteine thiols, produced by the DTT-dependent reduction of their disulfide, are two additional metal binding ligands that alter the metal specificity of the *M. jannaschii* agmatinase from Mn(II) to Fe(II).



Agmatinases (SpeB) are widely distributed enzymes that catalyze the hydrolytic release of urea from agmatine leading to putrescine (Figure 1) as one of the steps in a pathway

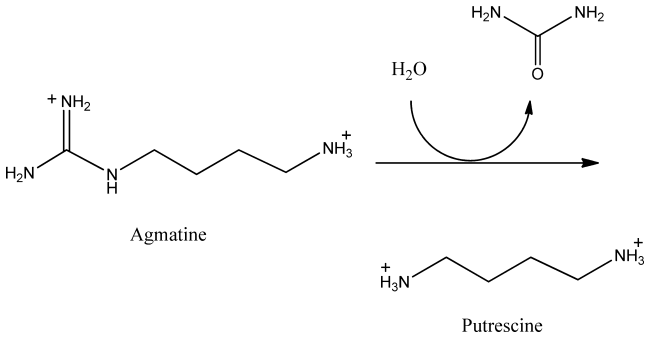


Figure 1. The reaction catalyzed by agmatinase.

leading to polyamines in *Archaea* and *Bacteria*.[1,2] Agmatinases are homologous to arginases, and both are members of the ureohydrolase superfamily of enzymes[3,4] that also includes formiminoglutamase and proclavaminate amidohydrolase.[5] All previously characterized agmatinases and arginases have been found to contain a binuclear Mn(II) center in their catalytic site.[6−8] Here we demonstrate that SpeB from the methanogenic *Archaea Methanocaldococcus jannaschii*, product of the MJ0309 gene, uses Fe(II) as the catalytic metal and requires reduction of two disulfide bonds in the protein for activity.

This is the first report of an iron dependent agmatinase. Homologues of the *M. jannaschii* SpeB have the same conserved Mn(II) metal binding ligands that have been established through crystal structures to be as shown in Figures 2 and 3.[8−10] The methanogenic agmatinases, however, also contain an additional conserved $CX_{68}CX_{14}CX_{48}C$ motif, where the second cysteine, C136, is found to be within the metal binding motif DAHCD. Here we report evidence that the first and third cysteines in this motif (C71 and C151) are iron binding ligands and the remaining two are involved in disulfide bond formation.

MATERIALS AND METHODS

Cloning, Overexpression, and Purification of the M. *jannaschii* MJ0309 Gene Product in *E. coli*. The MJ0309 gene (Swiss-Prot accession number Q57757) was amplified by PCR from genomic DNA using oligonucleotide primers MJ0309 Fwd (5′-GGTCATATGGAGGAGCAC-3′) and MJ0309 Rev (5′-CGGGATCCTTATAGAATCATCAAC-3′). PCR amplification was performed as described previously[11] using a 55 °C annealing temperature. Purified PCR product was digested with *Nde*I and *BamH*I restriction enzymes and ligated into compatible sites in plasmid pET19b. Sequence of the resulting plasmid, pMJ0309, was verified by DNA sequencing. Plasmid pM0309 was transformed into *Escherichia coli* strain BL21-CodonPlus(DE3)-RIL cells (Stratagene). The transformed cells were grown in LB medium (200 mL) supplemented with 100 μg/mL ampicillin at 37 °C with shaking until they reached an OD_{600} of 1.0. Recombinant protein production was induced by addition of lactose to a final concentration of 28 mM.[11] After an additional 2 h of culture, the cells were harvested by centrifugation (4000*g*, 5 min) and frozen at −20 °C. Induction of the desired protein was

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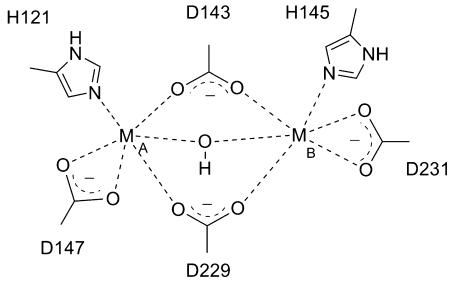
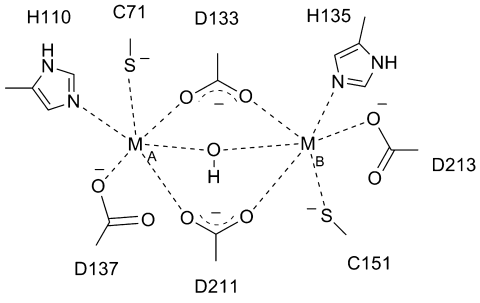


Confirmed metal binding ligands in *D. radiodurans*



Proposed metal binding ligands in *M. jannaschii*

Figure 2. Confirmed and proposed metal binding ligands in different agmatinases. Two metal ion binding site for the non-methanogenic agmatinase from *Deinococcus radiodurans* and a proposed two metal ion binding site methanogenic *M. jannaschii* agmatinase. The canonical agmatinases contain two Mn(II) as the metals where in the methanogens the metals would be two Fe(II) and with two thiol ligands. The top figure shows the amino acid numbers for the *D. radiodurans* agmatinase, and the bottom figure has the amino acid numbers for the *M. jannaschii* agmatinase showing the involvement of the thiol ligands.

confirmed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of total cellular proteins.

Site-Directed Mutagenesis. To test the possible function of conserved Cys71, Cys136, Cys151, and Cys229 residues of MJ0309 the mutants were generated. The Quick-Changed Site Directed Mutagenesis kit (Stratagene) was used according to the manufacturer's instructions with the template pMJ0309 (pET19b as the vector). The oligonucleotide primers (Invitrogen) used were MJ0309 C71-Fwd (5′-GATTTGGCA-GAATTAAAATACTCTGATTTAAAAGATTTAG-3′) and MJ0309 C71-Rev (5′-CTAAATCTTTTAAATCAGAG-TATTTTAATTCTGCCAAATC-3′), MJ0309 C136A-Fwd (5′-GAATTTGATGCCCATGCTGATTTGAGAGATG-3′), C136A-Rev (5′-CATCTCTCAAATCAGCATGGGCAT-CAAATTG-3′), C151-Fwd (5′-GTAATAAGCTCTCT-CATGGGTCTGTTATGAGGAGAGTTTATG-3′) MJ0309 C151-Rev (5′-CATAAACTCTCCTCATAACAGACGCAT-GAGAGAGCTTATTAC-3′), C229A-Fwd (5-CTGGAACTC-CAGAACCCGCTGGATTTTCAACAAGAG-3′), C229A-Rev (5′-CTCTTGTTGAAAATCCAGCGGGTTCTGGAGTTC-CAG-3′). DNA sequences of the mutated genes were confirmed by dye-terminator sequencing at DNA Sequencing Facility of Iowa University. Expression of these mutants and analysis of the proteins were done as described above.

Purification of Recombination SpeB and its Mutants. Frozen *E. coli* cell pellet (~0.4 g wet weight from 200 mL of medium) was suspended in 3 mL of extraction buffer (50 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), pH 7.0, 10 mM $MgCl_2$, 20 mM DTT) and lysed by sonication. SpeB and its mutants were found to remain soluble after heating the resulting cell extracts for 10 min at 70 °C followed by centrifugation (16000*g* for 10 min). This process allowed for the purification of SpeB and its mutants from the majority of *E. coli* proteins, which denature and precipitate under these conditions. The next step of purification was

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                          67     71                 83      110                 128
M. jannaschii             E---LKYCDLKDLDLY---GSQEE  GGEHSITYPIIK--AVKDIYDDFI
M. fervens                E---LKYCDLKDLDLY---GNQVE  GGEHSITYPIVK--AVKDVYNDFI
M. vulcanius              D---VKYCDLKDLDLY---GSQKE  GGEHSISFPIVK--AVKDVFGEVA
M. maripaludis (C5)       D---VKICDKYNLLME---GYQSE  GGEHSVTYPVVK--AVKSVYNDFA
M. vannielii              D---LKICDKYNISIE---GNQET  GGEHSVTYPMIK--AVKDVYSDFI
M. aeolicus               D---VPICDLHNISVD---GTQKD  GGEHSITYPVVK--SAKKKYDDIL
C. difficile (3LHL)       D---YNICDYGDLEISV--GSTEQ  GGEHLVTLPAFK--AVHEKYNDIY
C. difficile (NAP07)      D---YNICDYGDLEISV--GSTEQ  GGEHLVTLPAFK--AVYEKYNDIY
P. horikoshii             E---LKIADAGDVALP---VSIED  GGEHSMTYPPVK--VLEPKS----
E. coli (K12)             MRERLNVVDCGDLVYAF--GDARE  GGDHFVTLPLLR--AHAKHFGKMA
D. radiodurans            LQG-VTFADAGDVILPS--LEPQL  GGDHSVSYPLLR--AFADVP-DLH
D. radiodurans*           GDIEVALPETLDKHETGGLVFFEP  GGDHSVSMGTVTGNGLRGRPQRTG

                          129   136               151   204   211              229
M. jannaschii             VIQFDAHCDLRDEYLG-----NKLSHACVMRR  KPIYVTIDIDVLDPAYAPGTGTPEPCGFSTRE
M. fervens                VIQFDAHCDLRDEYLG-----NKLSHACVMRR  KPIYLTVDIDVLDPAYAPGTGTPEPCGFSTKE
M. vulcanius              VIQFDAHCDLRDEYLG-----NNLSHACVMRR  IPIYITLDIDVLDPAYAPGTGTPEPCGFTSRE
M. maripaludis (C5)       VIHFDAHCDLREEYMG-----NEQSHASVMRR  KPVYLTIDIDVLDPAFVPGTGTPEPCGFTPKE
M. vannielii              IIHFDAHCDLRNSYNG-----NEQSHASVIRR  KPIYITIDIDVLDPAFVPGTGTPEPCGFSPKE
M. aeolicus               LIQFDAHCDLRENYLN-----NKYSHASVIRR  KKIYITIDIDVLDPAFAPGTGTPEPCGFSSKE
C. difficile (3LHL)       VIHFDAHTDLREEYNN-----SKNSHATVIKR  KNIYLTIDLDVLDASVFPGTGTPEPGGVNYRE
C. difficile (NAP07)      VIHFDAHTDLREEYNN-----SKNSHATVIKR  KNIYITIDLDVLDASVFPGTGTPEPGGVNYRE
P. horikoshii             YVVFDAHLDLRDSYQG-----SRFNHACVARR  EPVYVSIDVDVFDLPLVPETGTPEPGGLGFWE
E. coli (K12)             LVHFDAHTDTYANGCE-----FDHGTMFYTAP  MPVYLTFDIDCLDPAFAPGTGTPVIGGLTSDR
D. radiodurans            VVQLDAHLDFTDTRND-----TKWSNSSPFRR  QNVYFSVDVDGFDPAVIPGTSSPEPDGLTYAQ
D. radiodurans*           VIWVDAHTDYNTPESSPSGNIHGMPVAHLTGR  ERLHVSFDADALDPGVCPGVGTPVPGGLSYRE

*Arginase
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Figure 3. Sequence alignment of agmatinases with highlighted canonical metal binding ligands (pink) and conserved cysteines (blue).

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performed by anion-exchange chromatography of the 70 °C soluble fractions on a MonoQ HR column (1 × 8 cm; Amersham Bioscience) using a linear gradient from 0 to 1 M NaCl in 25 mM Tris buffer (pH 7.5), over 55 min at a flow rate of 1 mL/min. Fractions of 1 mL were collected. Protein concentrations were determined by Bradford analysis.[12]

Measurement of the Native Molecular Mass of SpeB. The native molecular mass of *M. jannaschii* SpeB was determined by size exclusion chromatography on a Superose 12HR column (10 mm × 300 mm) developed with a buffer containing 50 mM TES (pH 7.2), and 150 mM NaCl at 0.5 mL/min with detection at 280 nm. Protein standards used to calibrate the column included cytochrome c (14 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), conalbumin (77.5 kDa), β-amylase (200 kDa), apoferritin (443 kDa), and blue dextran (2000 kDa).

Standard Enzymatic Assay. To test the activity of SpeB, 32−40 μg of the MonoQ purified protein was incubated at 70 °C for 30 min along with an initial concentration of 8.3 mM agmatine, 0.03 M DTT, 83 μM metal, 0.33 M NaCl, and 8.3 mM Tris at pH 7.5 in a total volume of 60 μL under an argon atmosphere. The high salt was included to match the salt concentration of the MonoQ fraction. The reaction was stopped by the addition of 2 μL 6 M HCl followed by a brief centrifugation (16000*g* for 5 min). The samples were then ready for TLC, direct infusion-electrospray ionization-mass spectrometry (DI-ESI-MS), or fluorescamine assays as described below.

Establishment of Enzymatic Activity and Product Analysis using thin layer chromatography (TLC). Samples from the incubation mixture were spotted onto the TLC plate along with known samples of agmatine and putrescine. The solvent system consisted of acetonitrile, water, formic acid (88%) (40:20:10 vol/vol/vol). Agmatine and putrescine were visualized by spraying the plate with 2% ninhydrin in ethanol followed by heating the plate to 140 °C. Agmatine gave a brown spot that had a higher migration (R_f = 0.32) than putrescine (R_f = 0.22), which gave an orange-brown spot, each the same as the known compounds.

Assay of Enzymatic Activity and Product Analysis Using DI-ESI-MS. At the completion of the incubation, samples were mixed with 1 mL of ammonium acetate buffer (1 g/L), and 20 μL samples were infused into AB SCIEX Triple Quad 3200 Qtrap LC/MS/MS. The ratios of agmatine and putrescine were then determined from the measured intensities of the MH^+ ion at 131.13 *m*/*z* for agmatine and 89.11 *m*/*z* for putrescine.

Assay of Enzymatic Activity using a Fluorescamine Based Assay. To 100 μL of 0.1 M borate buffer (pH 9.3), was added 10 μL of the enzymatic incubation mixture described above. Then 20 μL of a 10 mM solution of fluorescamine in dimethylformamide was added with rapid stirring. After 30 min at room temperature, the sample was diluted to 1 mL with water and 10 μL was assayed by HPLC.

The chromatographic separation and analysis of fluorescamine derivatives of the substrate agmatine and the product putrescine was performed on a Shimadzu HPLC System with a C18 reverse phase column (Varian PursuitXRs 250 × 4.6 mm, 5 μm particle size) equipped with a fluorescence detector. The elution profile consisted of 5 min at 95% sodium acetate buffer (25 mM, pH 6.0, 0.02% NaN_3) and 5% MeOH followed by a linear gradient from 50% to 100% MeOH over 28 min at 0.5 mL/min. Quantitation was based on fluorescent intensity with EX = 390 nm and EM = 475 nm. Under these conditions the major fluorescamine adducts of agmatine and putrescine eluted respectively at 13 and 15 min. Standard curves were generated from the observed ratios of the agmatine to putrescine against the known molar ratios.

In our analyses a small peak was observed just before the agmatine peak, eluting at 12.5 min. It was determined to be the monoderivative of the putrescine by measuring its production as a function of the ratio of fluorescamine to putrescine used in the assay. At lower ratios the relative amount of the first peak was much higher indicating that it was the monoderivative of the putrescine. Since this compound contained only a single fluorescent group, its area was multiplied by two and added to the area of the diderivative of the putrescine peak, the 15 min peak, to obtain the total amount of putrescine generated.

Analysis of Urea. Because it was possible that the enzyme could also catalyze the hydrolysis of the expected urea product to carbon dioxide and ammonia, the urea present in an incubation mixture was confirmed by converting it to 2-hydroxpyrimidine (2-HP) by reaction with 1,1,3,3-tetramethoxypropane and then identified by gas chromatography−mass spectrometry (GC-MS) of the monotrimethylsilyl derivative as previously described.[13]

Synthesis of 2-Hydroxypyrimidine. A known sample of 2-HP was prepared by mixing 60 mg of urea with 200 μL of 1,1,3,3-tetramethoxypropane and 3 mL of 6 M HCl.[14] The sample turned slightly yellow upon the addition of 1,1,3,3-tetramethoxypropane and then became more vibrant when HCl was added. The sample was mixed, incubated at room temperature for 1 h, and evaporated to dryness with nitrogen (N_2) gas to generate the crude product. Analytical preparative TLC was then performed on the material using the same solvent system as was used for the TLC activity assay of SpeB. The major UV absorbing spot about midway up the plate was removed and eluted with 50% methanol. The UV spectrum of this material (in water) showed an absorbance peak at 330 nm. Following addition of a small amount of 1 M NaOH (40 to 60 μL), the UV absorbance λ_{max} shifted to 292 nm. This is consistent with the reported value at 292 nm for 2-HP.[15] Mass spectrometry (EI-DI) showed a M^+ = 96 *m*/*z* for the isolated compound and the expected M^+ = 168 *m*/*z* and $M^+ - 15$ = 153 *m*/*z* for the monoTMS derivative.

Determination of the Kinetic Parameters. To determine the kinetic parameters wild type, C136A, and C229A enzymes were assayed under standard assay conditions except that the final concentration of agmatine was varied from 0.83 mM to 12.5 mM. The C71S and C151S mutants were assayed under standard conditions with the final concentration of agmatine varied from 0.83 mM to 50 mM.

Dependence of Enzymatic Activity on pH. To determine the optimal pH for SpeB activity, the standard assay conditions were followed except the buffer was replaced with 50 mM BIS-TRIS propane with pH from 6.5 to 9.0, 50 mM *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) from pH 7.5 to 9.0, or 50 mM diethanolamine from pH 8 to 10. The measured activity was about equivalent in the BIS-TRIS propane buffers to the standard assay buffer.

Inhibitors of SpeB. The activity of SpeB was tested under standard assay conditions with the initial concentrations of agmatine, arginine, and putrescine as indicated in Table 2. To test for the inactivation of the enzyme by *N*-ethylmaleimide (NEM), 42 μg of the MonoQ purified enzyme was incubated in 25 mM Tris (pH 7.5) 1 M NaCl with 0.2 μmol of NEM in the

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absence of DTT for 10 min at room temperature. At the end of the incubation, the sample was added to the standard assay mixture and the activity measured. By this process, the unreacted NEM was inactivated by the excess DTT (2 μmol) in the reaction mixture. To test for the inactivation of the enzyme by iodoacetamide (IAA), 42 μg of the MonoQ purified enzyme was incubated with 0.32 μmol of DTT at 70 °C for 5 min in a sealed tube under N_2. Next, 0.48 μmol of IAA (0.12 M) was added, sealed under N_2, and the sample was incubated for 30 min at room temperature. The sample was then added to the standard assay mixture except the final concentration of DTT was 2.7 mM (0.16 μmol).

Metal Ion Analysis of SpeB. Metal analysis of SpeB and mutants was performed at the Virginia Tech Soil Testing Laboratory using inductively coupled plasma emission spectrophotometry. Instrumentation included a Spectro CirOS VISION made by Spectro Analytical Instruments equipped with a Crossflow nebulizer with a Modified Scott spray chamber, nebulizer rate was 0.75 L/min. An internal yttrium standard (50 mg/L) was introduced by peristaltic pump. Samples were analyzed for cobalt, iron, manganese, nickel, and zinc.

Metal Titration of SpeB and Mutants. The metal titration of SpeB with Fe(II) and Mn(II) was performed with the wild type enzyme at a concentration of 60 μM. The assay was performed under argon using standard assay conditions with an increasing final concentration of iron or manganese of 0.03, 0.05, 0.075, 0.15, 0.23, and 0.3 mM. The metal titration was repeated with the C136A and C229A mutants with just Fe(II) in the same concentrations used for the wild type SpeB. The metal titration experiment was not done for the C71S and C151S SpeB because of their extremely low activity.

Absorbance Spectrum of the Fe(II) Enzyme. To 110 μL a solution of the MonoQ purified enzyme (1.14 mg/mL) was added 8 μL of a anaerobic solution of 0.1 mM Fe-$(NH_4)_2(SO_4)_2{\cdot}6H_2O$ in water. The absorbance spectra were recorded from 230 to 600 nm at different time periods to monitor any changes as a function of time. This procedure was repeated with each of the SpeB mutants. The spectra were recorded using a Shimadzu UV-1601 UV−visible spectrophotometer.

Effect of DTT and Metals on the Activity of SpeB. To determine the effect of DTT in the presence and absence of Fe(II) or Mn(II), the enzyme samples were assayed following the standard assay conditions and heated at 70 °C for 10, 15, 20, and 30 min for the wild type, C136A, and C229A mutants. The different assay periods were used to establish the time course of the reaction. The C71S and C151S mutants were only assayed with Fe(II) because of their low activity (0.0038 and 0.0042 μmol per min per mg, respectively) when Mn(II) was used. It was decided that the activity was too low to perform the assay with Mn(II) with and without DTT. To determine the dependence of SepB activity on the reduction potential of DTT the activity was tested in the presence of 0, 10, 50, 90, and 100% 4,5-dihydroxy-1,2-dithiane (DTTox) while the total concentration of DTTox plus DTT remained at 33 mM.

Testing Different Thiols for their Ability to Activiate SepB. To determine if DTT was specific for the activation of the enzyme other reducing agents were tried. The reaction was performed under standard reaction conditions except that the 33 mM DTT was replaced by other thiols/reducing agents at a final concentration of 33 mM for β-mercaptoethanol and 3-mercaptopropane-1,2-diol, 35 mM for 1,2-ethanedithiol, or 3.3 mM for tris(2-carboxyethyl)phosphine (TCEP).

Detection of Disulfides by SDS−PAGE. This assay was based on the known decrease in the mobility of proteins in SDS−PAGE analysis upon reduction of their disulfide bonds.[16] To determine if SpeB contains any disulfides as either the wild type or one of the four mutants, 20 μL of each was mixed with the loading buffer with and without β-mercaptoethanol (β-ME). The loading buffer typically consists of 475 μL Laemmli Sample Buffer (Bio-Rad) and 25 μL of β-ME. The samples were incubated for 5 min at 100 °C and then briefly centrifuged and then loaded onto a SDS−polyacrylamide gel for analysis.

Analysis of Disulfides in the Enzyme by Mass Spectrometry. The MonoQ purified wild type enzyme (50 μL of a 1 mg/mL solution) was placed in a glass vial, and the water was removed by evaporation with a stream of nitrogen gas. The resulting sample was dissolved in 50 μL of 6 M guanidine·HCl and split into two equal volumes in two vials under nitrogen. One sample was made 4.5 mM in DTT by the addition of 2.5 μL of a 50 mM solution of DTT in water under argon. Both samples were then heated at 57 °C for 10 min, cooled to RT and then 5 μL of 0.1 M NEM in dimethylformamide (DMF) was added to each sample to alkylate the cysteine thiols. After 30 min at 37 °C an additional 5 μL of 50 mM DTT was added to both samples to destroy the excess NEM. The excess reagents and guanidine·HCl were removed using a YM3 microcon (Millipore) followed by extensive washing with water. The membrane bound protein was recovered from the membrane with 80% formic acid. (This protein was retained very tightly on the membrane and could not be eluted with 0.2% SDS in 5 mM TES pH 7.5). The samples were then purified by SDS−PAGE, peptides were generated from the desired spots by in gel digestion with chymotrypsin, Glu C, and trypsin and analyzed by MALDI. The identity and positions of the cysteines alkylated were measured by MALDI mass spectral analysis of the excised protein band from the polyacrylamide gel, following in-gel digestion, using a 4800 MALDI Tof/Tof mass spectrometer (Applied Biosystems).

Quantitation of Disulfides and Free Thiols in Denatured SpeB Using mBBr and Fluorescence Spectroscopy before and after Reduction with DTT. The free thiols were determined by alkylation of the cysteines with monobromobimane (mBBr) followed by quantitation of the total thiols by measure of the proteins fluorescence. Thus, 50 μL of a solution of SpeB (2.1 mg/mL) was evaporated to 10 μL and the solution mixed with 90 μL of 6 M guanidine·HCl dissolved in 0.1 M Tris buffer (pH 8.0) with 5 mM EDTA. The solution was split into two equal parts and placed in vials under Ar. To one sample was added 5 μL of 25 mM tris(2-carboxyethyl)phosphine dissolved in the same buffer, and both samples were heated to 100 °C for 2 min. To the cooled sample was added 50 μL of 10 mM mBBr in acetonitrile with rapid mixing. After 15 min, the acetonitrile was evaporated from the samples and each sample was placed on a Sephedex G-25-40 column (0.5 × 5 cm) equilibrated in the 6 M guanidine·HCl 0.1 M Tris buffer (pH 8). Elution of the fluorescent compounds was visualized by exposing the column to UV light. The protein fraction eluted in ∼200 μL of solvent and the sample was diluted with additional buffer to give a volume of 1.0 mL for fluorescence measurement. This fraction was completely separated from unreacted mBBr that did not begin to elute for another 300 μL. The fluorescence of mBBr-derivitized

proteins have EX λ = 394 nm and EM λ = 478 nm. Calibration was accomplished using lysozyme that contains eight cysteines by the same procedure. Lysozyme (7.0 nmol) with its eight cysteines produced 98.6 units of fluorescence or 1.76 units of fluorescence per nmole of cysteine. This value was then used to calculate the cysteine content in the SepB samples. The spectra were recorded on Shimadzu RF-1501 spectrofluorometer.

Urease Activity of SpeB. To determine if SpeB had any urease activity, the standard assay was performed except the substrate used was urea at final concentration of 8.3 mM, instead of agmatine.

Assay of Ammonia by HPLC. To test if ammonia was a product of the urease assay described above, ammonia was assayed as the fluorescamine adduct as described above. A known sample of ammonia (0.77 mM) produced a single fluorescent peak with an area of 1×10^6, whereas the assay of the enzymatic reaction mixture produced no detectable peak (area $<2 \times 10^3$). The ammonia adduct was found to elute at 14.1 min. On the basis of these data, not more that 0.2% of the urea was converted into ammonia.

Phylogenetic Analyses of SpeB. The evolutionary history was inferred using the Neighbor-Joining method.[17] Phylogenetic analyses were conducted in MEGA4.[18] The optimal tree with the sum of branch length = 10.26694740 is shown. (next to the branches). The evolutionary distances were computed using the Poisson correction method[19] and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the data set (Complete deletion option). There were a total of 279 positions in the final data set.

RESULTS

Expression, Purification, and Analysis of SpeB and its Mutants. The MJ0309 gene from *M. jannaschii* and C71S, C136A, C151S, and C229A mutants were cloned and the corresponding proteins overproduced in *E. coli*. The recombinant proteins were extracted from the cells by sonication, purified by heating the extract at 70 °C for 20 min, followed by anion-exchange chromatography of the soluble proteins. The SDS−PAGE analysis with Coomassie staining of the purified SpeB and its mutants showed a single band corresponding to the predicted monomeric molecular mass of about 40 kDa with a purity of >95%. This molecular mass is consistent with the calculated monomeric molecular mass of 38073.7 Da for SpeB. On the basis of the intensities of the observed SDS−PAGE bands, the same amount of each protein was isolated from the wild type and each of the cysteine mutants, indicating that the mutations did not reduce the amount of protein or its heat stability. Attempts to measure the mass of the protein by MALDI-MS were unsuccessful. MALDI-MS of the tryptic peptides of the protein was used to confirm the presence of the desired protein.

Multimeric State of SpeB. Size exclusion chromatography of the *M. jannaschii* SpeB showed a molecular mass of 190 kDa consistent with the SpeB existing possibly as a homopentamer based on a monomeric mass of 38 kDa. However due to the inherent error in this measurement the enzyme could be either a tetramer or hexamer. This multimeric state is to be compared with an assortment of other agmatinases that range in size from a homodiamer from *E. coli*,[20] a homotetramer from *Pyrococcus horikoshii*,[21] and homohexamer from *D. radiodurans*.[9]

Identification of Reaction Catalyzed by SpeB. Incubation of SpeB with agmatine at 70 °C for 30 min in the presence of DTT and Fe(II) followed by analysis of the reaction mixture by TLC showed the production of a new ninhydrin positive spot with the same R_f as putrescine. Direct infusion-electrospray ionization-mass spectrometry (DI-ESI-MS) of the reaction mixture also confirmed the presence of agmatine and putrescine based on the observation of the expected ions at MH^+ = 131 m/z and MH^+ = 89 m/z, respectively. These same ions were also observed with known samples of these compounds. Analysis of the incubation mixture by HPLC after formation of the fluorescamine adducts showed two major fluorescent peaks. The first peak, eluting at 13 min, was determined to be agmatine and the second peak eluting at 15 min, was determined to be putrescine, the same retention times being observed using authentic compounds. Urea was confirmed as the other product of the reaction by its conversion to 2-hydroxypyrimidine followed by GC-MS analysis of the TMS derivative of this compound.

Determination of the Kinetic Parameters. The SpeB kinetics for the wild type enzyme and the C136A and C229A mutants were determined in the presence of 0.83−12.5 mM agmatine under standard assay conditions. The kinetics for the C71S and C151S mutants were determined in the presence of 0.83−50 mM agmatine under standard assay conditions. The results are summarized in Table 1.

Table 1. Specific Activities and Kinetic Data of SpeB and Its Mutants[a]

SepB	K_m (mM)	v_{max} (units mg^{-1})	k_{cat} (s^{-1}) × 10^{-2}	k_{cat}/K_m (mM $s)^{-1}$ × 10^{-2}
WT	2.4 ± 1.2	0.85 ± 0.13	3.2	1.3
C71S	11 ± 2.6	0.2 ± 0.019	0.75	0.069
C136A	9.2 ± 5.1	0.61 ± 0.18	2.3	0.25
C151S	6.2 ± 1.2	0.25 ± 0.015	0.95	0.15
C229A	5.8 ± 2.3	1.1 ± 0.19	4.1	0.77

[a]All assays were done under standard assay conditions in the presence of Fe(II) and DTT.

pH Dependence of SpeB Activity. The activity of SpeB was tested from pH 6.5 to pH 10.0. No activity was detected at pH 6.5 and the measured activity steadily increased to pH 10.0. Higher pH values were tested but the observation of activity was dependent on the buffer used and are not reported here. Agmatine showed no hydrolysis in the pH 10 and 11 buffers and assay conditions used. The *P. horikoshii* enzyme was reported to have an optimum activity at pH 11.0.[21]

Inhibition of SpeB. Wild type SpeB was found to be inhibited by the substrate analogue arginine at 16.7 mM, and by its product, putrescine at both 8.3 and 16.7 mM (Table 2). *M. jannaschii* SpeB is rendered inactive when the mono-Q purified enzyme was incubated with DTT followed by excess NEM or IAA. We propose that NEM and IAA alkylate C71 and C151 preventing Fe(II) from binding. That all the cysteines were alkylated to some extent was confirmed by MALDI-MS identification of the tryptic peptides containing the modified cysteines.

Metal Content of Recombinant SpeB. ICPES was used to determine the identity and quantity of metal ions present in the aerobically purified recombinant enzyme. As can be seen in Table 3 all samples were found to contain different amounts of iron with the most iron in the C71S mutant and the least in the wild type enzyme. Smaller amounts of Mn and Zn were also identified in some of the enzyme samples. No cobalt or nickel

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Table 2. Results of Inhibition Studies[a]

inhibitor	% activity
putrescine[b] 8.3 mM	57
putrescine[b] 16.7 mM	40
arginine 8.3 mM	97
arginine 16.7 mM	56
NEM[c]	0.6
IAA[c]	0.9

[a]All assays were done under standard conditions in the presence of 83 μM Fe(II) and 33 mM DTT and 8.3 mM agmatine. Activity in the absence of inhibitor was defined as 100%. [b]These calculations were done by subtracting the putrescine peak area of the control from the putrescine peak area of the incubated samples. [c]The reduced SpeB was alkylated with NEM and IAA before the addition of metals.

Table 3. Metal Analysis of SpeB and its Mutants[a]

	molar ratio of metal/SpeB (monomer)				
SpeB	iron	manganese	nickel	zinc	cobalt
wild type	0.65	<0.002	<0.006	0.37	<0.007
C71S	2.4	0.82	0.10	0.86	<0.007
C136A	1.4	0.046	<0.006	0.71	<0.007
C151S	1.34	0.082	<0.006	1.1	<0.007
C229A	0.90	<0.002	<0.006	0.383	<0.007

[a]"<" indicates concentrations below the detection limit.

was detected in most samples. The reason for detecting a molar ratio lower than four irons is a result of air oxidation of the Fe(II) to Fe(III) which is largely expelled from enzyme during purification.

Metal-Ion Dependency of SpeB Reaction. The specific activity of the recombinant *M. jannaschii* SpeB was determined in the presence of DTT and added Fe(II) and Mn(II). No activity was detected when Ni(II), Co(II), and Zn(II) were added to the assay mixture. SpeB was the most active (0.45 μmol min^{-1} mg^{-1}) in the presence of Fe(II). The only other metal supporting activity was Mn(II) which showed an activity (0.068 μmol min^{-1} mg^{-1}), 15% of the activity seen with Fe(II). The C136A and C229A mutants had 90% and 92% of the activity compared to wild type in the presence of Fe(II) (Figure 4). The fact that both mutants were still active means that these cysteines were not likely ligands to the catalytic metal(s). The C71S and C151S mutants, on the other hand, had only 4% and 6% of the activity compared to the wild type in the presence of Fe(II) (Figure 4). This information indicates that these two ligands are critical to the activity of SpeB and likely serve as metal ligands.

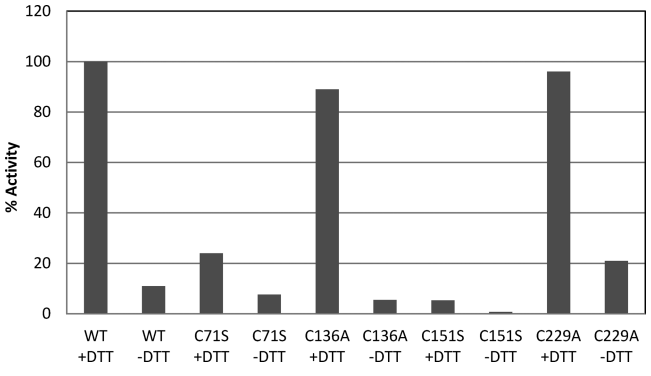

Figure 4. Effect of DTT on the enzymatic activity of SepB and its mutants.

Titration of the SepB with Fe(II) and Mn(II). Titration of the protein with Fe(II) in the presence of DTT showed that four mol of Fe(II) per mol of the SpeB subunit were required to obtain maximal activity (Figure 5). The titration with Mn(II) showed its relative activity when compared to the Fe(II) was minimal (Figure 5). No end point in the titration with Mn(II) was observed (data not shown). These data further demonstrate that SpeB from *M. jannaschii* requires Fe(II) for catalytic activity. Titration of the C136A and C229A mutants with Fe(II) in the presence of DTT also showed that four mol of Fe(II) per mol of the SpeB subunit were required to obtain maximal activity (data not shown). This result shows that these cysteines are not involved in iron binding and are involved in blocking activity by disulfide bond formation.

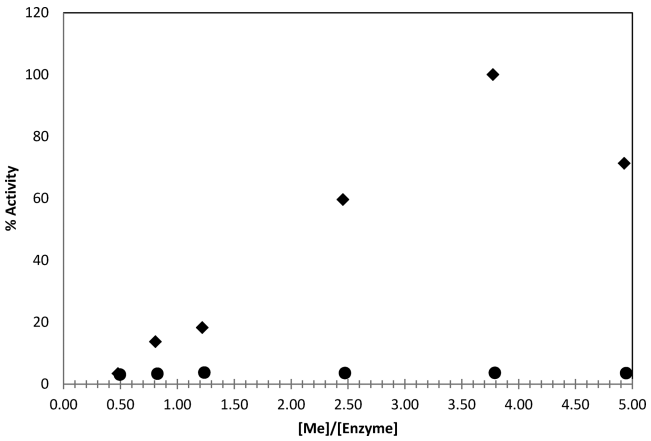

Figure 5. Metal titration of wt SpeB with Fe(II) and Mn(II) The activities with Mn(II) were all less that 1% of the Fe(II) data. (♦) Fe(II) and (●) Mn(II).

Effect of DTT and Metals on the Activity of SpeB. To test if DTT had an effect on enzymatic activity, the activity of the enzyme was measured with Fe(II) in the presence and absence of DTT. As can be seen in Figure 4 the activity of the wild type enzyme with Fe(II) and DTT was 10 times higher than Fe(II) alone. Some of the activity in the absence of DTT may be accounted for by the reduction of the disulfide bond by the added Fe(II).[22] The opposite effect was observed using Mn(II) where the addition of DTT inhibited the activity (data not shown). This same pattern of inhibition was also observed with the C136A mutant. These observations are consistent with the DTT promoting the incorporation of the Fe(II) into the active site and preventing the incorporation of Mn(II) into the active site.

Figure 4 also demonstrates that the C136A and C229A mutants have 89% and 96% of the activity of the wild type SpeB when DTT is present in the reaction. The C71S and C151S mutants, on the other hand, are still severely inhibited even when DTT is present in the reaction with only 24% and 5% of the wild type activity. Knowing that there are two disulfides in SpeB, the first disulfide between C136 and C229 is thought to be the in vivo redox active disulfide. When either of these cysteines is changed to an alanine this redox disulfide is forced to be in a "reduced state" because of the removal of one of its cysteines. However, when you look more closely at the activity trend to the SepB mutants with and without DTT you notice

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that C136A and C229A still require DTT for full activity. So, what is the DTT needed for if the disulfide between C136 and C229 has been prevented from forming by the mutation of either thiol to alanine? It is possible that when there is not Fe(II) in the active site another disulfide forms between C71 and C151. This second disulfide would then require the use of DTT to reduce this disulfide that prevents the binding of Fe(II). Only when the C71 and C151 disulfide is reduced is the active site opened allowing C71 and C151 to become metal ligands.

The C71S and C151S mutants are only weakly stimulated by DTT (Figure 4) even though by replacing these cysteines with a serine it would leave open the active site for Fe(II) binding, but the Fe(II) is unable to bind and form a catalytic efficient active site because of a required cysteine ligand, thus reducing activity.

In an effort to determine the redox potential of the disulfide in the enzyme that is responsible for the change in the activity of SpeB we used mixtures of DTT and DTTox to change the redox potential of the DTT and measured the rate of the reaction. The redox potential of the disulfide controlling activity was calculated to be −295 mV using a previously described method.[23] This value is close to that reported for thioredoxin.[24]

Activation of the Enzyme with Other Reducing Agents. To determine if reducing agents other than DTT would work to activate *M. jannaschii* agmatinase, the enzyme was assayed with β-mercaptoethanol, 3-mercaptopropane-1,2-diol, 1,2-ethanedithiol, and tris(2-carboxyethyl)phosphine substituting for DTT in the standard reaction assay. None of these reducing agents were as effective as DTT in activating the enzyme (Table 4). The specific activation of the enzymes by DTT reduction of their disulfide bonds is characteristic of enzymes activated by thioredoxin.[25]

Table 4. Activation of SpeB in the Presence of Different Reducing Agents

reducing agent (concentration)	% activity[a]
DTT (33 mM)	100
β-mercaptoethanol (33 mM)	6
3-mercaptopropane-1,2-diol (33 mM)	20
1,2-ethanedithiol (35 mM)	6
TCEP (3.3 mM)	1.4
no reducing agent	0.0

[a]Assays were performed under standard assay conditions with 83 μM Fe(II) with replacement of the DTT by the indicated reducing agent.

Analysis of Disulfides in the Enzyme by SDS−PAGE. MonoQ isolated enzyme samples were also reduced in nondenaturing conditions with DTT, and the relative mobility of the samples was compared to a nonreduced sample by SDS−PAGE. The wild type sample treated with DTT showed a molecular mass of 40.0 kDa, and the wild type sample not treated with DTT showed a molecular mass of 37.8 kDa. This experiment demonstrated that the reduced sample has a lower mobility, consistent with the oxidized sample having a more compact structure due to the presence of a disulfide(s).[16] When the same experiment was repeated with each of the mutants, the results showed that only the C71S and C151S showed a change in mobility on the gel a little less that seen for the wild type enzyme with the samples not treated with DTT showing a molecular mass of 38.7 kDa. This supports that the disulfide is between C136 and C229 because the C136A and C229A enzymes show no change in mobility, when in the presence or absence of the reducing agent.

Quantitation of the Disulfide Content of SepB. Analysis of the content of the cysteines present in 1.38 nmol of SepB denatured in 6 M guanidine·HCl before and after reduction with tris(2-carboxyethyl)phosphine (TCEP) using the fluorescence mBBr assay showed a relative fluorescence intensity of 0.88 before and 16.7 after TCEP reduction. From these data, it was calculated that denatured SepB contained 0.36 free cysteines before reduction and 6.8 after reduction with TCEP using the lysozyme data for calibration. Considering the errors in determining the protein concentrations of both SepB and the lysozyme, these results are consistent with the isolated enzyme containing less than one cysteine thiol that can react with mBBr and at least five cysteines thiols, all of which were involved in the formation of two disulfide bonds. It should be noted here that the *M. jannaschii* homologue has an additional cysteine at residue 16 in the sequence of SepB that is not found in any of the homologues of this enzyme resulting in the *M. jannaschii* enzyme having a total of five cysteines.

Absorbance Spectra of SepB. The inactive MonoQ isolated enzyme with 0.65 iron per subunit showed a small absorbance at wavelengths greater than 300 nm (Figure 6).

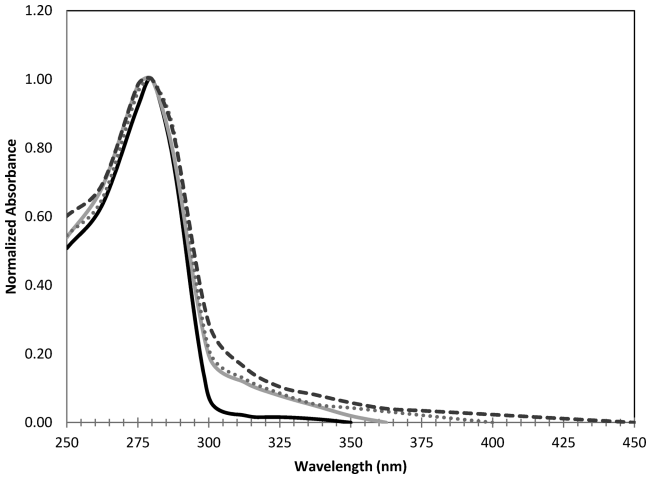

Figure 6. UV absorbance of SpeB. The spectra of each are indicated by (bold black line) BSA, (gray line) SpeB, (···) SpeB with four Fe(II), (---) SpeB with four Fe(II) and DTT.

Absorbance at these wavelengths is not a general characteristic of proteins, as demonstrated by the absorbance spectra of BSA (Figure 6), and likely represents the complex of the enzyme with Fe(III) formed by the oxidation of the Fe(II) during the aerobic purification. The appearance of a similar absorbance is observed in the air inactivated apo-dihydroxyacid dehydratase where the Fe(III) of the air exposed [4Fe-4S] cluster is present in the protein.[26] Addition of four equilivalents of Fe(II) to SpeB produced little change in the absorbance spectra (Figure 6). Whereas addition of 4 equiv of Fe(II) in the presence of DTT showed the generation of a small shoulder at ∼315 nm characteristic of a Fe(II) → sulfur charge transfer band[27−29] indicating that at least one of the Fe(II) was being coordinated by a cysteine ligand (Figure 6). As expected this absorbance band is weaker than those seen in previously studied systems, such as iron(II)−metallothionein, that contained multiple $Fe(II)(SCys)_4$ clusters.

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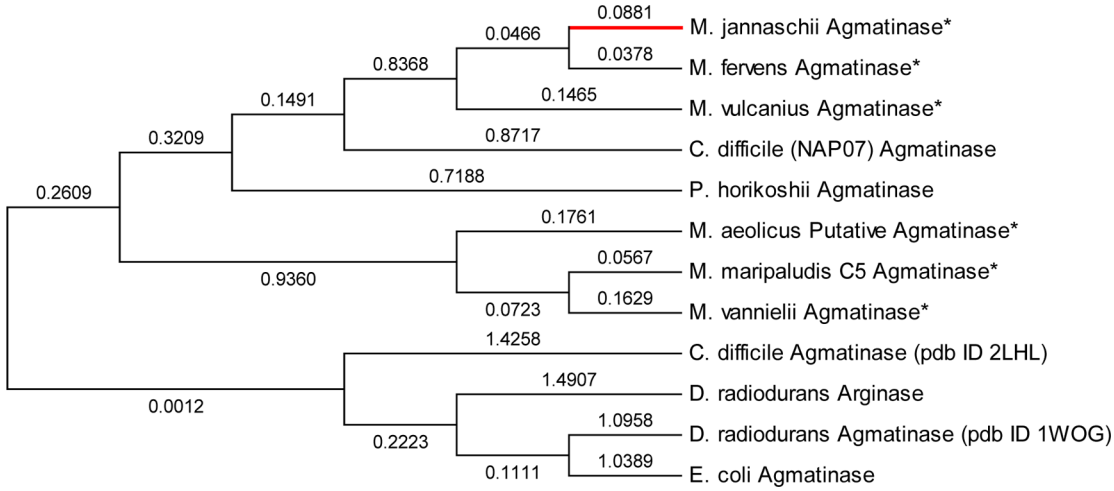

Figure 7. Evolutionary relationships of agmatinase. The * signifies that the agmatinase contains the three conserved cysteines found in the methanogenic homologues. Ordered locus tags for the agmatinases are as follows: *M. jannaschii* MJ0309, *M. fervens* Mefer_0143,*M. vulcaniz* Metvu_0240, *C. difficile* (NAP07) SpeB, *P. horikoshii* PH0083, *M. aeolicus* Maeo_1446, *M. maripaludis* (C5) MmarC5_1824, *M. vannielii* Mevan_0897, *C. difficile* CD0891, *D. radiodurans* DR_A0149, *E. coli* b2937, and *D. radiodurans* DR_0651 Arginase).

Urease Activity of SpeB. To establish if the *M. jannaschii* agmatinase could be involved in hydrolysis of the urea product of the enzyme to ammonia and carbon dioxide, we incubated the enzyme with urea and assayed for the formation of ammonia. No ammonia peak was observed in the reaction product indicating that SpeB contains no detectable urease activity.

Phylogenic Relations. Phylogenetic analysis of SpeB homologues identified at least two different groups of agmatinases in the methanogens (Figure 7). Each group contains the 3 main conserved cysteines, but the branch with the *M. jannaschii* SpeB contains an additional conserved cysteine (C151), which includes *M. fervens* and *M. vulcanius*.

DISCUSSION

Occurrence of Fe(II) Dependent Enzymes. Recent developments have prompted our search for enzymes functioning with Fe(II) as their catalytic metals. We recently showed that in the methanogenic *Archaea* the first two enzymes used for the biosynthesis of methanopterin and the first two enzymes involved in coenzyme F_{420} and riboflavin biosynthesis all require Fe(II) to function.[30−32] Three of these reactions hydrolyze amide like structures and the fourth hydrolyzes a five member-cyclic phosphate diester. It has also been shown that most of the metal containing proteins present in *Ferroplasma acidiphilium*[33] and in the anaerobic *Archaea Pyrococcus furiosus*[34] contain iron. Increasing numbers of Fe(II) dependent amidohydrolases have been demonstrated,[35] including cytosine deaminase,[36] peptide deformylase,[37] histone deacetylase,[38] *S*-ribosylhomocysteinase (LuxS),[39] UDP-3-*O*-[(*R*)-3-hydroxymyristoyl]-*N*-acetylglucosamine deacetylase,[40] and methionine aminopeptidase.[41] Also an increasing numbers of proteins once considered to be Zn(II) dependent are being found to be Fe(II) dependent, including the carbonic anhydrase from *Methanosarcina thermophila* that uses Fe(II) instead of Zn(II).[42] Since many of these enzymes/proteins were found in anaerobes this prompted our search for other Fe(II) dependent enzymes in *M. jannaschii*.

Possible Metal Binding Site in SepB. The discovery that *M. jannaschii* agmatinase is an Fe(II) dependent enzyme is unexpected considering that all of the conserved metal binding ligands for the canonical Mn(II) dependent enzyme are completely conserved in the methanogenic agmatinases. These ligands consist of histidine and aspartate side chains (Figure 2). These metal ligands were firmly established from the crystal structures of the enzymes from *Deinococcus radiodurans*[9] *Bacillus caldovelox*[10] and rat liver.[8] The question then arose as to what are the factors that have resulted in the substitution of Fe(II) for Mn(II)? Several possibilities come to mind. First is the possible involvement of the C71 and C151 thiols as additional metal binding ligands that may favor the use of Fe(II) (Figure 2). Absorbance spectra indicate the presence of a Fe(II) thiol charge transfer band showing that at least one of the irons is bound to cysteine (Figure 6). It could also be possible that minor changes in the protein structure could be responsible for the use of Fe(II) as has been observed for peptide deformylase.[43] Lastly, it must be considered that the original primordial enzyme may have contained four Fe(II) and as iron became much less abundant in the oxygenated environment then two-Fe(II) containing enzymes would have evolved and then these were finally replaced by two-Mn(II) containing proteins in aerobes.

The metal titration data (Figure 5) suggest that there are 4 mol of Fe(II) per monomer of protein. The crystal structure of *Clostridium difficile* agmatinase (pdb 3lhl) shows it too contains four metal ions, but only three were found to be ligated to the protein. In this crystal structure there is only one extra metal binding ligand, E257 (*M. jannaschii* numbering). This scenario could also be possible in our agmatinase, where the two extra iron ions are simply nonspecifically bound to the protein. However, we propose that the *M. jannaschii* agmatinase requires four active site bound Fe(II) for maximal activity. This statement can not be confirmed without X-ray crystal structure data.

The question arises as to what are the other ligands for the four Fe(II) and what is their geometric relationship. If we assume that all of the metal ligands found in the canonical enzymes, including the extra ligand in *C. difficile* agmatinase, plus two additional cysteines serving as ligands, then we would still need several more ligands to coordinate with the other two metals. By using the carboxylate ligands as bridging ligands as shown for D133 and D211 in Figure 8a, it is possible that the

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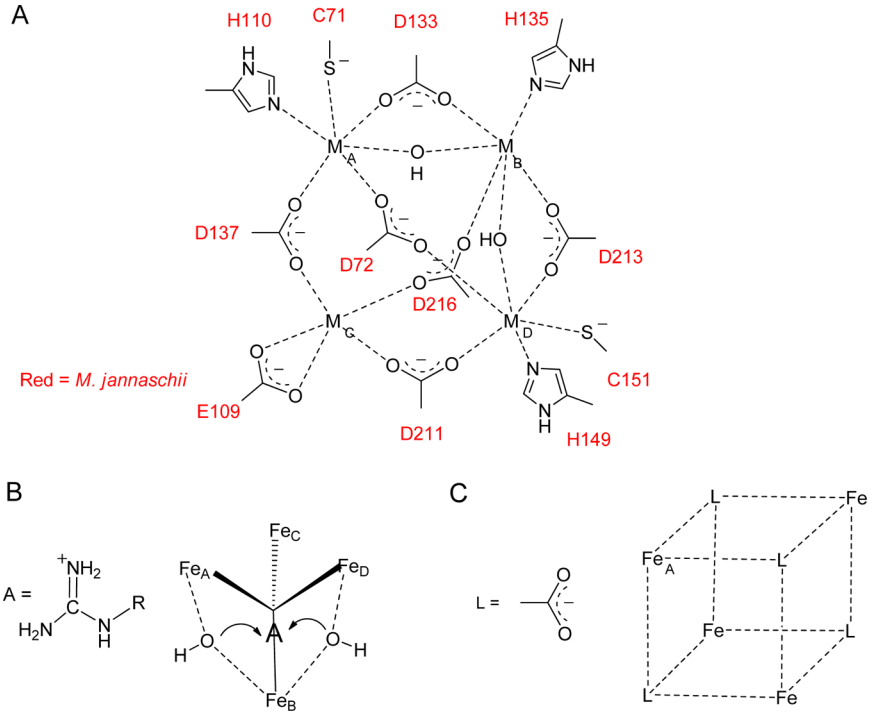

Figure 8. (A) A possible four metal binding site in SepB would be like a 4Fe-4S cluster but have carboxylate anions in place of sulfides. (B) Symmetry of 4 Fe(II) center with two possible nucleophilic oxo-groups. (C) Model of 4 Fe(II) center with carboxylates in replace of sulfides as would be found in a 4Fe-4S cluster.

irons could be arranged in a tetrahedral configuration as found in 4Fe-4S clusters. The proposed additional ligands were chosen because of their close proximity to the canonical metal ligands (Figure 3). In such a configuration each iron would be connected to each other through three different carboxylates with a total of six carboxylates being involved with Fe(II) binding. The involvement of such a structure is supported by the activity titration data that show a linear increase in enzymatic activity as up to four Fe(II) are added to SepB (Figure 5). Such a linear increase would only be produced if the binding of the first Fe(II) then initiates the rapid binding of the additional irons to the same protein molecule.

Considering the symmetry shown in Figure 8b it is possible that two nucleophilic hydroxyl groups could be bound in relationship to the substrate as shown. In this way a substrate could be bound symmetrically between these two groups and undergo the same catalytic mechanism regardless of which one was the nucleophile.

Although such structures have not yet been observed in proteins, several examples of tetranuclear iron complexes with bidentate carboxylate ligands and oxo-bridges are known. The closest to our proposed structure is the $[Fe_4O_2CC_2H_5)_7(bipy)_2]PF_6{\cdot}2H_2O$ (bipyr = 2,2′-bipyridine) complex.[44] As in our proposed structure each of the irons is ligated by three different carboxylate groups to each of the other irons, and two of the irons also contain a 2,2′-bipyridine ligand. These two bipyridine ligands would substitute for the two imidazole and two thiol ligands found in our structure. Another example is the $[Fe_4O_2CC_2H_5)_7(dpal)_2]PF_4{\cdot}H_2O$ complex that contains four irons coordinated by carboxylate ligands as well as two oxo-bridged irons.[45] Another interesting tetrairon complex is $[Fe_4(N_3O_2\text{-}L)_4(\mu\text{-}O)_2]^{4+}$ (L = 1-carboxymethyl-4,7-dimethyl-1,4,7-triazacyclononane) which is formed from the $[Fe_2(N_3O_2\text{-}L)_2(\mu\text{-}O)(\mu\text{-}OOCCH_3)]^{+}$ upon standing in acetonitrile solution.[46] This complex again has each iron bound to each other iron through a carboxylate bidentate ligand. The importance of the chemistry of this complex is that it shows how easily a two-iron cluster can be converted to a four-iron cluster without the addition of any additional carboxylates, since the carboxylates bind only a single metal in the two-iron complex and become bidentate in the four-iron complex. This could possibly help explain the origin of this four-iron containing agmatinase.

Use of Fe(II) by Other Agmatinases. The discovery that *M. jannashii*'s agmatinase is Fe(II) dependent prompted a search for other previously annotated Mn(II) dependent agmatinases that might also function with Fe(II). The *P. horikoshii* agmatinase requires divalent metals such as Co(II), Ca(II), and Mn(II), however, Fe(II) was not tested.[21] The *Plasmodium falciparum* arginase has been shown to work with Ni(II) at high concentrations but no activity was reported with Mg(II), Fe(II), Zn(II), or Ca(II).[47] The SpeB from *E. coli* was found to require Mn(II) for activity and was inhibited by Co(II), Ni(II), and Zn(II) but again Fe(II) was not tested.[6]

Disulfides Controling Enzymatic Activity. The conserved cysteines in many of the methanogenic agmatinases are found in the $C71X_{68}C136X_{14}C151X_{48}C229$ (*M. jannaschii* numbering), motif where C136 is found between two of the metal binding ligands. Since the C136A and C229A mutants retain activity, we conclude that these constitute the redox active disulfide. Since these cysteines are predicted to be too far apart to form a disulfide bond, based on the known crystal structures of agmatinases, a large rearrangement of the protein must occur with disulfide formation. This rearrangement is consistent with our SDS−PAGE data showing a more compact structure resulting from disulfide formation. The C71S and C151S mutants in contrast show a significant loss of activity with the C151S having essentially no activity. We propose that

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the C151 and possibly C71 are ligands for the irons. Their involvement as ligands may be the strongest explanation for the Fe(II) dependency of the enzyme.

Modeling of the expected positions of the cysteines in the protein based on known crystal structures of nondisulfide containing agamtinases indicates that the thiols of the cysteines in the *M. jannaschii* agmatinase are too far apart, greater than 3 Å, from each other to form disulfide bonds. The closest modeled pair of thiols was between C136 and C151, within 7.1 Å, followed by C136 and C229, within 16.2 Å, C229 and C71, within 36.3 Å, and C71 and C151, within 17.8 Å based on protein modeling with *Bacillus caldevelox* (pdb 1d3v) measurements made in PyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC). On the basis of these measurements inactivation of the enzyme by a disulfide bond formation would appear unlikely, indicating that a thiol based redox switch[48] may not be present in SpeB from *M. jannaschhii*. However, many excellent examples of conformational changes in redox pairs of proteins can be found in the PDB.[49] Portions of these have been referred to as allosteric regulators.[50] In these proteins the differences in the distances between the oxidized and reduced cysteines can be as much as 17 Å[51] and reduction involved large conformation changes.[49] It has been recently shown that the sulfur response regulator (SurR) from *Pyrococcus furiosus* is regulated by a sulfur-dependent redox switch.[52] Here the oxidation of the enzyme is caused by the oxidation of a CxxC motif by S° reduction. This oxidation to a disulfide changes the conformation of the enzyme which in turn changes its binding to DNA and regulation of gene expression.

Several classical examples of redox switches are known and include chloroplast fructose-1,6-bisphosphatase,[53] chicken liver fatty acid synthase,[54] and rat liver microsomal HMG-CoA reductase[55] but new ones are being found. These are being found experimentally since no protein cysteine motifs have been defined to identify a redox sensor that controls the activity of enzymes.

We have now observed this redox regulation in three other *M. jannaschii* enzymes that include FAD synthetase,[56] adenylosuccinate lyase (White, R. H. unpublished results), and adenine deaminase (White, R. H. unpublished results). We propose that these redox switches in these enzymes are controlled through a thioredoxin mediated regulation such as has been described in other systems where thioredoxin reduction of the disulfide(s) in the enzyme promotes activity.[57,58] The operation of such a system is supported by the presence of thioredoxin[59] (MJ0307) and thioredoxin reductase (MJ1536) in *M. jannaschii*.[60]

Analysis of Disulfides in the Enzyme by MALDI and SDS−PAGE. In an attempt to establish the positions of the disulfide bonds in SpeB, the enzyme denatured in 6 M guanidine·HCl was incubated in the presence and absence of DTT, then reacted with NEM and each sample digested with the three different proteases, chymotrypsin, Glu-C (a serine protease that cleaves at the c-terminus of aspartate and glutamate residues also known as *Staphylococcus aureus* protease V8, or endoprotease Glu-C), and trypsin. The resulting peptides were then analyzed by MALDI mass spectrometry. If the enzyme contained a disulfide(s) the number of NEM modified cysteine-containing chymotryptic, Glu-C, and tryptic peptides should increase in the sample treated with DTT over those not treated with DTT and the detection of these peptides would indicate the positions of the disulfides. In both cases, the same peptides containing each of the five of the cysteines were detected with the NEM modification. In addition no dipeptides linked by a disulfide were detected. This observation was consistent with the idea that the enzyme contained no disulfides since there was no change in the number of modified cysteines detected after DTT reduction. The detection of the same number of NEM modified cysteine containing peptides can be explained, however, by the occurrence of disulfide exchange occurring between the cysteines involved in the disulfide(s) and the free thiol containing cysteines when the samples were dissolved in guanidine·HCl under the conditions used. This would lead to the randomization of the disulfides among all the different cysteines and as a result, all cysteines would be labeled to some extent by the NEM. Thus this experimental result does not exclude the protein from containing a disulfide.

■ CONCLUSIONS

In conclusion we have determined that the *M. jannashii* SpeB is a Fe(II) dependent enzyme that requires four Fe(II) for maximum activity. Upon air oxidation most of the iron is lost from the protein and two disulfides bonds are formed to produce an inactive enzyme. We have proposed that the specificity of *M. jannaschii* SpeB for Fe(II) is due, in part, to having two cysteine ligands to the Fe(II) in addition to the canonical metal binding ligands found in the Mn(II) dependent enzymes. We propose that one of the disulfides formed upon air oxidation is formed from these two metal binding cysteines. The other disulfide formed is involved in regulating the activity of SpeB either by an allosteric change in the conformation of SpeB or is a redox sensor (switch). In an effort to confirm these observations the X-ray crystal structure of the *M. jannaschii* agmatinase is currently being undertaken.

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