

# Metal Ion Dependence of Recombinant *Escherichia coli* Allantoinase

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Allantoinase is a suspected dinuclear metalloenzyme that catalyzes the hydrolytic cleavage of the five-member ring of allantoin (5-ureidohydantoin) to form allantoic acid. Recombinant *Escherichia coli* allantoinase purified from overproducing cultures amended with 2.5 mM zinc, 1 mM cobalt, or 1 mM nickel ions was found to possess ~1.4 Zn, 0.0 Co, 0.0 Ni, and 0.4 Fe; 0.1 Zn, 1.0 Co, 0.0 Ni, and 0.2 Fe; and 0.0 Zn, 0.0 Co, 0.6 Ni, and 0.1 Fe per subunit, respectively, whereas protein obtained from nonamended cultures contains near stoichiometric levels of iron. We conclude that allantoinase is incompletely activated in the recombinant cells, perhaps due to an insufficiency of a needed accessory protein. Enzyme isolated from nonsupplemented cultures possesses very low activity ( $k_{\text{cat}} = 34.7 \text{ min}^{-1}$ ) compared to the zinc-, cobalt-, and nickel-containing forms of allantoinase ( $k_{\text{cat}}$  values of 5,000 and 28,200  $\text{min}^{-1}$  and 200  $\text{min}^{-1}$ , respectively). These rates and corresponding  $K_m$  values (17.0, 19.5, and 80 mM, respectively) are significantly greater than those that have been reported previously. Absorbance spectroscopy of the cobalt species reveals a band centered at 570 nm consistent with five-coordinate geometry. Dithiothreitol is a competitive inhibitor of the enzyme, with significant  $K_i$  differences for the zinc and cobalt species (237 and 795  $\mu\text{M}$ , respectively). Circular dichroism spectroscopy revealed that the zinc enzyme utilizes only the *S* isomer of allantoin, whereas the cobalt allantoinase prefers the *S* isomer, but also hydrolyzes the *R* isomer at about 1/10 the rate. This is the first report for metal content of allantoinase from any source.

Allantoinase (EC 3.5.2.5), present in a wide variety of bacteria, fungi, and plants, as well as a few animals, such as fish and amphibians, catalyzes the conversion of allantoin (5-ureidohydantoin) to allantoic acid by hydrolytic cleavage of the five-member hydantoin ring (33, 52). Allantoin is a key intermediate in nitrogen metabolism of leguminous plants, such as soybean (Fig. 1), where ureides provide the major transport form of symbiotically fixed dinitrogen (29, 43, 48, 55). Until recently, it was thought that allantoin was formed directly from uric acid by the action of uric acid oxidase (39); however, it has now been established that the true product of soybean uric acid oxidase is 5-hydroxyisourate (22), which is then acted upon by the enzyme hydroxyisourate hydrolase to yield 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (42). This compound is then converted to allantoin, most likely by a yet unidentified enzyme. The product of allantoin hydrolysis, allantoate, is sequentially converted in soybean to ureidoglycine, ureidoglycolate, and glyoxylate with the ammonia released during these reactions utilized for plant cell growth. Variations of this pathway occur in other organisms, including *Escherichia coli*, which can grow on allantoin as the sole nitrogen source, but not as the sole carbon source. The *E. coli* allantoinase gene is part of a 12-gene regulon involved in allantoin degradation and nitrogen assimilation, and these genes are expressed only when the cells are grown under anaerobic conditions (10).

On the basis of limited sequence similarities, allantoinase was suggested to belong to a broad family of metal-dependent amidohydrolases (17). Members of this family contain either

mononuclear metal sites, such as the zinc site found in adenosine deaminase (56) and the iron site in cytosine deaminase (57), or dinuclear sites, such as the di-nickel site documented to occur in urease (20) and the di-zinc sites of dihydroorotase (47) and hydantoinase (1, 2, 8). Some, but not all, dinuclear hydrolase family members have their two metals bridged by a carbamylated lysine ligand (1, 2, 4, 7, 8, 20, 47). Significantly, we find that allantoinase sequences align with the active site regions of crystallized hydantoinases (1, 2, 8), dihydroorotase (47), and urease (20), as well as with a subgroup of hydantoinases that have not been structurally characterized (Fig. 2). The ligands to the metal centers in these enzymes appear to be conserved in allantoinases, even including the lysine that is carbamylated in the crystallized enzymes. Thus, allantoinase is likely to possess a dinuclear metal site bridged by a carbamylated lysine side chain. Surprisingly, despite its purification from several sources (21, 24, 25, 33, 52, 55), the metal content of allantoinase has not been reported. Here we focus on recombinant allantoinase from *E. coli* and examine how supplementation of the medium with different metal ions affects the enzyme metal content, kinetic parameters, and substrate stereospecificity.

## MATERIALS AND METHODS

**Bacterial strains and media.** *E. coli* K-12 strain W3110 (16) was used as the source of genomic DNA for cloning the allantoinase gene. Production of recombinant allantoinase was performed with cells of *E. coli* strain C41(DE3) (31) as the host cells, which were grown in TB medium, consisting of 12 g of tryptone, 24 g of yeast extract (Difco), and 4 ml of glycerol per 900 ml (41) supplemented with 100 ml of filter-sterilized 0.5 M 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 7.2), in place of the standard phosphate buffer. The MOPS buffer allowed addition of high concentrations of metal ions without precipitation of the phosphate salts. All other recombinant DNA manipulations were done with

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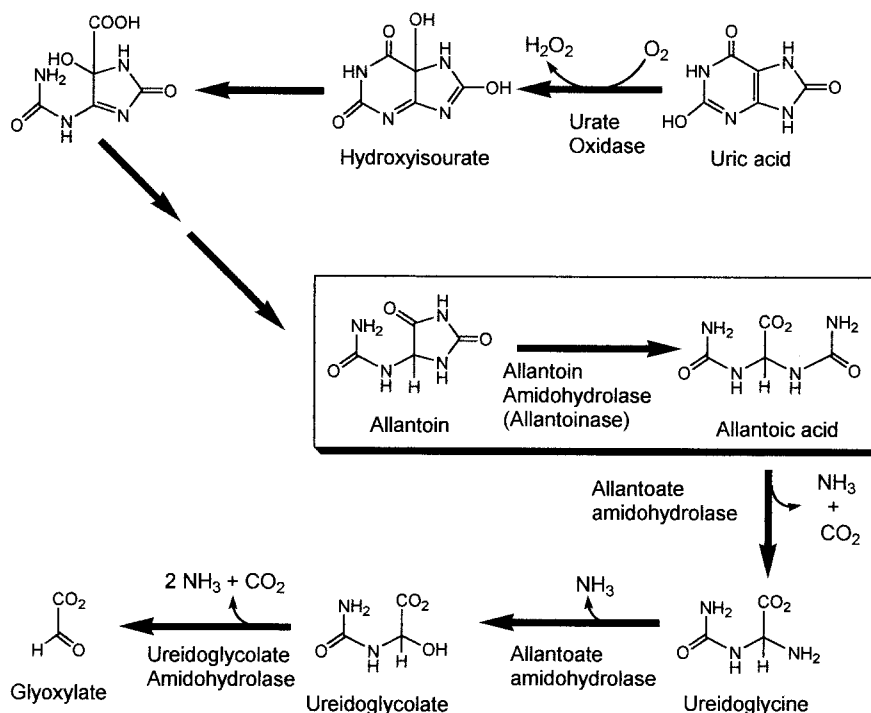


FIG. 1. Allantoinase reaction in the context of soybean uric acid metabolism. The boxed reaction is catalyzed by allantoinase.

*E. coli* DH5 $\alpha$  or XL1-Blue (Stratagene) and grown in Luria-Bertani (LB) medium according to standard protocols (3).

**PCR cloning and overexpression of allantoinase gene.** Genomic DNA was isolated from *E. coli* by standard methods (3). The PCR primers used were 5'-AAGAAACGTAGACCAGCAG-3' for the forward direction and 5'-GCAG AAGGTACCCTAGGAG-3' for the reverse, where the underlined bases represent base changes relative to the genomic sequence that introduced a *Kpn*I site before the 5' end of the allantoinase gene and an *Acc*I site after the 3' end. The amplified DNA fragment encoding the allantoinase gene was isolated from a 1% agarose gel by using QIAEX II gel purification reagents (Qiagen) and digested with *Kpn*I and *Acc*I. Vector pGEM-4Z (Promega) was also digested with *Kpn*I and *Acc*I, and the reaction products were ligated to produce plasmid pEA5. The entire allantoinase gene was sequenced by automated sequencing methods at the Michigan State University DNA Sequencing Facility.

The allantoinase gene was subcloned from pEA5 into pET11a to achieve a high level of expression. Site-directed mutagenesis was first performed with PCR with the complementary primers 5'-CAATCTGATTTTACAAGGAGTTTCA TATGCTTTTGATTTAATCATTA AAAACG-3' (forward), and 5'-CGTTTT TAATGATTAATCAAAGACATATGAAACTCCTTGTAAAATACAGA TTG-3' (reverse). The underlined bases represent base changes to introduce an *Nde*I site at the start ATG codon. The amplified circular plasmid products were transformed into *E. coli* XL1-Blue and analyzed for the presence of a new *Nde*I restriction site (one new site at the ATG start codon and one existing site after the 3' end of the gene in the pGEM-4Z vector). The allantoinase gene was resequenced to verify that no unintended mutations occurred, excised from pEA5 by using *Nde*I to produce a 1.1-kb fragment, isolated, and ligated into *Nde*I-cut pET11a (Novagen). The plasmid construct, designated pEA14, was checked by digestion with *Pst*I to verify proper insert orientation with respect to the T7 promoter.

**Allantoinase assays.** Enzyme assays were performed as previously described (40) by the *o*-phenylenediamine method. The standard assay mixture contained 50 mM allantoin (from Acros or Sigma) and 50 mM HEPES (pH 7.4) at 37°C. Allantoin solutions were prepared by adding the solid to hot water to aid in dissolution. When higher concentrations of allantoin were required (up to 125 mM), the solutions were held at 37°C and used within several min. For determination of  $K_m$  values, substrate concentrations ranged from 1.5 to 119 mM. Data were fit to the Michaelis-Menten equation with SigmaPlot (Jandell Scientific). Units of activity are defined as micromoles of allantoic acid produced per minute per milligram of protein. Values of  $k_{cat}$  were calculated with an  $M_r$  of 49,500 for the allantoinase monomer.

**Effect of metal ions on levels of enzyme activity in cell cultures.** Because allantoinase was suspected to be a metalloenzyme (see the introduction), the activity of recombinant enzyme was examined in crude extracts prepared from cultures grown in the presence of various metal ions. Cultures of *E. coli* C41(DE3)(pEA14) were grown aerobically at 30°C with rapid shaking in 10 ml of TB medium containing 250  $\mu$ g of ampicillin per ml or 100  $\mu$ g of carbenicillin per ml and 1 mM concentrations of the following metal salts:  $FeSO_4$ , Zn acetate,  $CoCl_2$ ,  $NiCl_2$ ,  $CdCl_2$ ,  $CuSO_4$ ,  $MgCl_2$ ,  $MnCl_2$ ,  $Na_2MO_4$ , and  $Na_2VO_4$ . Additional anaerobic cultures were grown either without added metal ions or with 2 mM ferrous ammonium sulfate in a sealed vial in which the headspace was replaced with nitrogen. When the cultures reached an  $A_{600}$  of 0.1 to 0.2, isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) was added to a final concentration of 100  $\mu$ M, and the cultures were moved to room temperature and grown overnight. Allantoinase activities were determined after centrifuging 1 ml of each culture, resuspending the pellets in 1 ml of 20 mM HEPES (pH 7.8), and disrupting them by sonication with a Branson Sonifier 250. Assays of the anaerobically grown cells were performed with an assay mix that was placed into centrifuge tubes and vials that were sealed and subjected to 10 cycles of evacuation and argon filling. The anaerobic cells were resuspended in degassed buffer containing 20 mM Tris (pH 8) with 0.1 mM EDTA, 0.1 mg of lysozyme per ml, and 1 mM sodium hydro-sulfite. After 20 min at room temperature, the cells were frozen and thawed twice, and aliquots were removed and assayed under aerobic and anaerobic conditions.

**Enzyme purification.** *E. coli* C41(DE3) cells were transformed with plasmid pEA14 and grown in TB medium supplemented with 250  $\mu$ g of ampicillin per ml plus either 2.5 mM zinc acetate, 1 mM cobalt chloride, or 1 mM nickel chloride at 30°C with rapid shaking. When the cultures reached an  $A_{600}$  of  $\sim$ 0.1, IPTG was added to 50  $\mu$ M, the flasks were shifted to room temperature, and growth continued for an additional 16 to 18 h. Preliminary studies aimed at optimizing expression conditions utilized 10- to 50-ml cultures, whereas routine large-scale purification was performed with 250-ml cultures grown in 2.8-liter Fernbach flasks. Cells were chilled on ice, harvested by centrifugation, and frozen at  $-20^\circ C$ . After thawing, cells were resuspended in 50 mM HEPES buffer (pH 8.1) and disrupted by sonication with ice cooling between pulses. The disrupted cell suspensions were adjusted to 0.5 mM in phenylmethylsulfonyl fluoride and 2% (wt/vol) in streptomycin sulfate, mixed thoroughly, and centrifuged at  $50,000 \times g$  for 25 min. The supernatant solutions were chromatographed at room temperature with DEAE Sepharose (2.5-cm diameter by 35 cm; Pharmacia). After being washed with 50 mM HEPES (pH 8.1), allantoinase was eluted with a 0 to 1 M KCl gradient in 50 mM HEPES (pH 8.1) in a total volume of 250 ml.

<i>E. coli</i>	All	PGMVDH <sup>59</sup> TH <sup>61</sup> ISEPGR	VGVVGFK <sup>146</sup> CFVATCG	GQPVLVH <sup>186</sup> CENAL	RLHVC <sup>239</sup> VSSPE	IDCLVSD <sup>315</sup> HSPC
<i>D. rad.</i>	All	PGVLDDH <sup>60</sup> VH <sup>62</sup> LNEPGR	CGVIGLK <sup>147</sup> AFMSHSG	GLVVATH <sup>183</sup> AESNE	ALHLVH <sup>239</sup> VSSGA	IDTVGSD <sup>312</sup> HSPA
<i>B. sub.</i>	All	PGVIDCH <sup>60</sup> VH <sup>62</sup> FNEPGR	AGAIGFK <sup>147</sup> AFLSKSG	GKILALH <sup>183</sup> AESDA	PVHFVH <sup>239</sup> ISTAK	IDMVSSD <sup>312</sup> HSPC
<i>B. hal.</i>	All	PGMVDVH <sup>63</sup> VH <sup>65</sup> FNEPGR	GGVIGFK <sup>150</sup> AFMSECG	GSILAVH <sup>186</sup> AESNE	PIHICH <sup>242</sup> VSSRK	IDLISSD <sup>315</sup> HSPS
<i>S. coe.</i>	All	PGLVDTH <sup>63</sup> VH <sup>65</sup> VNDPGR	AGVGFVK <sup>150</sup> AFLSPSG	DGLLIVH <sup>186</sup> AEDPH	RVHVLH <sup>238</sup> LSSSD	IDCVVTD <sup>312</sup> HSPS
<i>S. cer.</i>	All	PGLVDSH <sup>70</sup> VH <sup>72</sup> LNEPGR	AGVRGFK <sup>157</sup> GFLDSDG	DTMMMFH <sup>193</sup> AELPK	PVHIVH <sup>250</sup> LASMK	IGSVVSD <sup>322</sup> HSPC
<i>A. tum.</i>	Hyd	PGGIDVH <sup>57</sup> TH <sup>59</sup> VETVSF	LGITSEFK <sup>148</sup> VFMAYRG	GSLVMVH <sup>181</sup> AENGD	PIYIVH <sup>237</sup> LTCEE	LETVSSD <sup>313</sup> HCSW
<i>R. pic.</i>	Hyd	PGGIDVH <sup>57</sup> TH <sup>59</sup> VETVSF	LGITSEFK <sup>148</sup> VFMAYRG	GSLVMVH <sup>181</sup> AENGD	PIYIVH <sup>237</sup> VTCEE	FETVSSD <sup>313</sup> HCSW
<i>A. sp.</i>	Hyd	PGGVVDVH <sup>57</sup> TH <sup>59</sup> VETVSF	LGITSEFK <sup>148</sup> VFMAYRG	GSLVMVH <sup>181</sup> AENGD	PIYIVH <sup>237</sup> VTCEE	FETVSSD <sup>313</sup> HCSW
<i>S. coe.</i>	Hyd	PGGVDC <sup>65</sup> TH <sup>67</sup> MEMPF	EGVTSEFK <sup>156</sup> QFMAYPG	GGLIMMH <sup>189</sup> AENGI	PLYVVH <sup>245</sup> VSAE	LQVVSTD <sup>321</sup> HCPF
<i>B. mel.</i>	Hyd	PGGIDPH <sup>61</sup> TH <sup>63</sup> LQMPFM	RGINTFK <sup>151</sup> HFMAYKG	GAMPLVH <sup>184</sup> AENGD	PLYVVH <sup>240</sup> VSCAQ	LQCVATD <sup>317</sup> HCAF
<i>P. put.</i>	Hyd	PGGIDPH <sup>59</sup> TH <sup>61</sup> MQLPFM	HGVNSFK <sup>150</sup> HFMAYKN	GAVPTVH <sup>183</sup> AENGE	PLYVVH <sup>239</sup> ISSRE	LHTTATD <sup>316</sup> HCCF
<i>S. tok.</i>	Hyd	PGLIDGH <sup>60</sup> TH <sup>62</sup> MEFFPM	KGVVSEFK <sup>151</sup> LFMAYKN	GGVIGIH <sup>184</sup> AENGE	KMYIVH <sup>242</sup> TSTGE	IFTVGS <sup>316</sup> HCVY
<i>Therm.</i>	Hyd	PGFIDPH <sup>59</sup> VH <sup>61</sup> IYLPFM	DGISSFK <sup>150</sup> IFLSYKN	GVIVTAH <sup>183</sup> CENAE	TGYVVH <sup>239</sup> LSCPK	IDTVGTD <sup>315</sup> HCPF
<i>G. ste.</i>	Hyd	PGGIDPH <sup>58</sup> TH <sup>60</sup> LDMPF	EGITSEFK <sup>150</sup> VFMAYKD	GALVMVH <sup>183</sup> AENGD	QLYVVH <sup>239</sup> VSCAQ	LQTLGSD <sup>315</sup> QCSF
<i>A. aur.</i>	Hyd	PGVVDEH <sup>60</sup> VH <sup>62</sup> IIDMDL	AGAVGFK <sup>150</sup> SMMASV	GSVIVH <sup>183</sup> AENET	RLIVLH <sup>239</sup> VSNPD	IDTLGSD <sup>312</sup> HGGH
<i>E. coli</i>	DHO	RRPDDWH <sup>16</sup> LH <sup>18</sup> LRDGDM	GVFTAAL <sup>102</sup> LYPANAT	GMPLLVH <sup>139</sup> GEVTH	KVVFEL <sup>177</sup> ITTKD	RVFLGTD <sup>250</sup> SAPH
<i>K. aer.</i>	Ure	AGGIDTH <sup>134</sup> IH <sup>136</sup> WICPQQ	AGVIGLK <sup>217</sup> IHEDWGA	DIQVALH <sup>246</sup> SDTLN	TIHTFH <sup>272</sup> TEGAG	FSLTSSD <sup>360</sup> SQAM

FIG. 2. Sequence comparison of regions of selected amidohydrolases. Regions of sequence similarity are shown for allantoinases (All) of *Escherichia coli*, *Deinococcus radiodurans* (*D.rad.*), *Bacillus subtilis* (*B.sub.*), *Bacillus halodurans* (*B.hal.*), *Streptomyces coelicolor* (*S.coe.*), and *Saccharomyces cerevisiae* (*S.cer.*); hydantoinases (Hyd) of *Agrobacterium tumefaciens* (*A.tum.*), *Ralstonia pickettii* (*R.pic.*), *Agrobacterium* sp. strain IP I-671 (*A. sp.*), *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus* [*G.ste.*]), *S. coelicolor*, *Brucella melitensis* (*B.mel.*), *Pseudomonas putida* (*P.put.*), *Sulfolobus tokodaii* (*S.tok.*), a *Thermus* sp. (*Ther.*), and *Arthrobacter aurescens* (*A.aur.*); *E. coli* dihydroorotase (DHO), and *Klebsiella aerogenes* urease large subunit (*K.aer. Ure*). Crystal structures have been reported for the D-specific hydantoinases from *Thermus* (2) and *G. stearothermophilus* (8) (Protein DataBank accession no. 1GKP and 1K1D), the L-specific hydantoinase from *A. aurescens* (1) (accession no. 1GKR), *E. coli* dihydroorotase (47) (accession no. 1J79), and *K. aerogenes* urease (20) (accession no. 1FJW). The metalcenter ligands in these proteins are underlined with carbamylated lysines bridging the two metals.

Fractions were examined for the presence of allantoinase protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and those containing enzyme of sufficient purity were pooled and dialyzed in 50 mM HEPES (pH 8.1) at 4°C overnight. The dialyzed preparation was divided into two parts, and each was chromatographed on a Mono-Q HR 10/10 column by using a Pharmacia fast protein liquid chromatography system. Enzyme was eluted with a 0 to 1 M KCl gradient in a buffer of 50 mM HEPES (pH 8.1) with a two-step gradient of 0 to 0.4 M KCl in 80 ml followed by 0.4 to 1 M in 20 ml. The fractions were examined by SDS-PAGE analysis, and those containing >95% pure allantoinase were pooled and stored on ice. Protein concentrations for samples from early stages in the purification were determined with the Bio-Rad protein assay reagent with bovine serum albumin as the standard. The concentrations of purified enzyme samples were measured by using a calculated extinction coefficient at 280 nm of 35,700 M<sup>-1</sup> cm<sup>-1</sup> (35).

In a similar manner, allantoinase was purified from cell cultures of *E. coli* C41(DE3)(pEA14) to which no metal ions were added. Cultures grown in this way exhibited allantoinase activities comparable to those of background controls, yet showed significant allantoinase protein production based on SDS-PAGE analysis. The nearly inactive enzyme was isolated to 90% purity with the same DEAE-Sepharose/Mono-Q protocol described above, except that, for some preparations, buffers contained 0.1 mM dipicolinic acid to chelate trace metals in the solutions. Final purification, yielding at least 95% homogeneous protein, was achieved by concentrating fractions from the Mono-Q column in a Centricon 30 microconcentrator and chromatographing the sample on a 2.5-cm by 47-cm Superose 6 column equilibrated in 50 mM HEPES buffer (pH 8.1) containing 50  $\mu$ M EDTA and 50  $\mu$ M dipicolinic acid. The fractions of highest purity as judged by SDS-PAGE analysis were pooled and stored on ice.

**Effect of added metal ions on allantoinase activity.** Activation studies were performed to assess the effect of added metal ions on allantoinase activity. Buffers were treated with Chelex-100 resin to remove trace metal contaminants. Solutions of zinc sulfate and cobalt sulfate utilized Puratronic grade reagents (Johnson Mathey Chemicals, Ltd.). Typical experiments (100  $\mu$ l) contained 2.4 nmol of allantoinase in 50 mM Tris buffer (pH 8) containing 100  $\mu$ M zinc sulfate or 2 or 20 mM cobalt. In addition, some experiments included 1 to 3.6 M urea or 10 mM bicarbonate. After mixing allantoinase with the desired additives,

samples were incubated at room temperature and assayed periodically over several days.

**Characterization of allantoinase.** Metal contents of allantoinase preparations were measured by inductively coupled plasma emission-mass spectrometry in the Food Safety and Toxicology Center at Michigan State University or by inductively coupled plasma emission spectroscopy at the Chemical Analysis Laboratory of the University of Georgia. Prior to analyses, buffer components and excess metal ions were removed by several concentration/dilution steps in a Centricon-30 ultrafilter with metal-analysis-grade water. The resulting protein samples were made 5% in nitric acid (Fisher, TraceMetal grade) and centrifuged at 13,000 rpm for 10 min to remove the precipitated protein. The supernatants were used for metal analyses.

The pH dependence of allantoinase activity was measured in solutions of 50 mM allantoin containing 50 mM of the appropriate buffering agent. Because allantoin is a weak acid, the pH of each solution of allantoin was measured and adjusted by addition of NaOH as needed. Nonenzymatic degradation of allantoin, known to occur under acidic and alkaline conditions (51), was measured at pH values above 8.5. No significant nonenzymatic allantoin hydrolysis was observed under acidic conditions down to pH 5.4. The stability of allantoinase as a function of pH was measured by diluting enzyme samples into solutions containing 50 mM 2-(4-morpholino)ethanesulfonic acid (MES), HEPES, 2-[N-cyclohexylamino]ethanesulfonic acid (CHES), or 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) over a wide pH range. After exposure of the enzyme to the indicated pH conditions for 1 h at room temperature, the samples were tested for activity with the standard assay described above.

During preliminary experiments with allantoinase, it was found that thiols inhibited the enzyme. Allantoinase inhibition by dithiothreitol (DTT) was examined in detail for the cobalt and zinc forms by measuring enzyme activity over a range of DTT and allantoin concentrations.

**CD spectroscopy.** For circular dichroism (CD) spectroscopy, the enantiomeric specificities of the zinc and cobalt forms of allantoinase were examined by monitoring the changes in optical activity of solutions containing 1 mM racemic allantoin in the presence of 20 mM sodium phosphate buffer (pH 7) in a volume of 800  $\mu$ l. Samples were placed in a 1-cm quartz cuvette in a Jasco J-710 spectropolarimeter, and 0.3 U of either cobalt or zinc allantoinase was added and

mixed. The room temperature reaction was followed by taking spectra at various times or by continuous monitoring at a wavelength of 220 nm.

## RESULTS

**Effects of metal ions on recombinant allantoinase activity in cell cultures.** Using the genomic DNA sequence of *E. coli*, primers were designed to PCR amplify the segment containing the allantoinase gene and incorporate restriction sites for optimal placement in the T7 polymerase-controlled expression plasmid pET11a. The resulting construct, designated pEA14, was used for all subsequent experiments. When cultures were grown and induced by IPTG at 37°C, SDS-PAGE analysis of cell extracts revealed the production of some soluble allantoinase along with a larger fraction of insoluble enzyme (data not shown). Yields of total allantoinase were examined at IPTG concentrations of 0 to 0.5 mM with optimal amounts of protein observed at 50  $\mu$ M inducer. Growth at lower temperatures resulted in a larger fraction of soluble enzyme.

The TB medium used for cell growth contains metal-binding components that reduce the available concentrations of metal ions suspected to be required for allantoinase activity; thus, the effects of added metal ions were examined. At metal ion concentrations of 1 mM, only zinc, cobalt, and nickel provided activities (28, 35, and 6 U ml<sup>-1</sup>, respectively) that were significantly above that of the no-supplemental-metal control (1.3 U/ml). Specific activities of cultures grown in a range of zinc concentrations from 0 to 5 mM revealed maximal activity levels at 2.5 mM metal ion. Zinc concentrations above 2.5 mM and cobalt or nickel concentrations above 1 mM were inhibitory to cell growth. The conditions selected for routine purification of allantoinase from zinc-amended media used 2.5 mM zinc acetate. Similarly, 1 mM cobalt chloride or nickel chloride was added to cultures to obtain the cobalt or nickel forms of the enzyme. In all cases, cells were grown at 30°C until they reached an  $A_{600}$  of 0.1 to 0.2, at which time, IPTG was added to a final concentration of 50  $\mu$ M, and growth continued at room temperature overnight (22 to 25°C). Under these conditions, typical cultures contained 34,000 to 83,000 U (zinc supplemented), 35,000 to 90,000 U (cobalt supplemented), or 2,800 to 7,300 U (nickel supplemented) of allantoinase per liter.

Nonrecombinant *E. coli* allantoinase was previously reported to be synthesized only when cells were grown under anaerobic conditions (10), suggesting that the enzyme may be oxygen labile or that its regulation is controlled by the oxidation state of the cells. To test the effects of oxygen and added ferrous ions on recombinant allantoinase activity, extracts were prepared from cells grown anaerobically with various concentrations of ferrous ions and assays performed under anaerobic conditions. No significant activity was detected for any of these samples. In contrast, control experiments with cells grown in 2.5 mM zinc acetate under aerobic conditions and lysed by the anaerobic lysozyme method gave an activity of 21 U ml<sup>-1</sup>, while a parallel sample of cells disrupted by sonication yielded 34 U ml<sup>-1</sup>.

**Allantoinase purification.** Recombinant allantoinase was purified from sonicated extracts of cells grown in the presence of added zinc, cobalt, or nickel and from cultures with no metal supplementation. Each sample was purified by successive DEAE-Sepharose and Mono-Q chromatography steps. Typical

TABLE 1. Purification of *E. coli* allantoinase from cultures supplemented with zinc and cobalt

Purification step	Allantoinase			
	Protein (mg) <sup>a</sup>	Total U <sup>b</sup>	Sp act (U mg <sup>-1</sup> )	Recovery (%)
<b>Zinc-amended culture</b>				
Sonicated cells	985	21,600	22.0	100
Streptomycin SO <sub>4</sub>	643	23,300	36.1	100
DEAE-Sepharose	323	11,600	35.9	53.6
Mono-Q	53.7	4,090	76.1 <sup>c</sup>	18.9
<b>Cobalt-amended culture</b>				
Sonicated cells	670	44,300	66.0	100
Streptomycin SO <sub>4</sub>	330	46,500	142	100
DEAE-Sepharose	127	14,300	113	32.3
Mono-Q	20.0	8,200	410 <sup>c</sup>	18.5

<sup>a</sup> From 250 ml of culture.

<sup>b</sup> Measured at 50 mM allantoin.

<sup>c</sup> Concentration of final protein determined by  $A_{280}$  with a calculated extinction coefficient of 35,700 M<sup>-1</sup> cm<sup>-1</sup>.

purifications of allantoinases from zinc- and cobalt-amended cultures are shown in Table 1. Purifications were performed with buffers containing 50 mM HEPES (pH 8.1), and the pure enzyme samples were stored as pooled fractions directly off the Mono-Q column (estimated KCl concentration of 250 mM in 50 mM HEPES [pH 8.1]) or in 100 mM KCl–50 mM HEPES (pH 8.1). The allantoinase purified from zinc-amended cultures could be frozen and thawed at least four times without significant reduction of specific activity. The enzyme purified from cobalt-supplemented cultures was labile to freezing and lost about 50% of the initial activity within 1 week when stored at 4 to 8°C, with subsequent losses occurring at a somewhat slower rate. Purified allantoinase from either source was routinely stored on ice. In this way, the zinc form has been stored as long as 5 months and the cobalt form has been stored as long as 1 month without significant loss of activity. Short-term incubation with metal chelators, such as EDTA and dipicolinic acid, had negligible effects on either form of allantoinase activity at the pH of the storage buffer. Furthermore, long-term incubation of the enzyme isolated from zinc-amended cultures with 10  $\mu$ M cobalt did not lead to any increase in activity.

Allantoinase purified from cultures grown without added metal ions had a final specific activity of 0.7  $\mu$ mol min<sup>-1</sup> mg of protein<sup>-1</sup> (9.4 mg from a 250-ml culture). This form of the enzyme showed small, gradual increases in activity when incubated in buffers containing 2 or 20 mM cobalt, yielding enzyme with 3.9 and 8.1  $\mu$ mol min<sup>-1</sup> mg of protein<sup>-1</sup>, respectively, after 10 days at room temperature. No increase in activity was detected in samples incubated with 100  $\mu$ M zinc, even in the presence of mild concentrations of urea. Concentrations of zinc above 100  $\mu$ M could not be tested in this experiment due to precipitation of the protein. Inclusion of 10 mM bicarbonate to test the possible requirement that lysine carbamylation is required for enzyme activation as in urease (36) (and likely to be needed for dihydroorotase and hydantoinase) (1, 2, 8, 47) did not affect the allantoinase activity. For comparison, the activities of allantoinases purified from zinc- or cobalt-supplemented cultures were unaffected by incubation with these additives.

**Metal content.** The metal contents of representative allantoinase preparations are illustrated in Table 2. Growth condi-

TABLE 2. Properties of allantoinase purified from zinc-, cobalt-, and nickel-amended cultures and nonamended cultures

Culture	Allantoin $K_m$ (mM)	$k_{cat}$ ( $\text{min}^{-1}$ )	DTT $K_i$ ( $\mu\text{M}$ )	No. of mol of metal/mol of monomer <sup>a</sup>				Isomer preference
				Zn	Co	Fe	Ni	
Zinc amended	17.0 $\pm$ 2.7	5,000 $\pm$ 250	237	1.44	0.00	0.38	0.00	S
Cobalt amended	19.5 $\pm$ 1.3	28,200 $\pm$ 540	795	0.12	1.00	0.17	0.00	S (fast), R (slow)
Nickel amended	79.9 $\pm$ 5.5	200 $\pm$ 5	ND <sup>b</sup>	0.00	0.00	0.62	0.13	ND
Nonamended	ND	34.7 <sup>c</sup>	ND	0.03	0.00	1.08	0.00	ND

<sup>a</sup> Values are shown for representative samples.

<sup>b</sup> ND, not determined.

<sup>c</sup> Measured at 50 mM allantoin.

tions had a large impact on the metal content of the enzyme, with zinc, cobalt, nickel, and iron being the only metals found in significant quantities. Allantoinase prepared from cells grown in excess zinc contained 0.62 to 1.45 mol of metal per mol of monomer, as well as significant amounts of iron. No cobalt or nickel was detected in this form of the enzyme. Enzyme purified from cells grown in cobalt-supplemented medium contained approximately stoichiometric amounts of this metal (ranging from 0.81 to 1.0 mol per mol of monomer), but also had small amounts of zinc and iron. Unexpectedly, enzyme prepared from nickel-amended cultures contained only small amounts of this metal (0.13 mol of metal per mol of monomer) and larger amounts of iron (0.62 mol). Similarly, allantoinase purified from cultures grown without added metals had more than stoichiometric levels of iron and only trace amounts of zinc.

**Absorbance spectroscopy.** As described in the Discussion section, several cobalt-containing enzymes possess characteristic absorbance bands in the 500- to 700-nm-wavelength range. When examined by UV-visible spectroscopy, the cobalt-containing allantoinase showed a similar absorption band centered at 570 nm with an  $\epsilon_{570}$  of 209  $\text{M}^{-1} \text{cm}^{-1}$  (Fig. 3).

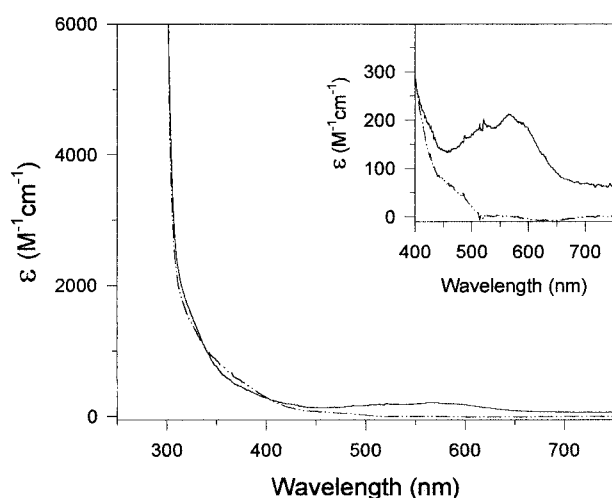


FIG. 3. Absorbance spectra of allantoinases prepared from cobalt (solid line), or zinc (dashed-dotted line)-amended cultures. (Inset) Expanded absorbance scale of the 400- to 750-nm region. The concentration of enzymes were 20  $\mu\text{M}$  for the zinc form and 15  $\mu\text{M}$  for the cobalt form in 50 mM HEPES (pH 7.8) buffer. The concentrations of enzymes in the inset were 400  $\mu\text{M}$  for the zinc form and 300  $\mu\text{M}$  for the cobalt form.

**Kinetic parameters.** Allantoinases purified from zinc-, cobalt-, and nickel-supplemented cultures were found to have significant differences in their kinetics, as shown in Table 2. The  $k_{cat}$  values were 5,000  $\pm$  250, 28,200  $\pm$  540, and 200  $\pm$  5  $\mu\text{mol min}^{-1}$  for the three species, respectively, while the corresponding  $K_m$  values were 17.0, 19.5, and 80 mM. Because the enzyme isolated from nonsupplemented medium exhibited only very low levels of activity, detailed kinetic analyses were not performed. Tests of the pH stability of the metal-supplemented allantoinase samples showed that the cobalt form of the enzyme (Fig. 4A, open symbols) is less stable at acidic pH than the zinc (Fig. 4A, closed symbols) or nickel (data not shown) forms. Each of the enzymes exhibited optimal activity at pH 7 to 8, with the high-pH end of the plots fitting nicely to theoretical curves yielding  $\text{p}K_a$  values of 8.8  $\pm$  0.1 for the zinc form and 9.0  $\pm$  0.1 for the cobalt form (Fig. 4B).

Alternative substrates and potential inhibitors of allantoinase activity were examined. No hydrolytic activity was observed with hydantoin or hydantoin-5-acetic acid, and no significant inhibition of allantoin hydrolysis was detected when assays were performed under standard conditions plus 20 mM hydantoin. The thiols  $\beta$ -mercaptoethanol and DTT were identified as inhibitors during tests to determine whether additives would alter allantoinase stability and activity. Since  $\beta$ -mercaptoethanol inhibited only weakly, inhibition of the zinc and cobalt forms of allantoinase by DTT was examined in detail. This compound exhibited competitive inhibition of both allantoinases with  $K_i$  values of 237  $\mu\text{M}$  for the zinc form and 795  $\mu\text{M}$  for the cobalt form (Fig. 5).

**Enantioselectivity of allantoinase.** When reaction progress curves were determined with the zinc form of allantoinase, only half of the allantoin was consumed, suggesting hydrolysis of a single isomer. The enantiomeric selectivity of the zinc- and cobalt-containing allantoinases was examined by CD spectropolarimetry, monitoring changes in the spectral range of 200 to 400 nm (data not shown) and at a fixed wavelength of 220 nm (Fig. 6). The reaction progress curves clearly show that the zinc form of allantoinase is enantioselective for the (+) or S allantoin. In contrast, the cobalt form of allantoinase consumes both isomers, but with the (-) or R enantiomer hydrolyzed at about 10% the rate of the (+) enantiomer.

## DISCUSSION

Allantoinase has been proposed to belong to a superfamily of metal-containing amidohydrolases related by evolution and predicted to have a common three-dimensional fold (17).

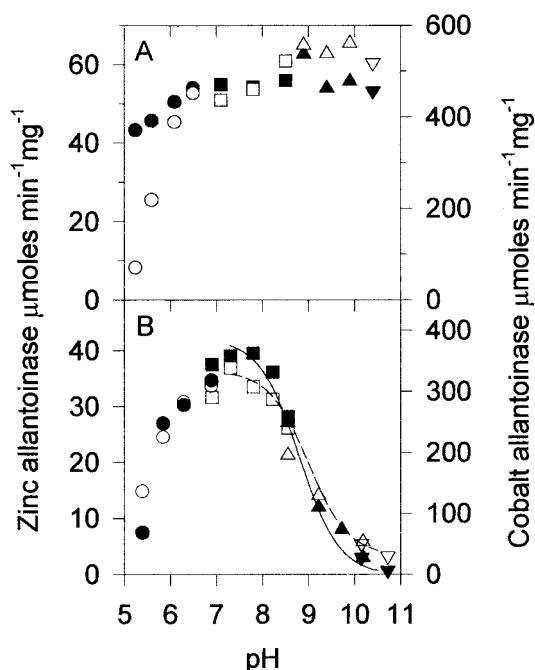


FIG. 4. Effects of pH on allantoinase stability and activity. Open symbols represent results for the cobalt form of the enzyme (corresponding to the right axis), and solid symbols represent those for the zinc form (corresponding to the left axis). The buffers used were MES (circles), HEPES (squares), CHES (triangles), and CAPS (inverted triangles). (A) Stability of allantoinases for 1 h at room temperature as a function of pH. (B) Activity as a function of pH. The curves are fit to the data in the range of pH 7.3 to 10.5 yielding  $pK_a$  values of 9.0 for the cobalt form of allantoinase (dashed line) and 8.8 for the zinc form (solid line).

More specifically, sequence comparisons versus crystallographically characterized proteins (Fig. 2) allow us to predict that allantoinase contains a dinuclear metal site bridged by a carbamylated lysine residue. To date, no report has appeared on the metal content of any purified allantoinase; however, stimulation of partially or completely purified allantoinases by

incubation with various metal ions has been reported (24, 53). Below, we discuss the effect of supplementing the cultures with various metal ions on the activity and metal content of recombinant *E. coli* allantoinase.

Our results confirm that *E. coli* allantoinase is a metalloenzyme. The highest levels of allantoinase activities are observed in cell extracts from cultures supplemented with zinc or cobalt to concentrations slightly below those found to be toxic to the cells. Of other added metals examined, only nickel yields activities above those of cells grown in medium without metal supplementation. Notably, supplementation of the medium with ferrous ions, even for anaerobically grown cultures, fails to enhance cellular allantoinase activity. Metal quantitation results for allantoinase species purified under the various culture conditions are consistent with the presence of a dinuclear metal active site in fully active enzyme. For example, protein purified from zinc-amended cultures typically contains more than stoichiometric levels of zinc, as well as significant amounts of iron. The high level of production of recombinant allantoinase (up to 1,000 mg liter<sup>-1</sup> or 29% of the soluble protein) is likely to result in enzyme with a low stoichiometry of bound metal, especially as we observed in protein isolated from the cobalt- and nickel-supplemented cultures and the nonsupplemented sample. This low stoichiometry may be due to factors such as limiting metal ion transport or the requirement for an accessory protein that is present in low abundance (described below). Thus, our purified allantoinase species likely represent populations of enzyme with dinuclear centers and populations with less than a full complement of active site metal ions.

The identity of the metal in nonrecombinant allantoinase is most likely to be zinc ions. Both the zinc and cobalt forms of the enzyme are active, with the cobalt form exhibiting nearly sixfold higher activity, but it is unlikely that cobalt has a biological role in vivo. For example, Walker and Bradshaw (54) have argued that yeast methionine aminopeptidase is a zinc-dependent enzyme, rather than cobalt dependent as previously reported, on the basis of the predicted low bioavailability of cobalt ions resulting in cytoplasmic concentrations that are 1,000-fold lower than that of zinc. Cobalt's ability to substitute

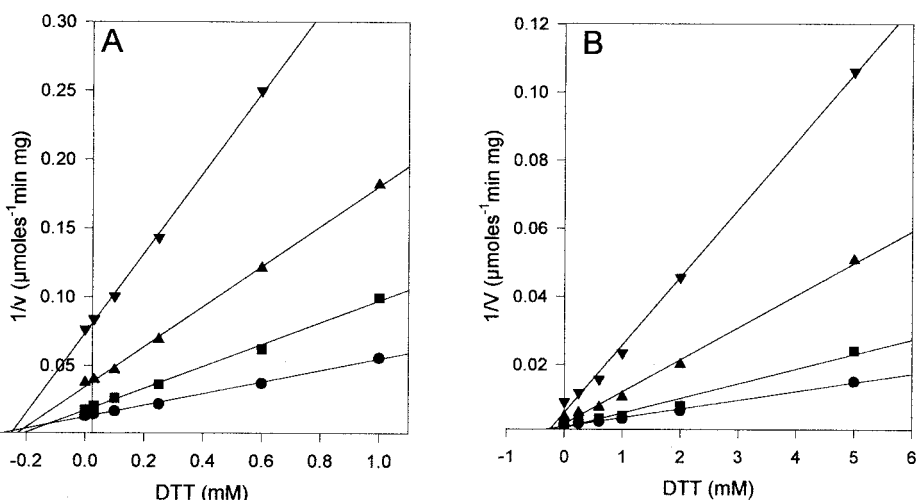


FIG. 5. DTT inhibition of allantoinase. (A) Zinc form of allantoinase. Allantoin concentrations were 50 mM (●), 30 mM (■), 15 mM (▲), and 7 mM (▼). (B) Cobalt form of allantoinase. The allantoin concentrations were 80 mM (●), 50 mM (■), 25 mM (▲), and 10 mM (▼).

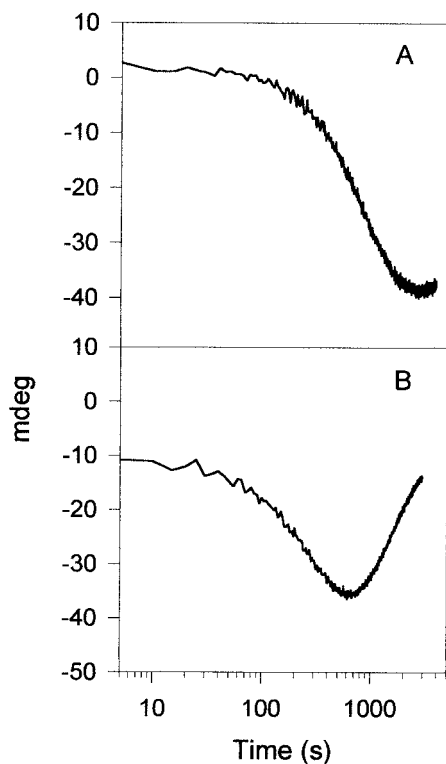


FIG. 6. Allantoinase assays monitored by CD spectropolarimetry. (A) Allantoinase prepared from zinc-amended cultures. (B) Allantoinase from cobalt-amended cultures.

for zinc and produce hyperactive enzymes is well known. For example, reconstitution of the apoenzyme form of hydantoinase with cobalt results in a 1.5-fold increase in activity compared to the level in the native zinc enzyme (28). Similarly, replacement of zinc with cobalt in untreated or *N*-ethylmaleimide-treated dihydroorotase results in an ~50% increase in hydrolytic activity (6, 18). In the case of adenosine deaminase, however, the activity of the cobalt-substituted enzyme is the same as that of the native zinc enzyme (9). Although not a member of the amidohydrolase superfamily, it is worth noting that cobalt-substituted alcohol dehydrogenase from *Saccharomyces cerevisiae* has 100-fold more activity than the native zinc enzyme (49). We conclude that cobalt substitutes for the native zinc in allantoinase to provide hyperactive enzyme when provided in the medium at high concentrations. If zinc and cobalt are not abundant, the allantoinase apoprotein appears to exhibit high affinity for iron, as shown by both the presence of stoichiometric amounts of this metal in enzyme purified from cultures grown in the absence of added metal ions and the detectable iron levels in other preparations. If not displaced or competed against by zinc or cobalt, this metal becomes locked into the enzyme. Perhaps related to this finding, *E. coli* cytosine deaminase is an iron-containing enzyme (19). Similarly, ferrous ions are found at the active sites of methionine aminopeptidase (12, 30) and peptide deformylase (38), whereas a zinc(II)/iron(III) dinuclear site is found in purple acid phosphatase (45), calcineurin (58), or serine/threonine phosphatase-1 (13). Thus, we cannot exclude the possibility that iron may play a significant role in allantoinase; however, we find

that protein containing only iron is inactive or possesses very low levels of activity and could not be activated by incubation under any conditions we could identify.

The UV/visible absorbance spectrum of the cobalt form of allantoinase provides insight into the coordination geometry of at least one of the metal sites. An absorption band centered at 570 nm with a shoulder at 520 nm resembles the absorption bands in cobalt-substituted dihydroorotase (6, 18), carboxypeptidase A (32), alcohol dehydrogenase, and sorbitol dehydrogenase (26). Such spectra, with  $\epsilon = 50$  to  $250 \text{ M}^{-1} \text{ cm}^{-1}$  as observed for allantoinase, are indicative of cobalt(II) *d-d* transitions associated with a pentacoordinate site (5). In contrast, no distinct ~350-nm transition, as found in the thiolate-ligated cobalt sites of  $\beta$ -lactamase II (11) or alcohol and sorbitol dehydrogenases (26), is observed in the cobalt form of allantoinase. We suggest that cobalt in the allantoinase active site is coordinated in a five-coordinate environment lacking cysteine ligation.

Two lines of evidence address the mechanism of assembly of the allantoinase metal center. Activity is not affected at pH 8.1 by incubation with EDTA or dipicolinic acid, suggesting that the metal is inaccessible to these metal chelators and deeply buried in the protein. Second, efforts to activate protein isolated from cultures not amended with metal ions proved unsuccessful, even in the presence of added bicarbonate (needed for lysine carbamylation) or mild concentrations of denaturant. This situation differs from those of several amidohydrolase family members, including hydantoinase (28), dihydroorotase (6, 18, 37), and adenosine deaminase (9). Bound metals are readily removed from and restored to these enzymes at neutral pH, consistent with an accessible active site. Our results also contrast with those from earlier studies of allantoinase in which chelators and metal additions exhibited a larger effect on activity (24, 52, 53). On the other hand, the properties of the apparently buried metallocenter of allantoinase somewhat resemble those of urease. Nickel ions bound at the urease active site cannot be removed by dialysis against various chelators, and formation of this buried metallocenter is a complex process requiring accessory proteins that appear to act as protein chaperones (15) and a metallochaperone (44). Thus, we speculate that allantoinase activation may also require the participation of an accessory protein. A requirement for an accessory protein may explain the observed low levels of activation in the overproducing recombinant, where the single-copy accessory gene is expressed at insufficient levels. Possible functions of such a protein include action in metal delivery or in the incorporation of  $\text{CO}_2$ /bicarbonate, which is suspected to occur as part of the activation step. The *E. coli* gene cluster containing the allantoinase gene does not include obvious candidates for such an accessory gene (10); however, it may be encoded elsewhere on the chromosome. Further efforts to identify an allantoinase accessory protein are warranted; however, the experimental observations noted above are also compatible with metal incorporation occurring during the protein folding process.

The kinetic parameters for *E. coli* allantoinase reported here differ from previously reported values. Working with partially purified allantoinase from *E. coli*, Vogels et al. (52) reported a specific activity of  $6 \mu\text{mol min}^{-1} \text{ mg of protein}^{-1}$ . More recently, Kim et al. (24, 25) examined purified recombinant *E.*

*coli* allantoinase and found specific activities of 5.7 to 19.9  $\mu\text{mol min}^{-1} \text{mg of protein}^{-1}$ . These values are significantly lower than the  $V_{\text{max}}$  of 101  $\mu\text{mol min}^{-1} \text{mg of protein}^{-1}$  presented here for the zinc form of the enzyme. In addition, the  $K_m$  of 17.0 mM for zinc allantoinase in this study is higher than the 4.2 and 7.4 mM values reported by Kim et al. (24, 25). Two possible explanations for the disparate kinetic parameters between our results and those reported earlier for purified *E. coli* enzyme relate to differences in enzyme metal content and assay conditions. No metal content of the purified enzyme was reported in the earlier studies and, perhaps significantly, no metal ions were added to the LB medium used for cell growth. Thus, the lower activity observed in the prior studies may be due to a large proportion of inactive enzyme arising from low levels of metal ions in their growth medium. In addition, the earlier assays were performed in the presence of 0.5 mM DTT, which we have shown to be a competitive inhibitor. The presence of a competitive inhibitor also would be expected to lead to a reduction in activity.

The most intriguing finding in this study was the observed difference in substrate stereospecificities between the zinc and cobalt allantoinases when examined by CD spectropolarimetry. The zinc enzyme utilized only the (+) *S* isomer, resulting in an initial negative phase (Fig. 6). A short upward phase at the end of the progress curve most likely results from spontaneous racemization, a process that occurs at neutral pH with a half-life of 10 h at room temperature (23). The cobalt enzyme reaction profile initially proceeded in the same direction, but faster because of the greater  $k_{\text{cat}}$ . The change in optical activity then reversed direction almost until the baseline was reached. This result clearly indicates that both isomers are utilized, but at rates that differ by about 10-fold. Precise kinetic parameters were not derived from these reaction profiles, since the spectropolarimeter response was unknown and the reactions were run at 1 mM allantoin, far below the  $K_m$ . Our results imply that cobalt allantoinase can hydrolyze both isomers of allantoin; however, the possibility exists that only one isomer is hydrolyzed and the enzyme possesses a slow racemase activity. Previous studies have suggested that crude preparations or partially isolated allantoinases from various sources are aspecific or favor the (+) isomer (14, 34, 50, 52). Care must be taken when interpreting these earlier studies, because allantoin is a substrate for D-hydantoinase (unpublished observations), a not surprising result considering that hydantoinases can hydrolyze a wide variety of 5-substituted hydantoins (46). Thus, contamination of partially purified allantoinases with hydantoinase could lead to incorrect conclusions about stereospecificity. The ability to affect the stereospecificity of allantoinase by simply replacing the metal cofactor could have important biotechnology implications. Illustrating this potential, studies have been undertaken to change the enantiomeric preference of hydantoinase by using directed evolution (27). The findings presented here indicate that metal substitution may be an alternative method to achieve this desired result.

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#### REFERENCES

1. Abendroth, J., K. Niefind, O. May, M. Siemann, C. Syldatk, and D. Schomburg. 2002. The structure of L-hydantoinase from *Arthrobacter aureescens* leads to an understanding of dihydropyrimidinase substrate and enantioselectivity. *Biochemistry* **41**:8589–8597.
2. Abendroth, J., K. Niefind, and D. Schomburg. 2002. X-ray structure of a dihydropyrimidinase from *Thermus* sp. at 1.3 Å resolution. *J. Mol. Biol.* **320**:143–156.
3. Ausubel, F. M. 1994. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
4. Benning, M. M., J. M. Kuo, F. M. Raushel, and H. M. Holden. 1995. 3-Dimensional structure of the binuclear metal center of phosphotriesterase. *Biochemistry* **34**:7973–7978.
5. Bertini, I., and C. Luchinat. 1994. The reaction pathways of zinc enzymes and related biological catalysts, p. 37–106. In I. Bertini, H. B. Gray, S. J. Lippard, and J. S. Valentine (ed.), *Bioinorganic chemistry*. University Science Books, Mill Valley, Calif.
6. Brown, D. C., and K. D. Collins. 1991. Dihydroorotase from *Escherichia coli*: substitution of Co(II) for the active site Zn(II). *J. Biol. Chem.* **266**:1597–1604.
7. Buchbinder, J. L., R. C. Stephenson, M. J. Dresser, J. W. Pitera, T. S. Scanlan, and R. J. Fletterick. 1998. Biochemical characterization and crystallographic structure of an *Escherichia coli* protein from the phosphotriesterase gene family. *Biochemistry* **37**:5096–5106.
8. Cheon, Y.-H., H.-S. Kim, K.-H. Han, J. Abendroth, K. Niefind, D. Schomburg, J. Wang, and Y. Kim. 2002. Crystal structure of D-hydantoinase from *Bacillus stearothermophilus*: insight into the stereochemistry of enantioselectivity. *Biochemistry* **41**:9410–9417.
9. Cooper, B. F., V. Sideraki, D. K. Wilson, D. Y. Dominguez, S. W. Clark, F. A. Quijcho, and F. B. Rudolph. 1997. The role of divalent cations in structure and function of murine adenosine deaminase. *Protein Sci.* **6**:1031–1037.
10. Cusa, E., N. Obradors, L. Baldoma, J. Badia, and J. Aguilar. 1999. Genetic analysis of a chromosomal region containing genes required for assimilation of allantoin nitrogen and linked glyoxylate metabolism in *Escherichia coli*. *J. Bacteriol.* **181**:7479–7484.
11. Davies, R. B., and E. P. Abraham. 1974. Metal cofactor requirements of beta-lactamase II. *Biochem. J.* **143**:129–135.
12. D'souza, V. M., and R. C. Holz. 1999. The methionyl aminopeptidase from *Escherichia coli* can function as an iron(II) enzyme. *Biochemistry* **38**:11079–11085.
13. Goldberg, J., H.-B. Huang, Y.-G. Kwon, P. Greengard, A. C. Nairn, and J. Kuriyan. 1995. Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. *Nature* **376**:745–753.
14. Gravenmade, E. J., G. D. Vogels, and C. van Pelt. 1969. Preparation, properties, and absolute configuration of (–)-allantoin. *Recl. Trav. Chim.* **88**:929–936.
15. Hausinger, R. P., G. J. Colpas, and A. Soriano. 2001. Urease: a paradigm for protein-assisted metallocenter assembly. *ASM News* **67**:78–84.
16. Hill, C. W., and B. W. Harnish. 1981. Inversions between ribosomal RNA genes of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **78**:7069–7072.
17. Holm, L., and C. Sander. 1997. An evolutionary treasure: unification of a broad set of amidohydrolases related to urease. *Proteins Struct. Funct. Genet.* **28**:72–82.
18. Huang, D. T. C., M. A. W. Thomas, and R. I. Christopherson. 1999. Divalent metal derivatives of the hamster dihydroorotase domain. *Biochemistry* **38**:9964–9970.
19. Ireton, G. C., G. McDermott, M. E. Black, and B. L. Stoddard. 2002. The structure of *Escherichia coli* cytosine deaminase. *J. Mol. Biol.* **315**:687–697.
20. Jabri, E., M. B. Carr, R. P. Hausinger, and P. A. Karplus. 1995. The crystal structure of urease from *Klebsiella aerogenes*. *Science* **268**:998–1004.
21. Janssen, D. B., R. Smits, and C. Vanderdrift. 1982. Allantoinase from *Pseudomonas aeruginosa*. Purification, properties and immunochemical characterization of its in vivo inactivation. *Biochim. Biophys. Acta* **718**:212–219.
22. Kahn, K., and P. A. Tipton. 1997. Kinetic mechanism and cofactor content of soybean root nodule urate oxidase. *Biochemistry* **36**:4731–4738.
23. Kahn, K., and P. A. Tipton. 2000. Kinetics and mechanism of allantoin racemization. *Bioorg. Chem.* **28**:62–72.
24. Kim, G. J., D. E. Lee, and H.-S. Kim. 2000. Functional expression and characterization of the two cyclic amidohydrolase enzymes, allantoinase and a novel phenylhydantoinase, from *Escherichia coli*. *J. Bacteriol.* **182**:7021–7028.
25. Kim, G. J., D. E. Lee, and H. S. Kim. 2001. High-level expression and one-step purification of cyclic amidohydrolase family enzymes. *Protein Expr. Purif.* **23**:128–133.
26. Maret, W. 1989. Cobalt(II)-substituted class III alcohol and sorbitol dehydrogenases from human liver. *Biochemistry* **28**:9944–9949.
27. May, O., P. T. Nguyen, and F. H. Arnold. 2000. Inverting enantioselectivity by directed evolution of hydantoinase for improved production of L-methionine. *Nat. Biotechnol.* **18**:317–320.



28. May, O., M. Siemann, M. G. Siemann, and C. Sydatk. 1998. Catalytic and structural function of zinc for the hydantoinase from *Arthrobacter aureescens* DSM 3745. *J. Mol. Catal. B Enzym.* **4**:211–218.
29. McClure, P. R., and D. W. Israel. 1979. Transport of nitrogen in the xylem of soybean plants. *Plant Physiol.* **64**:411–416.
30. Meng, L., S. Ruebush, V. M. D'souza, A. J. Copik, S. Tsunasawa, and R. C. Holz. 2002. Overexpression and divalent metal binding properties of the methionyl aminopeptidase from *Pyrococcus furiosus*. *Biochemistry* **41**:7199–7208.
31. Miroux, B., and J. E. Walker. 1996. Overproduction of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J. Mol. Biol.* **260**:289–298.
32. Moratal, J. M., J. Castells, A. Donaire, J. Salgado, H. R. Jimenez, and R. Domingo. 1994. Interaction of cobalt ions with carboxypeptidase A. *J. Inorg. Biochem.* **53**:1–11.
33. Noguchi, T., S. Fujiwara, and S. Hayashi. 1986. Evolution of allantoinase and allantoinase involved in urate degradation in liver peroxisomes. A rapid purification of amphibian allantoinase and allantoinase complex, its subunit locations of the 2 enzymes, and its comparison with fish allantoinase and allantoinase. *J. Biol. Chem.* **261**:4221–4223.
34. Okumura, I., K. Kondo, Y. Miyaki, K. Itaya, and T. Yamamoto. 1976. Stereospecificity of conversion of uric acid into allantoinic acid by enzymes of *Candida utilis*. *J. Biochem.* **79**:1013–1019.
35. Pace, C. N., F. Vajdos, L. Fee, G. Grimsley, and T. Gray. 1995. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* **4**:2411–2423.
36. Park, I.-S., and R. P. Hausinger. 1995. Requirement of carbon dioxide for in vitro assembly of the urease nickel metallocenter. *Science* **267**:1156–1158.
37. Pettigrew, D. W., R. R. Bidigare, B. J. Mehta, M. I. Williams, and E. G. Sander. 1985. Dihydroorotase from *Clostridium oroticum*. Purification and reversible removal of essential zinc. *Biochem. J.* **230**:101–108.
38. Rajagopalan, P. T. R., X. C. Yu, and D. Pei. 1997. Peptide deformylase: a new type of mononuclear iron protein. *J. Am. Chem. Soc.* **119**:12418–12419.
39. Reynolds, P. H. S., M. J. Boland, D. G. Blevins, D. D. Randall, and K. R. Schubert. 1982. Ureide biogenesis in leguminous plants. *Trends Biochem. Sci.* **7**:366–368.
40. Romanov, V., M. T. Merski, and R. P. Hausinger. 1999. Assays for allantoinase. *Anal. Biochem.* **268**:49–53.
41. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. Sarma, A. D., P. Serfozo, K. Kahn, and P. A. Tipton. 1999. Identification and purification of hydroxyisourate hydrolase, a novel ureide-metabolizing enzyme. *J. Biol. Chem.* **274**:33863–33865.
43. Schubert, K. R. 1981. Enzymes of purine biosynthesis and catabolism in *Glycine max*. I. Comparison of activities with N<sub>2</sub> fixation and composition of xylem exudate during nodule development. *Plant Physiol.* **68**:1115–1122.
44. Song, H. K., S. B. Mulrooney, R. Huber, and R. P. Hausinger. 2001. Crystal structure of *Klebsiella aerogenes* UreE, a nickel-binding metallochaperone for urease activation. *J. Biol. Chem.* **276**:49359–49364.
45. Sträter, N., T. Klabunde, P. Tucker, H. Witzel, and B. Krebs. 1995. Crystal structure of a purple acid phosphatase containing a dinuclear Fe(III)-Zn(II) active site. *Science* **268**:1489–1492.
46. Sydatk, C., O. May, J. Altenbuchner, R. Mattes, and M. Siemann. 1999. Microbial hydantoinases—industrial enzymes from the origin of life? *Appl. Microbiol. Biotechnol.* **51**:293–309.
47. Thoden, J. B., G. N. Phillips, T. M. Neal, F. M. Raushel, and H. M. Holden. 2001. Molecular structure of dihydroorotase: a paradigm for catalysis through the use of a binuclear metal center. *Biochemistry* **40**:6989–6997.
48. Thomas, R. J., and L. E. Schrader. 1981. The assimilation of ureides in shoot tissues of soybeans. I. Changes in allantoinase activity and ureide contents of leaves and fruits. *Plant Physiol.* **67**:973–976.
49. Vanni, A., E. Pessione, L. Anfossi, C. Baggiani, M. Cavaletto, M. Gulmini, and C. Giunta. 2000. Properties of a cobalt-reactivated form of yeast alcohol dehydrogenase. *J. Mol. Catal. B Enzym.* **9**:283–291.
50. Vogels, G. D. 1969. Stereospecificity in the allantoin metabolism. *Antonie Leeuwenhoek* **35**:236–238.
51. Vogels, G. D., F. E. de Windt, and W. Bassie. 1969. Hydrolysis and racemization of allantoin. *Recl. Trav. Chim.* **88**:940–950.
52. Vogels, G. D., F. Trijbels, and A. Uffink. 1966. Allantoinases from bacterial, plant and animal sources. I. Purification and enzymatic properties. *Biochim. Biophys. Acta* **122**:482–496.
53. Vogels, G. D., and C. van der Drift. 1966. Allantoinase from bacterial, plant and animal sources. II. Effect of bivalent cations and reducing substances on the enzymatic activity. *Biochim. Biophys. Acta* **122**:497–509.
54. Walker, K. W., and R. A. Bradshaw. 1999. Yeast methionine aminopeptidase. I. Alteration of substrate specificity by site-directed mutagenesis. *J. Biol. Chem.* **274**:13403–13409.
55. Webb, M. A., and J. S. Lindell. 1993. Purification of allantoinase from soybean seeds and production and characterization of anti-allantoinase antibodies. *Plant Physiol.* **103**:1235–1241.
56. Wilson, D. K., and F. A. Quijcho. 1993. A pre-transition-state mimic of an enzyme. X-ray structure of adenosine deaminase with bound 1-deaza-adenosine and zinc-activated water. *Biochemistry* **32**:1689–1694.
57. Yang, C., D. Carlow, R. Wolfenden, and S. A. Short. 1992. Cloning and nucleotide sequence of the *Escherichia coli* cytidine deaminase (*ccd*) gene. *Biochemistry* **31**:4168–4174.
58. Yu, L., A. Haddy, and F. Rusnak. 1995. Evidence that calcineurin accommodates an active site binuclear metal center. *J. Am. Chem. Soc.* **117**:10147–10148.