Subunit-Subunit Interactions in Trimeric Arginase

GENERATION OF ACTIVE MONOMERS BY MUTATION OF A SINGLE AMINO ACID*

The structure of the trimeric, manganese metalloenzyme, rat liver arginase, has been previously determined at 2.1-Å resolution (Kanyo, Z. F., Scolnick, L. R., Ash, D. E., and Christianson, D. W., (1996) Nature 383, 554–557). A key feature of this structure is a novel S-shaped oligomerization motif at the carboxyl terminus of the protein that mediates ∼54% of the intermonomer contacts. Arg-308, located within this oligomerization motif, nucleates a series of intramonomer and intermonomer salt links. In contrast to the trimeric wild-type enzyme, the R308A, R308E, and R308K variants of arginase exist as monomeric species, as determined by gel filtration and analytical ultracentrifugation, indicating that mutation of Arg-308 shifts the equilibrium for trimer dissociation by at least a factor of 10^5. These monomeric arginase variants are catalytically active, with k_{cat}/K_m values that are 13–17% of the value for wild-type enzyme. The arginase variants are characterized by decreased temperature stability relative to the wild-type enzyme. Differential scanning calorimetry shows that the midpoint temperature for unfolding of the Arg-308 variants is in the range of 63.6–65.5 °C, while the corresponding value for the wild-type enzyme is 70 °C. The three-dimensional structure of the R308K variant has been determined at 3-Å resolution. At the high protein concentrations utilized in the crystallizations, this variant exists as a trimer, but weakened salt link interactions are observed for Lys-308.

Arginase (EC 3.5.3.1) catalyzes the divalent cation-dependent hydrolysis of L-arginine to form L-ornithine and urea in the final step of urea biosynthesis. Liver arginase is one of the most important enzymes of mammalian nitrogen metabolism, since it comprises the principal route for disposal of excess nitrogen resulting from amino acid and nucleotide metabolism. The flux of nitrogen through this pathway is considerable, given that the average individual excretes about 10 kg of urea per year (1) (Scheme 1).

Liver arginase, also designated type I arginase, has been isolated from human and rat liver, and both enzymes have been expressed in Escherichia coli (2, 3). The physiological activator is Mn(II), and the fully active rat liver enzyme contains 2 Mn(II)/subunit (4, 5). The crystal structure of type I arginase from rat liver has been determined at 2.1-Å resolution (4). The key features of this structure include a trimeric quaternary structure, a binuclear manganese cluster, and an S-shaped tail composed of 19 amino acids at the C terminus (Fig. 1). The S-shaped tail at the C terminus of each monomer is located at the subunit-subunit interface (4), and 54% of the intersubunit interactions (van der Waals, hydrogen bonds, and salt links) are mediated by this region of the protein (1, 4).

In humans, type I arginase deficiency is a rare disorder that results in hyperargininemia characterized by episodic hyperammonemia and growth retardation, among other complications (6–10). Hyperargininemia is a heterogeneous disease resulting from point mutations throughout the type I arginase gene (11–17). An R291X mutation has been identified that would lead to the production of a truncated protein lacking the final 32 amino acids at the C terminus of the 323 amino acid subunit. The hyperargininemia patient with this mutation has both low levels of arginase activity and low levels of immuno-cross-reactive material (1, 11). This latter finding suggests that the R291X mutant may be characterized by decreased stability that results from loss of the S-shaped tail. This S-shaped tail appears to stabilize and maintain the native oligomeric state of the enzyme via a network of salt bridges nucleated by Arg-308. The Arg-308 residue forms an intramonomer salt bridge with Glu-262 and an intermonomer salt bridge with Asp-204 on the adjacent subunit (4). Mutations in this region may impact the oligomeric structure and stability of the enzyme as well as its kinetic properties.

The goal of the present study has been to utilize gel filtration, enzyme activity assays, EPR, analytical ultracentrifugation, differential scanning calorimetry, circular dichroism, and x-ray crystallography to elucidate the role of Arg-308, located at the S-shaped tail, in maintaining the overall oligomeric state and kinetic properties of rat liver arginase.

EXPERIMENTAL PROCEDURES

Materials—L-1-[guanidino-14C]Arginine (specific activity 1.9 GBq mmol⁻¹) was from PerkinElmer Life Sciences. isopropyl β-D-thiogalactopyranoside was from FisherBiotech. The Superdex-200 HiLoad 16/60 FPLC column was from Amersham Pharmacia Biotech. Reactive Red-120 dye ligand chromatography media, Chelex-100,

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1 The abbreviations used are: FPLC, fast protein liquid chromatog-
that no other mutations had been introduced during polymerase chain reaction.

**Overexpression and Purification of Mutant Enzymes**—The constructs for the R308A, R308E, and R308K arginase variants were used to transform competent BL-21(DE3) cells. Expression of the mutant proteins by the transformed BL-21(DE3) cells was induced by the addition of isopropyl β-D-thiogalactopyranoside (0.2 mg/ml final concentration) for 3 h after the cell culture had reached an A₆₀₀ of 0.8. The cells were then harvested by centrifugation at 5000 × g for 35 min and the cell pellet was stored at −70 °C overnight. The cells were then thawed and resuspended in 50 mM HEPES/KOH at pH 7.5, and the arginase variants were purified from the lysed cells by a modification of the method described by Cavalli et al. (2). The partially purified protein samples were chromatographed on a Sigma-Red dye ligand column (3.5 × 25 cm), instead of the Amicon-Green dye ligand column described previously (2) and eluted with a 0–0.3 M linear gradient of KC1 in 50 mM HEPES/KOH at pH 7.5. The purity of the proteins was determined by SDS-polyacrylamide gel electrophoresis under reducing conditions. All arginase samples were stored at 4 °C in ammonium sulfate at 85% saturation.

**Enzyme Assay**—The mutant R308A, R308E, and R308K enzymes and the recombinant wild-type enzyme were assayed for arginase activity by a modification of the method of Rüegg and Russell (18). A typical reaction mixture contained 200 mM CHES/NaOH at pH 9.0, 100 mM MnCl₂, 500 nM [³⁵S]arginine in a final volume of 40 μl per microcentrifuge tube. 5 μl of increasing concentrations (final concentrations of 0.5, 1, 2, 4, 10, 15, 20, and 25 mM) of nonradiolabeled L-arginine was added to separate reaction mixtures to give total volumes of 45 μl. The reactions were initiated by the addition of 5 μl of a 1 μg/ml solution of enzyme and incubated for 5 min for wild type or 20 min for the R308A, R308E, and R308K mutants. The reactions were stopped by the addition of 400 μl of a stop solution at pH 4.5 containing 0.25 M acetic acid, 7 M urea, and a 1:1 (v/v) slurry of Dowex 50 W-X8 in water and then vortexed. Reaction samples were then further gently mixed for 10 min and then centrifuged in a microcentrifuge at 6000 rpm for 10 min. 3 ml of Ecoscent solution was added to 200 μl from each supernatant for liquid scintillation counting in a Beckman LS5500 model LS5500E counter.

**EPR**—The arginase samples, stored as ammonium sulfate suspensions, were centrifuged at 14,000 rpm in a microcentrifuge for 20 min at 4 °C. The pellets were resuspended in 1 ml of 50 mM HEPES/KOH at pH 7.5 containing 10 mM MnCl₂ and then incubated at 60 °C for 10 min. The samples were further centrifuged for 20 min at 4 °C at 14,000 rpm in a microcentrifuge to remove any precipitate that might have formed during the heat activation step. The supernatants were diluted ex-

![FIG. 1. The crystal structure of rat liver arginase showing the trimeric quaternary structure, S-shaped tail in white at the subunit interface, and two manganese ions (spheres) at the active site of each subunit. Arg-308 is represented in the white ball and stick form.](Image 50x680 to 295x729)

EDTA, L-arginine, L-ornithine, MnCl₂, and all protein standards for gel filtration were from Sigma, except for transcription factor Rho, which was a gift from Dr. Barbara L. Stitt (Temple University). Synthetic oligonucleotide primers were prepared by Ransom Hill Bioscience. The QuikChange site-directed mutagenesis kit and E. coli BL-21(DE3) competent cells were from Stratagene. The DNA purification kit used was from Qiagen. Restriction enzymes were purchased from New England Biolabs. Centricon-30 microcentrators were obtained from Amicon. Ecoscent scintillation solution was from National Diagnostics. Dialysis tubing was purchased from BioDesign of New York, Inc. All other reagents were of highest quality commercially available.

**Site-directed Mutagenesis**—Using the QuikChange site-directed mutagenesis kit, mutations of Arg-308 to alanine, glutamate, or lysine were performed using the following oligonucleotides as mutagenic primers: 5’-GTCTTGGTTTGGAAAGCTGAGTATACATAGCAAAGC-3’ and 5’-CTGGCTTATGATTACCTGTTCTTCAGTCCAAAACAAGAC-3’ as the forward and reverse primers, respectively, for the Arg-308 to alanine mutation. Wild-type apo arginase cDNA in a pRSET-C T7-based expression vector (Invitrogen) was used as the DNA template for this site-directed mutagenesis procedure. The underlined codon, coding for arginine, was changed to GAA and TGC in the forward and reverse directions, respectively for the Arg-308 to alanine mutation. For the Arg-308 to lysine mutation, the underlined codon was changed to AAA and TTT in the forward and reverse directions, respectively. The mutant constructs were used to transform BL-21(DE3) cells. Plasmid DNA was isolated for each mutant construct using a Qiagen kit and analyzed by restriction mapping with NdeI and PstI, enzymes known to have unique restriction sites located outside the arginase cDNA. Positive constructs for each of the R308A, R308E, and R308K mutants, which had been identified by restriction mapping, were sequenced at the DNA Sequencing Facility at the University of Pennsylvania to verify that only the specifically introduced mutations were present and that no other mutations had been introduced during polymerase chain reaction.

**Gel Filtration**—The protein samples were prepared by exhaustive dialysis of the ammonium sulfate-suspended proteins in 50 mM HEPES/KOH at pH 7.5. The samples were then concentrated to 12 mg/ml in Centricon-30 microcentrators by centrifuging at 5000 × g (4 °C) for 30 min. The Superdex-200 column was equilibrated with 50 mM HEPES/KOH, 150 mM KC1 at pH 7.5, at a flow rate of 1.5 ml/min. 100-μl protein samples were chromatographed on the Superdex column using an Amersham Pharmacia Biotech FPLC system. Chromatography of the arginase solutions was also performed in 50 mM HEPES/KOH, 150 mM KC1 at pH 7.5 that contained 20 mM arginine and 20 mM MnCl₂. The Superdex column was standardized using gel filtration standards from Sigma as well as transcription factor Rho. The molecular mass for each arginase sample was determined using a standard curve generated from the elution volumes for transcription factor Rho (282,000 Da), β-amylase (200,000 Da), glucose-6-phosphate dehydrogenase (110,000 Da).
Active Monomers of Arginase

| Enzyme   | $K_m^{a}$ | $k_{cat}^{a,b}$ | $k_{cat}K_m^{a,b}$ | Mn/subunit$^{c}$ | Molecular mass$^{a,c}$ |
|----------|-----------|----------------|--------------------|------------------|----------------------|
| Wild type | 1.07 ± 0.04 | 220 ± 20 (100%) | (2.0 ± 0.1) × 10⁵ (100%) | 1.97 ± 0.04 | 94,400 ± 500 Da |
| R308A    | 2.5 ± 0.3  | 84 ± 10 (39%)   | (3.4 ± 0.2) × 10⁴ (17%) | 2.1 ± 0.1 | 30,000 ± 100 Da |
| R308E    | 2.6 ± 0.2  | 59 ± 5 (41%)    | (3.4 ± 0.1) × 10⁴ (17%) | 2.1 ± 0.3 | 30,000 ± 200 Da |
| R308K    | 2.7 ± 0.5  | 72 ± 7 (33%)    | (2.7 ± 0.2) × 10⁴ (13%) | 1.25 ± 0.04 | 34,000 ± 600 Da |

$^{a}$ For each enzyme six sets of data were determined.
$^{b}$ Percentage of wild-type value is shown in parentheses.
$^{c}$ For each enzyme three separate determinations were obtained, with the exception of R308K with 11 determinations.
$^{d}$ For each enzyme five determinations were obtained except R308K with eight determinations.

(a) Monomeric molecular mass for arginase is 35000 Da.

Da, bovine serum albumin (66,000 Da), carbonic anhydrase (29,000 Da), and cytochrome c (12,400 Da).

Analytical Ultracentrifugation—Sedimentation equilibrium analytical ultracentrifugation of wild-type and mutant arginases was carried out using a Beckman XL-1 analytical ultracentrifuge. Absorbance optics at 280 nm were used to measure the concentration of arginase. Enzyme was prepared for ultracentrifugation by exhaustive dialysis in 50 mM HEPES/KOH, pH 7.5, to ensure that the samples and blanks were of identical composition. Arginase concentrations for the analytical ultracentrifugation experiments ranged from 0.1 to 0.3 mg/ml. The centrifuge was operated at 20,000 rpm, 20 °C, and data were collected over a 40-h period with 2-h sampling intervals. Equilibrium was reached when no further difference in the exponential distribution data was observed between two consecutive data acquisitions. Equilibrium data were analyzed with software written using IGOR (WaveMetrics, Lake Oswego, OR). A partial specific volume of 0.743, determined using the sednterp program (19, 20), was used in the calculation of the species concentration in solution. All calculations were based on the molecular composition of a single subunit.

Molecular Weight Determinations—The native molecular mass for the wild-type enzyme had a $K_m$ of 1.1 mM for l-arginine, in good agreement with the values of 1–1.7 mM reported for rat liver arginase (2, 5, 28, 29). The mutant arginases showed $K_m$ values for l-arginine that were about 2.5-fold higher compared with wild-type enzyme. The catalytic activities ($k_{cat}$ of all the mutants ranged between 33 and 41% of that for the wild-type enzyme, and the $k_{cat}/K_m$ values ranged between 13 and 17% of that for wild-type enzyme.

Manganese Stoichiometry—Because an intact binuclear Mn(II) center is essential for maximal catalytic activity (2, 5), it is possible that the decreased activity of the R308A, R308E, and R308K mutants relative to wild-type enzyme resulted from altered manganese stoichiometries for the mutant enzymes. The Mn(II) stoichiometries for wild-type and the mutant arginases were determined by EPR spectroscopy using samples that were heat-activated in the presence of Mn(II) and then washed exhaustively to remove excess Mn(II). The results of the Mn(II) determinations are summarized in Table I. The wild-type enzyme had a $K_m$ of 1.1 mM for l-arginine, in good agreement with the values of 1–1.7 mM reported for rat liver arginase (2, 5, 28, 29). The mutant arginases showed $K_m$ values for l-arginine that were about 2.5-fold higher compared with wild-type enzyme. The catalytic activities ($k_{cat}$ of all the mutants ranged between 33 and 41% of that for the wild-type enzyme, and the $k_{cat}/K_m$ values ranged between 13 and 17% of that for wild-type enzyme.

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RESULTS

Purification of Arginase Variants—The mutant arginases were purified to apparent homogeneity using the standard protocol for the wild-type enzyme. No modifications of the protocol were required.

Kinetic Constants—The kinetic constants for wild-type arginase and the R308A, R308E, and R308K mutant arginases are summarized in Table I. The wild-type protein had a $K_m$ of 1.1 mM for l-arginine, in good agreement with the values of 1–1.7 mM reported for rat liver arginase (2, 5, 28, 29). The mutant arginases showed $K_m$ values for l-arginine that were about 2.5-fold higher compared with wild-type enzyme. The catalytic activities ($k_{cat}$ of all the mutants ranged between 33 and 41% of that for the wild-type enzyme, and the $k_{cat}/K_m$ values ranged between 13 and 17% of that for wild-type enzyme.

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molecular weights as described under “Experimental Procedures” and shown in Fig. 2A. The elution profiles for wild-type and the mutant arginase R308A are shown in Fig. 2B. The wild-type protein eluted as a single peak corresponding to the trimeric molecular weight, while the R308A mutant eluted as a single peak with a molecular weight consistent with a monomeric species. The protein profiles for the R308E and R308K mutant arginases were the same as the R308A mutant shown in Fig. 2B. The monomeric state of the mutants did not change when chromatography was performed in the presence of added arginine and MnCl₂ (data not shown). Wild-type arginase was determined to have a molecular mass of about 94–95,000 Da, which is consistent with previously reported molecular masses determined by gel filtration for the trimeric rat and human liver arginases (30, 31). In contrast, the molecular masses determined for the mutant arginases were about 30,000–34,000 Da, consistent with the monomeric molecular mass for rat liver arginase (31).

Analytical Ultracentrifugation Analysis—Equilibrium sedimentation analytical ultracentrifugation data (Fig. 3) for wild-type arginase were best fit to a trimeric species only. Because no significant amount of monomer could be fit using the wild-type data, the monomer-trimer equilibrium constant for this interaction could not be determined directly. Allowing the fitting program to fit for molecular weight rather than for oligomeric state resulted in a mass of 104,500 Da, matching that of trimeric arginase (31). Mutant arginases R308A, R308E, and R308K were found only in monomeric form. Introduction of a trimeric species into the fitting analysis resulted in very large error values and poor fit parameters. Fitting these data for molecular mass gave results indicative of monomeric species, with values of 35,000 ± 100 Da.

DSC—Fig. 4 shows DSC data measuring the thermal stabilities of wild type and the mutant forms of arginase. Some pretransition base-line variability is seen between the different temperature scans. This variability is instrumental in origin and is related to the low protein concentrations (0.4 mg/ml) used. All three mutant forms of arginase unfold with very similar apparent unfolding enthalpy changes of 190 ± 15 kcal/mol of subunit and similar midpoint temperatures of 65.5 °C (R308A), 64.1 °C (R308E), and 63.6 °C (R308K). In contrast, wild-type arginase unfolds at a higher temperature (70.0 °C) and also with a much larger enthalpy change of 280 kcal/mol of subunit. The additional thermal stabilization observed for wild-type arginase is at least partly due to the intersubunit, trimerization bonding energy (32, 33). These observed unfolding enthalpy changes agree with typical unfolding enthalpy changes observed for globular proteins (34), although, since unfolding of arginase was irreversible, the thermodynamic parameters may have contributions from side reactions such as aggregation in the unfolded form.

CD—To evaluate the integrity of the secondary structure of the arginase mutants, the circular dichroic spectra of the wild-type enzyme and the arginase variants were compared. The CD spectra of wild-type and mutant arginases were nearly identi-
cal over the wavelength range examined (data not shown). Minor differences in amplitude were observed, arising from slight variations in enzyme concentration, but the overall shapes of the curves were identical. These findings indicate that there are no gross structural changes, particularly in α-helical structure, occurring as a result of the mutagenesis, and this conclusion is consistent with the subsequently determined x-ray crystal structure of R308K arginase.

Crystallography—Overall, the refined structure of R308K arginase is very similar to that of the native enzyme; the root mean square deviation of 314 Ca atoms in the monomer is 0.38 Å. Although the trimeric quaternary structure of this variant is destabilized by the R308K substitution, the trimer is sufficiently stable at the high protein concentrations (14–18 mg/ml) employed for crystallization. The electron density map in Fig. 5 reveals clear and unambiguous density for the Lys-308 side chain, and the comparison of wild-type and R308K arginases in Fig. 6 reveals the structural basis for trimer destabilization, which is discussed further below.

Comparison of the active sites of trimeric wild-type and R308K arginases reveals no structural changes (data not shown). In R308K arginase, 2 Mn²⁺ ions are bound at the base of the active site cleft (Mn²⁺-Mn²⁺ separation = 3.4 Å) in identical fashion observed in the wild-type enzyme (Mn²⁺-Mn²⁺ separation = 3.3 Å (4)). Although this Mn²⁺ stoichiometry does not agree with that determined by EPR, 5 mM MnCl₂ was included in the crystallization solutions. This concentration of Mn²⁺ is sufficient to saturate the Mn³⁺ binding sites on the R308K enzyme. The nonprotein ligand, a bridging hydroxide ion, is not visible in the electron density map. This is probably a consequence of the moderate resolution of the map e.g. as found in the structure determination of H101N arginase (35).

**Discussion**

The present analytical ultracentrifugation and gel filtration studies, as well as results from x-ray crystallography (4), have shown that both the native and recombinant forms of rat liver arginase exist as trimeric species. An unusual “S-shaped oligomerization motif” which begins at Phe-304 and extends to the C terminus of the 323-amino acid polypeptide chain, was identified in the crystal structure of the enzyme. Since ~54% of intermonomer contacts are mediated via this S-shaped tail (4), it has been suggested that this motif is critical for maintaining the trimeric form of arginase (1). The sequence of this motif is highly conserved between rat, human, mouse, and *Xenopus* arginases as shown in Table II, suggesting a similar role in stabilizing the oligomeric states of these enzymes. In a recent report, the role of the S-shaped tail in stabilizing the trimeric human arginase was examined (30). No loss of enzyme activity and no change in the state of oligomerization were observed for a human arginase variant that lacks the final 14 amino acids of this motif but retains Arg-308 as the C-terminal amino acid. Thus, Mora et al. (30) conclude that the S-shaped tail is not important for maintaining the structural integrity of arginase or for optimal activity. Examination of the crystal structure, however, indicates that Arg-308 plays a critical role in nucleating a series of intra- and intersubunit interactions. In addition, this residue is conserved in the rat, human, mouse, and *Xenopus* arginase sequences.

In contrast to the study of Mora et al. (30), the results presented here have shown that retention of the S-shaped tail and mutation of a single amino acid, Arg-308, results in a shift in the oligomeric state of arginase from a trimeric species to a monomeric species, as determined by analytical ultracentrifugation and gel filtration. With the present data, it is not possible to determine equilibrium constants for the dissociation of the wild-type trimer into monomers (since only trimer was observed under all experimental conditions) or for the dissociation of mutant trimers into monomers (since only monomeric species were observed under the experimental conditions). However, limits on *K* for the dissociation of wild-type trimers or mutant trimers to form monomers can be estimated from the data in Fig. 3. For the wild-type enzyme, assuming detection limits that would permit observation of 5% of the total protein as the monomeric form, an upper limit of 8 × 10⁻¹⁴ M⁻² can be estimated for *K* for *K* for *K* for the dissociation of wild-type trimers or mutant trimers to form monomers (since only monomer species were observed under the experimental conditions). Although these are rough estimates, it is clear that mutation of Arg-308 shifts the equilibrium constant for trimer dissociation by at least a factor of 10⁶.

The monomeric forms of arginase, R308A, R308E, and R308K, are all catalytically active. Previous studies using nonmutagenic techniques such as acid or EDTA treatment (36) and glutaraldehyde cross-linking to a column support (37) have demonstrated that monomers of the enzyme are active. The three variants show modest increases in the *K* for arginine.

**Fig. 4.** Differential scanning calorimetry data for the unfolding of wild-type arginase (solid line), R308A (dashed and dotted line), R308E (dotted line), and R308K (dashed line) shown as excess molar heat capacity versus temperature. All scans are normalized per mole of arginase subunit. Conditions were as follows: 20 mM NaPO₄, 150 mM NaCl, 0.1 mM MnCl₂, pH 7.4, and 0.4 mg/ml arginase.

**Fig. 5.** Omit electron density map of R308K arginase for which the atoms of Lys-308 were omitted from the structure factor calculation. The map is contoured at 5σ, and selected residues are indicated. Atoms are color-coded as follows: carbon (yellow), oxygen (red), nitrogen (blue), and sulfur (green). Key hydrogen bond interactions are indicated by dotted red lines.
relative to the wild-type enzyme and ~3-fold decreases in $k_{cat}$ (Table I). In addition, the mutant enzymes show decreased thermal stability, particularly at low protein concentrations (data not shown). The three monomeric mutant forms of arginase unfold with very similar unfolding enthalpy changes and temperature midpoints as determined by DSC (Fig. 4). However, the native trimeric form of arginase is much more stable with respect to unfolding. Direct comparison of the thermal stabilities of the monomeric mutant arginase molecules to the trimeric native molecule is somewhat complicated by the predicted concentration dependence in the case of the trimer. In fact, for reversibly associated oligomeric proteins in general, the unfolding thermodynamics include a translational entropy component corresponding to conversion from folded oligomer to unfolded monomers (38). The difference in folding stabilities of the monomeric and trimeric forms is consistent with very tight trimerization interactions of the native form (33). However, because arginase unfolding was irreversible, it is not possible to calculate a trimerization constant from the DSC data using an equilibrium theory.

The activity differences between monomeric mutants and trimeric wild type cannot be attributed to decreased binding of the essential Mn(II), since both the R308A and R308E mutants contain a full complement of Mn(II). In contrast, the R308K mutant consistently displayed manganese stoichiometries of 1.25 Mn(II)/subunit but catalytic activities that were comparable with the other two variants. The origin of this discrepancy is unclear at present, and further Mn(II) EPR studies may be required to establish the distribution of Mn(II) between mononuclear and binuclear sites in this variant.

Similarly, it is unlikely that the mutations result in any gross conformational changes in structure, since each of the variants has considerable catalytic activity, and the CD spectra for the variants are very similar to that for the wild-type enzyme. It is more likely that the mutations have resulted in small perturbations in active site structure that have altered the catalytic activity as well as stability of the mutant enzymes. In this regard, it is interesting to note that the crystal structure for the arginase from Bacillus caldovelox does not show an S-shaped tail analogous to that found in rat liver arginase (39). This arginase, like many arginases from bacterial sources, is hexameric (a dimer of trimers) rather than trimeric, and the amino acid corresponding to Arg-308 in the rat enzyme is not conserved in the B. caldovelox enzyme. However, crystals of this enzyme grown in the presence of guanidinium hydrochloride have a guanidinium ion bound at the subunit-subunit interfaces in the trimer. Similarly, crystals grown in the presence of EDTA, to remove one of the catalytic metals, and arginase show two arginine binding sites. One molecule of arginine is bound at the active site, while the second is located at the subunit-subunit interface. In these structures, the guanidino group is stabilized by bidentate hydrogen bonding to Glu-256 (B. caldovelox numbering system) in one monomer and by a bifurcated hydrogen bond to Asp-199 of a second monomer. Both Glu-256 and Asp-199 are conserved in rat liver arginase (Glu-262 and Asp-204 in the rat numbering system) and form similar interactions with the guanidino moiety of Arg-308. Superposition of the structures for the rat liver enzyme and the B. caldovelox enzyme reveals that the guanidino group of Arg-308 in the rat enzyme superimposes with the guanidino group of the guanidinium ion or arginine in the B. caldovelox enzyme (39).

Comparison of crystal structures for the B. caldovelox enzyme determined in the presence or absence of guanidinium hydrochloride reveals that the binding of the guanidinium ion results in the rearrangement of a loop region composed of residues 12–20. Since these residues form part of the rim of the active site and their movement has a significant impact on other active site residues, it has been proposed that binding of the guanidinium ion or arginine to the external site at the monomer-monomer interface serves to activate the B. caldovelox enzyme. A similar role may be envisaged for Arg-308 in the rat liver enzyme, that of stabilizing the active conformation of the enzyme. Additional insights are provided by examination of the crystal structure of the mutant arginase R308K refined at 3.0 Å. In wild-type arginase, Arg-308 nucleates an alternating network of intra- and intermonomer salt links that stabilize trimer assembly. Specifically, Arg-308 donates intra-molecular hydrogen bonds to Glu-262 and intermolecular hydrogen bonds to Asp-204. In R308K arginase, the intramolecular hydrogen bond with Glu-262 is broken, and the intermolecular hydrogen bond with Asp-204 is present but weakened by poor geometry. Possibly, the new intramolecular hydrogen bond between Lys-308 and the carbonyl oxygen of Arg-255 hinders the formation of the crystal structure, which is similar to that found in rat liver arginase (39).
of an optimal intramolecular hydrogen bond with Asp-204. Therefore, the weakened intermolecular hydrogen bond between Lys-308 and Asp-204 accounts for the destabilization of trimer assembly in this variant. Comparison of the active sites of trimeric wild-type and R308K arginases reveals no significant structural changes. The origin of the 3-fold difference in activity of the Arg-308 mutants compared with that of wild-type remains unclear. Small (0.1—0.2 Å) movements of a catalytic residue would be difficult to discern at 3-Å resolution.

Furthermore, greater conformational flexibility for the Arg-308 mutation at Arg-308 weakens the network of intermonomer salt linkages, leading to the generation of catalytically active monomers as demonstrated by gel filtration and ultracentrifugation experiments. The modest changes in catalytic constants for the monomeric Arg-308 mutants relative to the wild-type enzyme are in stark contrast to the low levels of activity reported for the patient R291X mutant. This conclusion is supported by the finding that patients with the R291X mutation is expressed in only monomeric but that they are catalytically active. A single amino acid substitution at Arg-308 weakens the network of intermonomer salt linkages, leading to the generation of catalytically active monomers as demonstrated by gel filtration and ultracentrifugation experiments.

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REFERENCES

1. Ash, D. E., Scolnick, L. R., Kanyo, Z. F., Vockley, J. G., Cederbaum, S. D., and Christianson, D. W. (1998) Mol. Genet. Metab. 64, 243–249
2. Cavalli, R. C., Burke, C. J., Kawamoto, S., Soprano, D. R., and Ash, D. E. (1994) Biochemistry 33, 10652–10657
3. Ikemoto, M., Tabata, M., Miyake, T., Kono, T., Mori, M., Totani, M., and Murachi, T. (1990) Biochem. J. 270, 697–703
4. Kanyo, Z. F., Scolnick, L. R., Ash, D. E., and Christianson, D. W. (1996) Nature 383, 554–557
5. Reczkowski, R. S., and Ash, D. E. (1992) J. Am. Chem. Soc. 114, 10992–10994
6. Bernar, J., Hanson, R. A., Kern, R., Phoenix, B., Shaw, K. N., and Cederbaum, S. D. (1986) J. Pediatr. 108, 432–435
7. Brusilow, S. W., and Horwich, A. L. (1995) in Urea Cycle Enzymes (Sriver, C. R., Beaudet, A. L., Sly, W. S., and Valie, D., eds) pp. 1187–1242, McGraw-Hill Inc., New York
8. Cederbaum, S. D., Shaw, K. N., Spector, E. B., Verity, M. A., Snodgrass, P. J., and Sugarman, G. I. (1979) Pediatr. Res. 13, 827–833
9. Snyder, S. E., Sancar, C., Chen, W. J., Norton, P. M., and Phansalkar, S. V. (1977) J. Pediatr. 90, 563–568
10. Terheggen, H. G., Schwenk, A., Lewenthal, A., Sande, M. v., and Colombo, J. P. (1970) Z. Kinderheilkd. 107, 298–312
11. Grody, W. W., Klein, D., Doden, A. E., Kern, R. M., Wissmann, P. B., Goodman, B. K., Bassand, P., Marescau, B., Kang, S. S., Leonard, J. V., and Cederbaum, S. D. (1992) Am. J. Hum. Genet. 50, 1281–1290
12. Haraguchi, Y., Takiguchi, M., Amaya, Y., Kawamoto, S., Matsuda, I., and Mori, M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 412–415
13. Tabor, D. E., Vockley, J. G., Kern, R. M., Goodman, B. K., Wissmann, P. B., Grody, W. W., and Cederbaum, S. D. (1994) in Sixth International Conference of Inborn Errors of Metabolism, Milan, Italy (Abstr. 174)
14. Uchino, T., Haraguchi, Y., Aparicio, J. M., Mizutani, N., Higashikawa, M., Naitoh, H., Mori, M., and Matsuda, I. (1992) Am. J. Hum. Genet. 51, 1406–1412
15. Uchino, T., Snyderman, S. E., Lambert, M., Qureshi, I. A., Shapira, S. K., Sancar, C., Smith, L. M., Jakobs, C., and Matsuda, I. (1995) Hum. Genet. 96, 255–260
16. Vockley, J. G., Tabor, D. E., Kern, R. M., Goodman, B. K., Wissmann, P. B., Kang, D. S., Grody, W. W., and Cederbaum, S. D. (1994) Hum. Mutat. 4, 150–154
17. Vockley, J. G., Goodman, B. K., Tabor, D. E., Kern, R. M., Jenkinson, C. P., Grody, W. W., and Cederbaum, S. D. (1996) Biochem. Mol. Med. 59, 44–51
18. Ruegg, U. T., and Russell, A. S. (1986) Anal. Biochem. 152, 206–212
19. Laue, T. M., Shah, B., Ridgeway, T. M., and Pelletier, S. L. (1992) in Analytical Ultracentrifugation in Biochemistry and Polymer Science (Harding, E. S., Rowe, A. J., and Horton, J., eds) pp. 90–125, Royal Society of Chemistry, Cambridge, United Kingdom
20. Philo, J. (1994) in Modern Analytical Ultracentrifugation: Acquisition and Interpretation of Data for Biological and Synthetic Polymer Systems (Schuster, T. M., and Laue, T. M., eds) pp. 156–170, Birkhauser, Boston
21. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) Protein Sci. 4, 2411–2425
22. Kanyo, Z. F., Chen, C. Y., Daghigh, F., Ash, D. E., and Christianson, D. W. (1992) J. Mol. Biol. 224, 1175–1177
23. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
24. Navaza, J. (1994) Acta Crystallogr. A 50, 157–163
25. Collaborative Computational Project (1994) Acta Crystallogr. D Biol. Crystallogr. 50, 760–763
26. Brugner, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Goss, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. D Biol. Crystallogr. 54, 905–921
27. Jones, T. A., Zou, Y. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. A 47, 110–119
28. Garganta, C. L., and Bond, J. S. (1986) Anal. Biochem. 154, 388–394
29. Reczkowski, R. S., and Ash, D. E. (1994) Arch. Biochem. Biophys. 312, 31–37
30. Mora, A., del Ara Rangel, M., Fuentes, J., Soler, G., and Centeno, F. (2000) Biochim. Biophys. Acta 1476, 181–190
31. Pencinovk, M., Simon, J., and Wiame, J. (1979) Eur. J. Biochem. 94, 429–442
32. Backmann, J., Schafer, G., Wyns, L., and Bonisch, H. (1998) J. Mol. Biol. 284, 817–833
33. Brandis, J. F., and Lin, L. N. (1990) Biochemistry 29, 6927–6940
34. Privalov, P. L., and Khechinashvili, N. N. (1974) J. Mol. Biol. 86, 665–84
35. Scolnick, L. R., Kanyo, Z. F., Cavalli, R. C., Ash, D. E., and Christianson, D. W. (1997) Biochemistry 36, 10558–10565
36. Aiguiere, R., and Kasche, V. (1983) Eur. J. Biochem. 130, 373–381
37. Carvajal, N., Martinez, J., and Fernandez, M. (1977) Biochim. Biophys. Acta 481, 177–183
38. Tamura, A., and Privalov, P. L. (1977) J. Mol. Biol. 114, 1048–1060
39. Bewley, M. C., Jeffrey, P. D., Patchett, M. L., Kanyo, Z. F., and Baker, E. N. (1999) Structure 7, 435–448
40. Esnouf, R. M. (1997) J. Mol. Graph. Model 15, 132–134, 112–113
41. Merritt, R. A., and Bacon, D. J. (1997) Methods Enzymol. 277, 505–524