Disruption of a Salt Bridge Dramatically Accelerates Subunit Exchange in Duck δ2 Crystallin

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Intragenic complementation is a unique property of oligomeric enzymes with which to study subunit-subunit interactions. Complementation occurs when different subunits, each possessing distinct mutations that render the individual homomutant proteins inactive, interact to form a heteromutant protein with partial recovery of activity. In this paper, complementation events between human argininosuccinate lyase (ASL) and its homolog, duck δ2 crystallin, were characterized. Different active site mutants in δ2 crystallin complement the regeneration of native-like active sites as reported previously for ASL. The complementarity of the ASL and δ2 crystallin subunit interfaces was illustrated by the in vitro formation of active hybrid tetramers from inactive ASL and inactive δ2 crystallin mutants. Subunits of both ASL and δ2 crystallin do not dissociate and reassociate in vitro at room temperature, even after 6 days of incubation, indicating that the multimerization interface is very strong. However, disruption of a salt bridge network in the tetrameric interface of δ2 crystallin caused a drastic acceleration of subunit dissociation. Double mutants combining these interface mutants with active site mutants of δ2 crystallin were able to dissociate and reassociate to form active tetramers in vitro within hours. These results suggest that exchange of subunits may occur without unfolding of the monomer. Intragenic complementation in these interface mutants occurs by reintroducing the native salt bridge interaction upon hetero-oligomerization. Our studies demonstrate the value of intragenic complementation as a tool for investigating subunit-subunit interactions in oligomeric proteins.

The majority of naturally occurring proteins exist as oligomers. While surveying the Escherichia coli genome, Goodsell and Olsen (1) found that about 80% of the proteins were at least dimers if not higher order multimers. The primary amino acid sequence of a protein not only has to contain the information required to fold the polypeptide chain into its three-dimensional structure properly but also the subunit associations that allow it to homo- or hetero-oligomerize to its final quaternary state. Although there have been many advances in understanding how individual protein units fold (2–4), the study of how oligomeric enzymes coordinate folding with the assembly of subunits is still in its infancy. A unique property of oligomeric enzymes is their potential ability to exhibit intragenic complementation. Intragenic complementation is a phenomenon that can occur between different mutant subunits within a multimeric enzyme. Complementation occurs when certain combinations of mutant alleles produce an enzyme with greater catalytic activity than is observed in the homozygous state of either mutant. The occurrence of intragenic complementation in well characterized multimeric enzymes provides a valuable tool to study the subunit-subunit interactions of proteins.

Argininosuccinate lyase (ASL, 1 EC 4.3.2.1) is a ubiquitous enzyme that catalyzes the reversible cleavage of argininosuccinate to arginine and fumarate. Through a process called “gene sharing” ASL was recruited to the eye lens of birds and reptiles where it acts as the major lens crystallin (5). Subsequent gene duplication of δ crystallin has resulted in two nonallelic crystallin genes arranged in tandem which code for two different isomers (6–8). Although δ crystallin has retained ASL activity, δ1 crystallin is catalytically inactive (5, 9–12). The two isomers share 91 and 94% sequence identity in chickens and ducks, respectively (6–8). There is ~70% sequence identity between human ASL and the various δ crystallins (13). Both δ crystallin and ASL function as homotetramers with four identical multisubunit active sites (14–18). Extensive intragenic complementation is observed at the ASL locus in humans (19). By characterizing the effects of different pairs of mutations in ASL, two general mechanisms by which intragenic complementation can occur have been identified (20). Complete...
mentation can occur between two stable active site mutants by the formation of one or more functional native-like active sites or when a stable mutant subunit and an unstable mutant subunit form a hybrid protein with enough overall stability for catalysis to occur.

The physicochemical and spatial properties defining subunit and protein-protein interfaces have been the focus of several studies in recent years. Two separate studies (21, 22) have demonstrated that residues involved in the subunit interface are highly conserved across a series of homologous proteins compared with other surface residues. Interface conservation among protein homologs is based on the assumption that interface residues will be highly conserved if the oligomeric state is critical for biological function.

The ASL subunit interface has been shown to be extremely stable (20). The tetramers do not dissociate at room temperature. Subunits will exchange extremely slowly at 0 °C, reaching equilibrium only after several days (20). However, single amino acid substitutions of buried residues in the interface can vastly destabilize the protein. To gain more knowledge about the mechanisms of protein folding and subunit association, we have extended our complementation studies to the ASL homolog, duck ASL. Duck Crystallin is more thermostable than ASL (23) making it more amenable to *in vitro* analysis. A large library of duck ASL mutants also exists (24, 25) which can be used to study complementation.

In this paper, we have demonstrated how intragenic complementation can be used to study subunit-subunit interactions. By characterizing the complementation behavior of different ASL and duck ASL mutants, we have shown that the subunit interface of human ASL and duck ASL has been evolutionarily conserved. The 82 crystallin tetramer is extremely stable, yet disruption of the intersubunit salt bridges between Arg-302 and Glu-330 was sufficient to weaken the extensive subunit-subunit interface and suggests that exchange of subunits may occur without unfolding of the monomer. Complementation between the R302E and E330R mutants confirms the importance of this salt bridge in maintaining tetramer stability.

**MATERIALS AND METHODS**

**Expression Vectors**—The construction of pET-3d vectors expressing wild-type duck ASL crystallin and the D87N, N114D, T159D, and S281T single point mutants with a C-terminal His$_x$ tag was described previously (25). For the expression of 82 crystallin heterotetramers, vectors containing the full-length cDNA and T7 promoter regions for the N114D, T159D, or S281T mutant with the full-length cDNA and T7 promoter region for the D87N mutant were created. The pET-82-N114D-His plasmid was digested with Eco47III, and the resulting 1,898-bp fragment was ligated into the 4,818-bp fragment resulting from the digestion of the pET-82-D87N-His plasmid with Bst1107I and Bsp68I. This generated the pET-82-D87N-His/82-N114D-His coexpression vector. The pET-82-D87N-His/82-T159D-His and pET-82-D87N-His/82-S281T-His coexpression vectors were generated in a similar manner.

The construction of pET-3c vectors expressing wild-type human ASL and the D87G and Q286R single point mutants with a C-terminal His$_x$ tag was described elsewhere (20). For the expression of ASL/82 crystallin hybrid heterotetramers the ASL wild-type or mutant plasmid was digested with Eco47III, and the resulting 1,915-bp fragment was ligated into the 4,818-bp fragment resulting from the digestion of the 82 crystallin wild-type or mutant plasmid with Bst1107I and Bsp68I. This procedure generated the pET-wt/82-His/ wtASL-His, pET-wt/82-S281T-His/ASL-D87G-His, and pET-wt/82-D87N-His/ASL-Q286R-His coexpression vectors.

The *QuikChange* site-directed mutation kit from Stratagene was used to generate single point mutations in the pET-wt/82-His plasmid. The dioxgenucleotide primers, 5′-GCC AGC AAG GCC GGC gaa GTT GGA CGG C-3′ and 5′-G CAG GAG GAC AAG cgc gct GTC TTT GAT GTT GTG G-3′ and their complementary primers were used to replace the arginine at position 302 with glutamic acid (R302E) and the glutamate at position 330 with arginine (E330R), respectively. This created the plasmids pET-E330R-His and pET-E330R-His plasmids, respectively. The substituted nucleotides (lowercase) were designed to introduce both the desired codon (bold) and a unique restriction site (underlined), which was used as a screening tool to identify positive mutants.

**RESULTS**

Production of 82 Crystallin Mutant Heterotetramers in Vivo—To determine whether 82 crystallin could exhibit intragenic complementation by the regeneration of native-like ac-
active proteins exhibited Michaelis-Menten kinetics with no
0.1% wt/H9254
To examine the complementarity of the ASL and Vivo
E. coli were able to complement an ASL-deficient strain of
(H11006) (Table I). The wtASL-His/ASL-D87G-His hybrid protein
has 38.5 ± 1.4% wtASL-His activity (Table II) and intermediate thermal stability. The temperature midpoint for wtASL-His, wtASL-His, and the wtASL-His/ASL-D87G-His hybrid protein is 58, 69, and 63 °C, respectively (Fig. 2 and Table III).
To confirm further that the proteins were forming active hybrid proteins, the inactive S281T-His/H9254 mutant was coexpressed with the inactive ASL-D87G-His mutant and the inactive D87N-His mutant coexpressed with the inactive ASL-Q286R-His mutant. The S281T-His/ASL-D87G-His and S2-D87N-His/ASL-Q286R-His heteromutant hybrid proteins both exhibited in vivo ASL activity (Fig. 3) and had 10.5 ± 0.4 and 20.5 ± 1.5% wtASL-His/ASL-D87G-His hybrid activity, respectively (Table II). The D87N-His/ASL-Q286R-His heteromutant hybrid had thermal stability comparable with the wild-type hybrid with a midpoint at 63 °C, but the S281T-His/ASL-D87G-His heteromutant hybrid was slightly less stable, with a temperature midpoint of 59 °C (Fig. 2 and Table III).

In Vitro Complementation of S2 Crystallin Active Site Mutants—To compare the subunit association rates of ASL and S2 crystallin, pairs of the S2 crystallin active site mutants, S2-D87N-His, S2-T159D-His, and S2-T281T-His, were examined for their ability to undergo cold dissociation and exhibit intragenic complementation in vitro, as observed previously for ASL (20). Surprisingly, prolonged incubation of the protein samples at either 0 °C or room temperature did not result in the formation of active heterotetramers (Table I). S2 Crystallin does not undergo cold dissociation, indicating that its subunit interface is more stable than that of ASL.

Mutations in the Dimer-Dimer Interface of S2 Crystallin—Mutations were made in the subunit interface of S2 crystallin to examine the effect of intersubunit mutations on complementation. A number of charged residues in the dimer-dimer interface of S2 crystallin were identified as participating in a network of salt bridges. Two of the six residues in this network, Arg-302 and Glu-330, were targeted for site-directed mutagenesis and mutated to glutamic acid and arginine, respectively (Fig. 4). The charges of each residue were reversed both to weaken the subunit-subunit interaction within the homotetramer and to increase the affinity of the mutant subunits for each other (see below). Both mutants were expressed with C-terminal His6 tags to simplify purification. The catalytic activity and thermal stability of the purified proteins were then characterized. The S2-R302E-His and S2-E330R-His mutants had reduced catalytic activity with 13.5 ± 3.2 and 30.5 ± 6.9% wtASL-His activity, respectively (Table IV).

The S2-R302E-His and S2-E330R-His proteins did not introduce any gross changes in the far-UV scans (data not shown). The thermal stability of the mutant proteins, however, was decreased. The midpoint of the temperature-induced transition
Table I

| Protein                | In vitro activity | $K_m$ (μmol/min/mg) | $V_{max}$ (μmol/min/mg) | $h_{cat}$ (s$^{-1}$) | $h_{cat}/K_m$ (s$^{-1}$) | % of in vitro wt activity |
|------------------------|-------------------|---------------------|-------------------------|---------------------|--------------------------|--------------------------|
| wtδ2                   | +++++              | 0.05 ± 0.01         | 1.14 ± 0.05             | 3.96 ± 0.18         | (8.64 ± 0.10) × 10$^4$  | 98.4 ± 12.2              |
| wtδ2-His               | +++++              | 0.06 ± 0.01         | 1.35 ± 0.29             | 4.73 ± 1.01         | (8.60 ± 10.0) × 10$^4$  | 100.0 ± 1.2              |
| wtδ2/wtδ2-His          | +++++              | 0.04 ± 0.01         | 1.07 ± 0.05             | 3.76 ± 0.19         | (8.62 ± 0.57) × 10$^4$  | 100.3 ± 6.6              |
| δ2-D87N-His           | −                  | 1.39 ± 0.49         | 0.04 ± 0.01             | 0.13 ± 0.02         | (9.59 ± 3.70) × 10$^3$  | 0.2 ± 0.1                |
| δ2-N114D-His         | +                  | 0.40 ± 0.08         | 0.02 ± 0.01             | 0.06 ± 0.01         | (1.46 ± 0.23) × 10$^4$  | 0.2 ± 0.1                |
| δ2-Ti150D-His        | −                  | 0.03 ± 0.01         | 0.24 ± 0.02             | 0.86 ± 0.07         | (3.25 ± 0.28) × 10$^4$  | 37.8 ± 3.3               |
| δ2-D87N-His/δ2-Ti150D-His | +        | 0.02 ± 0.01         | 0.24 ± 0.05             | 0.85 ± 0.17         | (3.72 ± 0.67) × 10$^4$  | 43.3 ± 0.8               |
| δ2-D87N-His/δ2-S281T-His | NA               | 0.01 ± 0.06         | 0.34 ± 0.01             | 1.19 ± 0.03         | (2.35 ± 0.18) × 10$^4$  | 20.5 ± 1.5               |
| δ2-D87N-His + δ2-Ti150D-His | NA               | 0.01 ± 0.06         | 0.34 ± 0.01             | 1.19 ± 0.03         | (2.35 ± 0.18) × 10$^4$  | 20.5 ± 1.5               |

*Measured as the ability of the protein to recover growth of ΔASL E. coli cells on M9 minimal medium. ++++, growth of cells similar to wild-type; +, growth of cells less than wild-type; −, no growth.

Table II

| Protein                | In vitro activity | $K_m$ (μmol/min/mg) | $V_{max}$ (μmol/min/mg) | $h_{cat}$ (s$^{-1}$) | $h_{cat}/K_m$ (s$^{-1}$) | % of in vitro hybrid activity |
|------------------------|-------------------|---------------------|-------------------------|---------------------|--------------------------|-----------------------------|
| δ2-His                 | +++++              | 0.06 ± 0.01         | 1.35 ± 0.29             | 4.73 ± 1.01         | (8.60 ± 10.0) × 10$^4$  | 75.0 ± 0.9                 |
| ASL-D87G-His          | −                  | 0.12 ± 0.01         | 10.36 ± 0.90            | 34.5 ± 3.0          | (2.98 ± 0.19) × 10$^5$  | 259.5 ± 16.6               |
| δ2-D87N-His/δ2-His    | +                  | 0.07 ± 0.01         | 0.18 ± 0.01             | 0.60 ± 0.02         | (8.98 ± 0.84) × 10$^3$  | 7.8 ± 0.7                  |
| δ2-D87N-His/δ2-S281T-His | ++        | 0.09 ± 0.01         | 2.89 ± 0.08             | 10.13 ± 0.27        | (1.15 ± 0.43) × 10$^5$  | 100.0 ± 3.7                |
| δ2-D87N-His/δ2-S281T-His | +        | 0.10 ± 0.01         | 0.34 ± 0.01             | 1.20 ± 0.04         | (1.21 ± 0.05) × 10$^4$  | 10.5 ± 0.4                 |
| δ2-D87N-His/δ2-Ti150D-His | +        | 0.05 ± 0.01         | 0.34 ± 0.01             | 1.19 ± 0.03         | (2.35 ± 0.18) × 10$^4$  | 20.5 ± 1.5                 |

*Measured as the ability of the protein to recover growth of ΔASL E. coli cells on M9 minimal medium. ++++, growth of cells similar to wild-type; +, growth of cells less than wild-type; −, no growth.

Table III

| Protein                | $T_{1/2}$ °C |
|------------------------|-------------|
| wtASL-His              | 58          |
| ASL-D87G-His           | 56          |
| ASL-Q286R-His          | 58          |
| wtδ2-His               | 69          |
| δ2-D87N-His/δ2-His    | 67          |
| δ2-S281T-His          | 66          |
| δ2-R302E-His          | 62          |
| δ2-E330R-His          | 63          |
| δ2-D87N-R302E-His     | 59          |
| δ2-S281T,E330R-His    | 60          |
| δ2-His/ASL-His        | 63          |
| δ2-D87N-His/ASL-Q286R-His | 63       |
| δ2-S281T-His/ASL-D87G-His | 59        |
| δ2-E330R-His + δ2-E330R-His (0 °C)$^c$ | 68 |
| δ2-E330R-His + δ2-D87N-R302E-His (0 °C)$^c$ | 69 |

*Temperature at which half of the elliptical signal is lost at 222 nm.

**$T_{1/2}$ values from Yu et al. (20).**

**$T_{1/2}$ values from Sampaleanu et al. (25).**

**Protein samples were incubated at 0 °C for 6 days prior to temperature melt.**

Fig. 2. The temperature denaturation of ASL, δ2 crystallin, and ASL/δ2 crystallin hybrid proteins monitored as a function of the CD signal at 222 nm. The curves were normalized for comparison of data. •, wtASL-His homotetramer; ▲, wtδ2-His homotetramer; Δ, wtASL-His/wtδ2-His hybrid heterotetramers; ◊, ASL-D87N-His/δ2-S281T-His hybrid heterotetramers; □, ASL-Q286R-His/δ2-D87N-His hybrid heterotetramers.

The tetramers were cross-linked with glutaraldehyde in the presence of urea. wtδ2-His was first incubated with increasing glutaraldehyde concentrations under native conditions to determine the glutaraldehyde:protein ratio that would allow complete but not overcross-linking of the native tetramer (data not shown). The same glutaraldehyde:protein ratio was then used to cross-link the wild-type and mutant proteins under denaturing conditions. Even in the absence of urea, both δ2-R302E-His and δ2-E330R-His tetramers were less stable from native tetramer to aggregate was 62 and 63 °C for δ2-R302E-His and δ2-E330R-His, respectively (Table III).

To assess the effect of the mutations on the quaternary structure, the tetramers were cross-linked with glutaraldehyde in the presence of urea. wtδ2-His was first incubated with increasing glutaraldehyde concentrations under native conditions to determine the glutaraldehyde:protein ratio that would allow complete but not overcross-linking of the native tetramer (data not shown). The same glutaraldehyde:protein ratio was then used to cross-link the wild-type and mutant proteins under denaturing conditions. Even in the absence of urea, both δ2-R302E-His and δ2-E330R-His tetramers were less stable from native tetramer to aggregate was 62 and 63 °C for δ2-R302E-His and δ2-E330R-His, respectively (Table III).
ASL/δ Crystallin Subunit-Subunit Interactions

The far-UV scans and thermal denaturation melting curves for the δ-D87N,R302E-His and δ-S281T,E330R-His mixture, and the δ-R302E-His and δ-E330R-His mixture were collected after incubation at 0 °C for 6 days. The heterotetramers formed after subunit exchange were more stable compared with any of the individual mutant proteins with temperature midpoints closer to wild-type δ crystallin (Table III).

**DISCUSSION**

By using the phenomenon of intragenic complementation as a tool to study subunit dissociation/reassociation, we demonstrated previously that the subunit interface of ASL is extremely strong with subunit exchange in vitro occurring at a measurable level only after more than 24 h incubation at 0 °C (20). In this work, the same type of complementation event shown to occur in vivo and in vitro between the ASL mutants Q286R and D87G was reproduced in δ crystallin, a more stable homolog of ASL. The D87N mutant of δ crystallin was coexpressed with mutants in other regions that make up the active site, S281T and T159D. If the mutant subunits were completely inactive and could associate with equal probability, the resulting mixture of heterotetramers would be expected to have 25% wild-type activity. Complementation in δ crystallin of D87N with S281T, and D87N with T159D resulted in heterotetramers with 43.3 ± 3.3 and 37.8 ± 1.5% wtδ-His activity, respectively. It is unclear why the heteromutants recover higher activity than expected. This phenomenon was also seen in ASL (20) and may be explained by the different mutant subunits associating with unequal probability. The data clearly show that δ crystallin mutants exhibit intragenic complementation. The formation of hetero-oligomers by δ crystallin was first observed by Piatigorsky and co-workers (26, 27) when δ crystallin tetramers purified from the eye lenses of duck embryos were found to contain different ratios of δ1 and δ2 subunits. The regeneration of native-like active sites between two inactive mutants has also been shown to occur in the ASL superfamily member, adenylosuccinate lyase (28, 29), and δ crystallin mutants exhibit intragenic complementation between δ crystallin mutants has allowed us to characterize the subunit interactions of this enzyme.

A remarkable feature of the complementation reaction of δ crystallin compared with that of ASL is that it does not occur in vitro under any conditions tested, implying that the subunits of δ crystallin never dissociate. Thus, the intersubunit interactions of δ crystallin appear to be even stronger than those of ASL. The extremely low levels of subunit dissociation displayed by both ASL and δ crystallin may have evolved out of a necessity to maintain their tetrameric state inside the cell. Outside the eye lens where the concentration of ASL/δ crystallin is probably very low, if the enzyme dissociated, subunit reassociation would be very unlikely, and the enzyme would be inactivated.

Despite differences in the properties of the ASL and δ crystallin subunit interactions, our data demonstrate that ASL and δ crystallin subunits are able to associate with one another. As shown in Table II, coexpression of inactive ASL and δ crystallin mutants resulted in the production of hybrid tetramers with increased activity. These results indicate the complementarity of the ASL/δ crystallin subunit interfaces. Interestingly, the level of activity recovery when ASL and δ crystallin mutants were coexpressed was much lower than that seen when the δ crystallin mutants complemented each other (e.g. comparing ASL-D87G-His/δ-S281T-His (Table II) with activity was observed for the individual protein samples. The final level of wild-type activity for the mixture of δ-R302E-His and δ-E330R-His was 31.0 ± 1.4% (Table IV).

**Fig. 3. In vivo activity assay.** An ASL knock-out strain of E. coli was transformed with the following plasmids: pET-ASL-Q286R-His (1), pET-ASL-Q286R-His/δ-D87N-His (2), pET-δ-D87N-His (3), pET-ASL-D87G-His (4), pET-ASL-D87G-His/δ-S281T-His (5), and pET-δ-S281T-His (6), plated on LB medium (a), M9 minimal medium plus arginine (b), and M9 minimal medium alone (c) and grown at 37 °C for 48 h.

**Fig. 4. A close-up of the central α-helices of δ crystallin.** Each subunit is colored as in Fig. 1. Hydrogen bonds between Arg-302 and Glu-330 of each subunit are shown as dotted lines.
TABLE IV
Kinetic properties of 82 crystallin mutants before and after 6-day incubation at 0 or 25 °C

| Protein                  | Incubation temperature | $K_m$ ($\mu M$) | $V_{max}$ (μmol min$^{-1}$ mg$^{-1}$) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (μM$^{-1}$ s$^{-1}$) | % of in vitro wt activity |
|--------------------------|------------------------|-----------------|-------------------------------------|---------------------|----------------------------------|--------------------------|
| wt 82-His                | NA                     | 0.06 ± 0.01     | 1.35 ± 0.29                        | 4.73 ± 1.01         | 8.60 ± 0.10                      | 100.0 ± 1.2              |
| R302E-His                | NA                     | 0.07 ± 0.01     | 1.07 ± 0.05                        | 3.21 ± 0.17         | 5.98 ± 0.28                      | 32.5 ± 0.9               |
| R302E-His                | 25                     | 0.16 ± 0.03     | 0.61 ± 0.04                        | 1.18 ± 0.28         | 1.92 ± 0.26                      | 38.0 ± 2.3               |
| E330R-His                | 0                      | Inactive        |                                    |                     |                                  |                          |
| E330R + E330R-His        | 25                     | 0.21 ± 0.02     | 5.32 ± 0.14                        | 0.46 ± 0.23         | 2.14 ± 0.12                      | 63.0 ± 3.6               |
| R302E + E330R-His        | 0                      | Inactive        |                                    |                     |                                  |                          |
| 281T, E3302R-His         | 0                      | 0.01 ± 0.01     | 0.71 ± 0.04                        | 0.50 ± 0.23         | 2.34 ± 0.12                      | 75.0 ± 0.6               |
| D87N, R302E-His          | NA                     | 0.01 ± 0.01     | 0.87 ± 0.04                        | 0.60 ± 0.23         | 1.85 ± 0.12                      | 47.3 ± 1.9               |
| S281T, E3302R-His        | NA                     | 0.02 ± 0.01     | 1.17 ± 0.04                        | 0.83 ± 0.23         | 2.71 ± 0.12                      | 53.0 ± 1.9               |
| D87N, R302E-His + S281T-E3302R-His | 25 | 0.14 ± 0.03 | 0.48 ± 0.06 | 1.68 ± 0.22 | (1.24 ± 0.17) × 10^4 | 14.4 ± 1.9 |

* Enzyme kinetics were performed immediately after purification and prior to incubation.

As would be expected for such a strong subunit association, the monomers of 82 crystallin and ASL interact through an extensive interface. 33% of the surface area of the monomer or 7,129 Å$^2$ is buried on tetramerization. The ASL and 82 crystallin monomer is composed of three structural domains (16, 18). Five of the helices in the central domain of each monomer form a helical bundle. Two monomers associate, via mainly hydrophobic interactions between three of these five helices, to form a closely associated dimer. The tetramer displays D2 symmetry and consists of a dimer of dimers. The interactions between the two dimers of the tetramer are less extensive than between the two monomers of each dimer. One helix from each monomer interacts at the protein core to form a four-helix bundle. To assess whether the subunit interface of 82 crystallin could be perturbed to allow subunit dissociation and reassociation in vitro, we targeted the Arg-302 and Glu-330 positions for mutation. These residues are located in the central four-helix bundle of ASL and form salt bridges across the dimer-dimer interface (Fig. 4). These residues are conserved in all ASL/8 crystallin genes. Key interface residues are generally conserved in homologous enzymes if oligomerization is important for catalysis (21, 22).

The R302E and E330R substitutions had a dramatic effect on the oligomerization properties of 82 crystallin. The weakened subunit association of these mutants is clearly demonstrated in the cross-linking gels where they both display substantial levels of dimer even in the absence of denaturant (Fig. 5). The observed dissociation of these mutants is likely the cause of their reduced thermal stability (Table III) and catalytic activity (Table IV). Although both the R302E and E330R mutants appear to dissociate partially into dimers under normal buffer conditions (Fig. 5), their level of helicity as assessed by CD measurements (data not shown) was the same as the wild-type protein. This result implies that subunit dissociation of 82 crystallin can occur without appreciable unfolding of the monomers, and therefore subunit exchange leading to intragenic complementation can also occur without unfolding. Prolonged incubation of either mutant at 0 °C, conditions under which ASL subunit interactions are weakened (20), resulted in large decreases in activity that were not observed at room temperature (Table IV). This temperature-dependent effect may be the result of lowered solubility of the dimeric species at 0 °C, which leads to a slow aggregation process after tetramer dissociation.

When the 82 R302E-His and 82 E330R-His were mixed in vitro, heterotetramers with considerably greater activity than either of the homomutants were produced. The most striking case is the R302E mutant which was completely inactive when...
incubated on its own at 0 °C but displayed 47% wild-type activity when incubated with an equimolar amount of the E330R mutant (Table IV). In these heterotetramers, the electrostatic repulsion between like charges at positions 302 and 330 is alleviated. The salt bridge between dimers that is lost in the homomutant proteins may also be regenerated. Through these mechanisms, heterotetramers would be considerably more stable than homotetramers, thus skewing the distribution of possible tetromers toward those that contain both mutant subunits. The increased thermal stability of the 82-R302E-His/82-E330R-His heterotetramers compared with the homotetramers supports this hypothesis (Table III).

As a means to monitor conveniently the in vitro dissociation/ reassociation reaction of the R302E and E330R mutants, they were combined with the stable active site mutants, D87N and S281T, respectively. As expected, the 82-D87N,R302E-His and 82-S281T,E330R-His double mutants were completely inactive because of the mutations in the active site. When these mutants were mixed in vitro at either 0 or 25 °C, a rapid increase in activity was observed (Fig. 6), demonstrating that the subunits of these mutants were able to dissociate and reassociate within a few hours to create active heterotetramers. The behavior of these double mutants contrasts dramatically with that of the single D87N and S281T mutants, which when mixed showed no detectable reassortment of subunits under any conditions even after 6 days of incubation. These data prove that 82 crystallin subunits are able to dissociate and reassociate relatively quickly when the dimer-dimer interface is weakened by mutation and that the salt bridge between Arg-302 and Glu-330 plays an important role in mediating the slow dissociation rate seen for the wild-type 82 crystallin.

CONCLUSIONS

We have shown that intragenic complementation occurs in 82 crystallin and that the 82 crystallin tetramer is extremely stable because dissociation/reassociation does not occur either at room temperature or at 0 °C. Disruption of the salt bridge between Arg-302 of one monomer and Glu-330 of another is, however, enough to weaken the extensive subunit-subunit interface and allow subunit exchange to occur. Intragenic complementation between the R302E and E330R mutants occurs by reintroducing the native interaction upon hetero-oligomerization. Despite the predominance of oligomeric proteins in nature (1), protein folding studies are generally limited to small, single domain, water-soluble globular proteins. This arises from the fact that in vitro the reversible refolding and reassociation of proteins is studied rather than the folding and association that would occur normally in vivo. Multidomain proteins, the hydrophobicity of membrane-bound proteins, and the subunit-subunit interactions involved in oligomerization complicate the study of these types of proteins as aggregation during refolding is common. We have demonstrated how intragenic complementation can be used to study subunit-subunit interactions. Intragenic complementation is an attractive method because it does not require denaturation and refolding of the oligomer.

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