Intragenic Complementation at the Human Argininosuccinate Lyase Locus

IDENTIFICATION OF THE MAJOR COMPLEMENTING ALLELES*

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To determine the molecular and biochemical basis of intragenic complementation observed at the human argininosuccinate lyase (ASL) locus, we identified the ASL alleles in ASL-deficient cell strains with two unique complementation phenotypes: (i) frequent complementers, strains that participated in the majority of complementation events, and (ii) high activity complementers, strains in which complementation was associated with a relatively high level of restoration of ASL activity. Four mutations (Q286R, D87G, A398D, and a deletion of exon 13) were identified in the four strains examined. One of the two frequent complementers was homozygous, and the other heterozygous, for the Q286R allele. Similarly, one of the two high activity complementers was homozygous, and the other heterozygous, for the D87G allele. When the Q286R and D87G mutations were introduced by site-directed mutagenesis into wild-type ASL cDNA, each conferred loss of ASL activity in COS cell transfection assays. To test directly the hypothesis that intragenic complementation occurs at the ASL locus, one of the major complementation events observed previously, between strains carrying the Q286R and D87G alleles, was reconstructed in COS cell transfection assays. A partial restoration of ASL activity, comparable with the increase seen in the fibroblast complementation analysis, was observed on joint cotransfection of these two alleles. The results provide molecular confirmation of the major features of the ASL mutant complementation map, identify the Q286R and D87G alleles as the frequent and high activity complementing alleles, respectively, and provide direct proof of intragenic complementation at the ASL locus.

Argininosuccinate lyase (ASL, EC 4.3.2.1) deficiency is an autosomal recessive disorder of the urea cycle with substantial clinical and genetic heterogeneity (1–4). The clinical heterogeneity of the disease is manifested by variations in the age of onset and the severity of symptoms, with patients classified into three distinct clinical groups: neonatal onset, subacute onset, and late onset. The biochemical basis of this clinical variation is unclear, since there is only a partial correlation between the clinical phenotype and the residual enzyme activity detected in cultured fibroblasts (2) or other tissues (1). The ASL enzyme, which catalyzes the reversible breakdown of argininosuccinate into arginine and fumarate, is a homotetramer of 50-kDa subunits (5–7).

Genetic heterogeneity in ASL deficiency has been identified by complementation analysis using cultured fibroblasts from 28 unrelated patients (2). All ASL-deficient strains map to a single complementation group (i.e. affect a single locus), but the structure of the complementation map and the nature of the complementation events suggest that extensive interallelic complementation is present at this locus, and 12 distinct complementation subgroups were defined (2). The affected gene at the locus was confirmed to be ASL by the identification of the molecular defect in one cell strain belonging to the single complementation group (8). This strain (944) has a homozygous single substitution that converts an arginine to a cysteine residue in codon 95 (R95C) and produces a complete loss of ASL activity (8). Other mutations in the ASL gene have been described by Barbosa et al. (4). Additional evidence for genetic heterogeneity has been obtained from immunoblot studies of the abundance of ASL protein in patient fibroblasts (3). Although most strains examined have some detectable ASL cross-reacting material of normal size (50 kDa, ASL monomer), its abundance varies widely in cells from different patients.

To begin an analysis of the molecular basis of the intragenic complementation at the ASL locus, we have identified the ASL mutations in the two classes of ASL-deficient strains with the most unique complementation phenotypes: the “frequent” and “high activity” complementers (2). We chose to study the frequent complementer strains 926 and 1254 (2) because one or the other of them participated in almost all (30 of 32) of the positive complementation events observed. The designation “high activity” complementer was conferred on two other strains (1182 and 1253) because, when they were fused with either of the frequently complementing strains 926 or 1254, the restoration in ASL activity was significantly greater (~10-fold) than that seen (~3-fold) with any other complementation

PCR, polymerase chain reaction; SSGE, single-stranded gel electrophoresis; bp, base pair(s).
events (2). Strains 1182 and 1253 are the only members of the high activity complementer class, and they belong to the same complementation subgroup (2). The fact that the complementation behavior of a pair of strains is so similar suggests that each member of the pair shares at least one allele in common or that their ASL alleles have similar effects on the protein.

In this paper we report the identification of the alleles in ASL-deficient strains from the frequent and high activity complementers and demonstrate that strains with similar complementation behavior share the same alleles. The identification of the alleles in complementing ASL-deficient strains has also allowed us to obtain direct proof of intragenic complementation by reconstruction of one major complementation event in COS cells. Taken together, these results confirm major features of the intragenic complementation map constructed for the ASL locus (2). In addition, they establish that the Q286R and D87G alleles account for the frequent and high activity complementing phenotypes, respectively, of ASL-deficient cells that carry them. Each of these substitutions impairs enzyme function sufficiently to cause disease but not to the extent that some amelioration of the function of one mutant subunit by another is prevented in a heteroallelic ASL tetramer. Although the molecular basis of the increase in ASL activity that occurs in the complementing heteroallelic ASL tetramer is unknown, recent determination of the x-ray crystal structure of ASL suggests that the recovery of partial ASL activity may be due to the formation of one or more “native-like” active sites (9) rather than the conformational correction (9) of one mutant monomer by the other.

EXPERIMENTAL PROCEDURES

Cell Strains— Fibroblasts were cultured in α-minimal essential media, without antibiotics and supplemented with 10–15% fetal bovine serum as described previously (2, 3). The clinical phenotype and complementation behavior of all strains discussed in this paper have been reported previously (2).

Oligonucleotides—The oligonucleotide primers used for PCR amplification of single-stranded ASL cDNA, based on the ASL cDNA sequence of Todd et al. (16), are numbered using the internal nucleotide of the translated sequence as the first nucleotide. The complete coding sequence and short flanking sequences were amplified in three overlapping fragments. The 5′ fragment was amplified using primers 12 (nucleotides 27 to 31) and 22 (nucleotides 445–468) (Table I), the middle fragment using primers 32 (nucleotides 400–426) and 42 (nucleotides 943–966), and the 3′ fragment using primers 52 (nucleotides 910–936) and 62 (nucleotides 1396–1422). Each primer contained at its 5′ end the nine-base pair sequence, CCTGGATCC, containing a BamHI restriction site.

For single-stranded gel electrophoresis (SSGE) and direct sequencing, the cDNA was amplified a second time from one of the three restriction sites. The 5′ fragment was amplified using primers 9 (nucleotides 910–936) and 22 (nucleotides 1396–1422) (Table II). The Q286R mutation was introduced into the ASL cDNA sequence by site-directed mutagenesis using the method of Kunkel (15), and the mutagenized cDNA was cloned into the pESP-SVTEXP vector (pESP-Q286R). Since strain 1253 was found to be homozygous for the D87G allele, the D87G expression vector was created by PCR using primers 12 and 22 (Table I) to amplify the region of the 1253 cDNA (nucleotides 27 to 468) that contained the mutation.

After digestion of this fragment with MluI and Bsu36I, nucleotides 74–360 were then subcloned into the pESP-WT plasmid, replacing the normal sequence in this region (pESP-D87G). The entire ASL coding region of the control and mutant expression vectors and of the DNA flanking the ligitation sites was sequenced to ensure that no mutations, other than those introduced specifically, were present. An expression vector (pXGH5) (16) containing the mouse metallothionein-1 promoter controlling the full-length human growth hormone gene was used as a control for transfection efficiency of COS cells.

Direct Sequencing of Amplified cDNA—The PCR products were amplified as indicated above but without the use of α-labeled nucleotides. The PCR products were then partially purified and concentrated using Millipore Ultrafree-MC 30,000 NMWL filter units according to the manufacturer’s instructions. Approximately 100 ng of the partially purified PCR products were then asymmetrically amplified (17) under the same PCR conditions but with 100 ng of one primer only. The asymmetric PCR products were sequenced using standard conditions using the U.S. Biochemical Corp. Sequenase sequencing kit.

Transient Expression Analyses of ASL cDNAs—Transfection of the expression vectors was performed by the method of Chen and Okayama (18). Approximately 106 COS-1 cells were plated in a 100-mm Petri dish. One to two days later, the pESP-SVTEXP vector (20 μg) alone or this vector with either a normal or mutant (Q286R, D87G, or both D87G and Q286R) full-length ASL insert (20 μg) and p5 μg of pHG5 were transfected by calcium phosphate co-precipitation and harvested after 72 h. Following transfection, the cells were used to prepare RNA and crude cell lysates for protein and enzyme assays.

Cell lysis, protein electrophoresis, and ASL immunoblotting were done as described previously (3). The anti-ASL antibody has been described (6). Assays of human growth hormone were performed by the Allegro assay system, as suggested by the manufacturer (Nichols Institute Diagnostics). Protein was measured by the method of Lowry et al. (19) using bovine serum albumin as a standard.

Enzyme Assays of Cell Extracts—ASL enzyme activity was assayed as described previously (20). All ASL assays were performed in triplicate (three different dishes) for each expression vector. ASL activity was calculated by first subtracting the average ASL activity from mock-transfected COS-1 cells transfected with the normal and mutant alleles to remove background. ASL activity was then normalized for transfection efficiency by dividing the ASL activity of a cell extract by the relative human growth hormone activity of each plate. The specific activity of cell extracts was determined by dividing the ASL activity by the densitometry values of the ASL band from immunoblots of total protein from each dish. The densitometry values used for each transfection were the average ASL protein band intensity from the immunoblots of the three transfections performed in one experiment with each construct (see Fig. 3 and Table II).

Other Methods—Isolated restriction fragments were radioincubated with α-cDNA containing the described ASL activity greater than 10cpm/μg using random oligonucleotide primers (21). Radio labeling of oligonucleotides was done by the phosphatase exchange method as described by Sambrook et al. (13). Single-stranded DNA was prepared using Bluescript cloning by the method of Vieira and Messing (22) and sequenced by the dyeoxy method, using T7 or internal primers and Sequenase (23). SSGE was performed as described previously (24). Southern and RNA blotting were done using standard methods (15) and were hybridized with a full-length ASL cDNA (14).

RESULTS

Identification and Characterization of the ASL Mutations in the Two Major Complementation Phenotypes—To identify the mutations in the four cell strains being studied (the two frequent complementers 926 and 1254) and the two high activity complementers (1182 and 1253), we first established that gross alterations or rearrangements of the ASL gene were not present. EcoRI- or BglII-digested genomic DNA of all four strains was examined by Southern blot analysis. No changes in the size of the restriction fragments were seen in any strain (data not shown). The relative abundance and size of the ASL mRNA was also determined on blots of total RNA. All four strains had ASL mRNA of normal size and of an abundance comparable with controls (data not shown), indicating that at least one ASL allele in each strain produced near normal ASL enzyme activity.

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This mutation, an A nucleotide 857 of the noncoding strand, in exon 11 (Fig. 1)
only one consistent nucleotide change, a T
The sequence of seven clones of each fragment demonstrated
mine to arginine (Q286R).
second nucleotide of codon 286, changes the codon from glutamine
mRNA was reverse-transcribed, amplified in three overlapping
fore likely to be homozygous for a single mutant allele. The ASL
derived was the product of a consanguineous mating and there-
1254, a genomic fragment containing exon 11, exon 12, and the
(926 and 1254)—
We initially sought to identify the ASL mutation of both the control and mutant DNA is shown and the substituted base
indicated with an asterisk. a, Strain 1254, the noncoding strand was sequenced, but the amino acids corresponding to the sequence of the coding strand are shown. Codon 286 has been mutated from glutamine to arginine by the A
G transition at nucleotide 260. The absence of the 200-bp fragment, indicating that strain 1182 is heterozygous for the D87G allele.
The molecular size markers are 1-kilobase pair standards (1 kb stds) (Life Technologies, Inc.). b, the D87G mutation is present in both strains 1182 and 1253. SfiI restriction digest analyses were performed on genomic PCR products generated from region 3 (Table I) of the ASL cDNA. SfiI cuts the control PCR product into three fragments (Control lane (+)), including one of 200 bp. This 200-bp fragment is absent from the SfiI-digested PCR products of strain 1253 (1253 lane (+)), because the 200-bp band is digested to fragments of 105 and 95 bp due to the introduction of the mutation at nucleotide 286. The absence of the 200-bp band from the 1253 sample indicates that this strain is homozygous for the D87G mutation. SfiI digestion of the amplified DNA from strain 1182 generated the 105- and 95-bp bands (1182 lane (+)) but did not cut all of the 200-bp fragment, indicating that strain 1182 is heterozygous for the D87G allele.

tions at the ASL locus nor in 20 control alleles.3

generated both the ~175- and ~125-bp fragments (Fig. 2a), suggesting that this strain is heterozygous for the NciI site introduced by the A → G mutation and that it therefore also carries the Q286R allele.

The results obtained from the three diagnostic restriction enzyme digestions for strains 926 and 1254 were confirmed using DNA amplified in a second PCR reaction. The results of the digestion of the DNA with the three enzymes were identical to those obtained initially (data not shown). To confirm that codon 286 of strain 926 contained the identical substitution to that found in strain 1254, and to identify the mutation in the other allele of this strain, the entire cDNA was amplified in three overlapping ~500-bp fragments spanning the ASL cDNA, as described above. Gel electrophoresis of the 3’ fragment demonstrated the presence of a small (~450-bp) as well as a normal sized (~530-bp) band (data not shown); the other two fragments were of normal size. Direct sequencing of the smaller fragment, cut from the gel, showed that this allele lacked exon 13, a human ASL mutation reported previously by Barbosa et al. (4). All three ASL cDNA fragments of normal size were subcloned and sequenced. As expected, the only substitution present was that which creates the Q286R allele, the A → G transition at the second position of codon 286 (data not shown). The Q286R allele was not identified in any of the other 28 strains that had been examined by complementation analysis at the ASL locus nor in 20 control alleles.3

3 D. C. Walker, J. Christodoulou, H. J. Craig, L. R. Simard, L. Ploder, P. L. Howell, and R. R. McInnes, unpublished observations.

![FIG. 1. Partial sequence analysis of cloned ASL cDNA from the ASL-deficient strains 1254 and 1253.](image-url)
activity was greater than could be achieved by the transfection of ASL activity. We then reconstructed the complementation event between the products of either allele alone.

**Mutation Analysis of the High Activity Complementer Strains (1182 and 1253)—**To identify the ASL mutations in strain 1253, 10 overlapping fragments of ~225 bp each, using the primers shown in Table I, were amplified from the three major ASL cDNA fragments. The ~225-bp fragments were examined for mutations by SSGE. A homozygous single-stranded conformational variant was observed in the third cDNA fragment (Table I), which direct sequencing (Fig. 1b) revealed to be an A → T transversion at nucleotide 260, in exon 3. This substitution converts an aspartic acid to a glycine residue at codon 87 (D87G) and creates a novel SfaNI site. Restriction analysis was used to confirm that strain 1253 was indeed homozygous for the mutation (Fig. 2b).

Since strains 1253 and 1182 are the only two members of the high activity complementation subgroup (2), we examined the cDNA of strain 1182 for the D87G mutation using restriction analysis with SfaNI (Fig. 2b), which indicated that this cell strain is homozygous for the D87G mutation. This result was confirmed by direct sequencing (data not shown). Of the 28 ASL-deficient cell lines studied (2), 1182 and 1253 were the only two strains found to have the D87G mutation, and this allele was also absent from 20 control alleles. The mutation in the second allele of strain 1182 was identified by SSGE analysis and direct sequencing to be a C → A transversion at nucleotide 1193 in exon 3 (data not shown). This substitution destroys an existing HaeIII restriction site and results in an alanine to aspartic acid substitution at codon 398 (A398D). No other mutations were detected by SSGE and direct sequencing of the ASL cDNA of either strain 1182 or 1253.

**Reconstruction of the Q286R/D87G Complementation Event—**Since strain 1254 (frequent complementing phenotype) and strain 1253 (high activity complementing phenotype) are homozygous for the Q286R and D87G alleles, respectively, interaction between the Q286R and D87G polypeptides would appear to be responsible for the partial restoration of ASL activity (i.e. complementation) observed in cell fusions of fibroblasts carrying these alleles (2). To examine this hypothesis directly, we used transient expression assays in COS-1 cells to reconstruct the complementation event between the products of the Q286R and D87G alleles. We first demonstrated that the Q286R and D87G mutations produced significant reductions of ASL activity. We then reconstructed the complementation event between Q286R and D87G by co-transfecting these alleles into COS-1 cells to determine whether the resultant ASL activity was greater than could be achieved by the transfection of either allele alone.

**DISCUSSION**

Considerable indirect evidence indicates that extensive allelic heterogeneity characterizes human ASL deficiency. This evidence includes both the occurrence of intragenic complementation (2) as well as variation in the abundance of the ~50-kDa ASL monomer in patient fibroblasts (3). The impression of substantial genetic heterogeneity is further supported by the identification of four alleles (Q286R, D87G, A398D, and the exon 13 deletion) in the four strains studied here, by the description of six different mutations in the ASL gene in four
patients reported by Barbosa et al. (4), and by the demonstration of another allele, R95C, in an additional patient (8). That both strains 1253 and 1254 have homozygous ASL mutations reflects the derivation of these cells from patients whose parents were consanguineous. The D87G mutation was observed only in the two high activity complementer strains (1182 and 1253) and not in the other 26 ASL-deficient strains on which complementation analysis was performed (2). Although Q286R was also observed in one patient by Barbosa et al. (4), there is no evidence that the three ASL-deficient patients with the Q286R allele (two in our study and the patient of Barbosa et al.) are related, and its absence in 52 other ASL-deficient alleles that we examined indicates that it is not particularly common. The loss of activity in the Q286R protein demonstrated in the transient expression studies is clearly not due to a decrease in the subunit stability alone, since the monomer is present at an abundance of ~23% of the wild-type subunit, yet the protein has no measurable ASL activity (Fig. 3 and Table II). Consequently, the major effect of the Q286R mutation is to perturb the catalytic function of the ASL protein. The ablative effect of the Q286R substitution on ASL activity is consistent with all three patients with at least one Q286R allele (the donors of fibroblast strains 1254 and 926 from this study, and patient AR of Barbosa et al. (7)) having the severe neonatal onset form of the ASL deficiency.

The effect of the D87G mutation on catalytic activity, shown by the transient expression studies, is less severe than that of Q286R. The D87G ASL monomer was of reduced abundance (~22% of control) in the transient expression studies but still formed an ASL enzyme with ~5% of the activity of controls (Fig. 3 and Table II). The decreased but measurable ASL activity of the D87G protein is reflected in the moderately less severe clinical phenotype (the subacute form of the disease) of the patient homozygous for this allele, and in the mild (late onset) phenotype of the patient who is a D87G heterozygote. The very mild clinical phenotype of this D87G heterozygote, whose other allele is A398D, suggests that the latter allele may also have significant residual ASL activity.

Our studies demonstrate clearly that Q286R is the frequently complementing allele of human ASL deficiency. It is the only ASL allele present in one frequent complementer (strain 1254) and the only complementing allele present in the other frequent complementer studied (strain 926). Strain 926 also carries the exon 13 deletion allele. A deletion of exon 13 was reported in one case described by Barbosa et al. (4) and shown to result from a 13-bp deletion within exon 13 that leads to exclusion of this exon from the transcript; the exon 13 deletion seen in strain 926 is due to the identical 13-bp deletion. Although the removal of exon 13 from the transcript would maintain the reading frame to produce an ASL polypeptide shortened by 28 residues, this protein lacking exon 13 does not complement, since we have found this mutation in strains that do not complement at all, such as 1040 (2). Thus, the complementation behavior of strain 926, which carries both the 13-bp deletion of exon 13 and the Q286R alleles, can be attributed entirely to the latter. Neither the complementation properties of the Q286R allele nor the effect of this mutation on ASL activity were examined by Barbosa et al. (4).

The evidence that the D87G allele is the high activity complementation allele is similarly strong. It is the only ASL allele present in one high activity complementer (strain 1253), and it is also present in the only other strain (1182) that manifests the high activity complementation phenotype. We cannot exclude the possibility that the other allele carried by strain 1182, A398D, may also confer the property of high activity complementation in association with Q286R subunits. However, it is not necessary to invoke any complementation properties for the A398D allele to account for the high activity complementation behavior of strain 1182, which can be related solely to the D87G polypeptide.

The relatively high degree of restoration of ASL activity observed when cells with the D87G allele are fused with cells carrying the Q286R allele must therefore reflect interaction between Q286R and D87G ASL subunits. Formal proof of this interaction has been obtained from the transient co-expression of the Q286R and D87G alleles in COS cells. The co-expression of these two alleles leads to an approximate 10-fold increase in the observed ASL activity over the cells transfected with only one of these alleles alone (Table II). The ASL activity observed in the co-transfected cells was approximately 30% of that obtained with the wild-type ASL allele in the transient expression system. Since the complementation-related increase in ASL activity that resulted from the fusion of cells containing the Q286R and D87G alleles was substantially higher than any other increase observed (2), we conclude that the formation of the heteroallelic Q286R:D87G tetramer is the most successful intragenic complementation event that has been observed to date at the ASL locus.

The complementation patterns for strains 1254 and 926 are comparable but not identical. Strain 1254 complements 14 strains, while 926 complements 10 strains. Eight strains are complemented by both (2). The similar complementation behavior of these two strains and the fact they do not complement each other led us to predict that they would share an allele or at least have alleles with similar, if not identical, effects on the protein (2). The identification of the Q286R allele in these two

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**Table II**

| ASL allele in the expression vector | Relative amount of ASL protein | ASL activity | Relative ASL activity | ASL specific activity | Relative ASL specific activity |
|------------------------------------|-------------------------------|--------------|----------------------|----------------------|-----------------------------|
| Control                           | %                             | pmol/mg/min  | %                    | pmol/mg/min          | %                          |
| D87G                              | 100 ± 19                      | 1900 ± 150   | 100 ± 8              | 2000 ± 330           | 100 ± 16                    |
| Q286R                             | 18.5 ± 4.8                    | 15 ± 7.9     | 0.79 ± 0.41          | 92 ± 42              | 4.5 ± 2                     |
| Q286R/Q286R                       | 23.15 ± 2.0                   | <1.0         | <0.001               | 0.93 ± 0.15          | <0.05                       |
| D87G/Q286R                        | 6.9 ± 0.13                    | 130 ± 2.4    | 610 ± 89             | 30 ± 4.4             |                             |
| SVTEXP (vector)                   | 0                             | 0            | 0                    |                       |                             |

a Percentage of control ASL protein band intensity, normalized for transfection efficiency.
b The ASL activity normalized to the human growth hormone activity of COS cells transfected with the control cDNA construct and expressed as pmol of ASA formed/mg of cell protein/min.
c Percentage of control ASL activity.
d ASL activity normalized to the band intensity of ASL protein from immunoblots of the same extracts and expressed as pmol of ASA formed/mg of cell protein/min.
e Percentage of control ASL specific activity.
strains with comparable complementation behavior therefore partially confirms the complementation map at the molecular level.

Since strain 1254 carries only the Q286R allele and strain 926 carries one copy of Q286R together with a likely null allele (the exon 13 deletion), one might expect that both strains would have identical complementation patterns. The fact that they do not may be due to the presence of twice the number of Q286R subunits in strain 1254, which is homozygous for this allele, compared with strain 926, which is heterozygous. Thus, the failure to observe complementation between strain 926 and strains that complemented strain 1254 (2) may be a dosage effect, reflecting the comparatively lower amounts of Q286R polypeptides in strain 926. The reason that complementation was not observed between strain 1254 and two strains that complemented strain 926 (2) is not clear. One possible explanation is that the discrepancy may only be apparent because in the original complementation analysis the failure to detect complementation between two strains on an initial fusion was not re-evaluated in any subsequent experiment. Positive complementation tests, in contrast, were validated repeatedly (2). Alternatively, the ASL subunit produced from the other allele of strain 926 (the exon 13 deletion) may be capable of complementing some ASL alleles that the Q286R subunit cannot. However, given the frequent complementation phenotype of the Q286R allele and the severe alteration in subunit structure that would result from the absence of the 28 amino acids residues 327–355 of the ASL subunit) encoded by exon 13, this explanation seems unlikely.

One other allele, R95C, is known to be capable of complementing Q286R. Strain 944, which is homozygous for this allele (8), complements strain 1254 (homozygous for Q286R) (2), producing a small (−3-fold) but significant increase in ASL activity (2). The difference in the amount of ASL activity recovered from the Q286R:D87G (−10-fold) and Q286R:R95C (−3-fold) heteroallelic tetramers reflects, at least to some extent, differences in the stability of the individual mutant alleles involved, since transient expression studies of the R95C allele demonstrated that little or no R95C ASL monomer could be detected, and thus no ASL activity was measurable (8). The absence of complementation between strains with the R95C allele (strain 944) and those with the D87G allele is perhaps not surprising, since the proximity of the two affected residues may produce similar rather than complementary effects on the ASL protein.

ASL and δ-crystallins belong to a superfamily of metabolic enzymes that all function as tetramers and catalyze homologous reactions. Duck and chicken δ-II-crystallins, proteins found in the eye lenses of birds and reptiles, have been found to be directly related to ASL, not only in the primary sequences of the gene and protein (25, 26), but also functionally, since crystallins exhibit ASL activity (27−29). Apart from enzymes with ASL activity, members of this superfamily include fumarase (30), aspartase (30), adenylosuccinase (31), and 3-carboxy-cis,cis-muconate lactonizing enzyme (CMLE, E. coli fumarase C, and E. coli aspartase. The sequence homology between residues 85–101 in ASL (shown in b) is not present in the other members of the superfamily, and therefore these other proteins are not presented in b. Residues 286 and 87 are indicated by arrows. The alignment was performed using the GeneWorks program (37).

Fig. 4. Amino acid sequences of the highly conserved regions. Sequences of the highly conserved regions across the enzyme superfamily are shown a, residues 278–295; b, residues 85–101. The ASL sequences shown are those for human ASL, rat ASL, yeast ASL, duck δ-II-crystallin (duck D2C), and chicken δ-II-crystallin (chick D2C). Since residues 278–295 contain one of the three highly conserved stretches of amino acid sequences across the enzyme superfamily, representative sequences from the other members of the superfamily have also been included in panel a. Bacillus subtilis adenylosuccinase (AD5), Pseudomonas putida 3-carboxy-cis,cis-muconate lactonizing enzyme (CMLE), E. coli fumarase C, and E. coli aspartase. The sequence homology between residues 85–101 in ASL (shown in b) is not present in the other members of the superfamily, and therefore these other proteins are not presented in b. Residues 286 and 87 are indicated by arrows. The alignment was performed using the GeneWorks program (37).

putative active site of the enzyme2 (33, 34), these 11 residues have been suggested to play an important role in the catalytic mechanism (30, 33, 34). This suggestion is supported by the dramatic effect on catalysis of the Q286R mutation, which is located in this region. In addition, our preliminary data on the structure of ASL2 suggest that the loop on which Gin286e is located forms one part of active site bowl of the ASL tetramer. The aspartic acid residue at position 87 is close to the first conserved region (residues 110–121) of the whole ASL family of proteins (Fig. 4b), and this residue also appears to be located within the active site cleft.2

Intragenic complementation is a phenomenon that occurs between different mutants of multimeric proteins. Positive complementation, observed after fusion of two different mutant cell strains or by mixing cell extracts, produces an increase in functional activity that is greater than that seen in either mutant alone (35, 36). Crick and Orgel (9) suggested that the loop on which Gin286e is located forms one part of active site bowl of the ASL tetramer. The aspartic acid residue at position 87 is close to the first conserved region (residues 110–121) of the whole ASL family of proteins (Fig. 4b), and this residue also appears to be located within the active site cleft.2
three monomers, with four active sites per tetramer. Consequently, due to the symmetry of the molecule, it appears likely that in a Q286R:D87G heterodimeric tetramer at least one and possibly two "wild-type" active sites will be formed, a mechanism that is possible because in any one active site, residues 87 and 286 would be contributed by different monomers. Thus, if one active site of the hybrid tetramers contains both mutant residues, another will contain normal residues at both positions and will hence reconstruct a native active site. If this model of regeneration of a wild-type active site proves to be correct, it would also explain why D87G and R95C do not complement, since one or the other of these mutations would be present in every active site. Based on this hypothesis, one would also predict that complementation could occur between A398D and Q286R subunits. Tetramers constituted from A398D and Q286R subunits could have at least one normal active site in which residues 398 and 286 are contributed by different monomers.

The characterization of the molecular basis of intragenic complementation in human ASL deficiency outlined in this paper, together with the structural determination of ASL, should facilitate the use of ASL as a model for the study of genetic diseases affecting multimeric enzymes. The results of these two studies show that the interaction of the two alleles in a patient who is a genetic compound (as the majority of patients probably are (2)) may sometimes ameliorate the biochemical phenotype of the patient. The net residual ASL activity will not be the average of the two alleles, but it will be greater due to the complementation of the two alleles. When such complementation occurs, a clinical phenotype may be produced that is less severe than that resulting from either point mutation expressed homozgyously. Individuals with complementing alleles that lead to relatively high ASL activity may be biochemically normal and not have arginosuccinic aciduria. More generally, such interactions must be considered in any attempt to correlate patient phenotypes with the genotype of patients with mutations affecting multimeric proteins.

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