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Review

Intragenic complementation and the structure and function of argininosuccinate lyase

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Abstract. Argininosuccinate lyase (ASL) catalyzes the reversible hydrolysis of argininosuccinate to arginine and fumarate, a reaction important for the detoxification of ammonia via the urea cycle and for arginine biosynthesis. ASL belongs to a superfamily of structurally related enzymes, all of which function as tetramers and catalyze similar reactions in which fumarate is one of the products. Genetic defects in the ASL gene result in the autosomal recessive disorder argininosuccinic aciduria. This disorder has considerable clinical and genetic heterogeneity and also exhibits extensive intragenic complementation. Intragenic complementation is a phenomenon that occurs when a multimeric protein is formed from subunits produced by different mutant alleles of a gene. The resulting hybrid protein exhibits greater enzymatic activity than is found in either of the homomeric mutant proteins. This review describes the structure and function of ASL and its homologue δ crystallin, the genetic defects associated with argininosuccinic aciduria and current theories regarding complementation in this protein.

Key words. Argininosuccinate lyase; delta crystallin; argininosuccinic aciduria; intragenic complementation.

Introduction

The catabolism of amino acids and proteins produces large amounts of nitrogen in the form of ammonia. Ammonia is a highly toxic metabolite that is excreted by organisms in three different ways. Their water environment allows aquatic organisms to excrete ammonia directly in low enough concentrations to dilute its toxicity, while terrestrial organisms must convert their waste nitrogen to the nontoxic components, uric acid or urea [1]. Mammals are ureotelic animals and release their excess nitrogen as urea, which is easily excreted in the urine.

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The cyclic process of urea biosynthesis was first elucidated in 1932, when Hans Krebs and Kurt Henseleit implicated ornithine, citrulline, and arginine as participants in the synthesis of urea from aspartate and carbon dioxide [2]. Five enzymes are involved in the complete urea cycle, and the individual reaction catalyzed by each enzyme is shown in figure 1.

The first two enzymes of the cycle, carbamoyl phosphate synthetase I (CPS, EC 6.3.4.16) and ornithine transcarbamylase (OCT, EC 2.1.3.3), are mitochondrial matrix enzymes expressed almost exclusively in the liver [3–5]. This tissue-dependent expression localizes urea synthesis to this organ. Carbamoyl phosphate synthetase I is the only enzyme in the urea cycle with a regulatory cofactor and it catalyzes the formation of

one carbamoyl phosphate molecule from ammonium and bicarbonate at the expense of two ATP molecules [6]. The enzyme is catalytically active as a monomer with a molecular weight of 165 kDa [7–9], but in the absence of its allosteric activator, *N*-acetyl glutamate, the enzyme exists in a monomer–dimer equilibrium [10]. The second enzyme, ornithine transcarbamylase, is a trimer of identical 38-kDa subunits [11, 12]. Citrulline, the product of the OCT reaction, is exported out of the mitochondria to the cytosol [13–15] by facilitated diffusion through an ornithine/citrulline antiporter. Enzyme localization experiments and experiments with labeled substrates and intermediates indicate that the urea cycle operates as a metabolon spanning the two compartments with considerable channeling of intermediates from one enzyme to the next [16–18]. The three remaining enzymes, argininosuccinate synthetase (ASS, EC 6.3.4.5), argininosuccinate lyase (ASL, EC 4.3.2.1), and arginase (EC 3.5.3.1), are cytosolic. ASS and ASL function as homotetramers with monomer molecular weights of 46 and 50 kDa, respectively [19–21]. Human liver arginase is a trimer of identical 35-kDa subunits [22, 23]. Unlike the CPS and OTC enzymes, ASS, ASL, and arginase are expressed in a wider range of tissues.

The enzymes of the urea cycle are not limited to ureotelic animals but are ubiquitous in all organisms [24]. In mammalian tissues where urea synthesis does not occur, and in nonureotelic organisms, the primary role of these enzymes is the biosynthesis of arginine from citrulline and aspartate. Indeed, the urea cycle is suggested to have evolved from the addition of arginase to this preexisting arginine biosynthetic pathway [24].

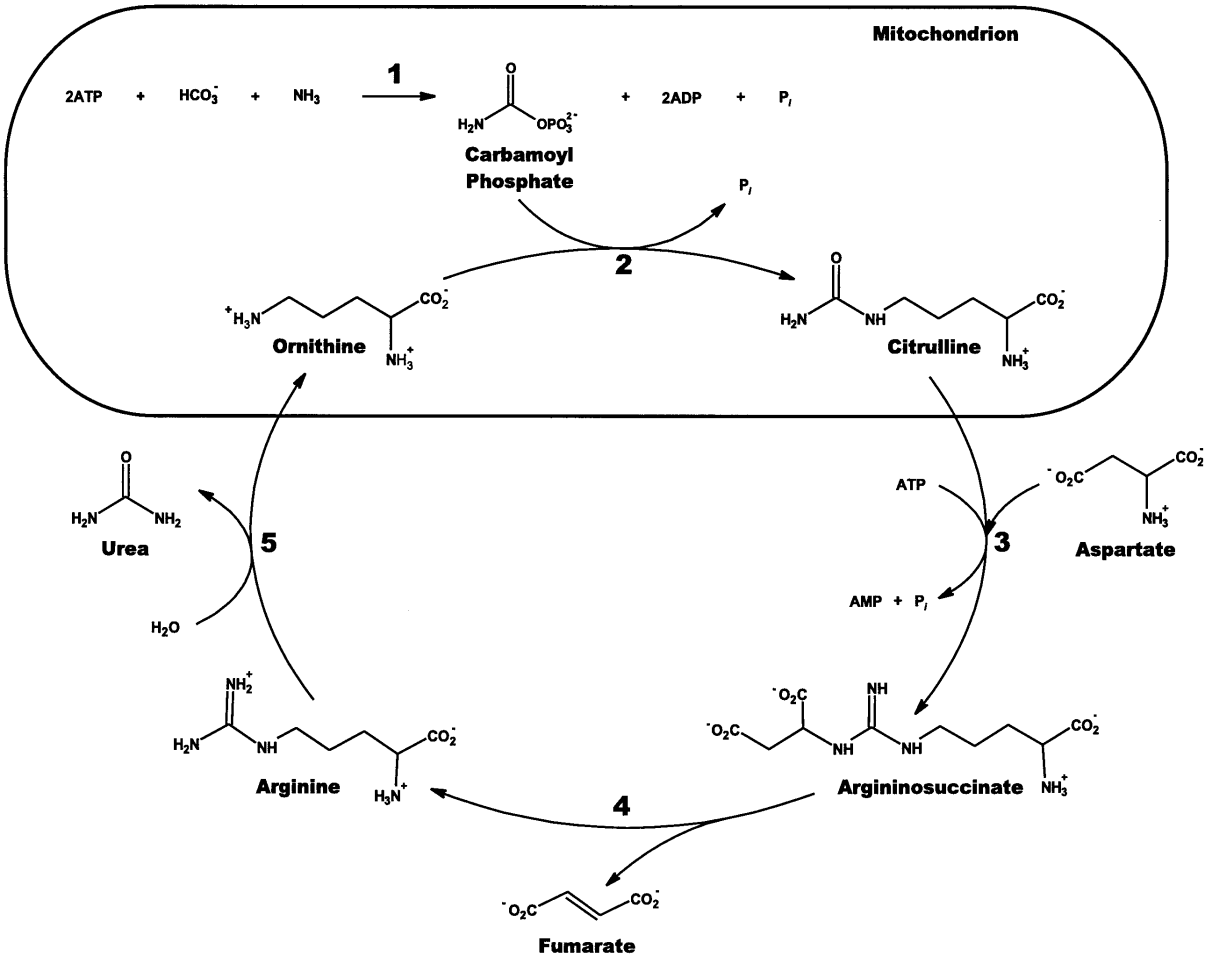


Figure 1. The enzymes of the urea cycle and their reactions. Carbamoyl phosphate synthetase I (1) and ornithine transcarbamylase (2) are mitochondrial matrix enzymes, while argininosuccinate synthetase (3), argininosuccinate lyase (4), and arginase (5) are cytosolic.

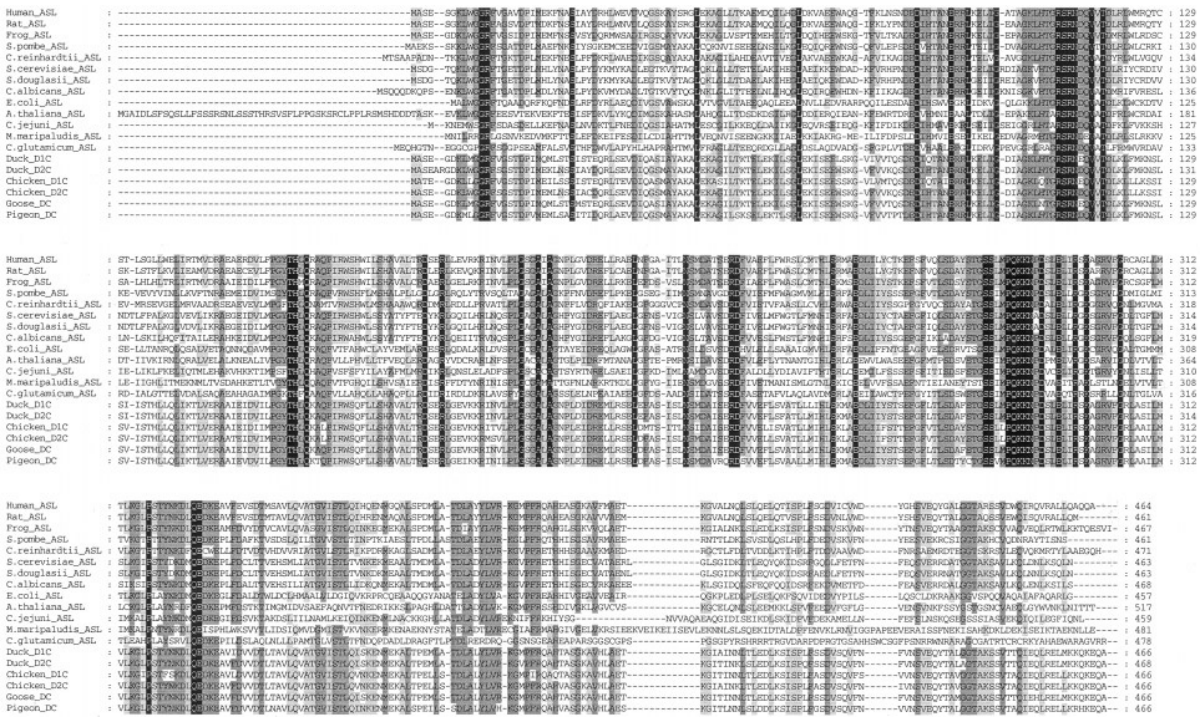


Figure 2. Multiple sequence alignment of ASL species. The alignment shading corresponds to 100% (black), 80% or higher (dark gray), and 60% or higher (light gray) amino acid sequence identity. ASL, argininosuccinate lyase; DC, δ crystallin; D1C and D2C, two different isoforms of duck and chicken δ crystallin: δ1 and δ2 crystallin, respectively. The alignment was performed using the program ClustalW [128].

Arginine production in nonhepatic tissues is important not only for protein synthesis but also for nitric oxide (NO) production. NO is a key cell-signaling molecule that has been found to elicit tumoricidal [25, 26], antiviral [27], bactericidal, and fungistatic [28] effects in the host defense system. NO is also a potent vasodilator, and overproduction of NO is therefore not entirely advantageous. Excess NO production is responsible for the hypotension associated with septic and cytokine-induced circulatory shock [29, 30]. NO is produced by the conversion of arginine to citrulline by nitric oxide synthetase (NOS) [31]. The rate-limiting factor for NO synthesis is the availability of arginine [32] and while possible sources of cellular arginine include uptake from plasma and intracellular protein degradation, the preferred source is its de novo biosynthesis from citrulline. The two urea cycle enzymes, ASS and ASL, in conjunction with NOS form the citrulline-NO or arginine-citrulline cycle, and hence provide the cell with a continuous source of cellular arginine for NO production.

This review focuses on the structure and function of ASL and the genetic defects in the ASL gene that result in the disease, argininosuccinic aciduria.

Argininosuccinate lyase

ASL was first described by Ratner and colleagues [33–35] as the second enzyme involved in the conversion of citrulline to arginine. The gene for ASL has now been identified from a variety of species including *Escherichia coli* [36], *Saccharomyces* [37–39], algae [40], amphibia [41], human [42], and rat [43, 44]. Overall, the amino acid sequences share approximately 42.9% identity (fig. 2). In all cases where the protein has been expressed and purified, the enzyme has been found to be active as a tetramer of identical subunits, with each monomer a single polypetide between 49–52 kDa [20, 45–49]. In humans, although the protein is expressed predominantly in the liver where it participates in urea synthesis, it is also found in skin fibroblasts [20], erythrocytes [50], kidney [51], pancreas and muscle [52], heart [53], and the brain [54, 55].

Bioautography in human-mouse somatic cell hybrids has located the gene for ASL to the pter $\rightarrow$ q22 region of human chromosome 7 [56]. The gene contains 16 exons and is approximately 35 kb in length. A clone for the human enzyme was identified by screening a cDNA library with antibodies specific for ASL [42]. The 1565-base pair clone had an open reading frame of 463

amino acids with a predicted molecular weight of 51.6 kDa.

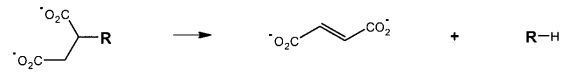
Kinetic properties

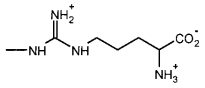
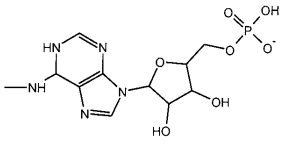
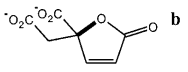
Human liver ASL was purified to near homogeneity in 1981 by O'Brien and Barr [20]. The enzyme exhibits normal Michaelis-Menten kinetics with specific activities of 10.3 μmol/min per milligram and 8.0 μmol/min per milligram for the forward and reverse reactions, with K_m values of 0.20 mM, 5.3 mM, and 3.0 mM for argininosuccinate, fumarate, and arginine, respectively.

Studying the positional isotope exchange of the ASL-catalyzed cleavage of ^{15}N-labeled argininosuccinate established that although the dissociation of products from the tertiary enzyme complex in the forward reaction is random and not rate limiting, fumarate is released approximately ten times faster than arginine [57]. In the reverse reaction, citrulline and succinate were found to be noncompetitive inhibitors of fumarate and arginine, respectively [58]. The order of addition of fumarate and arginine to the enzyme must therefore be random and the reaction catalyzed by ASL has a random, Uni-Bi mechanism.

The human and bovine enzymes purified from liver tissue have similar kinetic properties and also exhibit negative cooperativity [59]. This negative cooperativity, however, only occurs in phosphate and not in Tris buffer [60] and for the human enzyme also disappears with overnight storage of the enzyme in dilute solutions. The reasons for the dependence of negative cooperativity on the buffer type and the age of the enzyme sample are not known, and whether the observed negative cooperativity is actually due to additional activation sites remains undetermined. The larger K_m for higher concentrations of substrate have been hypothesized as due to a rate-dependent recycling of free enzyme through a series of conformational states [61]. A similar

Table 1. Members of the ASL superfamily and their substrates.



Enzyme	Substrate	R Group
Argininosuccinate Lyase δ Crystallin [a]	argininosuccinate	
Fumarase	malate	
Aspartase	aspartic acid	
Adenylosuccinate Lyase	adenylosuccinate	
3-Carboxy-*cis,cis*-muconate Lactonizing Enzyme	4-carboxymuconolactone	 b

[a] Argininosuccinate lyase and δ crystallin catalyze the same reaction.

[b] The reaction catalyzed by 3-carboxy-*cis,cis*-muconate lactonizing enzyme is a ring opening reaction. The bond shown in **bold** is cleaved. Fumarate is not released.

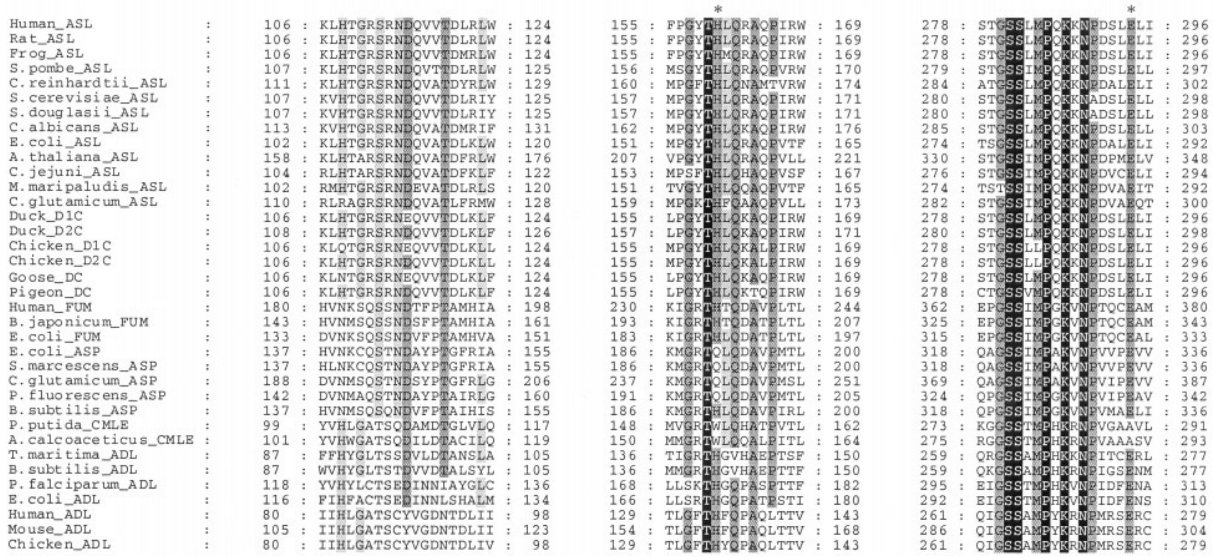


Figure 3. Consensus sequences of the ASL superfamily. The alignment shading corresponds to 100% (black), 80% (dark gray), and 60% (light gray) amino acid sequence identity. ASL, argininosuccinate lyase; DC, δ crystallin; ADL, adenylosuccinate lyase; CMLE, 3-carboxy-*cis*,*cis*-muconate lactonizing enzyme; FUM, fumarase; ASP, ammonia-aspartate lyase. See legend of figure 2 for definition of D1C and D2C. The alignment was performed using the program ClustalW [128]. The '*' represents the putative catalytic residues.

hypothesis has been suggested for fumarase, another member of the ASL superfamily. Fumarase also exhibits an increase in K_m with increasing substrate concentration [61]. Further study of this phenomenon is complicated by the fact that expressed recombinant ASL protein does not exhibit negative cooperativity [62–66].

ASL superfamily

ASL belongs to a superfamily of enzymes, which for the most part catalyze the cleavage of a C–N or C–O bond with the release of fumarate as one of the products (table 1). Other members of the family include class II fumarase [67], adenylosuccinate lyase [68], L-aspartase [67, 69], 3-carboxy-*cis*,*cis*-muconate lactonizing enzyme (CMLE) [70] and δ crystallin [42, 71, 72]. The overall amino acid sequence similarity between these enzymes is low, with a percent identity of approximately 15%. However, three regions of highly conserved residues across the superfamily have been identified as consensus sequences (fig. 3). These consensus sequences were suggested to be involved in the catalytic mechanism of these enzymes [73], a hypothesis that has now been confirmed with the structure determination of a number of members of the superfamily [64, 73–77].

Structure

The crystal structures of five members of the ASL superfamily [64, 73–78] reveal that all its members share a common protein fold (fig. 4). Each protein has a D_2 symmetric arrangement of monomers, with each monomer composed of three structural domains. Each domain is predominately α helical. In ASL and δ crystallin (fig. 4a, c, respectively) domains 1 and 3 have similar topologies consisting of two helix-turn-helix motifs stacked perpendicularly to each other. The central domain is composed of one small β sheet and nine α helices, five of which form a helical bundle arranged coaxially in an up-down-up-down-up topology. Three of these five helices from two monomers interact to form a closely associated dimer held together by mainly hydrophobic interactions. Two such dimers associate to form the tetramer with one helix of each monomer interacting at the core to form a four-helix bundle (fig. 4b). The less extensive interactions observed between the dimers agree with the experimental observations that tetrameric ASL undergoes cold dissociation via a dimer intermediate [79].

Active site cleft

The three superfamily consensus sequences are spatially removed from one another in the monomer (fig. 4a,

c–f) but come together at each of the four 'corners' of the tetramer to form four active site clefts (fig. 4b). Three different monomers contribute a different consensus sequence to each active site. This cleft was first identified as the putative active site in the structure of turkey $\delta 1$ crystallin [73] and was later confirmed when inhibitor- and substrate analogue-bound complexes of fumarase C [76, 77] and $\delta 2$ crystallin [80] were determined.

δ crystallins

Among all of the enzymes in the superfamily, ASL is the most closely related to δ crystallin with an amino acid sequence identity of 64–71% between human ASL and the various δ crystallins [72, 81]. Crystallins are a diverse family of water-soluble proteins found as structural components in the ocular lens of vertebrates. They are classified as either ubiquitous (α, β, γ) or taxon specific (ε, τ, δ, etc.). The taxon-specific crystallins are believed to have evolved from the recruitment to the lens of preexisting metabolic enzymes by a process called 'gene sharing' [72, 82–84]. This is a phenomenon whereby the same gene product functions as both a lens crystallin and as an enzyme in nonlens tissues. Hybridization studies provide strong evidence that this evolutionary relationship exists between the δ crystallins of avian and reptilian eye lenses and ASL [72]. After the recruitment of ASL to the lens, subsequent gene duplication and specialization resulted in two nonallelic, tandemly arranged δ crystallin genes (5′-$\delta 1$-$\delta 2$-3′) that code for two different isomers [85–87]. $\delta 1$ crystallin is catalytically inactive whereas $\delta 2$ crystallin has retained endogenous ASL activity [72,

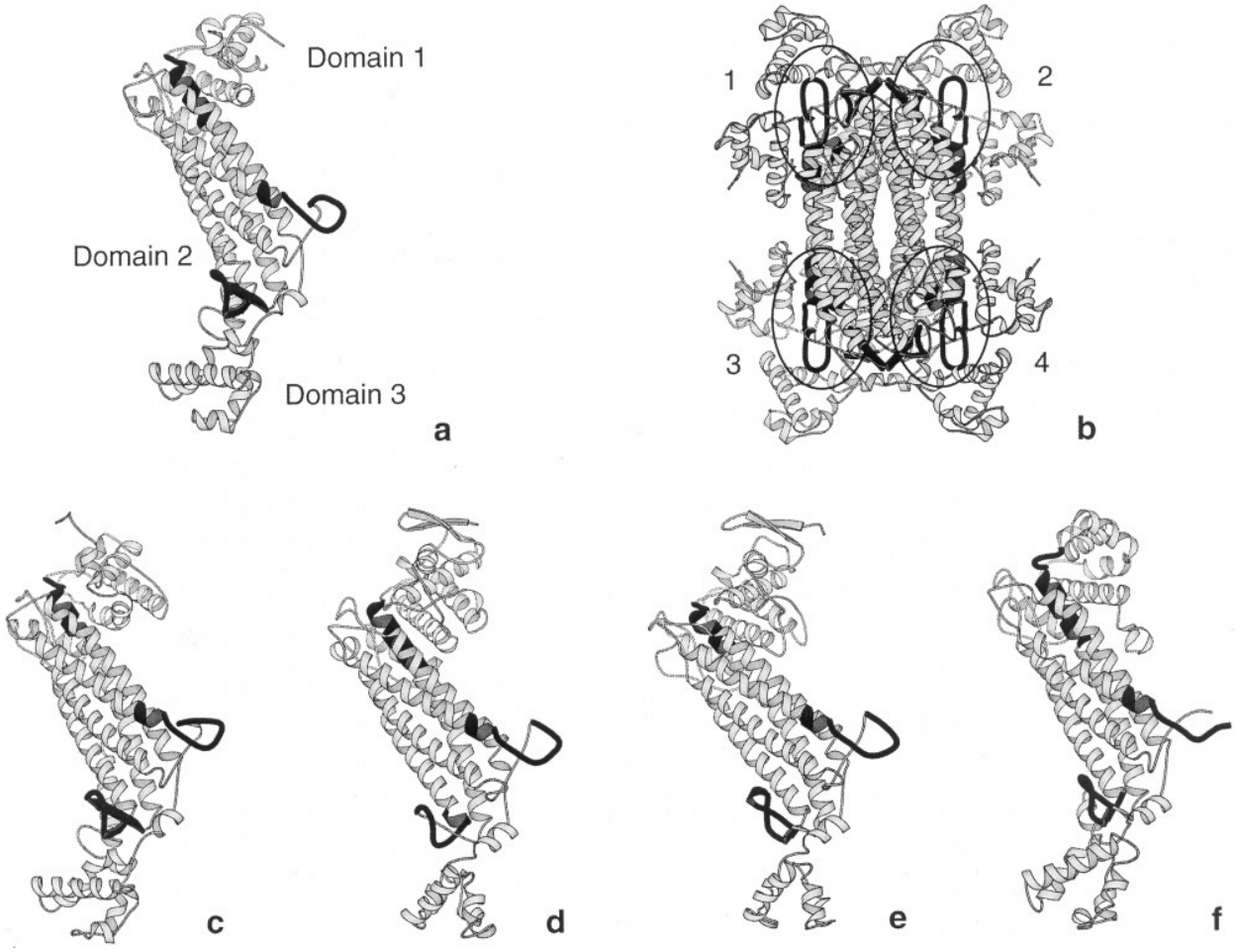


Figure 4. Schematic representation of the ASL monomer (*a*), ASL tetramer (*b*), and the turkey $\delta 1$ crystallin (*c*), fumarase (*d*), aspartase (*e*), and adenylosuccinate lyase (*f*) monomers. The highly conserved consensus sequences shown in figure 3 are colored black in each panel. In (*b*) the active tetrameric form of the ASL protein is depicted. The circles represent the location of the four active sites, numbered 1–4. This figure was prepared using the program Molscript [129].

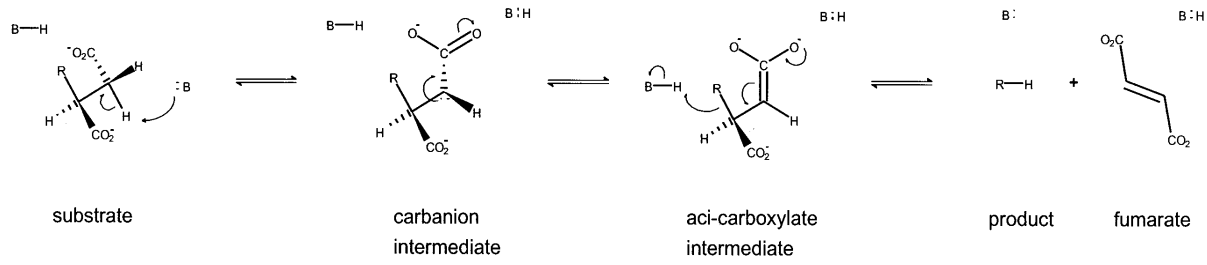


Figure 5. Proposed mechanism for the reaction catalyzed by ASL.

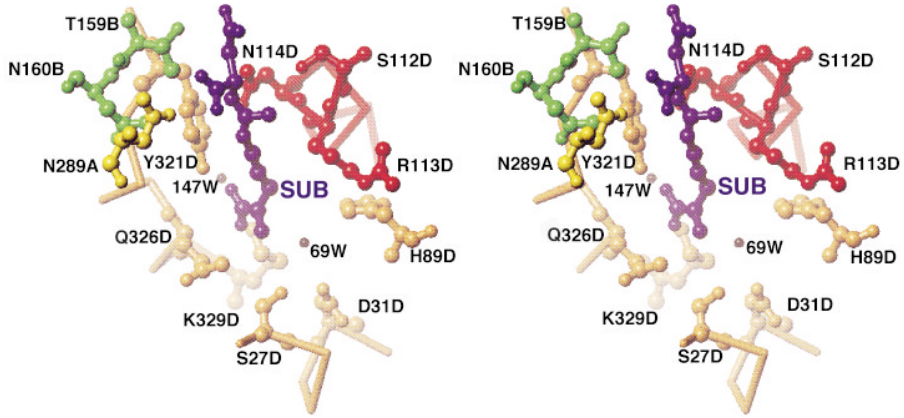


Figure 6. Stereoview of the argininosuccinate-binding site formed by monomers A, B, and D. Residues depicted in the figure from the conserved consensus sequences defined in figure 3 are colored red (residues 106–124), green (residues 155–169), and yellow (residues 278–296). The argininosuccinate substrate (SUB) and the water molecules (W) are colored purple. The amino acid residues are labeled with their one letter code, residue number, and the monomer (A, B or D), on which they are found.

88–92]. Despite the lack of activity in $\delta 1$ crystallin, the $\delta 1$ and $\delta 2$ isomers have an amino acid sequence identity of 91% in chicken [85, 86] and 94% in duck [87]. The loss of enzymatic activity in $\delta 1$ has to be the result of these variations in amino acid sequence. While these variations could affect a residue involved in the catalysis and/or the structure of the protein, the current hypothesis is that the loss of activity results from a structural perturbation that prevents substrate binding. This hypothesis is supported by structural comparisons of the inactive and active forms of the protein [64, 80] and by the fact that all the residues implicated in catalysis (see below) are conserved in the $\delta 1$ isomer. Comparative studies of the δ crystallins have been invaluable for understanding the enzymatic mechanism of the ASL reaction.

Catalytic mechanism

The formation of fumarate and arginine from argininosuccinic acid proceeds via a general acid-base mechanism. Evidence of a carbanion intermediate in the reaction pathway was first suggested when a nitro analogue of argininosuccinate, N^3-(L-1-carboxy-2-nitroethyl)-L-arginine, bound to the enzyme tighter than the actual substrate [58]. Nitro analogue inhibitors have been synthesized and tested for other members of the superfamily and also proven to be strong competitive inhibitors [93, 94], reinforcing the hypothesis that the overall catalytic mechanism for the superfamily is very similar. The reaction is initiated by the abstraction of a proton from the C_β position of argininosuccinic acid to form a carbanion intermediate (fig. 5). Redistribution of the negative charge onto the carboxyl group generates

an aci-carboxylate intermediate. Subsequent cleavage of the C_α–N bond requires the donation of a proton to the guanidinium nitrogen. Two separate acid-base groups are required for proton abstraction and donation due to the *trans*-stereochemistry of the reaction [95] and characteristic shape of the pH-rate profiles [90, 96]. The rate-limiting step of the reaction appears from kinetic isotope effect studies to be the cleavage of the C–N bond and not the abstraction of the proton [96–98]. While chemical modification [99] and pH-rate profile studies [100] can provide valuable clues to the identity of the catalytic residues, more definitive identification requires knowledge of the three-dimensional structure of the protein both in the presence and absence of bound inhibitors or substrate analogues.

Substrate-binding residues

In 1999, Vallée et al. [80] determined the X-ray structure of the enzymatically inactive H162N (His-160 in ASL) mutant of duck $\delta 2$ crystallin with bound argininosuccinate. (Note that the numbering used throughout this text, even for $\delta 2$ crystallin, is that of ASL. $\delta 2$ crystallin has a two-amino acid insert at residue 4; fig. 1.) In the crystal structure, the substrate was found to interact with residues from each of the three monomers that form the active site (fig. 6). In an active site comprised of residues from monomers A, B, and D, the amino and carboxyl groups of the arginine moiety were found to be oriented toward residues in either domain 1 or domain 2 of monomer D. Asn-114, Gln-326, and Tyr-321 from this monomer form hydrogen bonds with the arginine moiety directly whereas Asp-31, His-89, Arg-236, Leu-325, and Asp-328 interact with the arginine moiety via two water molecules (69W and 147W in fig. 6). Ser-27 and Lys-329 interact with the substrate both directly and indirectly via water molecules. The fumarate moiety is oriented toward residues located in the second and third conserved superfamily consensus sequences (fig. 3) and forms hydrogen bonds with Asn-289 of monomer A and Thr-159 of monomer B.

Mutational analysis of $\delta 2$ crystallin by Chakraborty et al. [65] confirmed the role played by various residues in substrate binding and catalysis. Point mutations of Arg-113, Asn-114, Thr-159, Ser-281, Glu-294, or Tyr-321 all abolished the catalytic activity. Thermodynamic characterization of these mutant proteins revealed that their stability is not significantly altered, and that the loss of catalytic activity is almost certainly due to the inability of the enzyme to bind or catalyze the substrate. Arg-113, Asn-114, Thr-159, and Tyr-321 were all shown in the crystal structure to interact with the argininosuccinate substrate. Arg-113 makes van der Waals contacts with the aliphatic part of the arginine moiety of argininosuccinate, while Asn-114, Thr-159, and Tyr-321 participate in hydrogen-bonding interactions with the substrate as mentioned above (fig. 6). Mutation of the Glu-294 residue affects catalysis by abolishing the His-160–Glu-294 interaction believed to be essential for initiating the reaction (see below). Although the exact role of Ser-281 is unknown, the conformation of the loop (residues 282–296) on which this residue is located appears to be important for substrate binding and catalysis. Mutation of two other residues on this loop also affects catalytic activity. In *E. coli* L-asparatase, mutation of the residue equivalent to Lys-287 results in a protein with only 0.3% of wild-type activity [101], while mutation of Gln-286 has been identified as causing the disease argininosuccinic aciduria [102]. Lys 287 is thought to be critical for stabilizing the carbanion intermediate.

Catalytic residues

In addition to defining residues involved in substrate binding, the H162N (His 160 in ASL) $\delta 2$ crystallin structure with bound substrate has enabled the identification of a number of residues involved in catalysis. Kinetic studies of the bovine liver ASL [100] and duck $\delta 2$ crystallin [99] had previously implicated a carboxyl group and a histidine residue as the acid and base, respectively. Mutagenesis studies had implicated His-160 as the catalytic base [103]. When histidines at residues 89, 108, 160, and 176 of duck $\delta 2$ crystallin were mutated to asparagine residues (H89N, H108N, H160N, and H176N) by site-directed mutagenesis, only H160N resulted in a complete loss of enzymatic activity [103]. Similarly, catalytic activity was abolished when the equivalent histidine, His-141, of *Bacillus subtilis* adenylosuccinate lyase was mutated separately to alanine, leucine, glutamate, and glutamine [104]. Crystal structures of ASL/$\delta 2$ crystallin reveal that a hydrogen bond exists between the $N_{\delta 1}$ of His-160 and the $O_{\varepsilon 1}$ of Glu-294 making this histidine more nucleophilic and therefore more capable of abstracting a proton to initiate the reaction [64, 75]. In the crystal structure of the inactive H162N (His-160 in ASL) mutant duck $\delta 2$ crystallin [80], the orientation of the side chain of the mutated residue is altered and the $O_{\delta 1}$ of Asn-160 forms a hydrogen bond with the backbone nitrogen of Lys-323 rather than interacting with Glu-294. This change in conformation prevents Asn-160 from mimicking the His-160–Glu-294 interaction and provides additional evidence for the importance of this interaction.

The equivalent histidine residues in *E. coli* fumarase C [76, 77] and *Thermotaga maritima* adenylosuccinate lyase [78] have similarly been proposed to have a role in a 'charge relay system.' In the case of *E. coli* fumarase C, the histidine is proposed to abstract a proton from a water molecule which subsequently acts as the catalytic base [76, 77]. There is no structural evidence of an analogous water

molecule in the structure of either ASL or $\delta 2$ crystallin suggesting, in this case, that the histidine acts directly on the substrate rather than exerting its effect via a water molecule. Although the working hypothesis is that all members of the superfamily would share a common reaction mechanism, the identification of this charge-relay pair presents a dilemma, as in CMLE, the equivalent histidine and glutamate residues have been replaced by tryptophan and alanine, respectively, while in all species of L-aspartase except that of *B. subtilis*, the equivalent histidine has been replaced by glutamine (see fig. 3).

To date, the catalytic acid has yet to be identified. In the substrate-bound H162N (His-160 in ASL) mutant duck $\delta 2$ crystallin structure, the fumarate moiety of the substrate is only partially defined due to the poor quality of the electron density in this region [80]. The uncertainty regarding the position of the substrate and its possible perturbation due to the H162N mutation prevents any definitive conclusions about the identity of the catalytic acid. There is stronger evidence for adenylosuccinate lyase that His-68 in this protein is the catalytic acid [104, 105]. However, in the structural superposition of ASL/$\delta 2$ crystallin with adenylosuccinate lyase, Arg-113 is closest in space to His-68 [78]. Although Arg-113 has been shown to be essential for catalytic activity [65], the extremely high pK_a of the guanidinium group, together with a lack of precedence for acid catalysis by arginine, makes Arg-113 an unlikely candidate for the catalytic acid. Similarly for fumarase C, Thr-100 is closest in space to His-68, again a residue unlikely to act as a catalytic acid. These observations have lead Toth and Yeates [78] to the counter-intuitive suggestion that the catalytic acid is not spatially conserved across the superfamily and that the substrate fumarate moiety binds in a different conformation in each enzyme. This suggestion coupled with the lack of sequence conservation of the catalytic base (His-160) across the superfamily would appear to suggest that while members of this superfamily may share a common reaction mechanism (i.e., β-elimination with cleavage of a C–N or C–O bond), how the fumarate moiety of the substrate binds and the location of the residues involved in catalysis in the active site may be different.

Argininosuccinic aciduria

Mutations in ASL result in the clinical condition argininosuccinic aciduria. This autosomal recessive disorder was first diagnosed by Allan et al. in 1958 [106] and has subsequently been found to be the second most common urea cycle disorder with an incidence of approximately 1 in 70,000 live births [107].

There is considerable clinical and genetic heterogeneity associated with the deficiency. The clinical heterogeneity is manifested by variations in the age of onset and the severity of the symptoms, with three distinct clinical phenotypes: neonatal, subacute, and late onset. In all cases, there is a full-term, normal pregnancy with an uneventful labor and delivery. Neonatal onset occurs within a few days of birth, with patients becoming lethargic, requiring stimulation for feeding, and exhibiting vomiting, hypothermia, and hyperventilation. Insufficient ammonia detoxification leads to hyperammonemia, which can cause the infant to become comatose and even die. The subacute- and late-onset phenotypes are less severe. Symptoms manifest themselves later in infancy and include vomiting, lethargy, disorientation, irritability, intermittent ataxia, seizures, and physical and mental retardation. *Trichorrexia nodosa*, a hair abnormality thought to be due to arginine deficiency, is a distinguishing feature of the late-onset form of argininosuccinic aciduria. There have also been reports of normal development, with asymptomatic individuals being diagnosed from the results of routine urine tests [108].

This clinical heterogeneity is common in all urea cycle disorders. Diagnosis of an inborn error of metabolism is suggested when an increased level of ammonium is detected in the plasma of patients. Elevated levels of argininosuccinic acid and its anhydrides, which are not usually found in the plasma of healthy individuals, easily distinguish patients with argininosuccinic aciduria from those suffering from other urea cycle disorders. Levels of argininosuccinic acid increase from undetectable to approximately 3 mg per 100 ml of plasma and up to 10 mg per 100 ml of cerebrospinal fluid [109]. Plasma citrulline levels will also increase to concentrations of 100–300 μM. Prevention of death or permanent neurological damage is dependent on an early diagnosis followed by appropriate therapy. Therapy is usually aimed at reducing both the requirement for ureagenesis by providing alternate routes for the excretion of nitrogen, and the levels of urea precursors by lowering the intake of protein in the diet. The symptoms, diagnosis, and treatment of argininosuccinic aciduria, as well as other urea cycle disorders, are reviewed in detail elsewhere [110–113].

Intragenic complementation

Extensive genetic heterogeneity was identified from the complementation analysis of 28 unrelated patients with argininosuccinic aciduria [114]. Incorporation of ^{14}C from L-[*ureido*-^{14}C]citrulline into acid-precipitable material was measured as an indirect assay of ASL activity in the heterokaryons of patient fibroblasts fused in all pairwise combinations. All the mutants mapped to a single complementation group (i.e., affected a single locus). Twelve distinct complementation subgroups were

defined, suggesting extensive interallelic complementation. Evidence that this complementation occurred at the ASL locus was provided by immunoblot analysis [115]. ASL cross-reactive material was detected in varying amounts and sizes in the mutant fibroblasts, suggesting that ASL deficiency is caused by mutations in the structural gene coding for the ASL monomer rather than in any regulatory gene. This was later confirmed when one of the mutant strains was identified to be homozygous for a single amino acid substitution. The arginine at codon 95 of the ASL monomer was found to be mutated to cysteine (R95C) [116].

In addition to the R95C mutation, seven other mutations in the ASL gene have now been identified (table 2) [63, 102, 116]. Of these, five are missense mutations, one is a small deletion, and the other is a splice defect. The residual enzyme activity in these mutants varies due to the heterogeneous effects that mutations can have on the protein. Mapping the mutations onto the three-dimensional structure of ASL provides insight into the potential effect of each mutation on the tetramer. Either the active site or the stability of the enzyme can be affected. A homotetramer with the glutamine at position 286 mutated to arginine (Q286R) has less than 0.05% of wild-type ASL activity despite its relative stability, implying that this mutation affected the active site of the enzyme [102]. The R95C mutation, on the other hand, produced substantially lower levels of protein, indicating that this mutation affected enzyme stability [116].

Complementation is a phenomenon that occurs in multimeric enzymes due to protein subunit interactions. Two distinct subunits are said to complement if they can interact to give a partially functional heteromer despite, individually, having no appreciable enzymatic activity as homomeric proteins. Intragenic complementation has been shown to occur in argininosuccinic aciduria [114], propionic acidemia [117, 118], and methylmalonic aciduria [119], but is a phenomenon believed to exist in all genetic diseases involving multimeric proteins. In 1964, Crick and Orgel [120] suggested that complementation in a dimeric protein between two monomers Ab and aB with different inactive regions (denoted by lowercase a and b) aggregate to form an inactive site ab and an active site AB, which results in a partial restoration of $\sim 50\%$ activity. While this scenario is observed in some complementation events, as seen below, Crick and Orgel dismissed this scenario from their general theory of complementation, assuming that because a residual amount of activity remained, such a protein would not be detected as bearing a mutation. Instead, they suggested that complementation occurs between mutant subunits because a misfolding in one subunit is compensated by an unaltered portion of the adjacent subunit, a theory that may, in time, prove to be correct for mutations that are located outside the active site region.

In complementation studies of ASL, Walker et al. [102] found that the Q286R and D87G mutations participate in the complementation event with the highest recovery of activity [102]. Homomeric proteins for either mutation result in little or no enzymatic activity in vivo [102] or in vitro [121]. However, hybrid proteins of the two mutants exhibit approximately 30% of wild-type protein activity [102, 121]. To understand the structural basis of the intragenic complementation event exhibited between the Q286R and D87G mutants, the mutated residues were mapped onto the tetrameric structure of ASL [75] (fig. 7). Although neither Gln-286 nor Asp-87 have been implicated in the catalytic mechanism, both are in close

Table 2. Mutations in argininosuccinic aciduria.

Mutation	Percent wild-type activity*	Percent buried surface area	Location in protein	Potential effect	Reference
D87G	5	92	helix 5, domain 1	conformation	[102]
R95C	<1	87	helix 5, domain 1	stability	[116]
R111W	<3	93	conserved region 1 loop, domain 1	conformation	[63]
R193Q	<3	92	helix 8, domain 2, dimer interface	stability	[63]
Q286R	<3	51	conserved region 3, loop, domain 2	catalysis	[63, 102]
A398D	<1	98	helix 18, domain 3	stability	[102]
Δ 13 bp	not tested; expression would produce truncated protein				[63, 102]
Δ exon 2	not tested; expression would produce truncated protein				[63]

* Measured in COS cell experiments.

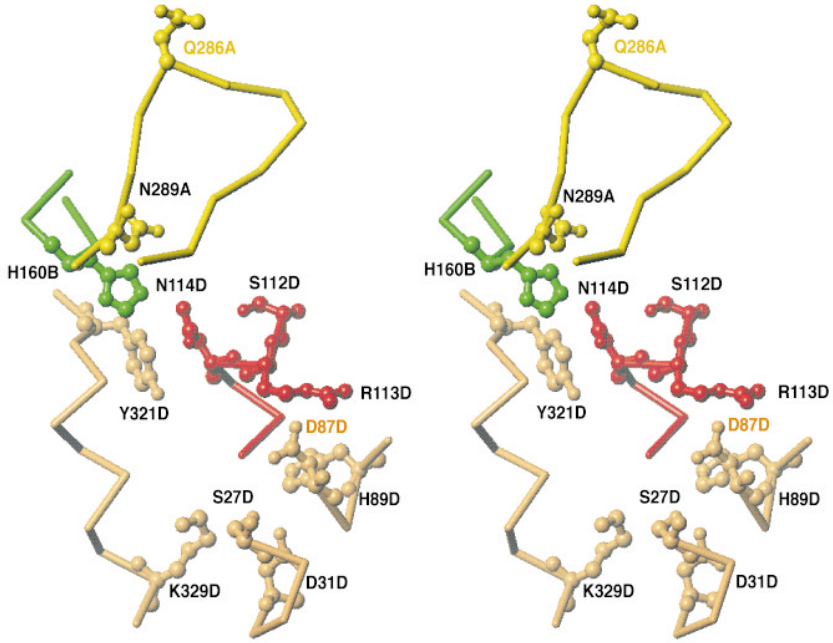


Figure 7. Stereoview of the active site of ASL comprised of monomers A, B, and D showing the relative location of Gln-286 and Asp-87. The active site is shown in the same orientation as in figure 6. Residues depicted in the figure from the conserved consensus sequences defined in figure 3 are colored red (residues 106–124), green (residues 155–169), and yellow (residues 278–296). All other residues are colored in orange. The amino acid residues are labeled with their one-letter code, residue number, and the monomer (A, B, or D) on which they are found.

proximity to residues that may be enzymatically important. In any one active site, D87 and Q286 are contributed by different monomers. Due to the symmetry of the enzyme, a heterotetramer of Q286R and D87G monomers could therefore contain active sites with one or both mutations, or active sites that are devoid of either mutation (fig. 8). The recovery of activity exhibited by complementation of the two mutant subunits is therefore believed to be due to the reconstruction of wild-type active sites [122]. This is supported by the observed catalytic activity of the heterotetrameric enzyme. Combination of the two mutants should theoretically yield a mixture of tetramers with Q286R to D87G ratios of 0:4, 1:3, 2:2, 3:1, and 4:0 in a 1:4:6:4:1 distribution and with an activity of 25% compared to the wild-type ASL. The greater activity seen experimentally can be attributed to the $\sim$ 5% of ASL activity exhibited by the D87G homotetramer. This type of complementation, the reconstruction of wild-type active sites, has also been observed in another member of the superfamily, adenylosuccinate lyase [104], as well as in the homotrimeric enzyme aspartate transcarbamoylase [123], and homodimeric proteins glutathione reductase [124], thymidylate synthase [125], mercuric reductase [126], and ribulose bisphosphate carboxylase/oxygenase [127].

The reconstruction of active sites clearly explains the complementation event observed between the Q286R and D87G mutations of ASL. This theory, however, cannot be used to explain all of the complementation events observed at the ASL locus because it does not take into account the mutations that occur outside the active site region (table 2). How mutations, such as A398D, which might affect the stability and/or folding of the protein, affect the catalytic activity and exhibit complementation with other mutants is under investigation. Clearly changes in monomer stability and/or subunit association would decrease the amount of active tetramer and also the level of recovered activity in the heterotetramer. Present hypotheses explaining the complementation events between these mutants need to be further investigated and proven for a full understanding of the phenomenon of intragenic complementation. Only then can attempts be made to understand the extensive heterogeneity observed in patients suffering from argininosuccinic aciduria and other genetic diseases associated with multimeric proteins.

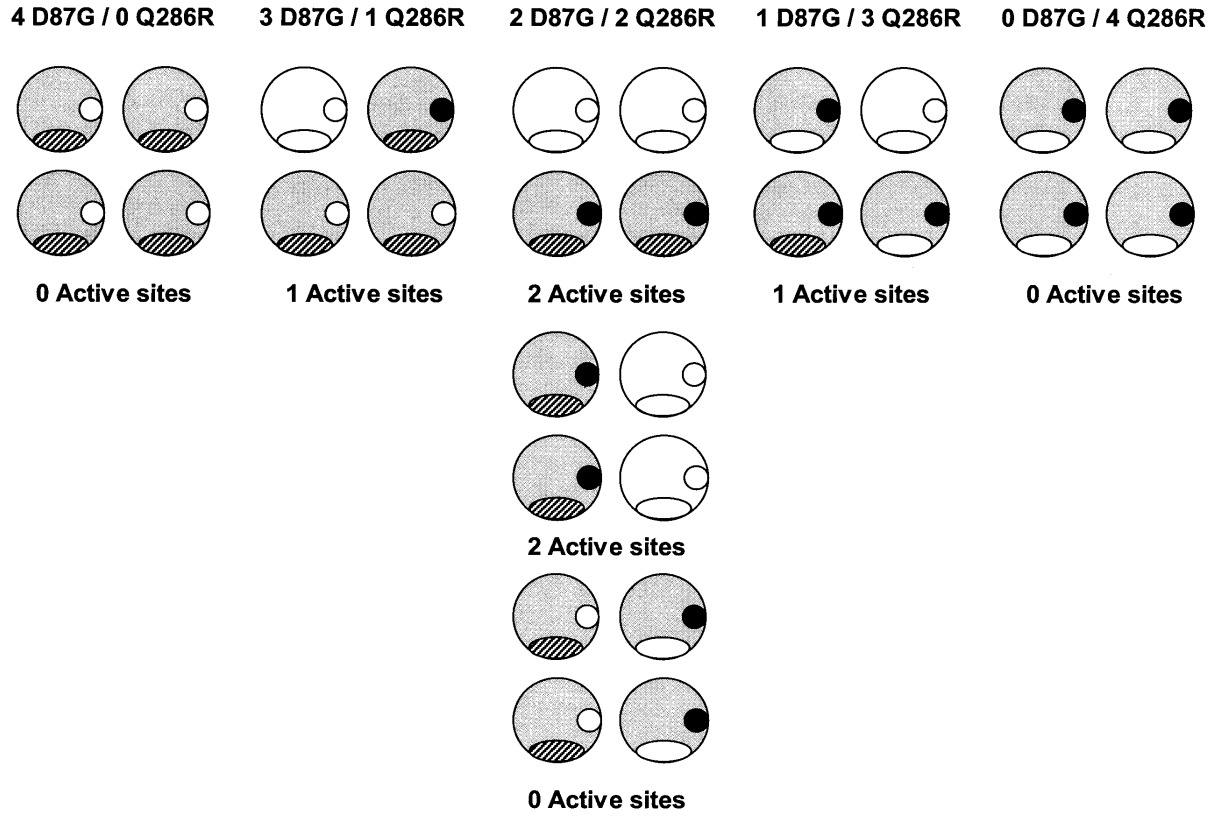


Figure 8. Pictorial representation of the actives sites of the statistically available combinations of mutants in the D87G/Q286R complementation event. For clarity, the diagram has been drawn to show the interaction of only D87 (⬭) and Q286 (○). The shading of these symbols represents the presence of the point mutations D87G and Q286R, respectively. Each large circle represents one of the four active sites found in the protein (see fig. 4b). Light-gray shading of the active site indicates that it contains at least one or more mutations and is therefore considered inactive. In each active site, residues 286 and 87 are always contributed from a different monomer. Due to the molecular symmetry of the tetramer, in the case of the 2D87G:2Q286R tetramer, there are three distinctly different ways of combining the monomers which will give rise to either two or zero native active sites.

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