Recovery of Argininosuccinate Lyase Activity in Duck δ1 Crystallin†

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ABSTRACT: δ-Crystallin, the major soluble protein component in the avian eye lens, is homologous to argininosuccinate lyase (ASL). Two δ-crystallin isoforms exist in ducks, δ1- and δ2-crystallin, which are 94% identical in amino acid sequence. While duck δ2-crystallin (dδc2) has maintained ASL activity, evolution has rendered duck δ1-crystallin (dδc1) enzymatically inactive. Previous attempts to regenerate ASL activity in dδc1 by mutating the residues in the 20s (residues 22−31) and 70s (residues 74−89) loops to those found in dδc2 resulted in a double loop mutant (DLM) which was enzymatically inactive (Tsai, M. et al. (2004) *Biochemistry 43*, 11672−82). This result suggested that one or more of the remaining five amino acid substitutions in domain 1 of the DLM contributes to the loss of ASL activity in dδc1. In the current study, residues Met-9, Val-14, Ala-41, Ile-43, and Glu-115 were targeted for mutagenesis, either alone or in combination, to the residues found in dδc2. ASL activity was recovered in the DLM by changing Met-9 to Trp, and this activity is further potentiated in the DLM-M9W mutant when Glu-115 is changed to Asp. The roles of Trp-9 and Asp-115 were further investigated by site-directed mutagenesis in wild-type dδc2. Changing the identity of either Trp-9 or Asp-115 in dδc2 resulted in a dramatic drop in enzymatic activity. The loss of activity in Trp-9 mutants indicates a preference for an aromatic residue at this position. Truncation mutants of dδc2 in which the first 8, 9, or 14 N-terminal residues were removed displayed either decreased or no ASL activity, suggesting residues 1−14 are crucial for enzymatic activity in dδc2. Our kinetic studies combined with available structural data suggest that the N-terminal arm in ASL/δ2-crystallin is involved in stabilizing regions of the protein involved in substrate binding and catalysis, and in completely sequestering the substrate from the solvent.

Crystallins account for the majority of soluble proteins in the eye lens where they contribute to its structural and refractive properties. Two categories of crystallins exist: the ubiquitous crystallins (α,β,γ) which are found in all vertebrate lenses, and the taxon- or species-specific crystallins. Taxon-specific crystallins are not specialized lens proteins, but were recruited to their role from a variety of metabolic enzymes through a process termed gene sharing (*1*). This phenomenon involves the acquisition of a novel secondary function by a gene usually through a modification in gene expression without loss of the primary function or duplication of the gene (*2*). Molecular evolution through gene sharing differs from the more widely recognized view that gene duplication, either complete (*3*) or partial (*4*) is required for the evolution of a new function. After recruitment of the enzyme to the lens, duplication of the gene can occur in response to evolutionary pressures or adaptive conflicts.

δ-Crystallin is uniquely expressed in the eye lens of birds and reptiles and is directly related to the enzyme argininosuccinate lyase (ASL).[1] Following the recruitment of ASL to the eye lens in ducks, a duplication event occurred resulting in two tandemly arranged δ-crystallin genes, δ1 and δ2-crystallin. The two isoforms are 94% identical in amino acid sequence[2] and 69 and 71% identical to human ASL (hASL), respectively. In humans, ASL is expressed predominantly in the liver, where it is involved in the

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[1] Abbreviations: ASL, argininosuccinate lyase; hASL, human argininosuccinate lyase; dδc2, duck δ2-crystallin; dδc1, duck δ1-crystallin; CX, conserved amino acid region X, where X corresponds to 1, 2, or 3; 211-chimera, a hybrid duck δ-crystallin protein, consisting of domain 1 from the active duck δ2-crystallin isoform and domains 2 and 3 from the inactive duck δ1-crystallin isoform; DLM, duck δ1-crystallin double loop mutant, where residues 22−31 and 74−89 have been mutated to those of duck δ2-crystallin; dδc2-TRUN (1−X), duck δ2-crystallin truncation mutant with residues 1−X removed, X corresponds to 8, 9, or 14; eASL, *E. coli* argininosuccinate lyase; ASU, asymmetric unit; rmsd, root-mean-square deviation.

[2] The amino acid numbering used throughout the text is that of dδc1. dδc2 has a two-residue insert at position 5, causing the amino acid numbering to be shifted by two relative to hASL and dδc1. In this paper to avoid confusion when comparing structurally equivalent residues, we have used the dδc1 numbering for both the dδc1 and dδc2 isoforms. When referring to the eASL protein, the numbering will be according to the eASL sequence which is shifted by four and six relative to hASL/dδc1 and dδc2, respectively, due to the shorter N-terminus in the protein.

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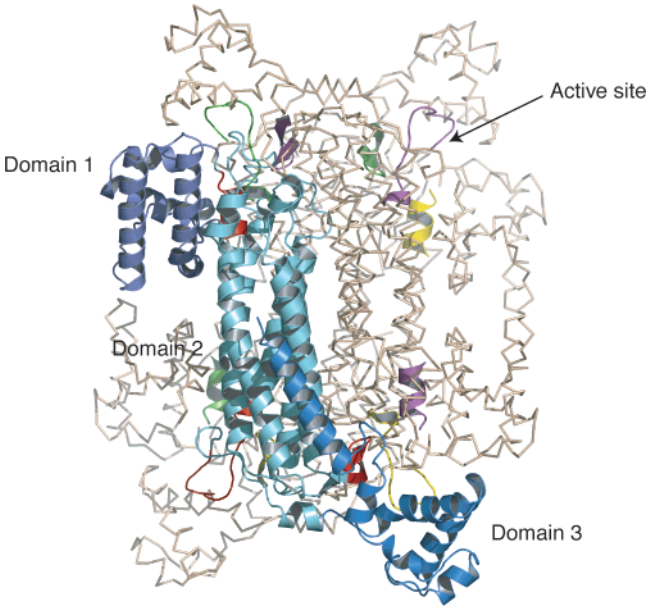

FIGURE 1: The structure of the DLM (*33*) showing the representative quaternary structure of the δ-crystallin proteins. Monomer A is shown in ribbon representation, with each structural domain colored in a different shade of blue. Domains 1−3 encompass residues 1−110, 111−361, and 362−466. In monomer A, the three regions of conserved amino acid residues are shown in red (C1 = 112−119, C2 = 157−166, C3 = 280−294). The conserved regions in each of the other monomers are shown in yellow, violet, or green. PyMol was used for figure preparation (*44*).

formation of urea. Although birds are uricotelic, the ASL/δ2 protein is found at lower levels outside of the duck lens where it acts as an enzyme in arginine synthesis.

ASL/δ2-Crystallin belongs to a superfamily of homotetrameric enzymes which includes class II fumarase (*5*), adenylosuccinate lyase (*6*), L-aspartase (*7*), and 3-carboxy-*cis*, *cis*-muconate lactonizing enzyme (*8*). Although these enzymes share only 15−30% overall amino acid sequence identity, there exists three regions of highly conserved amino acid residues (denoted C1−C3). Crystal structures are available for all members of the superfamily (*5*, *7*, *9*−*12*) and reveal a common fold, with each monomer comprised of three structural domains (Figure 1). The three conserved regions are found spatially remote in the monomer, but come together in the tetramer to form four multisubunit active sites. Each active site is found at the interface of three different monomers, with each monomer contributing residues from a different conserved region (Figure 1). Residues in the conserved regions of the superfamily members have been implicated in the acid−base mechanism of the enzymes (*13*−*24*).

The ASL/δ2-crystallin reaction is initiated by abstraction of a proton from the Cβ position of the substrate. Inhibition studies with a nitro-analogue of argininosuccinate have provided evidence for the formation of a carbanion intermediate in the reaction pathway (*25*). Subsequently, proton donation to the guanidinium nitrogen of argininosuccinate results in cleavage of the Cα−N bond and product release. Given that the reaction occurs with *trans*-stereochemistry (*26*), two separate groups are required for proton abstraction and donation. From extensive structural and biochemical studies, Ser-281 (*20*, *27*) and His-160 (*21*, *28*−*30*) have been implicated as the catalytic acid and base in the ASL/δ2-crystallin reaction mechanism, respectively.

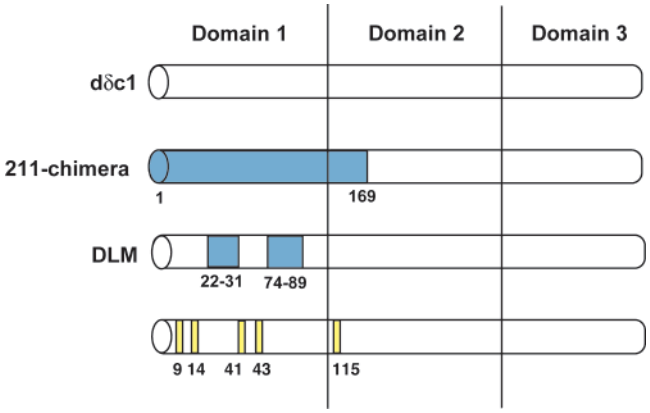

FIGURE 2: Schematic representation of dδc1, the 211-chimera, and the DLM. The residues in the 211-chimera or DLM which correspond to those found in dδc2 are colored blue, with the residue numbers indicated below. Domain 1 of the 211-chimera extends from residues 1−169, due to the restriction enzyme used to generate the protein (*32*). The positions of the remaining amino acid differences between the DLM and 211-chimera are shown in the final panel. Adapted from Tsai et al. (*33*).

Despite the fact that dδc1 and dδc2 differ by only 27 amino acid residues, only dδc2 has been shown to exhibit ASL activity (*31*). Therefore, the two isoforms represent an interesting system for investigating factors that contribute to both loss and gain of function in the protein, thus, providing us with insight into the enzymatic mechanism of ASL/δ2-crystallin. We had previously hypothesized that the dδc1 protein is enzymatically inactive due to its inability to bind the substrate, the result of amino acid substitutions in the 20s and 70s loops (residues 22−31 and 74−89, respectively). This hypothesis was based on the findings that: the putative catalytic residues are conserved between dδc1 and dδc2; 17 out of 27 amino acid differences between dδc1 and dδc2 reside in the N-terminal domain (domain 1); a protein consisting of domain 1 from dδc2 and domains 2 and 3 from dδc1 (211-chimera) (*32*) exhibited a relative catalytic efficiency of 29%; and structural comparisons of different δ-crystallin proteins revealed the largest conformational variability was localized to residues 22−31 and 74−89, the 20s and 70s loops. Consequently, a dδc1 double loop mutant (DLM) was constructed in which the residues which differed between dδc1 and dδc2 in the 20s and 70s loops were mutated to those found in dδc2. Although the DLM was expected to exhibit ASL activity, the protein was found to be enzymatically inactive, and while structurally similar to dδc2, isothermal titration calorimetry revealed that the DLM, like dδc1, is unable to bind argininosuccinate (*33*).

The DLM and 211-chimera differ by only five amino acid residues at positions 9, 14, 41, 43, and 115 (Figure 2). These residues are Met, Val, Ala, Ile, and Glu in the DLM/dδc1, respectively, and Trp, Ser, Gly, Met, and Asp in the 211-chimera/dδc2, respectively. The absence of enzymatic activity in the DLM must therefore be attributed to one or more of the amino acid substitutions at these positions. In an attempt to restore ASL activity in the DLM, the residues at positions 9, 14, 41, 43, and 115 were mutated either alone or in combination to those found in dδc2. Interestingly, the single Met-9 to Trp mutation was sufficient to recover ASL activity in the DLM. Furthermore, the activity of this DLM-M9W mutant is potentiated when Glu-115 is changed to Asp. The precise roles of Trp-9 and Asp-115 in wild-type dδc2

Table 1: Kinetic Parameters and *T*m Values

mutant	V_{max} ((μmol/min)/mg)	K_m (mM)	k_{cat} (s^{-1})	catalytic efficiency k_{cat}/K_m (s^{-1} M^{-1})	relative catalytic efficiency (%)	*T*m[a] (°C)
dδc2 wild type	0.980(±0.023)	0.045(±0.005)	3.266(±0.076)	7.35(±1.70) × 10^4	100	67
211-chimera	0.752(±0.023)	0.119(±0.017)	2.508(±0.023)	2.13(±0.14) × 10^4	29.0	57
			DLM Mutants			
M9W	1.162(±0.120)	2.724(±0.441)	3.874(±0.400)	0.144(±0.089) × 10^4	2.0	71
M9W/V14S	1.338(±0.051)	3.159(±0.183)	4.459(±0.170)	0.142(±0.093) × 10^4	1.9	63
M9W/E115D	1.075(±0.035)	0.294(±0.032)	3.585(±0.118)	1.252(±0.372) × 10^4	17.0	71
M9Y/E115D	0.738(±0.029)	0.610(±0.089)	2.460(±0.096)	0.404(±0.109) × 10^4	5.5	65
M9F/E115D	0.154(±0.017)	0.212(±0.043)	0.513(±0.026)	0.242(±0.060) × 10^4	3.3	65
A41G/I43M	inactive					72
I43M	inactive					71
E115D	inactive					69
			dδc2 Mutants			
W9A	0.040(±0.001)	0.112(±0.025)	0.155(±0.090)	0.136(±0.020) × 10^4	1.9	67
W9M	0.024(±0.001)	0.115(±0.026)	0.081(±0.005)	0.076(±0.019) × 10^4	1.0	67
W9R	0.044(±0.002)	0.069(±0.017)	0.147(±0.082)	0.220(±0.050) × 10^4	3.0	67
W9Y	1.188(±0.022)	0.106(±0.008)	3.960(±0.073)	3.640(±0.949) × 10^4	49.5	67
W9F	0.273(±0.009)	0.111(±0.015)	0.909(±0.032)	0.833(±0.209) × 10^4	11.3	67
D115A	2.529(±0.047)	0.255(±0.016)	8.431(±0.156)	3.306(±0.958) × 10^4	45.0	53
D115E	2.559(±0.035)	0.273(±0.012)	8.532(±0.116)	3.134(±0.920) × 10^4	42.6	71
TRUN[1−8]	0.661(±0.015)	0.083(±0.007)	2.204(±0.049)	2.713(±0.676) × 10^4	36.9	67
TRUN [1−9]	0.047(±0.002)	0.105(±0.018)	0.157(±0.006)	0.151(±0.032) × 10^4	2.1	67
TRUN [1−14]	inactive					69
			dδc1 Mutants			
M9W	inactive					69
M9W/E115D	inactive					67

[a] Temperature at which signal is half lost at 222 nm.

were examined using site-directed mutagenesis. While the N-terminal arm (residues 1−15) is conformationally flexible and has not been observed in any of the δ-crystallin structures determined to date (*20*, *21*, *27*, *33*, *34*), previous results (*35*, *36*) and the site directed mutagenesis and N-terminal truncation mutants (dδc2-TRUN [1−8], [1−9], and [1−14], respectively) presented here suggest that this region of ASL/δ2-crystallin is important for enzymatic activity. Our biochemical results together with the recently determined structure of *Escherichia coli* ASL (eASL) (*37*), suggest that the N-terminal arm in dδc2 is required for stabilizing regions of the protein involved in substrate binding and/or catalysis, and completely sequestering the substrate from solvent during catalysis.

MATERIALS AND METHODS

Construction of Site-Directed and Truncation Mutants. The Quick Change Site-Directed Mutagenesis method (Stratagene, La Jolla, CA) was used to construct all mutants in Table 1. To generate the truncation mutants, an additional *NcoI* site was engineered into the wild-type dδc2 gene (*32*) prior to the codon corresponding to Trp-9, Gly-10, or Gly-15 in the translated product. In each case, the dδc2-pET-3d vector containing the new restriction site was digested with the enzyme *NcoI*, resulting in three fragments. The fragments corresponding to the truncated dδc2 gene and empty pET-3d vector were recovered using a gel extraction kit (Qiagen), and the fragments were ligated together at the *NcoI* sites by incubating at 10 °C for 4 h with T4 DNA ligase. DNA sequencing (ACGT Corporation and The Center for Applied Genomics at The Hospital for Sick Children, Toronto) confirmed the presence of all desired mutations.

Protein Expression, Purification, and Kinetic Analysis. The C-terminal His-tagged proteins were expressed and purified as previously described (*32*). The eluted protein (15 mL) was collected in one fraction and dialyzed overnight at 4 °C against 4 L of buffer A (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA). All proteins were >95% pure as determined by SDS−PAGE (data not shown).

The mutants were assayed for ASL activity by monitoring at 25 °C the production of fumarate at 240 nm (ϵ = 2.44 mM^{-1} cm^{-1}) (*32*). Stock solutions of disodium argininosuccinate (Sigma) were prepared ranging from 0.2 to 20 mM. The stock solutions were subsequently diluted so that the final concentration in the reaction mixture (800 μL) ranged from 0.02 to 2.0 mM. The reactions were initiated by adding 10 μg of protein. In some instances where low levels or no ASL activity was detected, the assay was repeated with up to 200 μg of protein. All assays were performed in triplicate. The initial velocities were averaged and normalized to the amount of protein used. Kinetic parameters, K_m (mM) and V_{max} (μmol min^{-1} mg $enzyme^{-1}$) were obtained by fitting the data to the Michaelis-Menten equation using Sigma Plot (SSPM, Chicago, IL).

Circular Dichroism Spectroscopy. Circular dichrosim (CD) experiments were performed on an AVIV CD spectropolarimeter (model 62A DS). All scans were performed between 200 and 260 nm with a path length of 0.1 cm on protein solutions of 0.4 mg/mL in buffer A. The CD signal was normalized to the amount of protein used. To examine the thermal stability of each protein, the loss of ellipticity at 222 nm was monitored as the protein samples were heated from 25 to 101 °C. The temperature was increased in 2 °C increments, with 1 min preequilibration between readings.

Amino Acid Sequence Alignments and Structural Comparisons. Amino acid sequences were retrieved from the SWISS−PROT database, and Clustal W (*38*) was used for

the multiple sequence alignment. Structural alignments were performed with PROFIT (version 6.0) written by G. D. Smith. Structurally equivalent residues in five of the helices in domain 2 of the proteins were chosen and subjected to an iterative least-squares fitting procedure. The residues chosen for the δ-crystallin proteins were 124−142, 175−195, 238−262, 294−312, and 327−345. The numbering for the structurally equivalent residues in eASL is shifted by four due to the shorter N-terminus in this protein.

RESULTS

DLM and dδc1 Mutants. The inactive DLM differs from the active 211-chimera by only five residues in domain 1. These are Met-9, Val-14, Ala-41, Ile-43, and Glu-115 in the DLM/dδc1 and Trp, Ser, Gly, Met, and Asp, respectively, in the 211-chimera/dδc2 (Figure 2). Thus, the residues at the five positions were mutated either alone or in combination in the DLM to the residues found in the 211-chimera/dδc2. The two-amino acid insert at position five in dδc2 is not found in any other ASL/δ2-crystallin and is not thought to be important for enzymatic activity.

The mutations I43M, E115D, and A41G/I43M made in the background of the DLM did not lead to any detectable ASL activity (Table 1). A relative catalytic efficiency of ∼2% is measured for both DLM-M9W and DLM-M9W/V14S. The k_{cat} values for these mutants are similar to the wild-type enzyme, but a 60- and 70-fold increase in K_m is observed for DLM-M9W (2.72 mM) and DLM-M9W/V14S (3.15 mM), respectively. When the mutations M9W, M9Y, and M9F are made in the background of the DLM-E115D mutant, relative catalytic efficiencies of 17, 5.5, and 3.3% are observed, respectively.

No ASL activity is detected when the M9W and M9W/E115D mutations are made in the context of the dδc1 protein.

dδc2 Site-Directed Mutants. To investigate the role of Trp-9 in the active dδc2 protein, this residue was changed to Ala, Met, Arg, Tyr, and Phe. Mutating Trp-9 to Ala, Met, or Arg, resulted in mutant proteins with catalytic efficiencies that were <3% of the wild-type enzyme. For all three mutants, an increase in K_m is observed (1.5−2.5-fold), while a concomitant 22 (W9A and W9R)- and 40 (W9M)-fold decrease in k_{cat} is observed. When Trp-9 is mutated to the aromatic residues, Tyr and Phe, relative catalytic efficiencies of 49.5 and 11% are measured, respectively. A 2.5-fold increase in K_m is observed for both dδc2-W9Y (0.11 mM) and dδc2-W9F (0.11 mM). While the k_{cat} for dδc2-W9Y (3.96 s^{-1}) is similar to that of the wild-type enzyme, a 3.6-fold decrease is observed for dδc2-W9F (0.91 s^{-1}).

Since the relative catalytic efficiency of DLM-M9W/E115D was 8.5-fold higher than the single DLM-M9W mutant, Asp-115 was also targeted for mutagenesis in dδc2 to assess the importance of this residue for ASL activity in the protein. When Asp-115 is mutated to either Ala or Glu, proteins with relative catalytic efficiencies of 45 and 42.6% are observed, respectively. The K_m is increased ∼6-fold for both dδc2-D115A (0.25 mM) and dδc2-D115E (0.27 mM). The k_{cat} is also increased 2.6-fold for both mutants (8.5 s^{-1}).

dδc2 Truncation Mutants. To investigate the importance of the N-terminal segment in dδc2 for ASL activity, three truncation mutants were constructed in which the first 8, 9, or 14 residues were removed. When the first eight N-terminal residues are deleted in dδc2 (dδc2-TRUN [1−8]), a relative catalytic efficiency of 37% is observed. Removal of only one additional residue (dδc2-TRUN [1−9]) results in a more dramatic drop in ASL activity, with the mutant displaying a catalytic efficiency that is 2.1% of the wild-type enzyme. A 2.3-fold increase in K_m is observed for dδc2-TRUN [1−9] (0.11 mM) while the k_{cat} is decreased 21-fold (0.16 s^{-1}). Removal of five more residues from the N-terminus results in a protein (dδc2-TRUN [1−14]) with only minor residual activity. Low correlation coefficients for the measured rates were obtained for dδc2-TRUN [1−14] even when up to 200 μg of protein was used. Kinetic parameters could not be calculated with confidence, and therefore this mutant was considered to be inactive.

Stability of the Wild-Type and Mutant Proteins. To ensure that the differences in the enzymatic activities of the mutant proteins were not a consequence of conformational changes resulting from the mutations or truncations, the secondary structures and thermal stabilities of the mutant proteins were assessed using circular dichroism. For all proteins, similar spectra with minima at 208 and 222 nm were observed, characteristic of proteins with large α-helical content (data not shown). While not all spectra superimposed, the similar overall shape suggests that neither the mutations nor the truncations significantly altered the secondary structure of the proteins. Variations in the spectra may be the result of small errors in the protein concentration determination. The thermal stabilities of the proteins were investigated by monitoring at 222 nm the change in ellipticity as the temperature was increased from 25 to 101 °C. All transitions from the folded to unfolded state as a function of temperature were found to be cooperative and irreversible due to complete denaturation of the proteins. Although no thermodynamic parameters can be obtained from such irreversible reactions, this serves as a simple method to compare the thermal stabilities of the mutant proteins to that of the wild-type enzyme. The midpoint of transition for the majority of mutant proteins was similar to wild-type dδc2 (65−72 °C) (Table 1), suggesting the thermal stabilities were not grossly affected by the mutations or truncations. The exceptions were the 211-chimera and dδc2-D115A mutant which display decreased thermal stabilities, with transition midpoints of 57 and 53 °C, respectively.

DISCUSSION

Restoration of ASL Activity in the DLM. To determine which amino acid substitutions in domain 1 of dδc1 in addition to those in the 20s and 70s loops are responsible for the loss of ASL activity, a series of single and double mutants of the DLM was constructed in which Met-9, Val-14, Ala-41, Ile-43, and Glu-115 were mutated to the residues found in dδc2 (Table 1). Although the DLM exhibits a relative catalytic efficiency of 2% when Met-9 is mutated to Trp, this is significantly lower than the levels of activity measured for the 211-chimera (29%). Ser-14, Gly-41, Met-43, and/or Asp-115 must contribute in some manner to the enzymatic potential of the 211-chimera relative to the DLM-M9W mutant. The dramatic 8.5-fold increase in catalytic efficiency of DLM-M9W/E115D compared to the single DLM-M9W mutant was somewhat surprising given the conservative nature of the Asp to Glu substitution. This result demonstrates that, although a Trp residue is required at

position 9 for ASL activity in the DLM, the additional Glu-115 to Asp mutation significantly potentiates the activity of the single DLM-M9W mutant. The similar kinetic parameters for DLM-M9W and DLM-M9W/V14S suggest the Val substitution at position 14 does not contribute significantly to the absence of enzymatic activity in the DLM/dδc1. This result was expected as a valine residue is found at the equivalent position in hASL.

Since ASL activity can be restored in the DLM by changing Met-9 to Trp and is further increased when the additional E115D mutation is made, we were interested to see the effect of these mutations in the background of the dδc1 protein. Given that residues in the 20s and 70s loops of dδc2 have been implicated in substrate binding (*20*, *34*), we did not expect that the M9W or M9W/E115D mutations would be sufficient to regenerate enzyme activity in the dδc1 isoform. As anticipated, mutating Met-9 alone or in combination with Glu-115 to the residues in dδc2 did not lead to any detectable ASL activity in dδc1. The latter verifies the importance of the residues in the 20s and 70s loops for substrate binding and thus catalysis. However, mutating the amino acid residues in the 20s and 70s loops of dδc1 to those found in dδc2 are alone insufficient to restore ASL activity in the protein, as exemplified by the DLM. Recovery of enzymatic activity in dδc1 requires the additional mutation of Met-9 to Trp, as demonstrated by the current results.

The Role of Trp-9 in dδc2. A sequence alignment of 89 active ASL/δ2-crystallin proteins from the SWISS−PROT database indicates that the residues corresponding to Trp-9 and Asp-115 in dδc2 are 88 and 100% conserved, respectively. The importance of these residues for ASL activity in dδc2 is further demonstrated by the present mutagenic and kinetic results for the DLM. To gain additional insight into the roles of Trp-9 and Asp-115, these residues were targeted for site-directed mutagenesis in wild-type dδc2.

Mutation of Trp-9 to Met or Ala results in proteins with catalytic efficiencies that are less than 2% of the wild-type enzyme. These results were not so surprising given the nonconservative nature of the substitutions and suggest that the Trp-9 to Met substitution in dδc1 contributes significantly to the loss of activity in the protein. The more conservative substitutions, W9Y and W9F resulted in relative catalytic efficiencies of ∼50 and 11%, respectively. The hydroxyl group in Tyr appears to favor enzymatic activity. To further test the preference for Tyr over Phe at position 9, two additional DLM mutants were constructed in which Met-9 was changed to either Tyr or Phe in the background of DLM-E115D. The residue preference at position 9 in DLM-E115D was also determined to be Trp > Tyr > Phe. Interestingly, the sequence alignment of ASL/δ2-crystallins demonstrates that, in species where a tryptophan is not found at this position, the substitution is limited to Tyr or Arg. The only exception is ASL from *Halobacterium sp.* where a glycine is found. All ASLs with the Trp to Tyr or Arg substitution are from archaeal species. The Trp to Arg substitution is specific to ASLs from five anaerobic methanogens and one from an anaerobic sulfur-metabolizing organism. Charge−charge interactions have been shown to increase protein stability through the formation of salt bridges. The presence of polar residues has also been associated with improved thermal stability in proteins (*39*), and a strong preference for Tyr over Trp has been observed in thermophilic proteins in contrast to their mesophilic counterparts (*40*). The presence of Tyr or Arg in place of Trp may result in a more thermostable protein, a property required for the extreme conditions in which archaea are often found. However, in contrast to the relatively high levels of ASL activity observed for dδc2-W9Y, the dδc2-W9R mutant exhibited a catalytic efficiency of only 3% relative to the wild-type enzyme, suggesting a positively charged residue is not tolerated at this position in dδc2.

Interpretation of the site-directed mutagenesis results for Trp-9 is complicated by the limited availability of structural information. In all δ-crystallin structures determined to date (*9*, *20*, *21*, *27*, *34*), the first 15−17 N-terminal residues could not be modeled due to weak or absent electron density. However, in the structures of the Q286R hASL (*35*) and *E. coli* ASL (eASL) (*37*) enzymes, electron density of sufficient quality has allowed the N-terminal segment to be partially modeled. The contribution of Trp-9 and the N-terminal arm to the enzymatic activity of dδc2 is discussed below.

The Role of Asp-115 in dδc2. Asp-115 belongs to the first conserved region (C1, residues 112−119) and is absolutely conserved across all sequences of active ASL/δ2-crystallin proteins. In the present study, a >50% drop in catalytic efficiency is observed when Asp-115 is mutated to Glu or Ala in dδc2. The role of Asp-115 can be explained in light of the dδc2-S281A−substrate complex (*20*). Residues 113−115 are found at the beginning of one of the core helices in domain 2. This helix is preceded by a loop region formed by residues 109−112. Despite the fact that Asp-115 does not interact directly with the substrate, the neighboring residues Arg-113 and Asn-114 participate in binding the fumarate and guanidinium moieties of argininosuccinate (Figure 3). Ser-112 appears to be important for maintaining the conformation of Asn-114 as its Oγ-atom is within hydrogen-bonding distance (2.9 Å) of the backbone nitrogen atom of Asn-114. In turn, the Oδ1 atom of Asp-115 is observed to hydrogen-bond (3.3 Å) to the backbone nitrogen atom of Ser-112, while its Oδ2 atom interacts via a water molecule with the oxygen atom of Gly-110. Given these interactions, Asp-115 is likely involved in stabilizing the structural transition from loop to helix, residues Arg-113 and Asn-114, and thus ensuring precise positioning of argininosuccinate for catalysis. The fumarate moiety is common to all the substrates catalyzed by the ASL/Fumarase C superfamily, and the residues involved in binding this portion of the substrate are highly conserved. Not surprisingly, mutation of these residues has been shown to negatively impact substrate binding and catalysis (*20*, *41*). Although the charge of the residue is conserved in the dδc2-D115E mutant, the additional methylene group in the glutamate side chain may be sufficient to perturb the local structure of the region. In dδc2-D115A, the interactions contributing to the stabilization of this region would be lost, resulting in a protein with a compromised ability to bind the substrate.

As previously observed (*20*, *41*), the Asp-115 mutants demonstrate that even mutations to residues which do not interact directly with the substrate can have a significant impact on the enzymatic activity of the protein. The ASL/δ2-crystallin active site is complex, given that residues from three different monomers must work in a highly coordinated manner to bind the substrate. A complicated network of interactions involving residues both close to, and remote

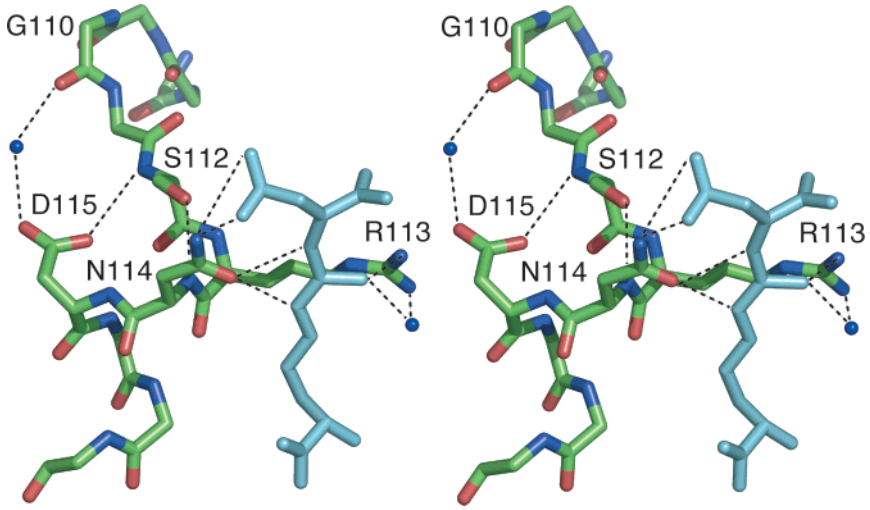

FIGURE 3: Stereoview of the interactions between Asp-115 and neighboring residues as observed in dδc2-S281 (*20*). Side chains are only shown for the indicated residues. The substrate argininosuccinate is shown in cyan. Hydrogen bonds and water molecules are shown as dashed lines and blue spheres, respectively. PyMol was used for figure preparation (*44*).

from, the active site is required for optimal binding and orientation of the substrate for catalysis (*20*). Thus, mutation of any of the residues involved in maintaining this network would be expected to negatively impact the enzymatic potential of the protein.

The N-terminus Is Important for Enzymatic Activity in dδc2. Despite its conformational flexibility, there is evidence to suggest that the N-terminal segment in ASL/δ2-crystallin is important for enzymatic activity in the protein. Our mutagenic results have shown that changing the identity of Trp-9 in dδc2 results in a drastic decrease in ASL activity. Furthermore, the mutation of Arg-12 to Gln in hASL has been identified in a patient with the genetic disease, *argininosuccinic aciduria* (*36*), and the corresponding R12Q hASL mutant exhibits only 10% wild-type activity in vitro (*35*). To investigate the effect of truncating portions of the N-terminal segment on the enzymatic activity of dδc2, three mutants were constructed in which the first 8, 9, or 14 N-terminal residues were deleted.

The sequence alignment of active ASL/δ2-crystallins revealed that several ASLs possessed shortened N-termini relative to dδc2. The ASL proteins with the shortest N-termini are from *Caulobacter crescentus* and *Pyrococcus furiosus*, where residue 2 in each case is equivalent to residue 9 in dδc2. To investigate the importance of the first eight N-terminal residues in dδc2 for ASL activity, a truncation mutant was constructed in which these residues were removed. Given that a relative catalytic efficiency of 37% is observed for dδc2-TRUN [1−8], residues 1−8 appear to be important, although not essential, for enzymatic activity in dδc2. On the other hand, the dδc2-TRUN [1−9] mutant exhibits a relative catalytic efficiency of only 2%, an 18.5-fold decrease relative to dδc2-TRUN [1−8]. This result provides further evidence that Trp-9 plays a crucial role in conferring enzymatic activity to dδc2. As residue 15 is the extent of the N-terminal arm that has ever been observed in the δ-crystallin structures, five more N-terminal residues were removed to generate the dδc2-TRUN [1−14] mutant. Not surprisingly, no ASL activity was detected for this protein. The truncation mutants demonstrate that, although the N-terminal segment in dδc2 is conformationally flexible, the residues in this region of the protein, and in particular Trp-9, contribute significantly to the catalytic potential of dδc2.

Available ASL Structures and Insight into the Role of the N-terminal Arm. The first hASL structure to be solved was a low 3.5-Å resolution structure (*12*), in which the first 19 N-terminal residues could not be modeled due to the absence of electron density for this region of the protein. A high-resolution structure of a Q286R hASL mutant (*35*) was subsequently determined in which residues 6−18 could be modeled for one of the two monomers in the asymmetric unit (ASU). This structure allowed an initial snapshot of the potential conformation of the N-terminal arm in ASL/δ2-crystallin. Trp-9 and Arg-146 are observed to participate in van der Waals interactions, while residues 10−16 formed a loop and were within hydrogen-bonding distance of Arg-141, Thr-142, Tyr-277, Gln-344, and Ser-351 from another monomer (*35*). In the conformation observed in Q286R hASL, the N-terminal arm is in close proximity to the active-site cleft of the second monomer, although it does not make direct interactions with residues forming the active site. In light of this, we had previously hypothesized that amino acid substitutions in this region could result in a conformation that does not allow for substrate binding and catalysis (*35*). In such a scenario, entry of the substrate into the active site may be prevented, and/or movement of the 280s loop and domain 3, events which are hypothesized to be important for catalysis, may be hindered.

Recently, the structure of the wild-type eASL protein was determined (*37*). A 45, 44, and 42% sequence identity exists between eASL and hASL, dδc2, and dδc1, respectively. The eASL structure was solved with two monomers in the ASU, and interestingly, two phosphate molecules were located in one of the two active sites. As a result, significant conformational changes were observed in three regions of the protein which formed this active site: the N-terminus of monomer A, the 280s loop (residues 274−286 in eASL) of monomer B, and domain 3 of symmetry related monomer C. Conformational changes in these regions do not occur in the absence of phosphate, as demonstrated by the second phosphate free active site. The phosphate-bound and -unbound sites will be referred to as the "A" and "B" active sites, respectively, for the remainder of the paper. Movement

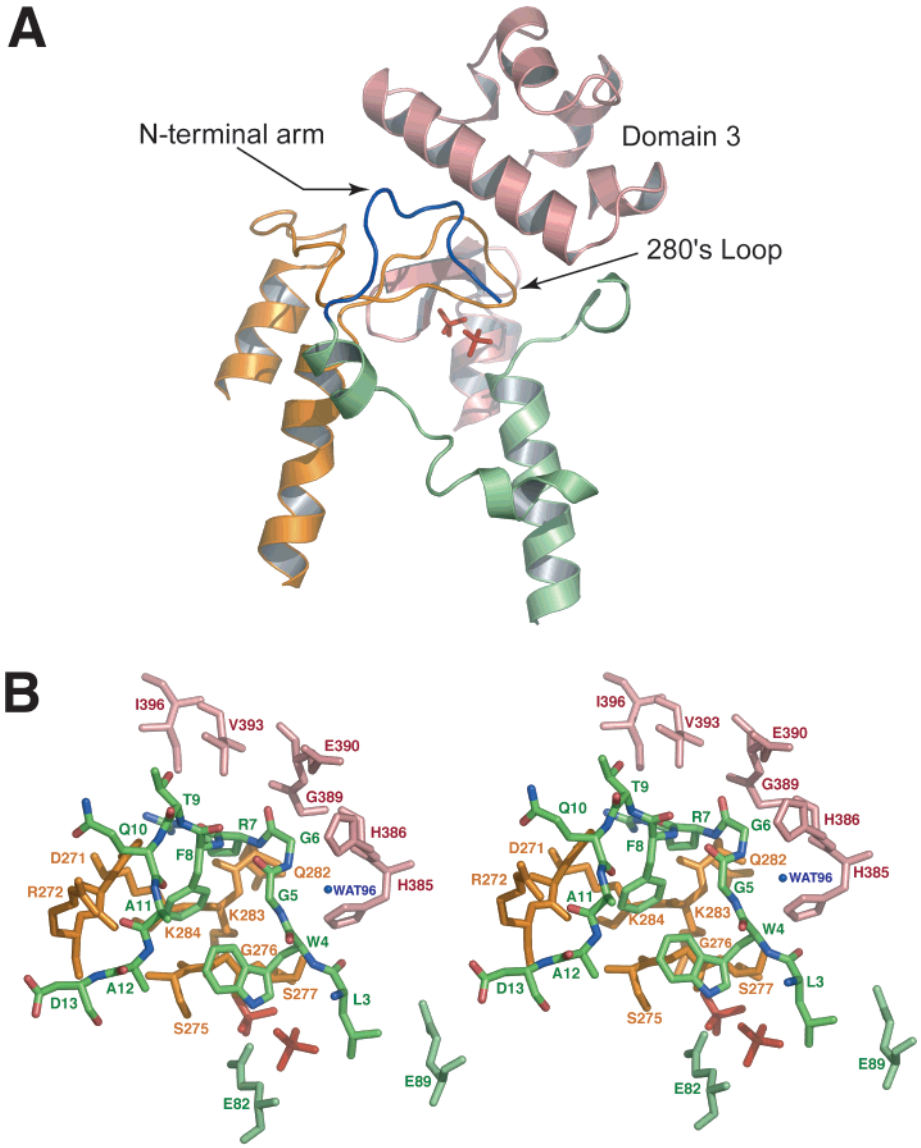

FIGURE 4: (A) The "closed" active site in eASL (*37*). Monomers A, B, and C, which contribute residues to the active site, are colored green, orange, and pink, respectively. The two phosphate ions are shown in red stick representation. The N-terminal arm (residues 3−13) is shown in dark blue for emphasis. (B) Interactions between the N-terminal residues of monomer A and key residues in neighboring monomers B and C in eASL. The orientation and coloring for the monomers is the same as in panel A. Each amino acid is labeled with the one letter code and numbered according to the eASL sequence. The label color for each residue corresponds to which monomer it belongs. PyMol was used for figure preparation (*44*).

of the N-terminal arm, the 280s loop, and domain 3 toward the active site upon phosphate binding results in a "closed" active site (Figure 4), similar to that observed in the sulfate bound dδc1 and DLM proteins (*27*) (Figure 5).

Phosphate binding in active site "A" causes the N-terminus of monomer A to become ordered, allowing residues 3−13 to be modeled (when referring to the eASL protein, the numbering will be according to the eASL sequence which is shifted by four and six relative to hASL/dδc1 and dδc2, respectively, due to the shorter N-terminus in the protein). Only residues 7−13 could be modeled for monomer B, which contributes to the formation of the "B" active site. A structural comparison of eASL and Q286R hASL demonstrates that the conformations of the N-termini in these two proteins are dramatically different (Figure 6). In monomer A of eASL, residues 3−13 are observed to "swing" into the active-site cleft in the presence of phosphate. While the N-terminal arm of the B monomer in eASL is not visible in its entirety, residues 7−13 appear to form an extended helical structure.

The large conformational change in the N-terminal arm of monomer A is coupled with closure of the 280s loop (monomer B) and movement of domain 3 (monomer C) toward the active site (Figure 4). A structural alignment between monomers A and B of eASL reveals Cα rms deviations of up to ~11 and 8 Å for the 280s loop and domain 3, respectively. When the structural alignment is performed with only the third domain independent of the rest of the protein, an average Cα rms deviation of ~2 Å is observed. This suggests that the large conformational movement of domain 3 in eASL is the direct result of phosphate binding. The magnitudes of the conformational changes in the 280s loop and domain 3 in eASL are similar to those observed in dδc1 and the DLM when sulfate is bound (*33*). Movement of these regions has been proposed to be important for sequestering the substrate from solvent (*27*),

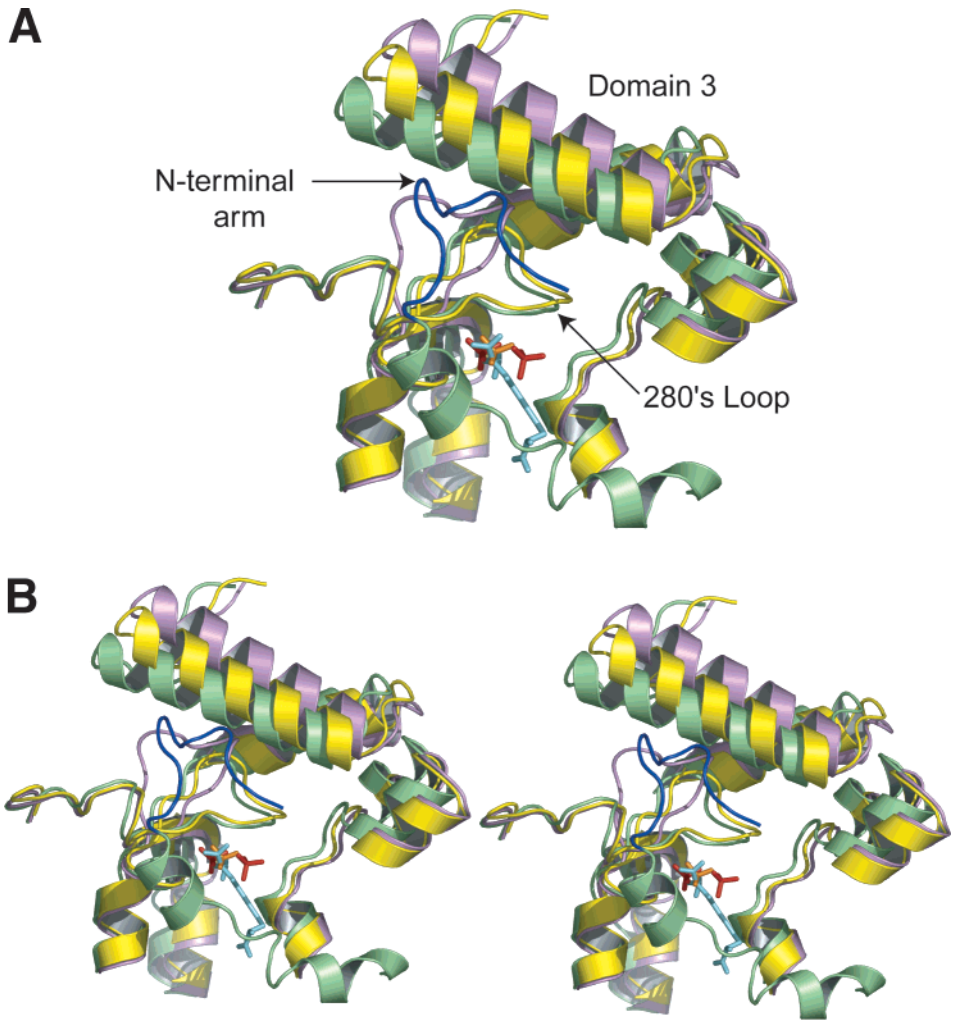

FIGURE 5: (A) Structural alignment of the dδc1 (yellow), dδc2-S281A (pink), and eASL (green) active sites. The N-terminal arm (residues 3−13) of eASL is colored dark blue. Also shown are the relative positions of sulfate (orange), phosphate (red), and argininosuccinate (cyan) in the active sites of the dδc1, eASL, and dδc2-S281A structures, respectively. (B) Stereoview of image in panel A. PyMol was used for figure preparation (*44*).

a phenomenon frequently observed in proteins during catalysis. Furthermore, a structural comparison of eASL and substrate-bound dδc2-S281A demonstrates that the spatial positions of the two phosphate ions and the fumarate moiety of argininosuccinate are very similar (Figure 5). The eASL structure represents the first example of an active ASL/δ2-crystallin protein in which the conformational changes in the 280s loop and domain 3 have been observed. This structure not only reinforces our previous hypothesis that these movements may be relevant to the enzymatic mechanism of ASL/δ2-crystallin but also allows us to draw insight into the conformation and role of the N-terminal arm during substrate binding and catalysis.

In the eASL structure, Trp-4 (Trp-9 in dδc2) is observed to make van der Waals interactions with Ser-275, Gly-276, and Ser-277 from monomer B (Figure 4B). These residues form part of the 280s loop and are absolutely conserved across the ASL/Fumarase C superfamily members. Ser-277 (Ser-281 in dδc2) is the proposed catalytic acid in the ASL/δ2-crystallin enzymatic mechanism (*20*). Furthermore, the Nε1 atom of Trp-4 makes a hydrogen bond with the Oε1 atom of Glu-82. The corresponding residue in dδc2 is Glu-86, which is found in the 70s loop and has been shown to stabilize the guanidine moiety of argininosuccinate via its interaction with Arg-113 (*20*). The importance of the interactions between Trp-4 and putative substrate binding/catalytic residues is exemplified by the dδc2-W9 and truncation mutants.

Residues other than Trp-4 in the N-terminal arm interact with the 280s loop of monomer B. For example, a hydrogen bond is observed between Arg-7 (Nη1) and Gln-282 (backbone oxygen atom). The Cζ atom of Arg-7 is also within van der Waals distance of the Cβ and Cδ atoms of Lys-284. These interactions are probably important for maintaining the conformation of the 280s loop so that Lys-283 is positioned correctly. Lys-283 is invariant across all the superfamily members. The equivalent residue in dδc2, Lys-287, has been proposed to stabilize the carbanion intermediate that is formed after abstraction of the Cβ-proton from the substrate. In the mutant-substrate complexes of dδc2, Lys-287 is the only positively charged residue in the vicinity of the substrate, and its mutation completely abolishes ASL activity in the wild-type enzyme (unpublished data). Hydrogen bonds are also observed between Thr-9 (N-atom) and Asp-271 (Oδ2), Ala-11 (O-atom) and Arg-272 (Nη1), and Asp-13 (N-atom) and Arg-272 (O-atom). Asp-271 and Arg-272 precede the 280s loop in eASL which starts at residue 274. The 280s loop is intrinsically flexible and

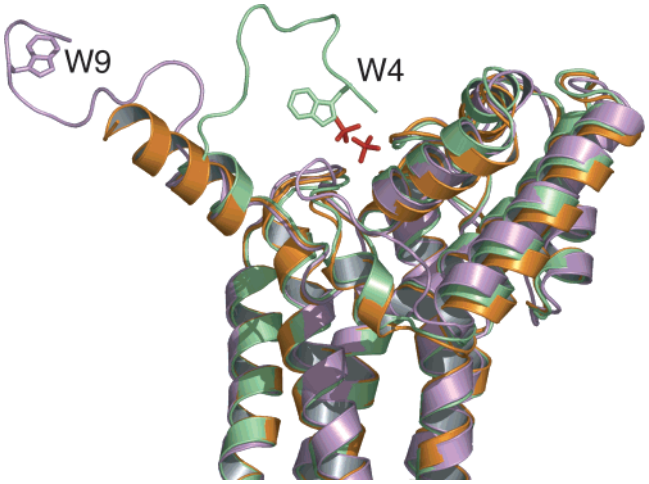

FIGURE 6: Structural superposition of monomer B of Q286R hASL (*35*) (violet) and monomers A (green) and B (orange) of eASL (*37*) highlighting the large conformational difference in the N-termini of the two proteins. Only the upper half of each monomer is shown. The side chains for Trp-9 and Trp-4 are shown for Q286R hASL and monomer A of eASL, respetively. The two phosphate ions bound in the active site of eASL are shown in red stick representation. PyMol was used for figure preparation (*44*).

has been associated with weak or absent density in the structures of various superfamily members (*10*, *21*, *42*, *43*). In eASL, the 280s loop in monomer B appears to be more rigid, as supported by the average *B*-factor of 15.5 Å^2 for this region of the protein, compared to 26.2 Å^2 for monomer A. Interaction of the 280s loop with residues in the N-terminal arm probably contributes to this more stable conformation. Upon catalysis and closure of the 280s loop over the active site, precise placement of residues for their role in the enzymatic mechanism is likely achieved in part through their interaction with the N-terminal arm.

Interactions are also observed between Gly-6 and Arg-7 and residues in domain 3 of monomer C. The backbone nitrogen atom of Gly-6 is within van der Waals distance of the Cδ2 atom of His-386 (3.7 Å), while its main chain oxygen atom makes several contacts with Gly-389 and Glu-390 (3.6−3.7 Å). Gly-6 also interacts indirectly with the Nδ1 atom of His-385 via its hydrogen bond with Wat-96. The Nϵ and Cζ atoms of Arg-7 are within van der Waals distance of the Cδ1 atom of Ile-396, while its backbone oxygen atom is 3.5 Å from the Cγ2 atom of Val-393 (Figure 4B).

The N-terminal residues in eASL appear to be important for stabilizing regions of the protein which contribute to the formation of the active site. The conformations of the 280s loop and domain 3 appear to be particularly influenced, as numerous interactions are observed between the N-terminal arm and residues in these two regions. It is therefore not surprising that removal of Trp-9 and additional residues from the N-terminus of dδc2 would result in a dramatic decrease or complete abolishment of ASL activity. The precise roles of the first eight residues in dδc2, however, remain somewhat elusive owing to the shortened N-terminus in eASL and lack of structural detail for residues 1−3.

Together, the Q286R hASL and eASL structures demonstrate that the N-terminal arm is able to undergo significant conformational movements. We previously hypothesized that substrate binding and catalysis in ASL/δ2-crystallin is accompanied by closure of the 280s loop and movement of

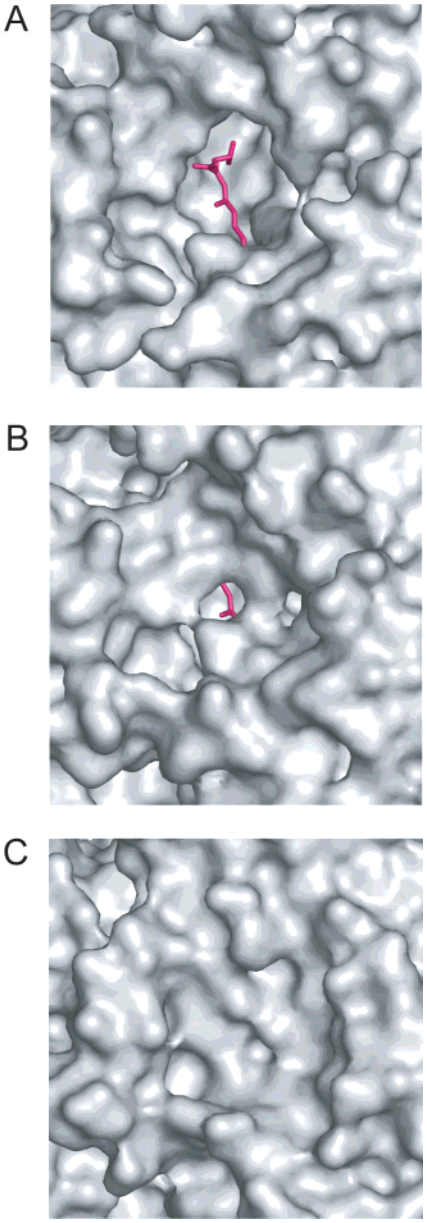

FIGURE 7: Surface representation of the active site regions of (A) dδc2, (B) dδc1, and (C) eASL. Argininosuccinate is shown in magenta. Although present, the substrate is not visible in panel C due to complete sequestration of the substrate in the eASL active site. PyMol was used for figure preparation (*44*).

domain 3 toward the active site (*27*). Surface representations of wild-type dδc1 and dδc2 with modeled argininosuccinate clearly demonstrate that the conformational changes in the 280s loop and domain 3 result in a more closed active site, allowing partial sequestration of the substrate from solvent (Figure 7A,B). The eASL structure suggests that, in addition to these events, the N-terminal arm "swings" into the active-site cleft into a position to help stabilize residues in the 280s loop and domain 3 for their role in substrate binding or catalysis. The consequence of the movement of the N-terminal arm is that the active site is now completely occluded, as shown in Figure 7C. These results suggest that catalysis in ASL/δ2-crystallin requires the complete sequestration of substrate from solvent, which is achieved through the combined conformational movements of the 280s loop, domain 3, and the N-terminal arm. After catalysis, product release likely requires the 280s loop and domain 3 to return

to their unbound or "open" conformations. In this event, the N-terminal arm must undergo another large scale motion to remove it from the active site region, as steric clashes would otherwise occur between the N-terminal residues and those in the 280s loop (Figure 5). This alternative conformation may be one that resembles the conformation of the N-terminal arm in the Q286R hASL structure.

CONCLUSIONS

We have shown that ASL activity can be recovered in the DLM by mutating Met-9 to Trp, the corresponding residue in dδc2. Furthermore, the activity of this single DLM-M9W mutant is significantly potentiated when Glu-115 is changed to Asp. The critical role of these residues in conferring ASL activity to dδc2 is supported by the dδc2-W9 and dδc2-D115 mutants. The truncation mutants demonstrate that, although the N-terminal arm is conformationally flexible in δ-crystallin, this region of dδc2 and, in particular Trp-9, is necessary for ASL activity. The phosphate-bound structure of *E. coli* ASL suggests that, upon substrate binding and catalysis, the N-terminal arm undergoes a significant conformational change in which this region of the protein "swings" into the active site cleft. This action occurs in concert with movement of the 280s loop and domain 3 toward the active site and allows complete sequestration of substrate during catalysis. The extensive interactions between the N-terminal arm and residues in the 280s loop and domain 3 suggest that the N-terminus of ASL/δ2 is critical for stabilization of these regions during substrate binding and catalysis.

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