Mutagenesis of Rat Liver Arginase Expressed in *Escherichia coli*: Role of Conserved Histidines†

R. Christopher Cavalli,‡ Carl J. Burke,§ Susumu Kawamoto,‖ Dianne Robert Soprano,‡ and David E. Ash*,‡

Department of Biochemistry and Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140, Department of Bacteriology, Yokohama City University School of Medicine, Yokohama, Japan, and Department of Pharmaceutical Research, Merck Research Laboratories, West Point, Pennsylvania 19486

Received January 27, 1994; Revised Manuscript Received June 13, 1994®

ABSTRACT: Rat liver arginase has been overexpressed in *Escherichia coli* using a T7-based expression system. The kinetic properties of the recombinant wild-type protein are essentially identical to those of the native rat liver enzyme. The recombinant wild-type protein contains six Mn(II) ions per trimer, in good agreement with results obtained with the fully active native enzyme. However, in contrast to the native enzyme which loses three Mn(II) per trimer upon extended dialysis, the recombinant protein binds Mn(II) tenaciously, and retains six Mn(II) per trimer even after extensive dialysis. Three histidine residues, corresponding to His101, His126, and His141 in the rat liver enzyme, are highly conserved in arginases from evolutionarily divergent species. The replacement of His101 and His126 with Asn by site-directed mutagenesis produced only modest effects on enzymatic activity when measured in the presence of Mn(II) ions. However, EDTA treatment of these mutant enzymes reduced activity to <0.2% of that for the wild-type enzyme. The activity of wild-type enzyme and the His141Asn mutant was unaffected by treatment with EDTA. Thus, His101 and His126 are proposed to be ligands to the binuclear Mn(II) center of the enzyme. The His141Asn mutation produced an enzyme which, in contrast to the native, wild-type, His101Asn, and His126Asn arginases, was not inactivated by diethyl pyrocarbonate. These results suggest a catalytic role for His141.

The final reaction in the urea cycle, the hydrolysis of L-arginine, is catalyzed by arginase (L-arginine amidinohydrolase, EC 3.5.3.1):

$$\text{L-arginine} + H_2O \rightarrow \text{L-ornithine} + \text{urea} \qquad (1)$$

Although arginase activity is most abundant in mammalian liver (Herzfeld & Raper, 1976), the activity is also found in nonhepatic tissues such as red blood cells (Kedra-Luboinska *et al.*, 1988), lactating mammary glands (Yip & Knox, 1972), and the kidney (Herzfeld & Raper, 1976). The function of arginase in those tissues lacking a complete urea cycle is thought to be the production of L-ornithine, a biosynthetic precursor of L-proline (Yip & Knox, 1972) and the polyamines (Tabor & Tabor, 1984). In addition, recent studies have suggested that arginase may function as a modulator of the immune response, since an inhibitor of the mixed-lymphocyte reaction purified from rat liver has high N-terminal sequence homology with rat liver arginase and also displays arginase activity (Shinomiya *et al.*, 1990).

Rat liver arginase is a trimeric protein composed of three apparently identical subunits of molecular mass 35 kDa (Penninckx *et al.*, 1974; Kanyo *et al.*, 1992). The isolated protein contains approximately three tightly bound Mn(II) per trimer (Reczkowski & Ash, 1992), and exhibits ~50% maximal activity when assayed in the absence of added metal. Full catalytic activity is observed upon incubation of the isolated protein with Mn(II) (Hirsch-Kolb *et al.*, 1971), and this activation is accompanied by an increase in the Mn(II) stoichiometry to 6 Mn(II)/trimer (Reczkowski & Ash, 1992). Electron paramagnetic resonance (EPR)[1] spectra for the activated enzyme are diagnostic of spin-coupled binuclear Mn(II) centers (Reczkowski & Ash, 1992), suggesting that a common protein-derived ligand and/or water molecule bridges the Mn(II) ions.

Although little is known about the catalytic mechanism of arginase, there is considerable evidence to suggest that a histidine residue plays a critical role in the active-site chemistry. Rat liver arginase is rapidly inactivated by diethyl pyrocarbonate (DEPC), and Reczkowski and Ash (submitted for publication) have determined that this inactivation results from the modification of a single histidine side chain. A chemical mechanism has been proposed in which the DEPC-sensitive histidine serves first as a base to activate a Mn(II)-bound water molecule for attack on the guanidino carbon of L-arginine and subsequently as an acid to protonate the product L-ornithine (Reczkowski and Ash, submitted for publication).

A comparison of the sequences of arginases from rat liver (Kawamoto *et al.*, 1987), human liver (Haraguchi *et al.*, 1987), *Xenopus laevis* liver (Xu *et al.*, 1993), yeast (Sumrada & Cooper, 1984), and the *Agrobacterium* TiC58 plasmid (Schrell *et al.*, 1989) shows that, of the nine histidine residues per subunit of rat liver arginase, three are conserved. These three residues correspond to His101, His126, and His141 in the rat liver sequence. The conserved histidine residues are found in

† This work was supported in part by NIH Training Grant 5 T32 DK07162 (R.C.C.), Biomedical Research Support Grant S07 RR05417 from the Division of Research Resources, NIH (D.E.A.), a grant-in-aid from Temple University (D.E.A.), and NIH Grants DK44841 (D.E.A.), DK44517 (D.R.S.), and DK41089 (D.R.S.).

* To whom correspondence should be addressed.
‡ Temple University School of Medicine.
§ Merck Research Laboratories.
‖ Yokohama City University School of Medicine.

® Abstract published in *Advance ACS Abstracts,* August 1, 1994.

[1] Abbreviations: CHES, 2-(cyclohexylamino)ethanesulfonic acid; CD, circular dichroism; DEPC, diethyl pyrocarbonate; EPR, electron paramagnetic resonance; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); IPTG, isopropyl β-D-thiogalactopyranoside; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

0006-2960/94/0433-10652$04.50/0

Table 1: Conserved Histidines of Rat, Human, *Xenopus*, Yeast, and *Agrobacterium* TiC58 Arginases

Rat	98	GGD**H**SMAIGS	107	122	WVDA**H**TDINT	131	137	SGNL**H**GQPVA	146
Human	98	GGD**H**SLAIGS	107	122	WVDA**H**TDINT	131	137	SGNL**H**GQPVS	146
Xenopus	98	GGD**H**SLAVGT	107	122	WVDA**H**ADINT	131	137	CGNL**H**GQPLS	146
Yeast	120	GGD**H**SIAIGT	129	144	WIDA**H**ADINT	153	159	SGNL**H**GCPVS	168
TiC58	112	GGD**H**SMSAGT	121	139	WLDA**H**TDLHT	148	155	SGNL**H**GTPVA	164

a 40-residue segment beginning at position 101 of the rat sequence and ending with residue 141 as shown in Table 1. It is possible that one of these conserved histidine residues is the catalytically essential residue detected in chemical modifications with DEPC.

In the present study, the role of each of the conserved histidine residues in rat liver arginase has been examined through construction, purification, and characterization of the H101N,[2] H126N, and H141N site-directed mutants of the enzyme. High levels of expression of rat liver arginase in *E. coli* have been obtained with a T7-based expression system. We have determined that His141 is the DEPC-sensitive histidine, and we have identified His101 and His126 as essential to the stability of the binuclear metal center.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes were purchased from New England Biolabs. The pRSET C expression vector was purchased from Invitrogen Corp., and Sequenase was obtained from United States Biochemical. L-[*guanidino*-^{14}C]Arginine (specific activity 2.5 GBq mmol^{-1}) was from NEN/Dupont, IPTG[1] was from Jersey Lab and Glove Supply, and Chelex-100 resin was from Bio-Rad Laboratories. Amicon Green dye ligand chromatography support was purchased from Amicon Inc. Synthetic oligonucleotide primers were prepared in the Nucleic Acid Resource Facility at Temple University, and protein sequencing was performed in the Fels Institute Protein Facility at Temple University. All other reagents were of the highest quality commercially available. The full-length clone of rat liver arginase cDNA was supplied by Dr. S. Kawamoto, Yokohama University City School of Medicine, Japan.

Construction of an Expression Vector for Wild-Type[3] *Arginase.* Using the previously isolated full-length clone of rat liver arginase cDNA, pARGr-2 (Kawamoto *et al.*, 1986), as a template, the polymerase chain reaction (PCR) was carried out using the synthetic oligonucleotides 5′CCCCCATATGAGCTCCAAGCCAAAG3′ (pARG-NdeI) and 5′CCCCGCTGCAGATGACTTTTATGCGA3′ (pARG-PstI) as primers. The PCR product obtained was a 1 kb fragment with an *Nde*I restriction site at the ATG start codon and a *Pst*I restriction site 24 bp downstream of the TAA stop codon. The fragment was purified using a Qiagen PCR purification kit, digested with *Nde*I and *Pst*I, and ligated into *Nde*I- and *Pst*I-digested pRSET C to obtain pARG-X. The ligation mixture was used to transform *Escherichia coli* DH5α. The DNA sequence of pARG-X was determined from the *Nde*I site through the entire coding sequence of the arginase cDNA by the method of Sanger *et al.* (1980) to ensure the appropriate reading frame and to ensure that the PCR did not introduce any random mutations.[4]

Mutagenesis. Site-directed mutagenesis was carried out by the method of Higuchi *et al.* (1988) to prepare 3 H*xxx*N mutants in which His101, His126, or His141 was replaced with Asn. The two primary PCRs were carried out using pARG-NdeI paired with the appropriate antisense mutagenic oligonucleotide primer (H101N, 5′TGCCATACTGTTGTCTCCACCC3′; H126N, 5′GATGTCAGTGTTAGCATCCACC3′; and H141N, 5′CGGTTGCCCGTTCAGATTCCCA3′) and pARG-PstI paired with the corresponding sense mutagenic oligonucleotide primer (H101N, 5′GGGTGGAGACAACAGTATGGCA3′; H126N, 5′GGTGGATGCTAACACTGACATC3′; and H141N, 5′TGGGAATCTGAACGGGCAACCG3′) using arginase cDNA as template. The two resulting PCR fragments were each isolated by electrophoresis on 5% polyacrylamide gels and recovered by electroelution. The secondary PCR was performed using pARG-NdeI and pARG-PstI as primers and the two PCR fragments from the primary reaction as template. The resulting full-length mutated cDNA was digested with *Nde*I and *Pst*I and ligated to *Nde*I- and *Pst*I-digested pRSET C, followed by transformation into *E. coli* DH5α. The DNA from positive colonies was sequenced as described above to confirm the mutation and reading frame and to ensure that the PCR had not introduced any random mutations. The DNA sequence of all of the mutants was the same as those of pARG-X and pARGr-2, except for the desired mutations.

Overexpression of Wild-Type and Mutant Arginases. The pRSET C vector carrying the arginase cDNA was used to transform *E. coli* BL21 (DE3) (Studier *et al.*, 1990). DNA was isolated from selected colonies (Goode & Feinstein, 1992) and analyzed by restriction mapping with *Nde*I, *Hin*dIII, and *Pst*I. A positive clone for wild-type and each of the mutant enzymes was grown overnight in ZB media. From this culture, 10% glycerol stocks were prepared and frozen at −70 °C. The frozen stock was used as the source of cells for subsequent expression of protein.

An aliquot of the frozen cell stock was streaked on LB plates containing 50 mg/L ampicillin and incubated overnight at 37 °C. One colony from this plate was used to inoculate 100 mL of ZB media containing 50 mg/L ampicillin. This culture was grown to an A_{600} between 0.5 and 1.0 and used to inoculate 12 L of M9ZB media that contained 50 mg/L Timentin. The fermentation was carried out at 37 °C in a MF-14 Microferm fermentor (New Brunswick Scientific). When the A_{600} of the culture reached ~0.8, the cells were induced to express the recombinant protein by adding IPTG to a final concentration of 0.2 mM. Three hours later the cells were harvested by centrifugation at 5000*g* for 30 min. The cells were washed once with 50 mM Hepes–KOH, pH 7.5, and the cell paste was then frozen in a dry ice/methanol slurry and stored at −70 °C.

[2] This notation indicates that the His at position 101 of the rat liver arginase sequence has been replaced by Asn.

[3] Wild-type is used to refer only to the recombinant enzyme; native enzyme refers to the protein isolated from rat liver.

[4] Sequence analysis of both pARG-X and the original template DNA pARGr-2 detected one discrepancy with the published sequence. The sequence reported by Kawamoto *et al.* (1987) shows a C at position 918, while our analysis indicates that the base at this position is a G. The deduced sequence places a proline at position 298, while our data indicate that this amino acid is alanine. All of the other published arginase sequences have an alanine at the position corresponding to 298 in the rat sequence, suggesting that the correct base at position 918 in the cDNA is G.

Protein Purification. The cells were thawed and suspended in 5 volumes of 50 mM Hepes–KOH, pH 7.5, containing 0.1 mM PMSF. Cells were lysed by the addition of lysozyme to a final concentration of 1.0 mg/mL, followed by incubation for 1 h at room temperature. This mixture was placed on ice and sonicated for 30 min, and then centrifuged at 20000*g* for 30 min. The supernatant was decanted into a stainless-steel beaker, and $MnCl_2$ was added to a final concentration of 10 mM. The protein solution was heated at 60 °C for 30 min, quickly cooled on ice, and then centrifuged at 20000*g* for 30 min. The supernatant was decanted and dialyzed exhaustively against 10 mM Tris-HCl at pH 7.5. The dialysate was centrifuged at 20000*g* for 30 min, and the supernatant was applied to an Amicon Green dye ligand column (3.5 × 25 cm) that had been prepared according to the manufacturer's instructions and equilibrated with 10 mM Tris-HCl, pH 7.5, at a flow rate of 1 mL/min. The column eluate was monitored at 280 nm. When the A_{280} of the eluate dropped to background levels, a linear gradient of 0–0.3 M KCl in 10 mM Tris-HCl at pH 7.5 was applied. The fractions containing arginase activity were pooled and precipitated by the addition of ammonium sulfate to 80% saturation. The purity of the protein was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970). Unless otherwise noted, protein concentrations were determined by the absorbance at 280 nm using an extinction coefficient of 1.09 mL mg^{-1} cm^{-1} (Schimke, 1970). The enzyme was stored as a suspension in ammonium sulfate at 4 °C.

Circular Dichroism Spectroscopy. CD spectra were recorded on a Jasco J-720 spectropolarimeter using a thermostated cuvette equilibrated at 20 °C. The reported spectra represent averages of two repeat scans employing a bandwidth of 1 nm using a cuvette with a path length of 100 μm. The protein concentration used for the scans was 7–17 μM enzyme trimer in 50 mM Hepes–KOH/50 μM $MnCl_2$ at pH 7.5. CD spectra were analyzed for secondary structure content by a variable selection algorithm implemented in the VARSELEC program (Manavalan & Johnson, 1987).

Thermal melts were performed on an Aviv 62DS spectropolarimeter at protein concentrations of 0.6–1.6 μM trimer in 50 mM Hepes–KOH/50 μM $MnCl_2$ at pH 7.5. The temperature was increased in 2 °C intervals with a 2 min equilibration time at each temperature. Thermal transitions were monitored by following the change in ellipticity at 222 nm. Analysis of the thermal melt profiles was performed by the program DENFIT written and provided by Dr. Leslie A. Holladay (Alza Corp., Palo Alto, CA).

Enzyme Assay. The arginase activity of wild-type and mutant enzymes was assayed by a modification of the method of Rüegg and Russell (1980). Assays were performed in 100 mM CHES–NaOH/100 μM $MnCl_2$ at pH 9.0. The reactions were initiated by the addition of 5 μL of a ~1 unit/mL enzyme solution to 45 μL of reaction mixture that contained the CHES buffer, the appropriate concentration of arginine (0.1–5 mM for wild-type, H101N, and H126N and 1–25 mM for H141N), and ~5.0×10^4 cpm of L-[*guanidino*-^{14}C]arginine. After 5.0 min, 200 μL of a stop solution containing 0.25 M acetic acid/7 M urea at pH 4.5 was added to the reaction. Arginase has essentially no activity at the low pH of the stop solution. [^{14}C]Urea was separated from unreacted L-[*guanidino*-^{14}C]-arginine by treatment with 200 μL of a 1:1 v/v slurry of Dowex 50 W-X8 in water, and quantitated by adding 200 μL of the supernatant from the Dowex treatment to 3 mL of Liquiscint (National Diagnostics) for liquid scintillation counting in a TM Analytical Delta 300 Liquid Scintillation System, Model 6891. The data were analyzed using double-reciprocal plots of the initial velocity measurements; standard errors were determined by regression analysis.

The sensitivity of the wild-type and mutant enzymes to ethylenediaminetetraacetic acid (EDTA) was assessed by the assay described above, except that the reaction mixture contained 10 mM EDTA and the enzymes were incubated with 10 mM EDTA at room temperature for 30 min prior to initiation of the reaction. Two control experiments were performed in order to assess the effect of EDTA on the activity and stability of mutant enzymes. The arginase activity of the mutant enzymes was determined (i) after the enzymes were incubated for 30 min in the absence of EDTA and (ii) after addition of 20 mM $MnCl_2$ to EDTA-treated samples.

Manganese Stoichiometry. Samples of wild-type and mutant arginase were prepared for manganese determinations by dialysis overnight against 50 mM Hepes–KOH at pH 7.5 containing 10 g of Chelex-100 resin per 100 mL. The samples were concentrated by ultrafiltration using a Centricon-10 concentrator (Amicon). Protein concentration was determined with the Pierce Coomassie protein assay. An equal volume of 6% perchloric acid was added to each protein solution to denature the enzyme and release bound Mn(II). Precipitated protein was removed by centrifugation at 10000*g* for 30 min. Manganese concentrations were determined by EPR, as described previously (Ash & Schramm, 1982), using a Bruker ER 200D-SRC electron paramagnetic resonance spectrometer.

Inactivation by DEPC. The sensitivity of the wild-type enzyme and the H*xxx*N mutants to inactivation by DEPC was measured by reacting each protein at a concentration of 2 mg/mL with 1.1 mM DEPC in 50 mM Hepes–KOH/100 μM $MnCl_2$ at pH 7.0. The reactions were initiated by the addition of DEPC to the enzyme. Aliquots of the reaction mixtures were removed at 45 s intervals and quenched by the addition of 10 mM imidazole at pH 7.0. The remaining enzyme activity was measured as described above except that the reaction mixture contained 100 mM Hepes–KOH, pH 7.0, and 20 mM arginine. This assay was performed at pH 7.0, rather than at the optimal pH of 9.0, because the DEPC modification of histidine is rapidly reversed at alkaline pH. The stoichiometry of the DEPC modification was determined from the change in absorbance at 248 nm using the extinction coefficient of 3200 M^{-1} cm^{-1} for the carbethoxyimidazole derivative (Miles, 1977).

Reversal of DEPC Inactivation with Hydroxylamine. Samples of inactivated enzyme were incubated with 1 M hydroxylamine in 50 mM Hepes–KOH, pH 7.0, at 25 °C for 18 h. Enzyme activity was measured at pH 7.0 as described above.

RESULTS

Expression and Purification. After induction, the level of arginase in the *E. coli* BL21(DE3) cells ranged from 4% to 10% of the soluble protein. The results of a typical purification of wild-type arginase are shown in Figure 1. The final yield of purified protein ranged from 8 to 10 mg/L of culture, although yields as high as 25 mg/L of culture have occasionally been obtained. Similar purification patterns were observed with the H101N, H126N, and H141N mutants. SDS–PAGE analysis of the purified wild-type and mutant proteins showed a single major band that migrated with native arginase isolated from rat liver at a molecular mass of 35 kDa. The purity of all protein samples was estimated as greater than 95%. Amino-terminal analysis of the wild-type protein showed that approximately 80% of the arginase began with the sequence

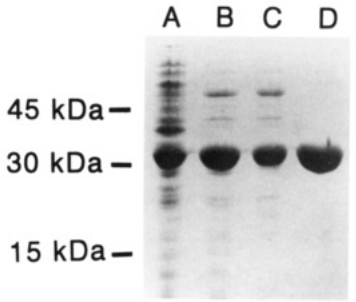

FIGURE 1: SDS–PAGE analysis of protein samples at different stages of the purification of wild-type arginase. 10 μg of protein was loaded on an 11% polyacrylamide gel: lane A, crude lysate from *E. coli* BL21(DE3); lane B, the preparation after treatment with 10 mM $MnCl_2$ and heating at 60 °C; lane C, the preparation after exhaustive dialysis against 10 mM Tris-HCl, pH 7.5; lane D, wild-type arginase after dye–ligand chromatography on Amicon Green resin. Molecular mass standards used were egg albumin (45 kDa), carbonic anhydrase (30 kDa), and egg white lysozyme (15 kDa).

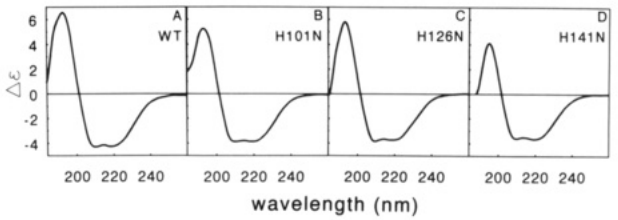

FIGURE 2: Circular dichroic spectra for wild-type and mutant arginases. The spectra were collected at 20 °C in 50 mM Hepes, pH 7.5, with 50 μM $MnCl_2$. All spectra are characterized by a positive peak at 196 nm and negative signals at 208 nm and are given in units of molar circular dichroism ($\Delta\epsilon$).

Ser-Ser-Lys-Pro-Lys, compared to the predicted sequence Met-Ser-Ser-Lys-Pro-Lys based on the cDNA sequence (Kawamoto *et al.*, 1987). The remaining 20% of the arginase was missing the initial serine residue and thus began with the sequence Ser-Lys-Pro-Lys.

CD and Predicted Secondary Structure. To characterize the secondary structure of the wild-type and mutant enzymes, UV CD spectra were recorded (Figure 2). The variable selection fit for secondary structure content predicts that the wild-type protein has the following structural content: 22% ± 3% helix, 28% ± 8% β-sheet, 17% ± 8% turn, and 33% other, where the errors are approximations associated with the fitting algorithm. Although small differences are observed in the CD spectra of the mutants, the intensity differences do not translate into significant changes in secondary structure.

In order to evaluate the conformational stability of the enzymes, circular dichroism at 222 nm was monitored as a function of temperature (Figure 3). The midpoints of the thermal transitions, T_m, were 77 ± 2, 68 ± 2, 70.5 ± 2, and 82 ± 2 °C, for wild-type, H101N, H126N, and H141N, respectively. Substitution of Asn for His at positions 101 and 126 tends to slightly destabilize the enzyme, whereas the mutation at position 141 has a small stabilizing effect.

Kinetic Constants. The wild-type and mutant arginases were each evaluated for catalytic activity. The kinetic constants for wild-type arginase expressed in *E. coli* and each of the mutant proteins are reported in Table 2. The K_m of the wild-type protein for L-arginine is 1.4 mM, in good agreement with the value of 1–1.7 mM reported for the native rat liver enzyme (Garganta & Bond, 1986; Reczkowski & Ash, 1994). Similarly, the k_{cat} values for the native and wild-type proteins are, within experimental error, identical under these assay conditions. The H101N and H126N mutants have K_m values of 1.0 and 3.4 mM, respectively, which are

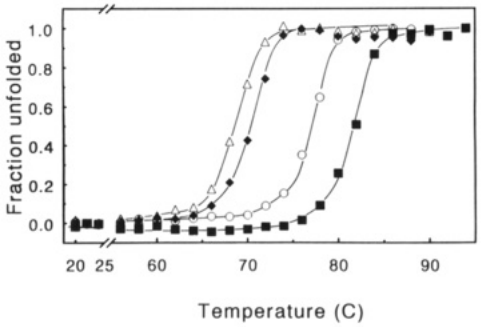

FIGURE 3: Thermal denaturation curves for wild-type and mutant arginases as measured by changes in circular dichroism. Linear baseline correction was applied to each of the data sets, and the fraction unfolded was calculated by dividing each CD data point by the final ellipticity at 222 nm for the denatured enzymes. Wild-type arginase, ○; H101N, △; H126N, ◆; H141N, ■.

only slightly different from the K_m for the wild-type enzyme. When the enzymes are assayed in the presence of 100 μM Mn(II), the H101N and H126N mutants have k_{cat} values that are 47% and 88%, respectively, of the k_{cat} for wild-type enzyme. The H141N mutant exhibited the largest changes in the kinetic parameters, with a K_m of 11.5 mM and a k_{cat} that is 11% of that for wild-type.

Effects of EDTA. The effect on catalytic activity of removing loosely bound Mn(II) from the wild-type and mutant arginases was examined by preincubation and assay in the presence of EDTA. Preincubation of the enzyme and assay in the presence of 10 mM EDTA had no effect on the kinetic constants of wild-type arginase and the H141N mutant (Table 2). The K_m values for arginine of the H101N and H126N mutants were slightly affected by EDTA; however, the k_{cat} values for these mutants were decreased dramatically. In the presence of EDTA, the H101N mutant retains only ~0.2% of wild-type activity, and the H126N mutant retains ~0.001% of wild-type activity. Control samples that were incubated for 30 min in buffer without EDTA showed no loss of arginase activity. Full catalytic activity was restored upon addition of excess $MnCl_2$ to the EDTA-treated H101N and H126N mutant enzymes.

Manganese Stoichiometry. The Mn(II) stoichiometries for wild-type arginase and the mutant proteins were determined after overnight dialysis in the presence of Chelex-100 chelating resin. Both wild-type and the H141N mutant contained the full complement of 6 Mn(II)/trimer. However, after the same treatment, the Mn(II) content of the H101N and H126N mutants was 3.3 and 3.6 Mn(II)/trimer, respectively.

Inactivation with DEPC. The sensitivity of the wild-type and mutant arginases to inactivation by DEPC is shown in Figure 4. The activity of the H141N mutant was unaffected by DEPC during a 3 min incubation. Wild-type arginase and the H126N mutant retained only 20% of their original activity after a 3 min incubation with DEPC, while the H101N mutant retained 40% of its original activity. The activities of the DEPC-treated wild-type, H101N, and H126N arginases were restored by treatment with 1 M hydroxylamine (data not shown). Treatment with this concentration of hydroxylamine had no effect on the activity of arginase that had not been chemically modified with DEPC.

Spectrophotometric measurements indicated that wild-type, H101N, and H126N arginases were modified by DEPC to a stoichiometry of three histidine residues per subunit, while the H141N mutant was only modified at two histidine residues per subunit. These results suggest that modification at His141 is responsible for the loss of activity upon treatment of the

Table 2: Comparison of Kinetic Parameters for Wild-Type and H*xxx*N Mutant Arginases

	100 μM $MnCl_2$			10 mM EDTA		
enzyme	K_m (mM)	k_{cat} (s^{-1})	% wild-type act.	K_m (mM)	k_{cat} (s^{-1})	% wild-type act.
wild-type	1.4 ± 0.3	250 ± 20	100	1.5 ± 0.3	245 ± 11	98
H101N	1.0 ± 0.2	118 ± 8	47	2.2 ● 0.5	0.51 ± 0.05	0.17
H126N	3.4 ± 0.2	220 ± 10	88	0.8 ± 0.02	0.0028 ± 0.0002	0.0011
H141N	11.5 ± 0.3	28 ● 2	11	9.2 ± .6	24 ± 2	10

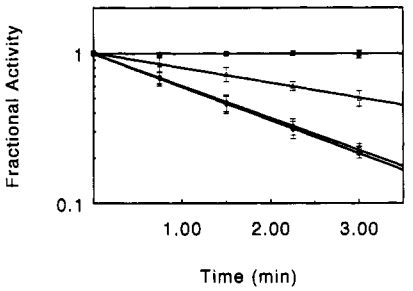

FIGURE 4: Time course for DEPC inactivation. The inactivations were carried out at room temperature using a ~20:1 ratio of DEPC to subunit of enzyme. Each point represents the average fractional activity as determined in three separate inactivation experiments. Wild-type arginase, ○; H101N, △; H126N, ♦; H141N, ■.

enzyme with DEPC.

DISCUSSION

The T7-based overexpression system for the production and purification of rat liver arginase in *E. coli* yielded approximately 10 mg of homogeneous protein per liter of bacterial culture media. *E. coli* does not contain arginase, and the related bacterial enzyme, agmatine ureohydrolase, does not utilize L-arginine as a substrate (Satishchandran & Boyle, 1986). Therefore, all of the arginase activity isolated from these cells is due to the recombinant protein. Introduction of mutations into the coding sequence for the rat liver enzyme had no discernible effect on the expression or yield of purified protein. The major increase in protein purity occurred in the heat step, during which many *E. coli* proteins are precipitated. The successful purification of the H*xxx*N mutant arginases with this protocol suggested that the variant enzymes possess thermal stabilities similar to those of the wild-type and native enzymes. These expectations were confirmed in thermal unfolding experiments which show that the secondary structure of all the mutant proteins retains a high degree of thermal stability, with the H141N mutant actually having a higher T_m than wild-type arginase.

Amino acid sequence analysis of the recombinant wild-type arginase expressed in *E. coli* indicates that the N-terminal methionine has been removed by proteolytic modification of the protein. This finding is in accord with the proposal that the second amino acid in the sequence determines the fate of the N-terminal methionine; amino acids such as serine and alanine at this position promote proteolytic cleavage of methionine (Miller, 1987). In addition, for about 20% of the arginase molecules, the serine adjacent to the N-terminal methionine has also been removed. N-Terminal sequence heterogeneity has also been observed with native rat liver arginase. For this enzyme, cleavage of the N-terminal methionine occurs frequently; for a smaller percentage of the enzyme, the N-terminal methionine is retained (Kawamoto *et al.*, 1987). The similarity of the CD spectra and kinetic constants of native rat liver and recombinant, wild-type arginase provides convincing evidence that the N-terminal anomalies of the recombinant protein do not have significant effects on the structure or function of the enzyme.

The kinetic and physical properties of the wild-type arginase expressed in *E. coli* are essentially identical to those for the native rat liver enzyme. Previously published CD spectra of rat liver arginase (Muszyńska & Ber, 1978) predicted a secondary structural content of 54% α-helix, 8% β-structure, and 38% random coil. The CD spectra shown in Figure 3 are nearly identical to those published spectra; however, our analysis of secondary structure for the wild-type enzyme indicates a larger contribution of β-structure, 28%, and a smaller contribution from α-helix, 22%. Manavalan and Johnson (1985) report that the reliability of the analysis for secondary structure improves when a larger spectral width is utilized. Therefore, the analysis presented here may be more accurate since a wider spectral range was employed and since the variable selection method is considered more reliable than the earlier methods (Manavalan & Johnson, 1985). It is clear from the spectra in Figure 3 that the H*xxx*N mutations have little effect on the secondary structure of the enzyme.

When fully activated with Mn(II), both native rat liver and wild-type arginases contain six Mn(II) ions per trimer. However, the Mn(II) binding properties of the wild-type recombinant enzyme differ considerably from those of the native rat liver enzyme. Extended dialysis of the recombinant protein at 4 °C has no effect on the enzymatic activity or the Mn(II) stoichiometry. However, the same treatment of the native rat liver enzyme results in a 50% reduction of the enzymatic activity and a decrease in the Mn(II) stoichiometry to three Mn(II) per trimer. Activation of the native enzyme requires extended incubations in the presence of millimolar concentrations of Mn(II), and results in the reversible binding of three additional Mn(II) ions per trimer to give the full complement of six Mn(II) per trimer. The K_d for the reversible binding of Mn(II) during activation is 50 μM (Hirsch-Kolb *et al.*, 1971). From a physiological standpoint, it is difficult to reconcile this relatively high K_d with the 0.25–0.7 μM concentration of free Mn(II) found in rat hepatocytes (Ash & Schramm, 1982). Kuhn *et al.* (1991) have reported that arginase in crude mouse liver homogenates has a K_d of 0.08–0.3 μM for Mn(II) during activation, and have proposed on the basis of these results that the enzyme may be "damaged" during the rigors of purification, resulting in altered metal binding properties of the purified enzyme.

The properties of the H101N and H126N mutants indicate that these conserved histidine residues are essential for the maintenance of the binuclear metal center. When assayed in the presence of excess Mn(II), both mutant enzymes have activities that are within a factor of 2 of that for wild-type enzyme. However, when assayed in the presence of EDTA, a treatment that has no effect on the activity of the wild-type enzyme or the H141N mutant, the k_{cat} for the H101N and H126N mutants is several orders of magnitude lower than the k_{cat} of the wild-type enzyme. After extended dialysis, both H101N and H126N mutants have approximately three Mn(II) bound per trimer, whereas after identical treatment wild-type enzyme and the H141N mutant retain six Mn(II) ions per trimer. Similar treatment of the fully Mn-activated

enzyme from rat liver reduces the Mn(II) content from six Mn(II) per trimer to three Mn(II) per trimer (data not shown), with a corresponding 2-fold decrease in activity. The metal binding properties of the H101N and H126N mutant arginases indicate that replacement of a potential metal ligand, the imidazole side chain of histidine, with asparagine, an amino acid less likely to participate in metal binding through its side chain, results in the labilization of one of the metals of the binuclear center. Although it is tempting to speculate that His101 and His126 are ligands to the binuclear Mn(II) center, the available data do not permit unambiguous assignment of this role to either of these histidine residues.

Despite the similarities in Mn(II) stoichiometries for the H101N, H126N, and native rat liver arginases after extensive dialysis, significant differences in activity are observed for these three enzymes. There are several possible routes for Mn(II) loss from the enzyme that could account for these activity differences, dependent upon the function of the individual metal sites and whether loss of Mn(II) leads exclusively to enzyme forms with intact binuclear centers, mononuclear centers, or a mixture of binuclear and mononuclear centers. It is clear from the data that the mutant enzymes lose Mn(II) via a different route than the native rat liver enzyme. The role of the binuclear metal centers in catalysis is currently under investigation by EPR spectroscopic studies of the H101N, H126N, and wild-type arginases.

Mutagenesis has identified His141 as the DEPC-sensitive residue that is critical in the catalytic mechanism. This histidine may serve as a general base catalyst, possibly to activate a metal-bound water molecule for attack at the guanidino carbon of arginine as proposed by Reczkowski and Ash (submitted for publication). It is somewhat surprising, however, that although the H141N mutant is no longer sensitive to inactivation by DEPC, this enzyme displays 11% of wild-type activity when assayed at pH 9. This unexpectedly high level of activity upon removal of an "essential" residue could be explained if, at this alkaline pH, specific base catalysis by OH^- is operative. If the enzyme does function via specific base catalysis at pH 9, a greater difference between wild-type and mutant activities should be observed at lower pH values. Preliminary kinetic studies at pH 7.5 indicate that the H141N mutant enzyme still has approximately 5% the activity of wild-type enzyme at this pH, raising concerns about the proposed role of His141 in the activation of a Mn(II)-bound water molecule. The pK_a of a Mn(II)-bound water molecule is 10.5 (Chaberek *et al.*, 1952); however, the pK_a of a water molecule bound to a binuclear manganese center would probably be considerably lower (Karlin, 1993). Therefore, while His141 may serve as a base during catalysis, this residue is not essential to the catalytic mechanism. Because of the lowered pK_a of the water bound to the binuclear center, His141 appears to play a stimulatory rather than an essential role in the mechanism of this enzyme. Additional site-directed mutants at this position are being constructed to clarify the role of His141 in catalysis.

ACKNOWLEDGMENT

We thank Dr. Leslie Holladay for generously providing the DENFIT program and Dr. Arazdordi Toumadje and Dr. W. Curtis Johnson for generously providing the VARSELEC program.

REFERENCES

Ash, D. E., & Schramm, V. L. (1982) *J. Biol. Chem. 257*, 9261–9264.

Chaberek, S., Courtney, R. C., & Martel, A. E. (1952) *J. Am. Chem. Soc. 74*, 5057–5060.

Garganta, C. L., & Bond, J. S. (1986) *Anal. Biochem. 154*, 388–394.

Goode, B. L., & Feinstein, S. C. (1992) *BioTechniques 12*, 374–375.

Haraguchi, Y., Takiguchi, M., Amaya, Y., Kawamoto, S., Matsuda, I., & Mori, M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 412–415.

Herzfeld, A., & Raper, S. M. (1976) *Biochem. J. 153*, 469–478.

Higuchi, R., Krummel, B., & Saiki, R. K. (1988) *Nucleic Acids Res. 16*, 7351–7367.

Hirsch-Kolb, H., Kolb, H. J., & Greenberg, D. M. (1971) *J. Biol. Chem. 246*, 395–401.

Kanyo, Z. F., Chen, C.-Y., Daghigh, F., Ash, D. E., & Christianson, D. W. (1992) *J. Mol. Biol. 224*, 1175–1177.

Karlin, K. D. (1993) *Science 261*, 701–708.

Kawamoto, S., Amaya, Y., Oda, T., Kuzumi, T., Saheki, S., & Mori, M. (1986) *Biochem. Biophys. Res. Commun. 136*, 955–961.

Kawamoto, S., Amaya, Y., Murakami, K., Tokunaga, F., Iwanaga, S., Kobayashi, K., Saheki, T., Kimura, S., & Mori, M. (1987) *J. Biol. Chem. 262*, 6280–6283.

Kedra-Luboinska, M., Zamecka, E., & Porembska, Z. (1988) *Biochem. Med. Metab. Biol. 39*, 247–257.

Kuhn, N. J., Talbot, J., & Ward, S. (1991) *Arch. Biochem. Biophys. 286*, 217–221.

Laemmli, U. K. (1970) *Nature 227*, 680.

Manavalan, P., & Johnson, W. C. (1985) *Proc. Int. Symp. Biomol. Struct. Interact., Suppl. J. Biosci. 8*, 141–149.

Manavalan, P., & Johnson, W. C. (1987) *Anal. Biochem. 167*, 76–85.

Miles, E. (1977) *Methods Enzymol. 47*, 431–442.

Miller, C. G. (1987) in *Escherichia coli and Salmonella typhimurium, Cellular and Molecular Biology* (Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M., & Umbarger, H. E., Eds.) pp 680–691, American Society for Microbiology, Washington, D.C.

Muszyńska, G., & Ber, E. (1978) *Int. J. Biochem. 9*, 757–759.

Penninckx, M., Simon, J.-P., & Wiame, J.-M. (1974) *Eur. J. Biochem. 49*, 429–442.

Reczkowski, R. S., & Ash, D. E. (1992) *J. Am. Chem. Soc. 114*, 10992–10994.

Reczkowski, R. S., & Ash, D. E. (1994) *Arch. Biochem. Biophys. 312*, 31–37.

Rüegg, U. T., & Russell, A. S. (1980) *Anal. Biochem. 102*, 206–212.

Sanger, F., Coulson, A. R., Barell, B. G., Smith, A. J. H., & Roe, B. A. (1980) *J. Mol. Biol. 143*, 161–178.

Satishchandran, C., & Boyle, S. M. (1986) *J. Bacteriol. 165*, 843–848.

Schimke, R. T. (1970) *Methods Enzymol. 17a*, 313.

Schrell, A., Alt-Moerbe, J., Lanz, T., & Schroeder, J. (1989) *Eur. J. Biochem. 184*, 635–641.

Shinomiya, T., Ohara, T., Wada, N., Omori, A., & Kamada, N. (1990) *J. Biochem. 107*, 435–439.

Studier, F. W., Rosenberg, A. H., Dunn, J. J., & Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60–89.

Sumrada, R. A., & Cooper, T. G. (1984) *J. Bacteriol. 160*, 1078–1087.

Tabor, C. W., & Tabor, H. (1984) *Annu. Rev. Biochem. 53*, 749–790.

Xu, Q., Baker, B. S., & Tata, J. R. (1993) *Eur. J. Biochem. 211*, 891–898.

Yip, M. C. M., & Knox, W. E. (1972) *Biochem. J. 127*, 839–899.