Mechanisms for Intragenic Complementation at the Human Argininosuccinate Lyase Locus†

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ABSTRACT: Argininosuccinate lyase (ASL) is a homotetrameric enzyme that catalyzes the reversible cleavage of argininosuccinate to arginine and fumarate. Deficiencies in the enzyme result in the autosomal, recessive disorder *argininosuccinic aciduria*. Considerable clinical and genetic heterogeneity is associated with this disorder, which is thought to be a consequence of the extensive intragenic complementation identified in patient strains. Our ability to predict genotype−phenotype relationships is hampered by the current lack of understanding of the mechanisms by which complementation can occur. The 3-dimensional structure of wild-type ASL has enabled us to propose that the complementation between two ASL active site mutant subunits, Q286R and D87G, occurs through a regeneration of functional active sites in the heteromutant protein. We have reconstructed this complementation event, both in vivo and in vitro, using recombinant proteins and have confirmed this hypothesis. The complementation events between Q286R and two nonactive site mutants, M360T and A398D, have also been characterized. The M360T and A398D substitutions have adverse effects on the thermodynamic stability of the protein. Complementation between either the M360T or the A398D mutant and the stable Q286R mutant occurs through the formation of a more stable heteromeric protein with partial recovery of catalytic activity. The detection and characterization of a novel complementation event between the A398D and D87G mutants has shown how complementation in patients with *argininosuccinic aciduria* may correlate with the clinical phenotype.

Intragenic complementation is a phenomenon whereby particular combinations of mutant alleles at a given locus produce a less severe phenotype than the same alleles do in the homozygous state, or in the presence of noncomplementing alleles. Intragenic complementation has been demonstrated to occur in a number of human metabolic disorders, including *argininosuccinic aciduria* (*1*), *propionic acidemia* (*2, 3*), and *methylmalonic aciduria* (*4*), and is believed to contribute to the extensive clinical heterogeneity observed among patients suffering from these diseases. In all cases examined, intragenic complementation has been found to involve genes encoding multimeric enzymes. Mutant subunits of these enzymes, each possessing distinct amino acid substitutions that render the homomutant proteins inactive, interact to form heteromutant multimers possessing partial activity. Since intragenic complementation may occur in all genetic diseases involving multimeric proteins, the accurate prediction of genotype−phenotype relationships in these diseases requires a complete understanding of the mechanisms by which this phenomenon can occur. In this study, the metabolic enzyme argininosuccinate lyase (ASL)[1] is used as a model system to investigate intragenic complementation.

Argininosuccinate lyase (ASL, EC 4.3.2.1), a homotetrameric protein of 50 kDa subunits (*5*−*7*), catalyzes the reversible breakdown of argininosuccinic acid into arginine and fumarate. This reaction is a required step in the urea cycle, the major pathway for the detoxification of ammonia. Mutations in the ASL gene cause *argininosuccinic aciduria*, an autosomal recessive disorder with considerable clinical and genetic heterogeneity (*1, 8*−*10*). The clinical heterogeneity of the disease manifests itself in the variation of the age of onset and the severity of the symptoms (*8*). Three distinct clinical phenotypes have been identified: neonatal, subacute, and late-onset. The biochemical basis of this clinical variation is unclear, as there is only partial correlation between the clinical phenotype and the residual enzyme activity detected in cultured fibroblasts (*1*) and other tissues (*8*).

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[1] Abbreviations: ASL, argininosuccinate lyase; kDa, kilodalton(s); bp, base pair(s); wt, wild-type; Q286R-his, C-terminal 6-histidine tagged argininosuccinate lyase with a glutamine to arginine mutation at residue 286; D87G-his, C-terminal 6-histidine tagged argininosuccinate lyase with an aspartate to glycine mutation at residue 87; A398D-his, C-terminal 6-histidine tagged argininosuccinate lyase with an alanine to aspartate mutation at residue 398; M360T-his, C-terminal 6-histidine tagged argininosuccinate lyase with a methionine to threonine mutation at residue 360; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; MW, molecular weight; CRM, cross-reactive material; NADP-GDH, nicotinamide adenine dinucleotide phosphate-linked glutamic dehydrogenase.

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The genetic heterogeneity at the ASL locus was characterized by complementation analysis of fibroblasts cultured from 28 unrelated patients with *argininosuccinic aciduria* (*1, 9*). These studies showed that the fusion of certain pairs of cell lines resulted in significant increases in ASL activity, which could be attributed to intragenic complementation. Intriguingly, subsequent analysis demonstrated that patient strains containing an ASL allele encoding a glutamine to arginine substitution at codon 286 (Q286R) participated in almost all of the observed complementation events (*11*). Intragenic complementation could not be detected in most other combinations of patient strains. In addition, fusions of the Q286R-containing strains and those containing a mutant allele encoding an aspartate to glycine substitution at codon 87 (D87G) resulted in 3-fold higher levels of activity than that seen in any other complementation event. The existence of intragenic complementation between the Q286R and D87G mutants was directly demonstrated in COS cell transfection experiments (*11*). Plasmids expressing normal ASL, the Q286R mutant, and the D87G mutant were transfected into COS cells in various combinations, and the rate of conversion of ^{14}C-labeled fumarate into ^{14}C-labeled argininosuccinate was measured. The D87G and Q286R mutants showed little (~4.5%) or no (<0.05%) activity, respectively, when transfected on their own. However, COS cells transfected with both the D87G and Q286R mutants simultaneously were found to exhibit approximately 30% of wild-type ASL activity.

The determination of the high-resolution 3-dimensional structures of human ASL (*12, 13*) and the homologous eye lens protein, duck δ-crystallin (*14*−*17*), has suggested a mechanism for the Q286R:D87G complementation event (*12, 18*). Each of the four active sites of the ASL protein is located in a cleft formed by three different monomers (*12, 14*−*17*). Both D87 and Q286 lie in the active site region, but in any one active site, each residue is contributed by a different monomer. A homotetramer of D87G or Q286R would be inactive since all four active sites would have an amino acid substitution. Heterotetramers, containing subunits with either the D87G substitution or the Q286R substitution, could have 0, 1, or 2 active sites with all wild-type residues depending on the number and orientation of the different mutant subunits in the tetrameric protein. If the subunits had equal probability of associating, a mixture of five tetramers would form with D87G to Q286R ratios of 4:0, 3:1, 2:2, 1:3, and 0:4 in a 1:4:6:4:1 distribution. By calculating the number of active sites containing all wild-type residues in each tetramer and assuming that these nativelike active sites are fully functional and that both individual mutants are completely inactive, the mixture of heterotetramers should have 25% of the catalytic activity of the wild-type protein. This value is consistent with the 30% of wild-type activity observed for the Q286R/D87G heterotetramers in the COS cell experiments described above (*11*).

Thirteen additional mutations in the ASL gene associated with *argininosuccinic aciduria* have been identified (*11, 19*−*22*). In contrast to the Q286R and D87G substitutions, which are located in the ASL active site, the majority of these other disease-causing substitutions are dispersed throughout the ASL structure. Nevertheless, the Q286R mutation was found to participate in complementation events with almost all of these ASL mutants, regardless of their location in the tertiary structure (*1*). In all cases, however, the recovered wild-type ASL activity was only about one-third of that for the Q286R:D87G complementation event (*1*).

The work presented in this paper is aimed at answering three questions raised by the previous studies on intragenic complementation at the ASL locus: (1) Why do most of the ASL alleles fail to display intragenic complementation in the fibroblast assay? (2) Why does the Q286R mutant complement almost all other ASL mutants? (3) Why does Q286R:D87G complementation result in much higher activity than any other observed ASL complementation event? To address these questions, we have characterized the effects of the Q286R and D87G substitutions, as well as an alanine to aspartate substitution at codon 398 (A398D) (*11*) and a methionine to threonine substitution at codon 360 (M360T) (*21*), on the recombinant ASL protein. The latter two mutants were identified in patient strains that complement with Q286R. The A398 and M360 residues are removed from the active site of ASL. By coexpressing combinations of these mutants, we have been able to purify mutant heterotetramers and assess their complementation behavior in vitro. We have also assessed the thermal stability of the homo- and heteromutant proteins. The results of these experiments have allowed us to identify two general mechanisms by which intragenic complementation occurs at the ASL locus. First, complementation can occur by the regeneration of functional nativelike active sites in heterotetramers of two distinct, stable, active site mutants. Furthermore, these stable, active site mutants can also oligomerize with unstable mutant subunits to form hybrid tetramers with enough stability for catalysis to occur.

MATERIALS AND METHODS

Expression Vectors. A pET-3c vector (T7 promoter, amp^r, pBR322 origin) containing human ASL (pET-wtASL) was obtained from Dr. M. Hershfield (*23*). The pET-3c D87G (pET-D87G) and Q286R (pET-Q286R) expression vectors were constructed using restriction digestion and the pESP-SVTEXP expression vectors pESP-D87G and pESP-Q286R constructed previously (*11*). The pESP-D87G plasmid was digested with *Mlu*I and *Spe*I, and nucleotides 74−743 were subcloned into the pET-wtASL plasmid, replacing the normal sequence in this region. Similarly, the pESP-Q286R plasmid was digested with *Spe*I and *Sfi*I, and nucleotides 743−1211 were subcloned into the pET-wtASL plasmid. To simplify purification, plasmids that expressed a C-terminal histidine-tagged version of the protein were also constructed. The pET-wtASL-his plasmid was constructed by amplifying the 5′ end of the gene using PCR with the addition of a six-histidine tag. The PCR product and the pET-wtASL were digested with *Sfi*I and *Bam*HI, and the digested PCR product was ligated into the analogous region of the pET-wtASL plasmid to construct pET-wtASL-his. The pET-D87G-his and pET-Q286R-his plasmids were constructed as described above for the pET-D87G and pET-Q286R plasmids except that pET-wtASL-his was used instead of pET-wtASL.

The pET-3c A398D-his (pET-A398D-his) and M360T-his (pET-M360T-his) expression vectors were constructed by site-directed mutagenesis using the Unique Site Elimination kit from Pharmacia. The oligonucleotides (Gibco BRL) CC TCC GGA AAA GCT GTG TTC ATG **GAC** GAG ACC

and CAA GAG AAC **ACG** GGA CAG GCC TTA AGC CC were used to generate the A398D and M360T mutations, respectively. The above mutagenic primers were used to introduce both the desired codon (boldface) and a unique restriction site (underlined), *Kpn*2I or *Bsp*TI, respectively, in the region of the mutation. Screening for the presence of the new restriction site by restriction enzyme mapping identified positive mutants. All plasmids were sequenced to ensure that each mutation had been correctly introduced and that no additional mutations existed.

For the in vivo complementation studies, coexpression vectors containing the full-length cDNA and T7 promotor site for either the D87G-his, A398D-his, or M360T-his mutant with the full-length cDNA and T7 promotor site for the Q286R mutant were created. The pET-D87G-his plasmid was digested with *Bgl*II and *Bam*HI, and the resulting 1537 bp fragment was subcloned into the *Bgl*II site of the pET-Q286R plasmid to generate the pET-D87G-his/Q286R plasmid. The pET-A398D-his/Q286R and pET-M360T-his/Q286R plasmids were generated as above.

Protein Expression and Purification. Plasmids containing C-terminal histidine-tagged proteins were overexpressed in the *E. coli* strain BB101, genotype *ara* Δ*(lac proAB)* Δ*slyD (kan*^{r}*) nalA argEam rif thi* F′*[lacI*^{q} *proAB*$^+$*]* (λDE3), using the T7 polymerase system. Cells were harvested 3−4 h post-induction (1 mM IPTG, 37 °C) for wtASL-his, Q286R-his, D87G-his, and D87G-his/Q286R, and 20 h post-induction (1 mM IPTG, 25 °C) for A398D-his, M360T-his, and heterotetramers containing either the A398D-his or the M360T-his subunit. Cells were stored at −20 °C until required. Expression of the full-length A398D-his and M360T-his mutant proteins was confirmed by western blot analysis (*24*) using the QIA*express* Penta•His antibody (Qiagen).

The frozen cells for the homomutant proteins were lysed by sonication in 20 mL of Buffer A (20 mM Tris-HCl, pH 8, 0.5 M NaCl) with 0.5 mM PMSF. Cells were sonicated for a total of 10 min using a pulse sequence for 30 s interspersed with 90 s of cooling on ice. The sonicated cells were centrifuged (JA-20 rotor in a Beckman J2-21 centrifuge) at 15 000 rpm for 30 min at 4 °C. The pellet was resuspended in 20 mL of Buffer A and sonicated and centrifuged as described above. The supernatant from both spins was pooled and applied to a Ni-affinity column (His-Bind^R resin, Novagen) preequilibrated with Buffer A. The column was washed with 30 mL of 5 mM imidazole in Buffer A and 12 mL each of 30 and 60 mM imidazole in Buffer A. The protein was eluted with 15 mL of 100 mM imidazole in Buffer A and the eluted fraction dialyzed overnight at 4 °C against 4 L of dialysis buffer (20 mM potassium phosphate, pH 6.5, 1 mM EDTA, 1 mM DTT, 300 mM NaCl).

The frozen cell pellets from the coexpressed heteromutant proteins were lysed and applied to the Ni-affinity column as described above for the homomutant proteins. The column was washed with 30 mL of Buffer A, 30 mL of Buffer A with 5 mM imidazole, and 12 mL each of Buffer A with the following imidazole concentrations: 10, 20, 30, 60, 100, and 200 mM. The fractions eluted with 30 and 60 mM imidazole were pooled and dialyzed overnight at 4 °C against 4 L of dialysis buffer. All proteins were approximately 95% pure.

Circular Dichroism Spectroscopy. The CD spectra for all proteins were collected in a cell with a path length of 0.1 cm on an AVIV Circular Dichroism spectrophotometer (model 62A DS). The protein solution (0.15−0.20 mg/mL in 20 mM potassium phosphate, pH 7.5, 1 mM EDTA, 1 mM DTT) was scanned from 200 to 260 nm in 1 nm increments. To examine the thermal stability of each protein, the loss of ellipticity at 222 nm was monitored as the protein sample was heat-denatured. The temperature was increased from 25 to 101 °C in 2 °C increments with 1 min equilibration before each reading.

ASL Activity Assays. The ability of plasmid-borne ASL mutants to complement an *E. coli* strain with its endogenous ASL gene deleted served as a simple in vivo assay for ASL activity. *E. coli* strain W3678 (ΔgalT, LAM-, IN(rrnD-rrNE)1, ΔargH, F-) was obtained from V. L. Chan (*25*) and lysogenized using Novagen's λDE3 Lysogenization Kit. The resulting ΔASL cells transformed with various ASL-expressing plasmids were assayed on plates containing M9 minimal media (*24*) with and without arginine supplementation. The streaked plates were incubated at 37 °C for 48 h. The level of growth of the transformed cells on M9 minimal media was compared to that for cells transformed with the plasmid carrying the wild-type ASL protein.

The in vitro ASL enzyme activity of the wild-type and mutant proteins was determined spectrophotometrically; 10× stock solutions of sodium argininosuccinate (Sigma-Aldrich) in reaction buffer (20 mM potassium phosphate, pH 7.5, 1 mM EDTA) were prepared such that the final concentration of substrate in the reaction ranged from 0.02 to 2.0 mM. The reaction was initiated by adding 20−30 μL of the protein solution (10−20 μg of enzyme) to the reaction mixture for a total reaction volume of 500 μL. The reaction was monitored by measuring the increase in fumarate (λ_{max} = 240 nm; ϵ = 2.44 mM^{-1} cm^{-1}) concentration accessed by UV absorption at 240 nm on a Milton Roy 3000 spectrophotometer. All ASL assays were performed in triplicate at room temperature. Initial velocities were used to determine the kinetic parameters.

In Vitro Complementation Studies. Equal quantities of the D87G-his and Q286R-his ASL mutants (0.2 mg/mL) were mixed together and incubated at either 0 °C or room temperature (25 °C). Aliquots (50 μL) were taken from the mixture at various intervals of time over a 6-day period and diluted 10 times and allowed to equilibrate at room temperature for 30 min prior to performing the activity assay. The diluted sample (50 μL) was assayed for activity in the presence of 5 mM substrate. The enzyme activity of all samples was assayed in triplicate at 25 °C. The activities of wild-type ASL, individual D87G-his and Q286R-his homomutants, and the D87G-his/Q286R coexpressed protein, incubated at both 0 °C and 25 °C, were monitored as controls. The total protein concentration of the incubated mixtures was 0.4 mg/mL. The results obtained were expressed as the percentage of wild-type activity recovered over time.

RESULTS

Production of Q286R/D87G Heterotetramers by in Vivo Coexpression. To determine whether the ASL protein expressed in *E. coli* could be used as a model system for studying the phenomenon of intragenic complementation observed at the ASL locus, we first established that mutant heterotetramers would form when coexpressed in *E. coli*.

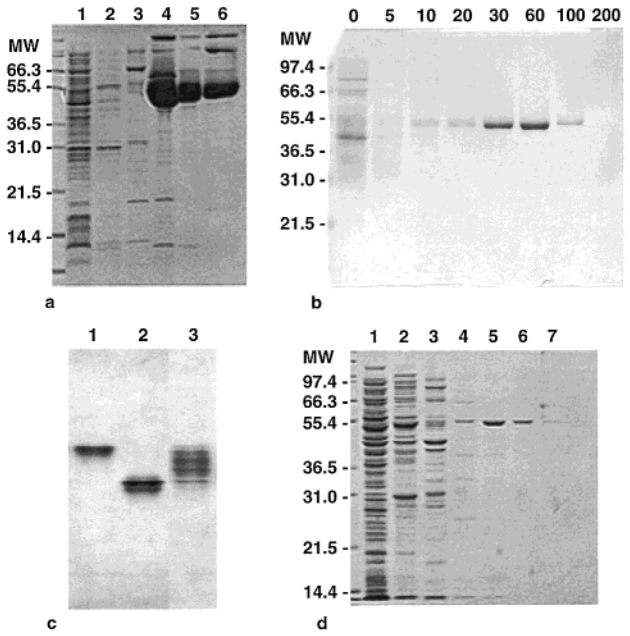

FIGURE 1: Expression and purification of ASL proteins. (a) Purification of wtASL-his by Ni-affinity chromatography. Lane 1, flow-through; lanes 2−6, washes with 5, 60, 100, 100, and 200 mM imidazole, respectively. The protein was eluted in the 100 and 200 mM imidazole fractions (lanes 4−6). (b) Purification of the coexpressed Q286R/D87G-his heterotetramers by Ni-affinity chromatography. The protein was eluted with increasing imidazole concentrations. The protein eluted with 30 and 60 mM imidazole was pooled and run on an isoelectric focusing gel. (c) Isoelectric focusing gel illustrating the heterogeneous mixture of the coexpressed protein. Lane 1, D87G-his homotetramer; lane 2, Q286R homotetramer; lane 3, Q286R/D87G-his-coexpressed heterotetramers. (d) Purification of M360T-his mutant ASL by Ni-affinity chromatography. Lane 1, flow-through; lanes 2−7, washes with 5, 30, 60, 100, 100, and 200 mM imidazole, respectively. The protein eluted in the 100 mM imidazole fractions (lanes 5 and 6).

To facilitate purification of wild-type and mutant ASL proteins, the proteins were expressed with C-terminal 6-His tags. 6-His-tagged wild-type ASL and Q286R and D87G ASL mutants (wtASL-his, Q286R-his, and D87G-his, respectively) were expressed in *E. coli* and yielded approximately 10−20 mg of 95% pure protein per liter of cultured cells (Figure 1a). Plasmids were constructed to coexpress various pairs of mutants within the same *E. coli* cell. On each plasmid, only one of the mutant ASL genes encoded a C-terminal 6-His tag. The objective of this approach was to produce heterotetramers in vivo, which could subsequently be purified by Ni-affinity chromatography. There are five different heterotetramers that can be formed from the combination of two different monomers (mutants 1 and 2). These tetramers would contain mutant 1 and mutant 2 polypeptides in the following ratios: 0:4, 1:3, 2:2, 3:1, 4:0. Since only one of the two mutants has a 6-histidine tag, the tetramers produced are expected to have varying affinities for a Ni column based on the number of histidine-tagged subunits present.

Putative heterotetramers, produced in cells where the Q286R and D87G-his mutants were coexpressed, were purified by Ni-affinity chromatography. ASL-containing fractions were found to elute at imidazole concentrations between 30 and 60 mM (Figure 1b). These fractions were presumed to contain heterotetramers of tagged and untagged ASL monomers because homotetramers of 6-His-tagged ASL elute at 100 mM imidazole (Figure 1a). A homotetramer with none of its subunits tagged does not bind to the column (data not shown). Isoelectric focusing gels were used to confirm the presence of heterotetrameric enzymes. Since the additional histidines will increase the isoelectric point of a tagged monomer, the isoelectric point of heterotetramers will increase with the number of histidine-tagged subunits present. Homotetramers containing only Q286R or D87G-his homotetramers should migrate as single bands at distinctly different positions on an isoelectric focusing gel (Figure 1c). To date, we do not know why the Q286R tetramer appears to run as a doublet, although this observation is not unique to this study. In previous immunoblot experiments, two bands of ∼51 and ∼49 kDa were seen for patient strains that carried the Q286R substitution (*9*). In contrast, ASL purified from the Q286R/D87G-his coexpression strain migrates in multiple bands with mobilities that are intermediate between the pure mutant homotetramers. These data demonstrate that heterotetramers with varying ratios of tagged to untagged monomers are present in this enzyme preparation.

Coexpression Studies with the M360T and A398D Mutants. Unlike the Q286R and D87G mutants, both the M360T-his and A398D-his mutants were found to accumulate in insoluble aggregates when expressed in *E. coli* at 37 °C. Western blot analysis confirmed that the full-length proteins were expressed at high levels similar to wild-type (data not shown), but remained in the pellet after cell lysis. Pure soluble M360T-his mutant protein (2 mg per liter of cultured cells) was obtained when the mutant protein was expressed at room temperature (Figure 1d). Varying the temperature at which the A398D-his protein was expressed, from 15 to 37 °C, failed to produce any soluble protein, preventing further investigation of this homomutant protein in vitro.

The solubility of the M360T-his and A398D-his mutants dramatically increases when they are coexpressed with the Q286R mutant. Most remarkably, soluble subunits of the A398D-his mutant protein were observed when coexpressed with Q286R, even though the A398D-his protein accumulates exclusively in insoluble aggregates when expressed on its own (data now shown). These results indicate that the M360T and A398D substitutions cause defects in protein folding which result in precipitation inside the cell. Coexpression with the Q286R mutant leads to a rescue of these insoluble mutants by the formation of more stable heterotetramers. Consistent with this scenario, coexpression of the two putative unstable mutants, M360T and A398D, did not increase the solubility of the proteins (data not shown).

Stability of Wild-Type and Mutant Proteins. To determine the effects of these substitutions on the structure and stability of the protein, the wild-type and mutant ASL proteins were analyzed by circular dichroism (CD) spectroscopy. All the ASL proteins in the present study have a typical α-helical spectrum with the characteristic minima at 208 and 222 nm (Figure 2). The variations in the total amount of ellipticity may reflect subtle alterations in the structure of some mutants. In this regard, it is notable that the homotetramer of the unstable M360T-his mutant displays the least ellipticity.

The unfolding of each protein was monitored by the change in ellipticity at 222 nm as the temperature of the sample was increased. All transitions were found to be

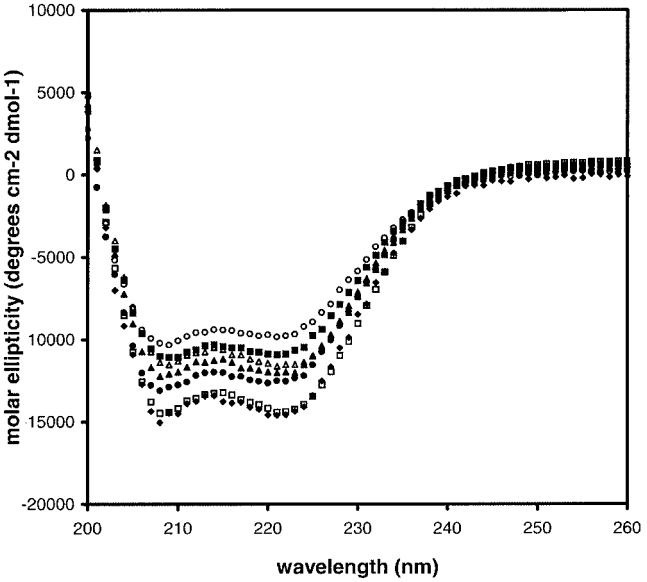

FIGURE 2: Circular dichroism spectra of wild-type and mutant ASL proteins. Proteins (0.15−0.20 mg/mL) were scanned from 200 to 260 nm, and the resulting spectra were superimposed. Key to plot: (◆) wtASL-his homotetramer; (□) Q286R-his homotetramer; (△) D87G-his homotetramer; (○) M360T-his homotetramer; (▲) Q286R/D87G-his heterotetramer; (●) M360T-his/Q286R heterotetramer; (■) A398D-his/Q286R heterotetramer.

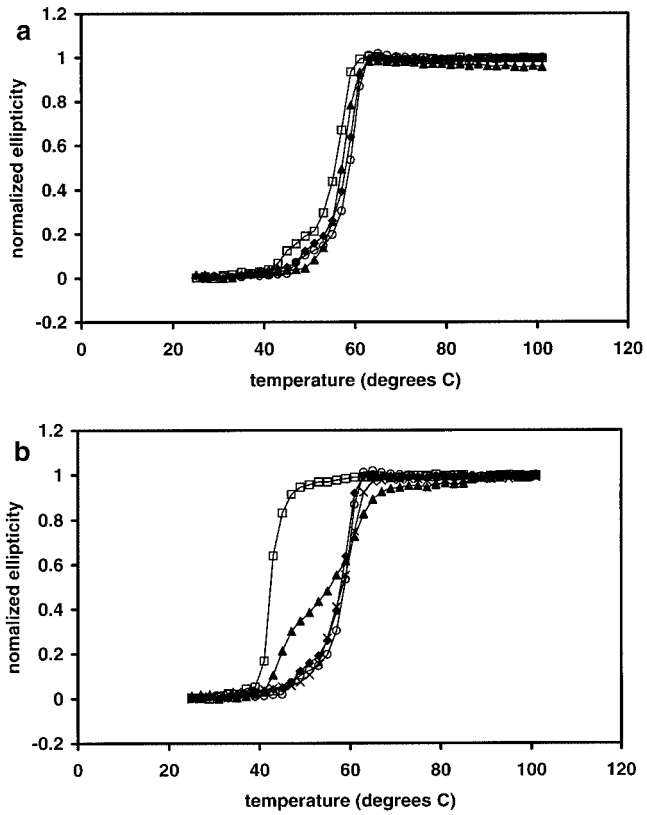

FIGURE 3: Temperature denaturation of ASL proteins monitored as a function of the circular dichroism signal at 222 nm. The curves were normalized for comparison of data. (a) Temperature denaturation of (◆) wtASL-his, (○) Q286R-his, (□) D87G-his, and the coexpressed protein (▲) Q286R/D87G-his. (b) Temperature denaturation of (◆) wtASL-his, (○) Q286R-his, (□) M360T-his, and the coexpressed proteins (▲) M360T-his/Q286R and (×) A398D-his/Q286R.

cooperative and irreversible. Although no thermodynamic parameters can be obtained from irreversible reactions, monitoring the loss of structure as a function of temperature serves as a simple method for comparing the stability of the different proteins. The wtASL-his, Q286R-his, and D87G-his proteins were all found to have comparable thermal stability with the midpoint of the transition ranging from approximately 56 to 58 °C (Figure 3a). Similarly, there was no change in the transition for the D87G-his/Q286R-coexpressed heterotetramers (Figure 3a). In contrast, the M360T-his protein exhibited a dramatic decrease in stability with the midpoint of the transition at approximately 42 °C (Figure 3b). The transition for the M360T-his/Q286R-coexpressed heterotetramers was less cooperative but exhibited greater resistance to thermal denaturation than the M360T-his homomutant protein. The thermal stability observed for the A398D-his/Q286R-coexpressed heterotetramers was near wild-type (Figure 3b).

Enzymatic Activity of Wild-Type and Mutant Proteins. To characterize the effect that the individual amino acid substitutions have on the function of the ASL protein, the enzyme activity was assayed. The in vivo catalytic activity of the mutant proteins was tested by assessing their ability to support growth of a ΔASL *E. coli* strain on minimal media. These cells do not carry a functional gene for ASL and therefore require an external source of arginine for growth on minimal media. In the absence of arginine, human wild-type ASL was found to complement growth of this *E. coli* strain. To determine the minimum level of catalytic activity required to complement the ΔASL strain, cells were transformed with a number of duck δ2 crystallin mutants (*26*). δ2 crystallin is an enzymatically active ASL homologue found in birds and reptiles (*27*). Using wild-type duck δ2 crystallin and the previously characterized δ2 crystallin mutants, the ΔASL strain was found to require a minimum threshold of 3% ASL activity to be able to grow on minimal media without an external arginine source (data not shown). The level of catalytic activity above or below this threshold could not be quantified by the screen. Compared to wild-type ASL, cells transformed with either M360T-his or Q286R exhibited slower growth as seen by the small colony size (Table 1). The A398D-his and D87G-his mutants were completely unable to support growth on minimal media. Strikingly, cells transformed with a plasmid coexpressing A398D-his with D87G could support growth, indicating that partial enzymatic activity was restored. This result demonstrates that intragenic complementation has occurred between these mutant alleles. Moreover, when transformed with any coexpression plasmid, with the exception of M360T-his/A398D, enough arginine was produced by the cells to permit growth on minimal media at levels similar to wild-type (Table 1). These data demonstrate that intragenic complementation between ASL alleles can be observed in vivo in *E. coli*, and that all pairs of mutants except M360T-his/A398D are able to complement.

The in vitro activity of the purified proteins was determined spectrophotometrically by measuring the rate of fumarate production. All active enzymes obeyed Michaelis−Menten kinetics with no evidence of negative cooperativity. The Michaelis−Menten plots for wild-type ASL and the Q286R-his mutant are shown in Figure 4a and b, respectively. A comparison of native and histidine-tagged wild-type proteins confirmed that the tag does not affect catalysis

Table 1: Kinetic Properties of Wild-Type and Mutant ASL Proteins

protein	in vivo activity[a]	K_M (mM)	V_{max} (μmol min^{-1} mg^{-1})	K_{cat} (s^{-1})	K_{cat}/K_M (M^{-1} s^{-1})	% of in vitro wt activity
wt ASL	++	0.12 ± 0.01	10.36 ± 0.90	34.5 ± 3.0	(2.98 ± 0.19) × 10^5	100 ± 6.4
Q286R-his	+	0.07 ± 0.01	0.18 ± 0.01	0.60 ± 0.02	(8.98 ± 0.84) × 10^3	3.0 ± 0.3
D87G-his	−					inactive
M360T-his	+					inactive
A398D-his	−					inactive
Q286R/D87G-his	++	0.06 ± 0.01	1.90 ± 0.09	6.34 ± 0.30	(1.05 ± 0.86) × 10^5	35.2 ± 2.9
A398D-his/Q286R	++	0.11 ± 0.01	1.52 ± 0.04	5.05 ± 0.13	(4.62 ± 0.10) × 10^4	15.5 ± 0.4
M360T-his/Q286R	++	0.06 ± 0.01	0.51 ± 0.02	1.71 ± 0.05	(3.07 ± 0.25) × 10^4	10.3 ± 0.9
A398D-his/D87G	++	0.03 ± 0.01	0.37 ± 0.02	0.12 ± 0.05	(3.67 ± 0.50) × 10^4	12.3 ± 1.7
M360T-his/D87G	++	0.06 ± 0.03	0.29 ± 0.09	1.02 ± 0.32	(1.89 ± 0.74) × 10^4	6.3 ± 2.5
M360T-his/A398D	+					inactive

[a] Measured as the protein's ability to recover growth of ΔASL *E. coli* cells on M9 minimal media. ++, growth of cells similar to wild-type; +, growth of cells slower than wild-type; −, no growth.

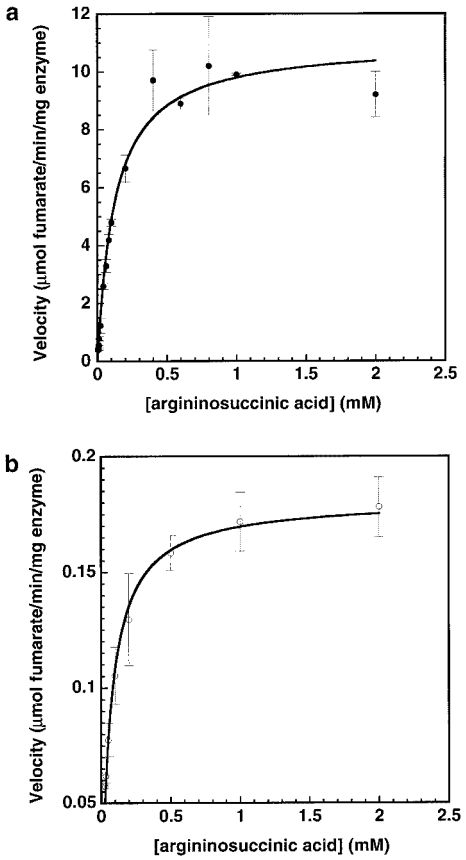

FIGURE 4: Michaelis-Menten plot of kinetic data for (a) wtASL and (b) Q286R-his homotetramers.

(data not shown). A summary of the kinetic parameters obtained is presented in Table 1. The D87G-his mutant protein was inactive, while the Q286R mutant exhibited 3.0 ± 0.3% residual wild-type activity. The coexpression of any two mutants resulted in an increase in ASL activity relative to either of the expressed homomutants. The largest increase was observed when the Q286R and D87G-his proteins were coexpressed, consistent with in vivo data (*1, 11*). A recovery of 35.2 ± 2.9% activity was observed. The A398D-his mutant, which is insoluble on its own, produced heterotetramers with either the Q286R or the D87G mutant with 15.5 ± 0.4% and 12.3 ± 1.7% wild-type activity, respectively. In contrast to the low level of in vivo activity described above, the M360T-his mutant exhibited no enzyme activity in vitro. However, heterotetramers of the inactive M360T-his mutant with either Q286R or the inactive D87G mutants recovered 10.3 ± 0.9% and 6.3 ± 2.5% wild-type activity, respectively.

Reconstruction of the Complementation of D87G and Q286R in Vitro. ASL has been reported to undergo reversible cold dissociation (*28, 29*). This phenomenon was exploited to reconstruct the complementation event in vitro using purified protein. When equal amounts of Q286R-his and D87G-his were mixed and incubated at room temperature, no increase in catalytic activity was observed (Figure 5). However, when the proteins were mixed and incubated at 0 °C, the amount of ASL activity increased over time, indicating that at 0 °C a reassortment of subunits occurred to form active heterotetramers. The level of activity for the Q286R/D87G-his-coexpressed proteins or the individual homomeric proteins by themselves did not vary with the temperature at which they were incubated. The final percentage of wild-type activity recovered after the 6 day incubation is represented in Figure 5b. The final level of activity recovered by the mixture of individually purified Q286R-his and D87G-his corresponded to that of the Q286R/D87G-his-coexpressed proteins. These experiments show that the purification procedure used to obtain the coexpressed heterotetrameric proteins did indeed produce a mixture of heterotetramers.

DISCUSSION

The Q286R Substitution in Frequent Complementing Strains. Walker et al. (*11*) identified the Q286R substitution in patient strains classified as frequent complementers. These strains participated in the majority of complementation events seen at the ASL locus (*1*). We have studied the effect of the Q286R substitution on the recombinant ASL protein, both by itself and with other mutant subunits in heteromeric proteins, to determine how it is able to participate in most complementation events.

Structural analysis by circular dichroism reveals that there are no gross alterations in the secondary structure or any difference in the thermal stability of the Q286R mutant compared to wild-type ASL. The loss of activity seen in the Q286R mutant has previously been attributed to the location of the substituted residue in the active site of the protein (*12*). Q286 is adjacent to K287, a residue that appears to be crucial for stabilizing the enzyme−substrate complex (*15, 30*). To understand the structural basis for the loss of activity in the Q286R homomutant protein, its 3-dimensional

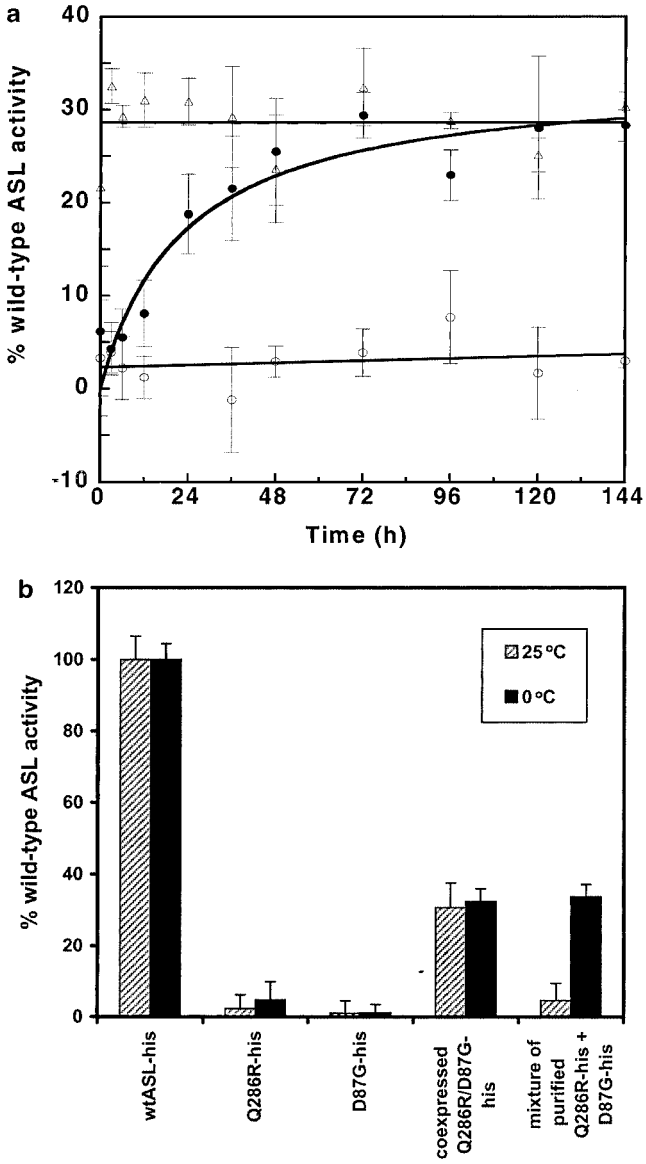

FIGURE 5: In vitro complementation between Q286R-his and D87G-his. (a) Equal quantities of purified Q286R-his and D87G-his mutant proteins were mixed together and incubated at either 0 °C (●) or 25 °C (○). The enzyme activity was measured at various time intervals and compared to that for the wild-type protein incubated under the same conditions. The Q286R/D87G-his-coexpressed heterotetramers (△) incubated at 0 °C were used as a control. (b) The percentage of wild-type activity recovered after the 6 day incubation at 25 °C (striped) and 0 °C (black) for the wtASL-his, Q286R-his, and D87G-his homotetramers, the Q286R/D87G-his-coexpressed heterotetramers, and the Q286R-his + D87G-his homotetramer mixture.

structure has been determined by X-ray crystallography [see preceding paper (*13*)]. While we are unable from this structure to determine unambiguously why the Q286R mutant is inactive, it is likely that the substitution hinders a conformational change, which is thought to be required for catalysis to occur. The loss of enzyme activity is therefore primarily due to the location of the amino acid substitution in the active site region of the protein.

Fifteen naturally occurring mutations in the ASL gene have been identified to date (*10, 11, 19, 21, 22*). The majority of mutations occur in buried positions outside the active site. Substitutions of these residues are likely to affect the stability and/or the conformation of the protein (*31*). The effect of most of these substitutions on catalysis will therefore be indirect since a change in monomer stability and/or subunit association would decrease the amount of active tetramer. Our investigation of two such mutants, M360T and A398D, confirms they have considerably less stability than wild-type ASL. The A398D substitution likely prevents folding of the protein since no stable, soluble tetramers are present in vivo in *E. coli* to rescue growth of the ΔASL cells on minimal media (Table 1). The A398D mutant accumulated in inclusion bodies even when expressed at 15 °C. The M360T mutant protein was found to have enough residual in vivo activity to permit slow growth of ΔASL cells on minimal media. The purified protein, however, was completely inactive (Table 1). In thermal denaturation experiments monitored by CD, the M360T-his mutant aggregated near 40 °C, approximately 20 °C below the wild-type protein (Figure 3b). This result is consistent with our observation that the M360T mutant was completely insoluble in *E. coli* when expressed at 37 °C, but was partly soluble at room temperature. The lack of in vitro activity for the M360T mutant is perplexing since the assays were preformed at room temperature, conditions under which this protein is folded (Figure 3b) and soluble. In addition to its destabilizing effect, the M360T substitution must cause some other conformational alteration that results in loss of activity. The low level of in vivo activity detected for the M360T mutant (Table 1) likely reflects some difference in the intracellular environment. The fact that aggregation occurs near physiological temperatures explains why the mutant protein aggregates when expressed at 37 °C and why substitution causes ASL deficiency in humans.

Our results show that the M360T and A398D mutants produce unstable proteins but when coexpressed with Q286R are able to form heterotetramers with increased stability and partial recovery of catalytic activity. Neither A398 nor M360 is positioned at the subunit interface, and both are removed from the Q286 residue in adjacent subunits (Figure 6). The flexibility of the loop (*16, 17*) on which Q286 is located, the 280's loop, allows the substitution to arginine to be tolerated without any detectable loss of stability. We surmise that the Q286R mutant is able to participate in complementation events with a variety of mutants because it is able to associate with unstable mutant subunits and generate heteromeric proteins with enough stability to be active. The increased stability of M360T-his/Q286R heterotetramers compared to M360T-his homotetramers (Figure 3b) confirms that the association of stable and unstable ASL monomers can produce a tetramer with intermediate stability.

No complementation was observed between the unstable mutants, M360T and A398D (Table 1). Clearly, unstable mutants must oligomerize with a stable mutant, like Q286R, for complementation to occur. These observations may explain why most of the ASL alleles failed to display intragenic complementation in the fibroblast assay. The location of most of the other disease-causing substitutions in ASL suggests that these mutant proteins would have reduced stability and therefore their inability to complement one another is not surprising.

Complementation due to "stabilizing" mutant subunits was previously observed in the hexameric nicotinamide adenine dinucleotide phosphate-linked glutamic dehydrogenase

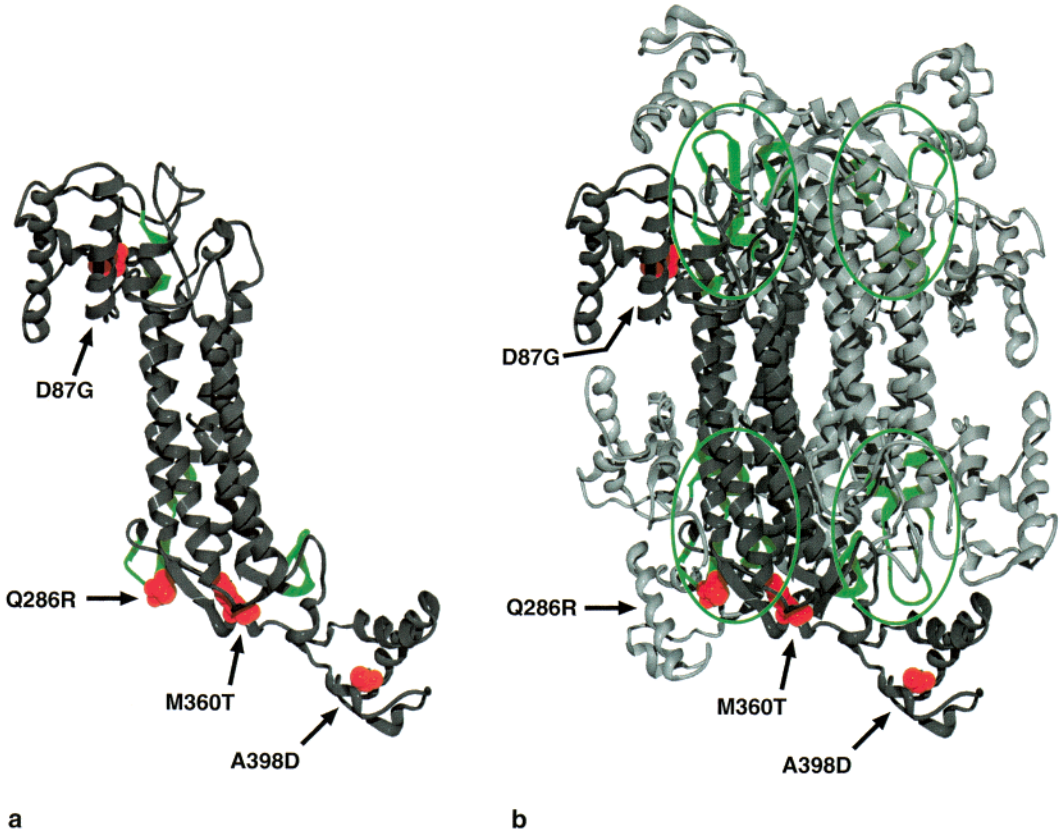

FIGURE 6: Schematic representation of the ASL (a) monomer and (b) tetramer. The highly conserved consensus sequences of the ASL superfamily are colored green in each panel. In (a), the locations of D87, Q286, M360, and A398 in the monomer are shown in red. In (b), the monomer shown in (a) is depicted in the active tetrameric form of the ASL protein. The green circles represent the location of the four active sites.

(NADP-GDH) of *Neurospora crassa* (*32*). The mutant am[1] appeared to be conformationally stable but unable to bind NADP for catalysis (*32*), much like Q286R is conformationally stable but catalytically inactive. Initial investigation of mutant am[1] indicated that the mutant subunit complemented with a variety of other am mutants, and heterohexamers were active over a much wider range of conditions; that is, they were more stable than the homomeric form of the second am mutant (*33*). Complementation was observed to occur in NADP-GDH only when a certain minimum content of the "stabilizing" am[1] subunit was present in the heterohexamer (*34*). Mutant am[14], which produced no detectable NADP-GDH hexamers, was found to form active heteromers with am[1] (*32*, *35*), mirroring the A398D:Q286R complementation event in ASL.

The D87G Substitution in High-Activity Strains. In their complementation analysis, McInnes et al. (*1*) found that two of the positive complementation tests produced a 3-fold higher increase in ASL activity than any other fusion. These two fusions occurred between the frequently complementing (Q286R-containing) strains and those that were classified as high-activity strains, which were subsequently found to be either homozygous or heterozygous for the D87G substitution (*11*).

Like Q286R, the inactivity of the D87G mutant is due to its location in the active site cleft (*12*). D87 binds the peptidic end of the argininosuccinate substrate via a water molecule (*36*). D87 is located at the end of a flexible loop and forms a cap at the beginning of helix α5. Analysis of various δ-crystallin structures has suggested that a specific conformation of residues 74−89 is required for catalysis (*17*). The absence of the carboxylic group in the D87G mutant could therefore eliminate the interaction with the substrate as well as cause conformational changes that prevent the substrate from binding and hence render the enzyme inactive.

We hypothesize that the Q286R:D87G complementation event results in higher activity than any other complementation event because both of these mutants possess wild-type stability. A recovery of at least 25% wild-type activity suggests that all possible heterotetramers containing these two mutants have an equal probability of occurring. The activity of the Q286R/D87G-his heterotetramers should actually be slightly greater than 25% due to the residual 3% activity seen in the Q286R mutant (Table 1). Complementation between either M360T or A398D mutant subunits and the Q286R mutant does not produce as high activity in vivo (*1*) or in vitro. Judging by their positions, M360T and A398D, as well as the other amino acid substitutions that cause *argininosuccinic aciduria*, likely cripple the enzyme by reducing its stability in some way. The lower levels of recovered activity seen during complementation with Q286R could perhaps be due to the unstable subunits not being as efficiently incorporated into heterotetramers as the stable mutants, or the heterotetramers formed with unstable subunits having aberrant active site conformations due to structural alterations caused by the substitutions. These observations are supported by results from immunoblot studies of ASL in patient fibroblasts (*9*). The majority of the patient strains exhibited cross-reactive material (CRM) of lower molecular weight, indicating degradation. The lower weight CRM was

not present in patient strains containing the Q286R substitution. It was also observed that the amount of normal molecular mass CRM (49−51 kDa) present in a patient strain correlated strongly with the frequency in which that patient strain participated in complementation. This correlation agrees with our hypothesis that the more stable mutants are better able to complement.

The experiments performed in this work demonstrate that the D87G mutant complements unstable mutants, such as M360T and A398D, almost as well as Q286R (Table 1). This result was expected because we hypothesize that stable active site mutants, such as D87G, should be able to complement most nonactive site unstable mutants. The D87G-containing fibroblast strains, however, were not found to be frequently complementing strains. At present, it is difficult to explain the very different behaviors of the D87G and Q286R mutants in the in vivo complementation analysis (*1*). Conformational changes that alter substrate binding in D87G homotetramers may also affect the ability of the D87G mutants to form active tetramers with unstable mutants. The relatively efficient D87G-mediated complementation that we observed in this work may be the result of much higher concentrations of protein being present in vitro and in *E. coli* as compared to the levels present in fibroblasts. The lower concentrations of ASL within fibroblasts could exacerbate any defects in multimerization possessed by the D87G mutant.

The A398D mutation was identified by Walker et al. as the substitution in the second allele in a patient strain heterozygous for the D87G substitution (*11*). Walker et al. suggested that the A398D allele had significant residual ASL activity due to the mild clinical phenotype of the heterozygote (late-onset) compared to a D87G homozygote (sub-acute) (*11*). Our results suggest that this is unlikely to be true since the A398D homomutant protein is completely inactive, cannot be expressed in a soluble form, and does not support growth of the ΔASL *E. coli* strain. There has been no reported case of an individual homozygous for the A398D substitution, probably due to the severe nature of the amino acid substitution on the homomeric protein. We hypothesize that in this particular case, the difference in the clinical phenotype observed is due to complementation between D87G and A398D.

Mechanisms for Intragenic Complementation. We have shown that intragenic complementation occurs in ASL between stable, active site mutants by the formation of nativelike active sites in the hybrid protein. Figure 7 shows schematically how two distinct active site mutants of a homodimeric protein could recover activity. This type of complementation has also been observed in another member of the ASL superfamily, adenylosuccinate lyase (*37*), as well as in the homotrimeric enzyme aspartate transcarbamoylase (*38*) and homodimeric proteins glutathione reductase (*39*), thymidylate synthase (*40*), mercuric reductase (*41*), and ribulose bisphosphate carboxylase/oxygenase (*42*).

The explanation that complementation occurred by the formation of "native" functional active sites was first suggested by Crick and Orgel in 1964 (*43*), but was dismissed from their general theory of intragenic complementation since it could not account for the complexity of most complementation maps. That is, the probability that all mutant subunits that complement involve substitutions

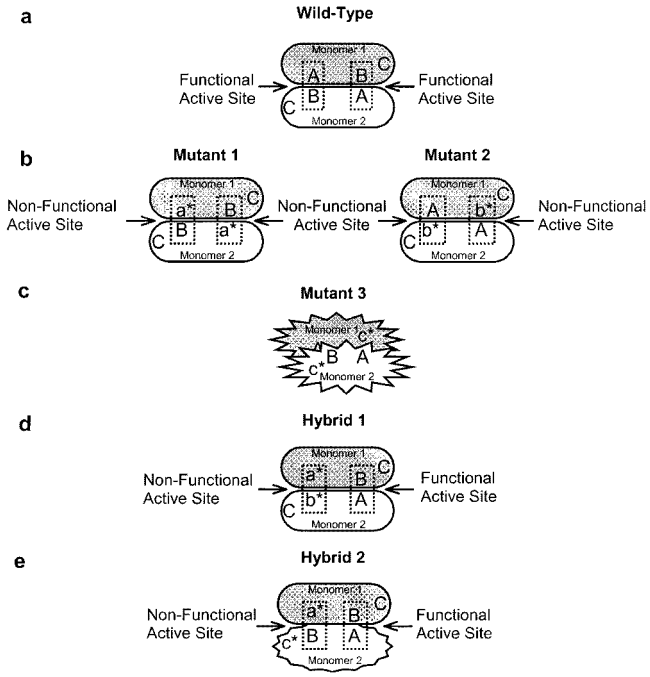

FIGURE 7: Schematic representation of the effects of different amino acid substitutions on a homodimeric protein. (a) The wild-type protein. The active site is represented by the dashed box and is formed by residues (A and B) from both monomers. There are two active sites per molecule. Residue C represents any amino acid removed from the active site. (b) Two distinct stable homomutant proteins. a* and b* represent substitutions of active site residues A and B, respectively. (c) An unstable homomutant protein. c* represents the substitution of nonactive site residue C. (d) The regeneration of nativelike active sites in a heterodimeric protein formed from two stable mutants. Hybrid 1 consists of monomer 1 from stable Mutant 1 and monomer 2 from stable Mutant 2 and has one functional active site. (e) The regeneration of nativelike active sites in a heterodimeric protein formed from one stable and one unstable mutant. In Hybrid 2, the stable monomer from Mutant 1 oligomerizes with the unstable monomer from Mutant 3 to form a hybrid protein with intermediate stability.

of active site residues is too small to be the basis for a general theory to account for all the complementation events observed. Additionally, intragenic complementation has been identified in enzymes, such as alkaline phosphatase, whose active sites do not lie in subunit interfaces (*44*). Crick and Orgel suggested that complementation generally occurs between mutant subunits because a local misfolding in one subunit is compensated for by the unaltered homologous region of the adjacent subunit.

Our understanding of protein folding has advanced considerably since Crick and Orgel put forward their hypothesis. Protein folding and unfolding occur cooperatively (*45*). Although there are several cases in which folding intermediates have been identified (*46*−*48*), it is unlikely that only a local unfolding, caused by an amino acid substitution, could occur and at the same time be corrected for by a neighboring region on an adjacent subunit. Instead, the majority of amino acid substitutions most likely affect the global stability of the oligomeric protein. We have shown that complementation can occur between thermolabile mutants and stable mutants by stabilizing the active oligomeric form of the hybrid enzyme as depicted schematically in Figure 7. In this case, however, an intersubunit active site is not strictly required, nor do the mutations have to lie near the subunit interface.

This mechanism for complementation therefore extends Crick and Orgel's original theory by including global destabilizing mutants. We propose that both mechanisms reported here can be used to broadly explain all of the complementation events observed at the ASL locus.

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REFERENCES

1. McInnes, R. R., Shih, V., and Chilton, S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4480−4484.
2. Gravel, R. A., Lam, K. F., Scully, K. J., and Hsia, Y. (1977) *Am. J. Hum. Genet. 29*, 378−388.
3. Gravel, R. A., Akerman, B. R., Lamhonwah, A. M., Loyer, M., Leon-del-Rio, A., and Italiano, I. (1994) *Am. J. Hum. Genet. 55*, 51−58.
4. Qureshi, A. A., Crane, A. M., Matiaszuk, N. V., Rezvani, I., Ledley, F. D., and Rosenblatt, D. S. (1994) *J. Clin. Invest. 93*, 1812−1819.
5. Lusty, C. J., and Ratner, S. (1972) *J. Biol. Chem. 247*, 7010−7022.
6. O'Brien, W. E., and Barr, R. H. (1981) *Biochemistry 20*, 2056−2060.
7. Palekar, A. G., and Mantagos, S. (1981) *J. Biol. Chem. 256*, 9192−9194.
8. Brusilow, S. W., and Maestri, N. E. (1996) *Adv. Pediatr. 43*, 127−170.
9. Simard, L., O'Brien, W. E., and McInnes, R. R. (1986) *Am. J. Hum. Genet. 39*, 38−51.
10. Barbosa, P., Cialkowski, M., and O'Brien, W. E. (1991) *J. Biol. Chem. 266*, 5286−5290.
11. Walker, D. C., Christodoulou, J., Craig, H. J., Simard, L. R., Ploder, L., Howell, P. L., and McInnes, R. R. (1997) *J. Biol. Chem. 272*, 6777−6783.
12. Turner, M. A., Simpson, A., McInnes, R. R., and Howell, P. L. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 9063−9068.
13. Sampaleanu, L. M., Vallee, F., Thompson, G. D., and Howell, P. L. (2001) *Biochemistry 41*, 15570−15580.
14. Abu-Abed, M., Turner, M. A., Vallee, F., Simpson, A., Slingsby, C., and Howell, P. L. (1997) *Biochemistry 36*, 14012−14022.
15. Simpson, A., Bateman, O., Driessen, H., Lindley, P., Moss, D., Mylvaganam, S., Narebor, E., and Slingsby, C. (1994) *Nat. Struct. Biol. 1*, 724−734.
16. Vallee, F., Turner, M. A., Lindley, P. L., and Howell, P. L. (1999) *Biochemistry 38*, 2425−2434.
17. Sampaleanu, L. M., Vallee, F., Slingsby, C., and Howell, P. L. (2001) *Biochemistry 40*, 2732−2742.
18. Howell, P. L., Turner, M. A., Christodoulou, J., Walker, D. C., Craig, H. J., Simard, L. R., Ploder, L., and McInnes, R. R. (1998) *J. Inherit. Metab. Dis. 21*, 72−85.
19. Walker, D. C., McCloskey, D. A., Simard, L. R., and McInnes, R. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9625−9629.
20. Barbosa, P., Wistow, G. J., Cialkowski, M., Piatigorsky, J., and O'Brien, W. E. (1991) *J. Biol. Chem. 266*, 22319−22322.
21. Craig, H. J. (1993) in *Molecular and Medical Genetics*, p 77, University of Toronto, Toronto.
22. Linnebank, M., Homberger, A., Rapp, B., Winter, C., Marquardt, T., Harms, E., and Koch, H. G. (2000) *J. Inherit. Metab. Dis. 23*, 308−312.
23. Turner, M. A., Achyuthan, A. M., Hershfield, M. S., McInnes, R. R., and Howell, P. L. (1994) *J. Mol. Biol. 239*, 336−338.
24. Sambrook, J., Frtisch, E., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
25. Hani, E. K., and Chan, V. L. (1994) *J. Bacteriol. 176*, 1865−1871.
26. Chakraborty, A. R., Davidson, A., and Howell, P. L. (1999) *Biochemistry 38*, 2435−2443.
27. Piatigorsky, J., O'Brien, W. E., Norman, B. L., Kalumuck, K., Wistow, G. J., Borras, T., Nickerson, J. M., and Wawrousek, E. F. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3479−3483.
28. Havir, E. A., Tamir, H., Ratner, S., and Warner, R. C. (1965) *J. Biol. Chem. 240*, 3079−3088.
29. Schulze, I. T., Lusty, C. J., and Ratner, S. (1970) *J. Biol. Chem. 245*, 4534−4543.
30. Saribas, A. S., Schindler, J. F., and Viola, R. E. (1994) *J. Biol. Chem. 269*, 6313−6319.
31. Yu, B., and Howell, P. L. (2000) *Cell. Mol. Life Sci. 57*, 1637−1651.
32. Fincham, J. R., Kinsey, J. A., Fuentes, A. M., Cummings, N. J., and Connerton, I. F. (2000) *Genet. Res. 76*, 1−10.
33. Fincham, J. R. S. (1962) *J. Mol. Biol. 4*, 257−274.
34. Coddington, A., and Fincham, J. R. S. (1965) *J. Mol. Biol. 12*, 152−161.
35. Sundaram, T. K., and Fincham, J. R. S. (1968) *J. Mol. Biol. 95*, 787−792.
36. Sampaleanu, L. M., Yu, B., and Howell, P. L. (2002) *J. Biol. Chem.* (in press).
37. Lee, T. T., Worby, C., Bao, Z. Q., Dixon, J. E., and Colman, R. F. (1999) *Biochemistry 38*, 22−32.
38. Wente, S. R., and Schachman, H. K. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 31−35.
39. Scrutton, N. S., Berry, A., Deonarain, M. P., and Perham, R. N. (1990) *Proc. R. Soc. London, Ser. B: Biol. Sci. 242*, 217−224.
40. Pookanjanatavip, M., Yuthavong, Y., Greene, P. J., and Santi, D. V. (1992) *Biochemistry 31*, 10303−10309.
41. Distefano, M. D., Moore, M. J., and Walsh, C. T. (1990) *Biochemistry 29*, 2703−2713.
42. Larimer, F. W., Lee, E. H., Mural, R. J., Soper, T. S., and Hartman, F. C. (1987) *J. Biol. Chem. 262*, 15327−15329.
43. Crick, F. H. C., and Orgel, L. E. (1964) *J. Mol. Biol. 8*, 161−165.
44. Hehir, M. J., Murphy, J. E., and Kantrowitz, E. R. (2000) *J. Mol. Biol. 304*, 645−656.
45. Tanford, C. (1968) *Adv. Protein Chem. 23*, 121−282.
46. Kim, P. S., and Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459−489.
47. Kim, P. S., and Baldwin, R. L. (1990) *Annu. Rev. Biochem. 59*, 631−660.
48. Englander, S. W. (2000) *Annu. Rev. Biophys. Biomol. Struct. 29*, 213−238.

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