Mutational Analysis of Duck δ2 Crystallin and the Structure of an Inactive Mutant with Bound Substrate Provide Insight into the Enzymatic Mechanism of Argininosuccinate Lyase*

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The major soluble avian eye lens protein, δ crystallin, is highly homologous to the housekeeping enzyme argininosuccinate lyase (ASL). ASL is part of the urea and arginine-citruilline cycles and catalyzes the reversible breakdown of argininosuccinate to arginine and fumarate. In duck lenses, there are two δ crystallin isoforms that are 94% identical in amino acid sequence. Only the δ2 isoform has maintained ASL activity and has been used to investigate the enzymatic mechanism of ASL. The role of the active site residues Ser-29, Asp-33, Asp-89, Asn-116, Thr-161, His-162, Arg-238, Thr-281, Ser-283, Asn-291, Asp-293, Glu-296, Lys-325, Asp-330, and Lys-331 have been investigated by site-directed mutagenesis, and the structure of the inactive duck δ2 crystallin (dδ2) mutant S283A with bound argininosuccinate was determined at 1.96 Å resolution. The S283A mutation does not interfere with substrate binding, because the 280's loop (residues 270–290) is in the open conformation and Ala-283 is more than 7 Å from the substrate. The substrate is bound in a different conformation to that observed previously indicating a large degree of conformational flexibility in the fumarate moiety when the 280's loop is in the open conformation. The structure of the S283A dδ2 mutant and mutagenesis results reveal that a complex network of interactions of both protein residues and water molecules are involved in substrate binding and specificity. Small changes even to residues not involved directly in anchoring the argininosuccinate have a significant effect on catalysis. The results suggest that either His-162 or Thr-161 are responsible for proton abstraction and reinforce the putative role of Ser-283 as the catalytic acid, although we cannot eliminate the possibility that arginine is released in an uncharged form, with the solvent providing the required proton. A detailed enzymatic mechanism of ASL/dδ2 is presented.

δ Crystallin is a taxon-specific crystallin that represents the major soluble protein in the eye lenses of birds and terrestrial reptiles. This crystallin was recruited from the housekeeping enzyme argininosuccinate lyase (ASL)1 through a process called gene sharing (3, 4). ASL is a cytosolic enzyme that catalyzes the reversible breakdown of argininosuccinate to arginine and fumarate. Gene recruitment of ASL was followed by gene duplication and resulted in two proteins, δ1 and δ2 crystallin. In ducks, there is 94% amino acid sequence identity between the δ1 and δ2 isoforms (dδ1 and dδ2), and 69 and 71% identity to human ASL, respectively (2, 5). During evolution, the δ1 isoform has presumably become a more specialized lens protein and has lost ASL activity (6–8), while the δ2 isoform has retained enzymatic activity and is the duck orthologue of ASL in non-lens tissues (9, 10).

ASL and δ crystallin are members of a superfamily of enzymes that are active as homotetramers and catalyze similar β-elimination reactions in which a C–N or C–O bond is cleaved with the subsequent release of fumarate as one of the products. Members of the superfamily include class II fumarate (11), aspartase (12), adenylosuccinate lyase (13), and 3-carboxy-cis,cis-muconate lactonizing (CMLE) enzyme (14). Although the overall amino acid sequence homology between these proteins is low (20–30%), there are three highly conserved regions that are remote from each other in the monomer structure but which cluster together in the tetramer to form the four active sites (15–18). The location of the active site has been confirmed with the structure determination of various superfamily members with bound inhibitors, substrate analogues, or substrates (19–22).

Structural and biochemical studies (23, 24), as well as site-directed mutagenesis data (25), have suggested that His-162, directly (15, 17) or via a water molecule (21), is responsible for proton abstraction (base catalysis), whereas Lys-289 has been proposed to stabilize the carbanion intermediate (15, 16). Lys-289 belongs to the flexible 280's loop (residues 270–290), and, although this lysine’s role in catalysis is supported by its strict conservation across superfamily, the same is not true for His-162. This histidine is replaced by glutamine in all aspartases and by tryptophan in CMLE (see Fig. 1). There is also no consensus in the superfamily regarding the catalytic acid. Our

1 The abbreviations used are: ASL, argininosuccinate lyase; dδ1, duck δ1 crystallin; dδ2, duck δ2 crystallin; tδ1, turkey δ1 crystallin; S283A dδ2, duck δ2 crystallin Ser-283* to Ala mutant; H162N dδ2, duck δ2 crystallin His-162* to Asn mutant; H91N dδ2, duck δ2 crystallin His-91* to Asn mutant. (*)To facilitate referencing to previously published papers, the amino acid numbering used throughout this manuscript corresponds to dδ2, where the two-residue insertion at amino acid 5 results in a shift of numbering relative to dδ1/ASL.

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The atomic coordinates and structure factors (code IKTW) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/). 

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recent structural (26) and biochemical (27) studies of duck δ1 and δ2 crystallin suggested that a number of conformational changes occur during catalysis and that the strictly conserved Ser-283 might be the putative catalytic acid.

To gain further insight into the catalytic mechanism of δ2 crystallin/ASL, a number of active site residues were investigated by site-directed mutagenesis, and the crystal structure of the inactive S283A δ2 crystallin mutant was solved at 1.96 Å with bound substrate. The argininosuccinate substrate was found in all four active sites. The conformation of the substrate with bound substrate. The argininosuccinate substrate was from pET3d (a gift from Dr. W. E. O’Brian, Baylor, Texas) into the XhoI and HindIII sites of the pUC19 vector (Amerham Biosciences, Inc.). The pUC19-d δ2 vector was subsequently used as a template for all mutagenesis reactions. When the oligonucleotide primers were designed, in addition to the mutation in the desired codon, silent mutations were introduced to either activate or inactivate a restriction endonuclease site in the template sequence. The endonuclease site served as a useful screening tool for identifying positive mutants. The mutated cDNA was then subcloned back into the pET3d vector, in-frame with a 3′, six-histidine tag (28). Selection of positive mutants was performed by restriction enzyme mapping and subsequently confirmed by DNA sequencing (Advanced Protein Technology Center at The Hospital for Sick Children, Toronto).

**Protein Expression and Purification**—The mutant d δ2 cDNA cloned into the pET-3d expression vector was transformed into Escherichia coli DH5α a strain with the genotype pro* lacY* (bas*) nala argE argE rif rific (lacrI proAB) (DE3). The slyD deletion prevents the expression of a 21-kDa histidine-rich E. coli protein that binds strongly to the nickel-nitrilotriacetic acid affinity resin (Novagen) used for purification. In the ADE3 prophage, the gene for T7 RNA polymerase is under the control of the isopropyl-1-thio-D-galactopyranoside-inducible promoter. The protein was expressed and purified as described previously for the C-terminal histidine-tagged d δ2 (28). The affinity-purified protein was eluted with 15 ml of 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 100 mM imidazole, and the eluate was collected in two 7.5-ml fractions. The first 7.5-ml fraction contained most of the purified protein, as determined by absorption readings at λ = 280 nm as well as the presence of the expected 50-kDa band on a SDS-PAGE gel (data not shown). Only the first 7.5-ml fraction was dialyzed overnight at 4 °C in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. Each 1 liter of bacterial culture yielded ~25 mg of ~98% pure protein. Silver-stained native gels (6% acrylamide) of the wild type and mutant proteins were also run to assess the oligomeric state of the proteins.

**Circular Dichroism of Wild Type and Mutant d δ2 Proteins**—Circular dichroism (CD) experiments were performed on an AVIV CD spectropolarimeter (model 62A DS). All scans were performed between 200 and 260 nm (0.1-cm path length) on protein solutions of 0.4 mg/ml in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. The samples were scanned five times and averaged. To examine the thermal stability of each protein, the loss of ellipticity at 222 nm was monitored, as the protein sample was heat-denatured. Protein solutions of 0.2 and 0.4 mg/ml in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA were used, and the readings were averaged. The temperature was increased from 25 to 101 °C in 2 °C increments with 1 min of equilibration before each reading.

**Enzyme Kinetics Assay**—The ASL activity was assayed by monitoring the release of fumarate at 240 nm (extinction coefficient of 2.44 mmol −1 cm −1) as described in a previous study (28). All assays were performed in triplicate. The initial velocities were averaged and normalized to the amount of protein used. Michaelis-Menten plots were used to determine the kinetic parameters K_m (millimolar) and V_max (μmol min −1 mg −1 protein).

**Crystallization, Data Collection, and Structure Determination**—The S283A δ δ2 crystal was crystallized at room temperature using the hanging drop vapor diffusion method. The protein (0.9 mg/ml in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA) was mixed with 2.75 mM argininosuccinate (disodium salt, Sigma Chemical Co.). Subsequently, 5-μl drops of the protein-argininosuccinate solution were mixed with an equal amount of the precipitating solution (12% w/v polyethylene glycol 2000 MME, 300 mM MgCl2, 100 mM Hepes, pH 7.4) and suspended over a 1-ml reservoir of the precipitating solution. Rectangular crystals grew to a maximal size of 0.9 × 0.7 × 0.4 mm. X-ray diffraction data were collected on a flash frozen crystal (100 K) using an MAR 345 image plate detector at station X8C (λ = 0.96 Å) at the National Synchrotron Light Source, Brookhaven, NY. The crystal was soaked prior to flash freezing for ~2 min in a 15% v/v glycerol/precipitant solution. The crystal diffacted to 1.94 Å resolution, and the data measured were processed using the DENZO SCALEPACK software package (29). The data collection statistics are listed below in Table I.

The structure of S283A δ δ2 was determined by molecular replacement using the program CNS (30) and the wild type d δ2 tetramer (26) as the search model. The molecular replacement solution for the four molecules in the asymmetric unit had a correlation coefficient of 0.71 and a packing value of 0.5.

**Refinement and Model Building**—The structure was refined using the program CNS (30) with a maximum likelihood target function (31, 32), a flat bulk solvent correction (33), and no low resolution or α cutoff applied to the data. Ten percent of the reflections were randomly selected and used to compute a free R value (Rfree) for cross-validation of the model (31). Each refinement step consisted of torsion angle simulated annealing (34), grouped and individual B-factor refinement, and the subsequent calculation of α-weighted 2Fo − Fc and Fc − Fo electron density maps (35, 36). These maps were then used to correct the model by manual rebuilding in TURBO-FRODO (37).

Non-crystallographic symmetry restraints were initially applied and gradually relaxed during the refinement. A cis-peptide was modeled between Ser-321 and Thr-322 in all monomers. Water molecules with proper hydrogen-bonding coordination and electron densities higher than 0.1 σ [Fo − Fc] or [Fc − Fo] α-weighted maps were progressively introduced while monitoring the decrease in Rfree. Strong α-weighted [Fo − Fc] electron density allowed the substrate to be modeled in all four active sites. XPLO2D (38, 39) was used to generate the topology, parameter, and energy minimization files for the substrate. PROCHECK (40) was used to analyze the stereochemistry of the model. The final refinement statistics are presented in Table I.

**Amino Acid Sequence and Structural Alignments**—The amino acid sequences for the superfamily members were retrieved from the SwissProt data base, and ClustalX (41) was used to perform the multiple sequence alignment. An initial profile was determined by aligning δ crystallin and ASL sequences, and the rest of amino acid sequences were subsequently aligned to this profile. For simplicity, only a few representative sequences of each enzyme in the superfamily are shown in Fig. 1.

The structural comparison between monomers belonging to different or to the same δ crystallin structure was performed using the RIGID option in the program TURBO-FRODO (37). Structurally equivalent residues located in the core helices, at the beginning and the end of each helix in domain 2, were selected, and an iterative least-squares fitting procedure was performed.

**RESULTS**

**Selection of Mutagenic Targets**—The H162N d δ2 structure with argininosuccinate bound (19) identified a number of residues being important in substrate binding and specificity. Our previous mutagenesis studies had determined the role of a number of these residues (27). To obtain a comprehensive understanding of the role of every residue shown to be involved in substrate binding, we therefore constructed the following mutants: S29A, D33N, D89N, R238Q, T281V, N291L, D293N, K325N, D330N, and K331Q. In addition, our previous mutagenesis results had shown that the N116L, S283A, T161V, and E296Q mutants were either inactive or exhibited low residual ASL activity (27). Asn-116 is implicated in substrate binding because its O61 is within hydrogen-bond distance of...
the nitrogen of the C–N bond cleaved during the reaction mechanism (19), whereas structural comparisons of wild type d&c1 and d&c2 suggest that Ser-283 and Thr-161, in addition to His-162 and Glu-296, are involved in catalysis (26). To better understand our previous mutagenesis results and to confirm the role each of these residues plays in substrate binding and determine whether these variations are significant or whether they just reflect changes in the way these proteins come out of solution, they just reflect changes in the way these proteins come out of solution.

Kinetic Characterization of d&c2 Mutants—The ASL activity of wild type and mutant d&c2 proteins was quantified by monitoring at 25 °C the release of the fumarate product (λ = 240 nm). All active proteins displayed linear kinetics. The Michaelis-Menten equation was used to determine the kinetic parameters $k_m$ and $V_{max}$ (Table II). The catalytic efficiency of all mutants was compared with the wild type d&c2. Mutations D33N, R238Q, S283C, E296D, and D330N have thermal stabilities that vary from that of the wild type protein. Only the S283C mutation results in a more thermostable protein ($T_m = 80^\circ$ C). Given that the thermal denaturation is irreversible, it is difficult to determine whether these variations are significant or whether they just reflect changes in the way these proteins come out of solution.

### Table I

| Data collection statistics | P 2₁ |
|----------------------------|------|
| Space group                |      |
| Cell dimensions (Å, °)     | a = 93.8 Å, b = 98.6 Å, c = 106.2 Å, β = 101.3° |
| Molecules/asymmetric unit  | 4    |
| Resolution limits (Å)      | 1.94 |
| Total reflections          | 692,205 |
| Unique reflections         | 127,416 |
| Mean redundancy            | 5.4  |
| Completeness (%)           | 94.1(±0.8)% |
| Average Font(I)            | 10.9 |
| % reflections with I > 2σ(I)| 84.3(±0.8)% |
| $R_{sym}$<sup>b</sup>     | 0.08 (0.34)% |

| Refinement statistics      |      |
|----------------------------|------|
| Resolution range used (Å)  | 20–1.96 |
| $R_{cryst}$<sup>c</sup>/$R_{free}$<sup>d</sup> | 20.725/3 |
| No. of reflections used in the refinement | 125,073 |
| No. of reflections used to compute $R_{free}$ | 12,440 |
| No. of non-hydrogen atoms  |      |
| Protein                    | 13,936 |
| Solvent                    | 707   |
| Argininosuccinate          | 80    |
| Mean B factor (Å²)         |      |
| Protein                    | 30    |
| Per monomer: A/B/C/D       | 28/33/28/30 |
| Per domain<sup>e</sup>: Domain 1 | 28/34/38/43 |
| Domain 2                   | 23/27/22/25 |
| Domain 3                   | 43/45/32/33 |
| Sovent                     | 29    |
| Argininosuccinate<sup>f</sup> | 39/47/39/39 |

<sup>a</sup> Last resolution shell extends from 2.03 Å to 1.96 Å.
<sup>b</sup> $R_{sym} = \sum |I - \langle I \rangle|/\sum I$, where I is the measured intensity for symmetry related reflections and $\langle I \rangle$ is the mean intensity for the reflection.
<sup>c</sup> $R_{cryst} = \sum |F_{o} - \langle F_{o} \rangle|/\sum |F_{o}|$; $R_{free} = \sum |F_{o} - \langle F_{o} \rangle|/\sum |F_{o}|$, where "<sup>f</sup>" refers to a subset of data not used in the refinement, representing 10% of the total number of observations.
<sup>e</sup> Domain A: residues N terminus to 112, Domain 2: residues 113–372, Domain 3: 371 to C terminus.
<sup>f</sup> Argininosuccinate molecules bound in the four active sites were labeled AS1 to AS4.
ble in magnitude to the detection limit of the assay. These mutants were, therefore, considered to be inactive. Mutations T161S and T281V led to a partial loss of activity. The T161S mutation had no effect on $K_m$ and decreased slightly the $K_{cat}$, whereas for the T281V mutant an increase in $K_m$ and a decrease in $K_{cat}$ were observed. The S29A mutation had only a minor effect on activity, because both $K_m$ and $K_{cat}$ values were similar to that found for the wt docr2 protein (Table II).

**FIG. 1.** Comparison of a representative number of amino acid sequences of the ASL/fumarase C superfamily. Amino acids residues that show 100%, 80% or greater, or 60% or greater conservation are shaded in black, dark gray, and light gray, respectively. The figure was prepared using the program GeneDoc (47). Abbreviations used: CRD1, $\delta$1 crystallin; CRD2, $\delta$2 crystallin; ARLY, argininosuccinate lyase; PUR8, adenylosuccinate lyase; FUMC, fumarase C; ASPA, aspartase; and PCAB, 3-carboxy-cis,cis-muconate lactonizing enzyme. The following letters represent the species names abbreviated according to the Swiss-Prot nomenclature (e.g. ANAPL corresponds to Anas platyrhynchos or the domestic duck).
Crystal Structure of S283A dōc2 Mutant with Bound Argininosuccinate—S283A dōc2 has the same overall architecture as that described previously for other δ crystallin (15, 17, 19, 26) and human ASL (18, 42) structures (Fig. 2). The N terminus of each monomer appears to be flexible, because there is generally poor quality electron density for these residues, and, as a consequence, variable numbers of residues (17 to 18) at the N terminus and also residues 466–468 at the C terminus are missing in each monomer. Ser-283 belongs to the most conserved amino acid region in the ASL superfamily (C3 in Fig. 1). The C3 residues map to a highly flexible region, denoted the 280s loop, which is often difficult to locate in electron density maps (19, 43). In the S283A dōc2 structure, the loop was modeled in all four monomers, although the quality of the electron density in this region varied from reasonably good (monomers C and D) to poor (monomers A and B). The electron density corresponding to the residue at position 283 indicates the presence of an alanine side chain, in agreement with the mutagenesis results.

The location of the active site cleft in the ASL superfamily was first suggested from the wild type tōc1 structure (15) and later confirmed by structural studies of fumarase C (20, 21, 44) and H162N dōc2 (19). In the present structure, argininosuccinate was bound in all four active sites of S283A dōc2. As seen in Fig. 3A, the [Fo]–[Fc] omit maps unambiguously identified the location and orientation of the substrate. Although the electron density for the arginine moiety is very good for all four substrate molecules, it is of variable quality for the fumarate moiety. The interactions between the enzyme and bound substrate are shown for active site C in Fig. 4. Similar interactions occur in the other active sites.

### DISCUSSION

The S283A dōc2 Active Site—The presence of bound substrate in all four active sites was unambiguously determined by the good quality of the electron density in these regions (Fig. 3A). The substrate establishes a number of interactions with active site residues (Fig. 4), and the importance of these interactions will be discussed in the context of the site-directed mutagenesis results. The conformation of the substrate in the S283A dōc2 structure differs from that seen previously in the H162N dōc2 structure (Fig. 3B). The variation in the conformation of argininosuccinate, especially the fumarate moiety, is thought to be the consequence of the different mutations present in the two structures. In wild type dōc2, His-162 forms a charge relay with Glu-296 (26). In the H162N structure, the side chain of Asn-162 does not interact with Glu-296, and the
resulting structural perturbation is, therefore, likely to alter how the fumarate moiety of the substrate binds to this mutant. The H162N mutation is not expected to interfere with the binding of the arginine moiety of the substrate and indeed the conformation of this part of the substrate is comparable in both structures (Fig. 3B). The S283A mutation does not interfere with substrate binding, because Ala-283 is ~7 Å from the substrate in the S283A ðc2 structure. The S283A ðc2 structure therefore represents a better model of how the substrate binds to the active site.

Conformational changes induced by argininosuccinate binding were investigated by comparing the wild type and the S283A ðc2 structures. As shown in Fig. 5A, residues in the 25–34 and 76–91 loops of domain 1 shift ~2 Å toward the substrate. These loops have previously been hypothesized to be important for substrate binding as the conformational changes observed between the enzymatically inactive ðc1 and the active ðc2 are localized to these regions (26). The S283A mutation has very little effect on the conformation of the 280s loop (Fig. 5A), because in both wild type ðc2 and the mutant structure, the 280s loop is in a very similar conformation. A number of water molecules become more structured upon substrate binding.

Characterization of AS Binding—There are a number of residues in the active site that interact either directly or via a water molecule with the substrate (Figs. 4 and 6A). Although the roles of Tyr-323, Ser-114, Arg-115, Asn-116, and His-91 have been investigated previously (17, 27), no information was available on the role residues Ser-29, Asp-33, Asp-89, Arg-238, Thr-281, Asn-291, Asp-293, Lys-325, Asp-330, and Lys-331 play in substrate binding. The current mutagenesis study found that most of the mutations of putative substrate binding residues had a significant impact on the enzymatic activity. The following discussion analyzes the results of site-directed mutagenesis in the light of the structural data provided by the S283A ðc2 structure.

The carboxyl end of the arginine moiety of the substrate is anchored in the active site by direct interactions with residues Lys-331 and Tyr-323 and indirect interactions with Asp-330 and Arg-238 (Figs. 4 and 5A). Lys-331 is hydrogen-bonded to the O atom of the argininosuccinate, and, when this interaction is abolished, as would be the case in the K331Q mutant, there is no detectable ASL activity (Table II). The N atom of the substrate binds to the protein's active site. The N atom of Lys-331 appears to be "locked" into the proper orientation for substrate binding by its interactions with Asp-330 (Fig. 4). Asp-330 is, in turn, hydrogen-bonded to Arg-238 and Lys-331.
All participate in coordinating a water molecule (W53). This water molecule interacts directly with the substrate (Fig. 4). This network of interactions appears to be crucial for substrate binding, because both the D330N and R238Q mutants are also enzymatically inactive (Table II). Any mutation in these residues will have a negative effect on the local environment and hence on the enzymatic activity, indicating the combined importance of the interactions between the substrate and this segment of the protein.

Direct interactions with Ser-29 and Glu-326, and indirect interactions with Asp-33, Asp-89, and His-91 (Figs. 4 and 6A) anchor the amino end of the arginine moiety (N41). Asp-33 binds the substrate via a water molecule and when mutated to asparagine, an increase of ~70-fold in $K_m$ was observed (Table II). In contrast, although Ser-29 interacts directly with the substrate (Fig. 4), the replacement of the hydroxyl group has only a minor affect, because the S29A mutant has almost wild type ASL activity (Table II). The main chain of Ser-29, which belongs to the 25–34 loop, may play a more important role, because it establishes main-chain interactions with Gln-88, a residue in the neighboring 76–91 loop. The maintenance of the interactions between these two conformationally flexible loops, both of which move on substrate binding (Fig. 5A), have been suggested to be important for catalysis to occur (19, 26). The N41 atom of the substrate also interacts via a water molecule (W219) with Asp-89 and His-91. His-91 and Asp-89 in turn interact via their Nδ1 and Oδ1 atoms, respectively (Fig. 4). The importance of residues in the 76–91 loop for substrate binding is confirmed by the D89N mutation that renders the protein inactive (Table II), and the H91N mutation that results in a loss of 90% of the ASL activity (8, 17). Asp-89 is also within hydrogen bonding distance of Arg-115, a residue that has been proposed to stabilize the flexible tail of argininosuccinate by establishing hydrophobic interactions with the aliphatic region of the arginine moiety (19, 27). The C1, C2, and N2 atoms of the arginine moiety are also stabilized by van der Waals interactions with the phenyl ring of Tyr-323. Although the hydroxyl group of Tyr-323 also forms a hydrogen bond with the O52 atom of the arginine moiety (Fig. 4), it is the phenyl group that is the greater binding determinant (27).

The active site of ASL/δ2 crystallin lies at the interface of three different monomers. All the residues involved in binding the substrate’s arginine moiety belong to the same monomer (e.g. monomer C in Fig. 4). The arginine moiety of the substrate is unique to the ASL/δ crystallin family and differs from the substrates used by other members of the ASL/fumarase superfamily. Given that these substrate-binding residues are highly conserved in various ASLs but not across the superfamily (Fig. 1), it is anticipated that these residues are responsible for defining the substrate specificity of ASL and by extension each superfamily member.

Residues belonging to three different monomers are responsible for binding the guanidine end of the substrate’s arginine moiety as well as its fumarate moiety. These residues are more conserved across the superfamily as would be expected given that the fumarate moiety is common to all substrates. Interpreting the site-directed mutagenesis results for residues Asn-116, Thr-161, His-162, Thr-281, Asn-291, Asp-293, and Lys-325 (Fig. 4) is complicated by the fact that any small local structural perturbation may result in the failure to correctly bind and orient this part of the substrate (19). This would affect catalysis, because the ability of the enzyme to catalyze the substrate will depend on the precise positioning of this end of argininosuccinate. Asn-116 belongs to the conserved region C1 (Fig. 1) and its main-chain N atom is stabilized by interactions with Ser-114. The Nδ2 atom of Asn-116 forms hydrogen bonds with the Oδ1 and Oδ2 atoms of the fumarate moiety, whereas the Oδ1 atom of Asn-116 interacts with the guanidine atoms N2 and N1 (Fig. 4). N1 is part of the scissile bond (N1–Co). The importance of the interactions between the substrate and this residue were highlighted by our previous mutagenesis results where we found that mutation of Asn-116 to Leu abolished enzymatic activity (27). In the present study, we mutated Asn-116 to Asp and found that the mutant still exhibits residual activity (Table II). The N116D mutant has an unfavorable effect on both $K_m$ (28-fold increase) and $V_{cat}$ (28-fold decrease) with the net effect of reducing the catalytic efficiency of the enzyme to less than 1% of the wild type δc2 protein (Table II). This reduction in activity is probably the result of repulsion between the Oδ2 of the Asp-116 side chain and the Oδ1 and Oδ2 atoms of the fumarate moiety.

Lys-325 interacts via a water molecule with the N3 atom of the guanidine moiety of the substrate. This residue is conserved in the ASL family but not across the superfamily (Fig. 5).
The N3 atom also interacts directly with Arg-115 (see above). The K325N mutation has a significant effect on substrate binding as shown by the 72-fold increase in $K_m$ and the 6-fold decrease in $k_{cat}$ (Table II). The presence of the two positively charged residues, Lys-325 and Arg-115, in the vicinity of the N3 atom suggests a weaker than expected positive charge or no charge on this atom of the argininosuccinate. Lys-325 also establishes a hydrogen bond with Asp-293 from a neighboring monomer. Asp-293, in turn, interacts via a water molecule with the O/H9253 atom of the substrate. When Asp-293 is mutated to asparagine, only 1% of relative catalytic efficiency is maintained. This mutation appears to affect $k_{cat}$ more than $K_m$ (Table II). However, the absence of a direct or indirect contact with the N1 atom of the scissile bond and the lack of conservation of this residue across the superfamily, strongly suggests that Asp-293 is involved in substrate binding rather than catalysis. To help position the carboxyl group of Asp-293, the O/H9254 atom interacts with Thr-281. When this threonine is mutated to valine, 21% of enzymatic activity is retained (Table II). This result suggests that the identity of this side chain may not be critical and that the importance of this residue may be the interaction that its main chain O makes with the N/H9254 of Asn-291. Asn-291 interacts both directly and via a water molecule with the fumarate moiety of the substrate (Fig. 4). The importance of Asn-291 is highlighted by the N291L mutation, which completely abolishes activity (Table II). This residue also belongs to the C3 signature sequence and is one of the few residues that is strictly conserved across the superfamily (Fig. 1).

His-162 and Thr-161 appear to play a dual role. Not only are these residues implicated in catalysis (see next section) but they also help anchor the carboxyl groups of the substrate’s fumarate moiety. His-162 is within hydrogen bonding distance of the O/H92 atom of the fumarate moiety, whereas Thr-161 interacts with the substrate’s O/H92 atom. In the inactive mutants H162N (25), H162E (Table II), T161V (27), and T161D (Table II) these interactions would be disrupted. It is, however, difficult to assess whether these disruptions or the interference with other interactions between His-162 or Thr-161 and the substrate is the cause for loss of activity. What is clear from the results presented in this study is that a complex network of interactions is involved in substrate binding. This network is comprised of both active site residues and structured water molecules. Small changes to these residues, even remote ones that are not directly involved in substrate binding, have a significant effect on the catalytic activity of the protein.

**Roles of Active Site Residues in Catalysis**—We have used site-directed mutagenesis and a structural comparison of S283A dS2 and dS1 with argininosuccinate and sulfate bound, respectively, to investigate the roles of Thr-161, His-162, Glu-296, Ser-283, and Lys-289 in catalysis. His-162 has previously been hypothesized to be involved either directly (15, 17) or via a water molecule (21) in abstracting a proton from the C/H92 atom. The charge-relay interaction of His-162 with Glu-296 adds additional support to the hypothesis that His-162 is the catalytic base (21). In the S283A structure however, His-162 is close not only to the C/H atom of the fumarate moiety but also to the Cα atom (3.24 Å) and O/H atom of the substrate.
Mutagenesis of either His-162 or Glu-296 has a significant effect on catalysis. Replacement of His-162 with either asparagine (25) or glutamate (Table II) completely abolishes activity, whereas the E296Q (27) and E296D (Table II) mutants have 0.01% catalytic activity or no activity, respectively. The effect of each of these mutations is expected to be similar to that observed in the H162N structure where the charge-relay interaction between His-162 and Glu-296 was disrupted and the local environment perturbed (19). Although the mutagenesis data has highlighted the importance of His-162 and Glu-296 and the interaction between them, the role of His-162 as the catalytic base is disputed by the observation that this histidine is not completely conserved in the superfamily. This histidine is replaced by a glutamine in all aspartases and by tryptophan in CMLEs (Fig. 1). Given the proximity of the Nε2 of His-162 to the Co atom of the substrate and the partial negative charge on His-162 due to its interaction with Glu-296, His-162 may play an alternative role. His-162 might induce a repulsive force that helps in the second part of the reaction, namely the cleavage of the Nε-Cα bond of the negatively charged argininosuccinate intermediate.

Examination of the S283A structure reveals that Thr-161 is 3.52 Å from the Cβ atom of the fumarate moiety. Thr-161 is highly conserved across the superfamily, and could also be the potential catalytic base. In the adenylosuccinase family a serine residue occasionally replaces Thr-161 (Fig. 1). When this conservative substitution was reproduced in dδc2, the T161S mutant retained almost wild type enzymatic activity (Table II).

In contrast, when Thr-161 was mutated to aspartate (Table II) or valine (27), both substitutions completely abolished activity. When the active site of the S283A dδc2 structure was examined, we found that there are steric constraints that limit the type of residue at this location. Even the replacement of threonine with valine, a fairly isomer substitution, appears to be unfavorable. Small perturbations in this region would interfere with substrate binding in the orientation observed in the structure. The activation of Thr-161’s hydroxyl oxygen for proton abstraction could potentially be accomplished by its interaction with Lys-289, the only positively charged strictly conserved residue in the active site. In the S283A dδc2 structure this interaction is mediated by a water molecule (W197) (Fig. 4) whereas in the dδc1 structure this water is displaced and Thr-161 is hydrogen bonded directly to Lys-289 (Fig. 5B). In the dδc1 structure the 280s loop is in the closed conformation. In the dδc1 structure Thr-161 is also closer to the Cβ atom of the fumarate moiety (2.85 Å). The orientation of the 280s loop seen in the dδc1 structure represents a conformational change that we have hypothesized occurs during catalysis in ASL/dδc2 (26).

Closure of the loop would sequester the substrate from the solvent. The putative role of Lys-289 and the S283A structure support this hypothesis. Lys-289 is proposed to stabilize the negatively charged carbanion intermediate (15, 16). The importance of Lys-289 is highlighted by its mutation to arginine in E. coli aspartase, which results in only 0.3% of the enzymatic activity being retained (16). Examination of the S283A structure reveals that the Nε atom of Lys-289 is 6 Å from the Cβ atom of the substrate. In contrast, in the dδc1 structure where the 280s loop is in the closed conformation, the Nε atom of Lys-289 is 3.5 Å from the Cβ atom (Fig. 5B), in a position to stabilize the negative charge on the carbanion intermediate.

There is currently no consensus across the superfamily regarding the identity of the catalytic acid. We have previously suggested that Ser-283 may be involved in proton donation (26). We have shown in this report that the S283A mutant is capable of binding argininosuccinate (Fig. 3A), indicating that this residue is not involved in the initial substrate-binding event. The S283A dδc2 structure also reveals that the 280s loop is in the open conformation, with the Cβ atom of Ala-283 ~7.75 Å from the Cβ atom of the substrate. In contrast, in the dδc1 structure where the 280s loop is in the closed conformation, the Oγ atom of Ser-283 is only 4.47 Å from the substrate (Fig. 5B).

Ser- or Ala-283 are also on the opposite side of the substrate from His-162 and Thr-161, as required for the trans β-elimination reaction (45).

To further investigate the role of Ser-283, we generated four addition mutants (Table II). Even the more conservative changes of Ser-283 to Cys or Thr as well as the less conservative substitutions to Asp or His, had drastic effects on the enzymatic activity. The fact that all mutants analyzed were inactive reinforces the strict requirement for a serine residue at this position and suggests that Ser-283 plays a critical role in catalysis. We cannot exclude, however, the possibility that Ser-283 may be responsible for maintaining the structural integrity of the 280s loop. If this is the case, then another residue might be responsible for acid catalysis, although no obvious candidate can be identified from the current study. Alternatively, if the leaving group arginine is neutral, there may be no need for an acid catalyst as the solvent could provide the required proton. This hypothesis is supported by the observation that there are no negative charges surrounding the substrate’s guanidine nitrogen atoms but rather water molecules and neutral (Asn-116, Tyr-323) or positively charged (Arg-115, Lys-325) residues (Figs. 4 and 6A). The lack of negative charge in this region could also aid product release.

Conclusions—The structural and mutagenesis studies presented here show that a complex network of interactions is responsible for binding the argininosuccinate substrate. This network involves not only amino acids that directly interact with the substrate but also residues that help orient their side chains and residues that interact via water molecules with the substrate (Figs. 4 and 6A). The results suggest the following enzymatic mechanism (Fig. 6). After substrate binding, Thr-161 or His-162 abstract a proton from the Cβ atom of argininosuccinate to form the carbanion intermediate. Either during or following the base catalysis step, the 280s loop closes over the active site and sequesters the substrate from the solvent. The negative charge developed on the substrate is stabilized by Lys-289. The movement of the 280s loop, together with the change in hybridization state of the substrate’s Ca and Cβ atoms from sp3 to sp2, place Ser-283 in a position to donate a proton to the N1 atom. After proton donation, the unstable negatively charged Ser-283 will need to be reprotaded and this might be the driving force for the loop to return to the open conformation, where Ser-283 could acquire a proton from the solvent. The loop movement would also allow the products, fumarate and then arginine, to be released (46). Alternatively, if there is no requirement for an acid group, an uncharged arginine would be produced, and the solvent could donate the proton required to produce charged arginine (Fig. 6).

This study highlights the complexity of substrate binding and the effects that small perturbations can have on catalysis. A detailed catalytic mechanism has been proposed that we are currently testing.

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Mutational Analysis of Duck δ2 Crystallin and the Structure of an Inactive Mutant with Bound Substrate Provide Insight into the Enzymatic Mechanism of Argininosuccinate Lyase
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