Interactions between Residues in Staphylococcal α-Hemolysin Revealed by Reversion Mutagenesis*

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α-Hemolysin (αHL), a pore-forming polypeptide of 293 amino acids, is secreted by Staphylococcus aureus as a water-soluble monomer. Residues that play key roles in the formation of functional heptameric pores on rabbit red blood cells (rRBC) have been identified previously by site-directed mutagenesis. αHL-H35N, in which the histidine at position 35 of the wild-type sequence is replaced with asparagine, is nonlytic and is arrested in assembly as a heptameric prepare. In this study, second-site revertants of H35N that have the ability to lyse rRBC were generated by error-prone PCR under conditions designed to produce single base changes. The analysis of 22 revertants revealed new codons clustered predominantly in three distinct regions of the H35N gene. One cluster includes amino acids 107-111 (four revertants) and another residues 144-155 (five revertants). These two clusters flank the central glycine-rich loop of αHL, which previously has been implicated in formation of the transmembrane channel, and encompass residues Lys-110 and Asp-152 that, like His-35, are crucial for lytic activity. The third cluster lies in the region spanning amino acids 217-228 (eight revertants), a region previously unexplored by mutagenesis. Single revertants were found at amino acid positions 84 and 169. When compared with H35N, the heptameric prepares formed by the revertants underwent more rapid conversion to fully assembled pores, as determined by conformational analysis by limited proteolysis. The rate of conversion to the fully assembled pore was strongly correlated with hemolytic activity. Previous work has suggested that the N terminus of αHL and the central loop cooperate in the final step of assembly. The present study suggests that the key N-terminal residue His-35 operates in conjunction with residues flanking the loop and C-terminal residues in the region 217-228. Hence, reversion mutagenesis extends the linear analysis that has been provided by direct point mutagenesis.

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The abbreviations used are: αHL, α-hemolysin (α-toxin) of Staphylococcus aureus; IVTT, in vitro transcription and translation; PCR, polymerase chain reaction; rRBC, rabbit red blood cell; WT-αHL, wild-type α-hemolysin; BSA, bovine serum albumin.

EXPERIMENTAL PROCEDURES

Error-prone PCR—The template for error-prone PCR (14) was the αHL-H35N gene in the plasmid pT7PH-8S (13), which was linearized with EcoRI. The 5′ primer was 5′-CGGGATCCTAATACGACTCACTATAGGG-3′, the last 20 nucleotides of which are complementary to the T7 promoter region upstream from the NdeI site (C′-ATAATG) in pT7PH-8S that contains the initiation codon for αHL. The 3′ primer was 5′-AAACATCATTGCAGGCTCTTAGG-3′, the last 20 nucleotides of which are complementary to a sequence in the proximal 3′-untranslated region that contains a HindIII site (15). Random mutations were introduced by: 1) using a large number of PCR cycles (fifty) using a high concentration of dNTPs (400 μM), which increased the error rate of tag-polymerase (16). Upon screening, a reversion frequency of 1 in 200 was obtained (see below). The PCR product was digested with NdeI and HindIII, gel purified, and ligated to gel-purified pT7PH-8S vector that had been digested with the same restriction enzymes. The ligation product was precipitated with ethanol and resuspended in 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, for...
identification of Revertants of αHL-H35N Produced by Mutagenesis with Error-prone PCR—Mutations were introduced into the αHL-H35N gene by error-prone PCR. Revertants of the inactive H35N were identified by screening colonies “fixed” on nitrocellulose for their ability to lyse rRBC in blood agar plates. A fraction of the expressed αHL that is immobilized on the nitrocellulose filter must leach out to cause lysis of the cells. Twenty-two revertant genes were sequenced (Table I). Of the 22, four exhibited the same base substitution and hence the same amino acid change (Asp-108 → Gly). This over-representation suggests that the mutation occurred during an early PCR cycle. Only the 19 apparently independent revertants were further studied. Seventeen of these had sequence changes that produced single amino acid changes. The remaining two (H35N/D227N/R236S and H35N/T11A/D227A) had a second amino acid substitution. Because these two revertants contain mutations at a common site (Asp-227) and because a neighboring mutation, Phe-228 → Leu (Table I), also resuscates H35N, it is assumed that the changes at position 227 are responsible for the activity of these mutants. One revertant, designated Δ222*, was missing three bases (TTC or TCT) in the sequence TCT- TCA that encodes Ser-221 and Ser-222, resulting in replacement of the Ser-Ser doublet by a single serine residue.

Hemolytic Activity of H35N Revertants—To determine the efficiency of translation of the revertants, the 35S-labeled IVTT products were quantitated by electrophoresis and autoradiography. All the revertants were translated as well as WT-αHL and the H35N mutant (data not shown). Three revertants had two bases (CA) missing from the Ndel (C↓-ATATG) cloning site (Table I). This change did not affect the efficiency of translation of these mutants, despite its proximity to the initiator methionine codon.

A portion of each IVTT mix was used to compare the hemolytic activity of the revertants. In a quantitative assay, the 19 mutants showed a wide range of activities (Table I). As expected from the screening procedure, all were more active than H35N and a few had activity comparable with that of WT-αHL.

Binding of Revertants to rRBC and Subsequent Oligomerization—A portion of the same IVTT mix was used to study the binding and subsequent oligomerization of the revertant polypeptides on rRBC. WT-αHL forms SDS stable oligomers that are conveniently analyzed by SDS-polyacrylamide gel electrophoresis. Binding was carried out for 1 h at 20°C, which is sufficient for maximal binding and oligomerization in the case of WT-αHL. All the revertants bound to rRBC and formed oligomers that were stable in SDS (Fig. 1).

Limited Proteolysis of the Oligomerized H35N Revertants—Pelleted cells or membranes to which the mutant αHL had bound (see above) were resuspended in K-PBSA (360 μl). Half of the sample was treated with proteinase K (1 μl, 1 mg/ml) for 5 min at room temperature, while the other half was treated with water (1 μl). The reaction was stopped by treatment with phenylmethylsulfonyl fluoride (1 mM final) for 5 min at room temperature followed by the addition of 5 X SDS loading buffer. The samples were heated to 95°C for 5 min to dissociate oligomers and subjected to electrophoresis in a 12% SDS-polyacrylamide gel, followed by autoradiography of the dried gel.

**RESULTS**

**Identification of Revertants of αHL-H35N Produced by Mutagenesis with Error-prone PCR**—Mutations were introduced into the αHL-H35N gene by error-prone PCR. Revertants of the inactive H35N were identified by screening colonies “fixed” on nitrocellulose for their ability to lyse rRBC in blood agar plates. A fraction of the expressed αHL that is immobilized on the nitrocellulose filter must leach out to cause lysis of the cells. Twenty-two revertant genes were sequenced (Table I). Of the 22, four exhibited the same base substitution and hence the same amino acid change (Asp-108 → Gly). This over-representation suggests that the mutation occurred during an early PCR cycle. Only the 19 apparently independent revertants were further studied. Seventeen of these had sequence changes that produced single amino acid changes. The remaining two (H35N/D227N/R236S and H35N/T11A/D227A) had a second amino acid substitution. Because these two revertants contain mutations at a common site (Asp-227) and because a neighboring mutation, Phe-228 → Leu (Table I), also resuscitates H35N, it is assumed that the changes at position 227 are responsible for the activity of these mutants. One revertant, designated Δ222*, was missing three bases (TTC or TCT) in the sequence TCT- TCA that encodes Ser-221 and Ser-222, resulting in replacement of the Ser-Ser doublet by a single serine residue.

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**DISCUSSION**

Point mutagenesis, especially systematic scanning mutagenesis (20), is valuable for obtaining information about the functional roles of individual residues and short sequences of residues in a polypeptide, provided that supporting evidence confirming the structural integrity of the mutant molecules is...
obtained. A summary of the results obtained by scanning point mutagenesis of αHL is given in the accompanying paper (4), in which residues involved in binding to rRBC, oligomerization, and pore formation are identified. Despite its utility, point mutagenesis does not usually provide definitive information about interactions between residues that lie far apart in the linear sequence of the polypeptide chain. One way of obtaining such information is to obtain second-site revertants of an inactive protein by “random” mutagenesis (21–24). In this study, we have located 16 residues that interact with the key residue

| Revertant | Amino acid position | Base change(s) | Amino acid change(s) | Lytic activity | Comments |
|-----------|---------------------|----------------|----------------------|--------------|---------|
| F84L*     | 84                  | TTT → CTT      | Phe → Leu            |              |         |
| I107M*    | 107                 | ATT → ATG      | Ile → Met            |              |         |
| D108G*    | 108                 | GAT → GGT      | Val (silent)         |              |         |
| T109I*    | 109                 | ACA → ATA      | Thr → Ile            |              |         |
| H144R*    | 144                 | ATT → ATG      | Ile → Met            |              |         |
| P151L*    | 151                 | GCT → CTT      | Pro → Leu            |              |         |
| S217C*    | 217                 | AGT → TGT      | Ser → Cys            |              |         |
| L219P*    | 219                 | TCA → TCA      | Leu → Pro            |              |         |
| D227N*    | 227                 | GAC → AAC      | Asp → Asn            |              | 227 likely site of reversion (see text) |
| D227A*    | 227                 | AGA → AGT      | Thr → Ala            |              | 227 likely site of reversion (see text); CA bases missing from the NdeI site |
| F228L*(A)| 228                 | TTT → TTA      | Phe → Leu            |              |         |
| F228L*(B)| 228                 | TTT → TTA      | Phe → Leu            |              |         |

Revertants of an Inactive α-Hemolysin Mutant

Table I
Revertants of αHL-H35N generated by error-prone PCR

Key to lytic activity (see accompanying paper): +++, activity within two wells of WT-αHL activity in the microtiter assay; +++, activity within four wells; +, activity within six wells; +, very weak activity after 1 h, detectable to within four wells of WT-αHL in 3 h. Details of the assay are under “Experimental Procedures.”

Fig. 1. Binding of WT-αHL, αHL-H35N and the revertants of H35N to rRBC and subsequent oligomerization. In vitro translated αHL polypeptides, radiolabeled with 

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α, oligomeric αHL; α, monomeric αHL.

obtained. A summary of the results obtained by scanning point mutagenesis of αHL is given in the accompanying paper (4), in which residues involved in binding to rRBC, oligomerization, and pore formation are identified. Despite its utility, point mutagenesis does not usually provide definitive information about interactions between residues that lie far apart in the linear sequence of the polypeptide chain. One way of obtaining such information is to obtain second-site revertants of an inactive protein by “random” mutagenesis (21–24). In this study, we have located 16 residues that interact with the key residue...
Hemolytic activity and limited proteolysis of the oligomerized αHL-H35N revertants. For each αHL polypeptide, the window shows a hemolysis assay of the intact molecule, as monitored for 1 h at 20 °C in an automated microplate reader. The in vitro translated protein was diluted 40-fold in the assay mix. Below each assay trace is an autoradiogram of a 12% SDS-polyacrylamide gel showing the proteolytic pattern of the 35S-labeled αHL polypeptide after assembly on rRBC for 1 h at 20 °C at the same dilution used in the hemolysis assay. Treatments were with water (−) or proteinase K (+) at 50 μg/ml for 5 min. Oligomers were dissociated by heating before SDS-polyacrylamide gel electrophoresis. The revertants are designated as described in Fig. 1 (legend). The last two shown are, in order, F228*L(A) and F228*L(B). α1, undigested αHL; p, proteinase K fragments generated by cleavage near the N terminus (2, 3).

In many cases, rather subtle changes, e.g. Ser-217 → Cys, Asp-227 → Asn, restored activity to αHL-H35N. This may reflect the fact that H35N is poised at the brink of activity. H35Q, which contains an additional methylenegroup, has substantial activity (13). H35N is correctly folded as demonstrated by limited proteolysis in solution, and the defect is in a single late step of assembly (3). It seems likely that reversion mutagenesis would be favored by such a situation of minimal disablement. We were also aided by the development of a powerful screening procedure and a high reversion frequency provided by the large number of acceptable reversion sites.

Fourteen of the 16 amino acids affected in the revertants are clustered in three regions of the polypeptide chain (Fig. 3). Four revertants had mutations between amino acids 107 and 111 inclusive, while five revertants (at four amino acid positions) had mutations between amino acids 144 and 155. These two clusters flank the central loop, which plays an important role in channel formation (3–5, 8, 9, 25, 26). Further, Lys-110 and Asp-152, which are critical for lytic activity (4), are located in the two clusters (Fig. 3). These findings are in keeping with the demonstration that the N terminus of αHL and the central loop cooperate in the final step of assembly (3). Of five residues identified as crucial for pore formation by cysteine scanning mutagenesis (4), three are interconnected by this study (His-35, Lys-110, and Asp-152). The integrity of these three residues and Asp-24, which was not identified here, is required for the final step of assembly. The fifth mutant, E70C, is defective in binding and therefore would not be expected to be linked with His-35. A third cluster of 8 revertants (at six amino acid positions) is located between amino acids 217 and 228, a region that was not explored in previous studies.

If the interactions revealed by reversion mutagenesis take place within a single polypeptide chain, the findings imply that the N- and C-terminal thirds of αHL cannot be considered as completely independent domains, although they contain distinctive distributions of functional residues (4). Interactions between the N and C termini of monomeric αHL have been demonstrated directly in experiments in which they are synthesized separately and recombined to form a functional hemolysin (25, 27). Perhaps the regions around His-35 and residues 217–228 form a point of contact between the two halves. Helix contacts in membrane proteins such as the α subunit of E. coli F′FATPase (28) and the E. coli lactose permease (29, 30) have been proposed, based on the existence of second-site revertants. Alternatively, because the fully assembled pore is a heptamer, it is quite possible that intersubunit interactions are corrected in the revertants.
Therefore, His-35 and all three clusters may be in close proximity in the prepore (Structure 3), either within individual subunits or at intersubunit contact sites, or they may be brought into proximity during formation of the active pore (Structure 4). Accordingly, the cysteine in H35C becomes unreactive toward a water-soluble sulfhydryl reagent during formation of the prepore (12). Proximity of the residues in question would lend a ready explanation for the revertants as beneficiaries of compensating mutations that through direct interaction repair a defect in the final step of assembly. However, it is by no means certain that the restoration of activity is the outcome of such proximity. For example, while second-site revertants of a defective triose phosphate isomerase were clustered near the primary mutation at the active site (24), reversion of other mutant proteins such as staphylococcal nuclease (23) and phage lambda repressor (22) can be brought about by amino acid substitutions distant from the primary site. Therefore, it will be most interesting to examine the placement of His-35, the three clusters and the two lone mutations at amino acid positions 84 and 169 in a three-dimensional structure of αHL.

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