Key Residues for Membrane Binding, Oligomerization, and Pore Forming Activity of Staphylococcal \(\alpha\)-Hemolysin Identified by Cysteine Scanning Mutagenesis and Targeted Chemical Modification

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The \(\alpha\)-hemolysin (\(\alpha\)HL) polypeptide is secreted by Staphylococcus aureus as a water-soluble monomer that assembles into lipid bilayers to form cylindrical hemolytic pores 1-2 nm in effective internal diameter. We have individually replaced each charged residue (79 of 293 amino acids) and four neutral residues in \(\alpha\)HL with cysteine, which is not found in the wild-type protein. The properties of these mutants have been examined before and after modification with the 450-Da dianionic sulfhydryl reagent 4-aminooctanoate-4’-((iodoacetyl)aminomethyl) stilbene-2,2’-disulfonate (IASD). This modification was highly informative as 28 of 83 modified polypeptides showed substantially reduced pore forming activity on rabbit erythrocytes (rRBC), while only five of the unmodified cysteine mutants were markedly affected. Through detailed examination of the phenotypes of the mutant and modified hemolysins, we have pinpointed residues and regions in the \(\alpha\)HL polypeptide chain that are important for binding to rRBC, oligomer formation and pore activity. Residues in both the N-terminal (Arg-66 and Glu-70) and C-terminal (Arg-200, Asp-254, Asp-255, and Asp-276) thirds of the protein are implicated in binding to cells. The His-35 replacement mutant modified with IASD was the only polypeptide in this study that failed to form SDS-resistant oligomers on rRBC. Altered hemolysins that formed oligomers but failed to lyse rRBC represented the most common defect. These alterations were clustered in the central glycine-rich loop, which has previously been implicated as a component of the lumen of the membrane-spanning channel, and in the regions flanking the loop. Alterations in mutant and modified hemolysins with the same defect were also scattered between the N terminus and His-48, in keeping with previous suggestions that an N-terminal segment and the central loop cooperate in the final step of pore assembly.

\(\alpha\)-Hemolysin (\(\alpha\)-toxin, \(\alpha\)HL) is secreted by Staphylococcus aureus as a water-soluble monomer, which binds to membranes and forms oligomeric cylindrical pores (1). The 293-amino-acid polypeptide provides a useful model for examining the structural changes that occur when a water-soluble protein assembles into a lipid bilayer (2). In addition, \(\alpha\)HL has been used as a tool in cell biology for the permeabilization of mammalian cells (3). \(\alpha\)HL may also have applications in biotechnology, e.g. in drug delivery or as a component of biosensors (4). Further, \(\alpha\)HL contributes to the pathogenicity of \(S\). aureus in certain diseases (1).

A working scheme for the assembly of \(\alpha\)HL summarizes an accumulation of experimental data (Structures 1-4).

\[
\begin{align*}
\text{Soluble monomer} &\rightarrow \text{Membrane-bound monomer} &\rightarrow \text{Membrane-bound heptamer} &\rightarrow \text{Fully assembled transmembrane pore} \\
1 &\rightarrow 2 &\rightarrow 3 &\rightarrow 4
\end{align*}
\]

Structures 1-4

Rabbit erythrocytes (rRBC) carry an unidentified receptor for \(\alpha\)HL that facilitates pore formation (5, 6). The receptor is not required for assembly as \(\alpha\)HL can form pores in protein-free bilayers (7, 8). Early studies revealed that the pore is an oligomer (e.g. Refs. 9, 10). More recent studies by gel-shift electrophoresis and X-ray crystallography have shown that the pore is a heptamer (11). Based on their circular dichroism, both the water-soluble monomer and the assembled pore are largely \(\beta\)-sheet (10, 12). Conformational analysis by limited proteolysis has suggested that the \(\alpha\)HL monomer consists of two regions of approximately equal mass connected by a central glycine-rich loop that encompasses residues 119-143 (10, 13). The loop becomes resistant to proteolysis when \(\alpha\)HL binds to membranes (10, 14) and, in the fully assembled pore, it is likely to penetrate the bilayer, as evidenced by fluorescence spectroscopy with derivatized single-cysteine mutants (15), and to line a segment of the lumen of the transmembrane channel, as evidenced by the introduction of a metal ion-binding site that is accessible from both sides of the membrane (16). Assembly intermediates have been demonstrated by the availability of mutants that accumulate as membrane-bound monomers (Structure 2) or as nonlytic oligomers (Structure 3) (14, 17, 18). The nonlytic "pre pore" 3 is also a heptamer (18). Because mutations in both the N terminus and the central loop can yield molecules that arrest as nonlytic heptamers (18, 19), it seems likely that the N terminus and the loop cooperate in the final step of assembly: conversion of the heptameric prepore to the fully assembled pore (Structure 3 → Structure 4). The fully assembled pore (Structure 4) might contain a 14-stranded \(\beta\)
barrel formed by the loop by analogy with the porins, which contain 16- or 18-stranded barrels (20–22). Such a barrel could accommodate (23) the essentially nonselective channel of 1-2 nm internal diameter that is formed by αHL (7, 9).

Here, we use scanning mutagenesis and targeted chemical modification of αHL to correlate sequence with function. Residues and regions of the polypeptide that are important for binding to rRBC, heptamer formation, and pore activity are delineated. The study also reveals the residues in αHL that will be available for chemical modification in investigations designed to produce pores with novel properties.

EXPERIMENTAL PROCEDURES

Mutagenesis—Single-cysteine mutants of αHL were made for each one of the 79 charged residues (26 aspartic acid, 11 glutamic acid, 28 lysine, 10 arginine, and 4 histidine) by oligonucleotide-directed mutagenesis using a method based on the procedure of Kunsel (24). Unless noted below, the template was pT7-NPH8S(K8A) (25). The αHL mutant K8A, encoded by this plasmid, has the properties of native αHL, but is less susceptible to inactivation by adventitious proteases (25). H35C, H48C, H144C, and H259C were derived from pT7NPH8S(WT) (25). T129C was derived from pT7NPH8(WT), which contains an earlier version of the αHL gene with a mutation, Ser-217 → Asn, that is corrected in pT7NPH8S(WT) (25). S217N has the wild-type phenotype. S3C and T292C were obtained from pT7NPH8(WT) by polymerase chain reaction with mutagenic 5’ and 3’ primers and inserted into the vector pT7Sf1A (26). Mutations were confirmed by DNA sequencing through the altered region. A second, independent isolate was obtained for most of the mutants and used to confirm the phenotype. In a few cases, where difficulties were encountered in obtaining the second isolate, the entire coding region was sequenced. No differences from the template DNAs were observed, other than those present in the mutagenic primers.

Coupled in Vitro Transcription and Translation—The experiments described here were performed with αHL mutants expressed from supercoiled plasmid DNAs by in vitro transcription and translation (IVTT) in an Escherichia coli S30 extract (Promega No. L4500) supplemented with T7 RNA polymerase, rifampicin, and [ 35S]methionine (26). Separate reactions with the complete amino acid premix and the premix minus methionine were incubated for 1 h at 37°C and then mixed. This maneuver yielded mutant polypeptides at concentrations above 10 μg/ml with specific radioactivities sufficient for rapid autoradiography or PhosphorImager analysis.

Exhaustive Modification by IASD—[ 35S]-Labeled single-cysteine mutant (24 μl of IVTT mix) was diluted with 0.5 M sodium phosphate, pH 8.5 (60 μl), followed by the addition of 10 mM dithiothreitol (12 μl). After 5 min at room temperature, 100 μl of IASD (Molecular Probes, Eugene, OR) in water (24 μl) was added. After 2 h at room temperature, the remaining IASD was inactivated with 120 mM dithiothreitol (24 μl). Unmodified mutants were produced by an identical treatment, except for the substitution of water for the IASD solution. A portion of the modification reaction (7.5 μl) was heated at 95°C for 5 min with 2 × gel loading buffer (7.5 μl) and analyzed by SDS-PAGE (27) in a 40 × 14-cm gel run at 200 V for 44 h (19).

Hemolysis Assays—Modification reaction mix (100 μl) was subjected to 2-fold serial dilution in microtiter wells with 20 μl K2HPO4, 150 μM NaCl, pH 7.4, containing 1 ng/ml bovine serum albumin, and 1 mM βME (K-PBSA/βME). An equal volume of 1% rRBC in K-PBSA/βME was then added to each well. The extent of hemolysis in the wells was monitored for 1 h at 20°C with a Bio-Rad model 3550-UV microplate reader, by observing the decrease in light scattered by the cells at 595 nm. All functional estimates of hemolysis were made at 3 and 24 h.

αHL Oligomer Formation on rRBC and Thermal Stability of the Oligomers in SDS Solution—The modification reaction mix (30 μl) was added to 12.5% rRBC in K-PBSA/βME (120 μl) and incubated at 20°C for 1 h. After centrifugation for 5 min at 16,000 × g, the resulting pellet of cells or membranes was resuspended in K-PBSA/βME (500 μl), centrifuged again, and then solubilized in 1 × Laemmli gel loading buffer (90 μl). The sample was divided into three 0.5-ml polyethylene tubes, which were placed into a thermocycler. One set of tubes was removed after 10 min at 30°C, a second set after a further 10 min at 50°C, and the final set after an additional 10 min at 80°C. The samples were loaded directly onto 12% Laemmli gels and run overnight at 66 V.

RESULTS

Overview—The properties of 83 single-cysteine mutants of αHL are reported here. Most were derived from αHL-K8A, a protease-resistant mutant of αHL with functional properties that are closely similar to the wild-type hemolysin (25). The remainder were derived from the wild-type sequence. In this paper, for convenience, we refer to both the unaltered sequence and K8A as wild-type αHL (WT-αHL). Both unmodified polypeptides and polypeptides derivatized at cysteine with IASD were studied, a total of 166 altered hemolysins. Modification with IASD adds a mass of 450 Da that contains two sulfonate groups that are fully ionized in aqueous solution (pK a = ~6.5). Hence, IASD modification is a far more drastic change than the replacement with cysteine itself.

Each cysteine mutant was obtained in [ 35S]-labeled form by in vitro transcription and translation (IVTT). Half of the sample was modified with IASD, while the other half was subjected to a mock reaction, which had little effect on the properties of the hemolysins. Portions of the modified and unmodified αHL were examined by SDS-PAGE to ensure that similar concentrations of each mutant were obtained by IVTT (they were), to measure the gel shift produced by the IASD modification (19), to determine the extent of modification (a few mutants were incompletely modified, see below), and to ensure that the modification was confined to the single cysteine. The reaction conditions (20 mM IASD, 2 h, room temperature, pH 8.5) were the most extreme that can be used without significant derivatization of additional functional groups on αHL. Prolonged modification gives a diffuse band on SDS-PAGE, rather than a clean shift in mobility, presumably because several additional nucleophiles (e.g., the ε-amino groups of lysine) are slowly derivatized.

An example of the analysis is displayed (Fig. 1).

Additional portions of the mutant and modified αHL polypeptides were used to measure pore forming activity by determining the rate of lysis of rRBC in a quantitative microtiter assay. An example of such an experiment is shown that exemplifies a variety of mutant phenotypes (Fig. 2). Further portions of the polypeptides were incubated with rRBC at 20°C for 1 h. SDS-PAGE was then used to determine the extent of binding to the cells, the extent of oligomer formation, and the heat stability of the oligomers. An example of this analysis is displayed (Fig. 3) for the same set of mutants shown in Fig. 2.

The results of measurements on all 166 altered hemolysins are compiled in Table I, which can be perused in conjunction with the primary sequence of αHL (Fig. 4). The results are also
summarized graphically to emphasize the distribution through the length of the polypeptide chain of residues involved in the major functions of α-HL: cell binding, oligomerization, and pore formation (Fig. 5, A and B).

Pore Formation on rRBC—Only five of the unmodified cysteine mutants show greatly reduced lytic activity toward rRBC: D24C, H35C, E70C, K110C, and D152C. E70C does not bind well to rRBC, and it is likely that this explains its low activity (Fig. 3). Interestingly, the small fraction of E70C that does bind oligomerizes efficiently (Fig. 3). The other four mutants bind and oligomerize (see below). His-35 is a crucial residue that has been examined previously (18, 19, 28–30). Most mutants with replacements at position 35 are inactive, but the nonnatural amino acid S-carboxamidomethylcysteine, which is closely similar in volume to histidine and has similar polarity and hydrogen bonding potential, is tolerated (30). H35N and H35C are arrested as heptameric preprores (Structure 3), as demonstrated by conformational analysis by limited proteolysis (18, 19). In other His-35 replacements, oligomerization does not occur or the oligomers are SDS-sensitive (28, 30). The present data suggest that D24C, K110C, and D152C have a similar phenotype to H35C.

As expected, the properties of the IASD-derivatized mutants are more dramatically affected than the properties of the cysteine mutants themselves. Twenty-eight showed greatly reduced pore forming activity. Few cysteine mutants in the central one-third of the polypeptide and none in the glycine-rich loop itself remain active after treatment with IASD. Residues affected by IASD attachment are also scattered through the most N-terminal 50 amino acids, but there are also many unaffected residues in this region. Again, the most common phenotype is arrest as a nonlytic oligomer, presumably similar to the assembly intermediate Structure 3.

Binding to rRBC—Not all of the altered hemolysins with reduced hemolytic activity are arrested as preprores (Structure 3). Unmodified R66C and more noticeably E70C do not bind efficiently to rRBC (Fig. 3). When R66C, E70C, R200C, D254C, D255C, and D276C are derivatized with IASD binding is greatly reduced, and this is likely to account for their lack of activity (see Figs. 2 and 3 for the first four of these six hemolysins). The density of IASD-sensitive positions in the C-terminal one-third of the polypeptide is far lower than that in the remainder of the molecule and in each case (R200C-IASD, D254C-IASD, D255C-IASD, and D276C-IASD) modulation is associated with failure to bind efficiently to rRBC. These positions are surrounded by sites for which activity is retained after IASD modification. We note that subtle changes in affinity linked to small reductions in lytic activity would not have been...
Properties of single-cysteine mutants of α-HL before and after modification with IASD

The symbol on the lefthand side of the table (e.g. O) is a quick guide to the phenotypes of the mutants. Where the open circle (O) is replaced by a filled circle (●), a property differs from that of WT-α-HL. Symbol 1 filled: the cysteine mutant was consistently difficult to modify with IASD to an extent of more than 75%; symbol 2 filled: poor binding to RBC (see full table entry for the effects of IASD); symbol 3 filled: low hemolytic activity of unmodified cysteine mutant; symbol 4 filled: low hemolytic activity of cysteine mutant after modification with IASD. Entries represent data from two independent clones of each mutant sequenced through the altered bases or a fully sequenced clone. At least three experiments were carried out for each entry. Unless otherwise stated, the extent of modification with IASD was ≥95% as determined by gel-shift electrophoresis. Key: binding: ++, similar to WT-α-HL; +, binding reduced to ≤25% of WT-α-HL; −, weak but detectable binding; −−, no detectable binding. Lysis: +++, activity within two wells of WT-α-HL 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-α-HL in 3 h; −, no activity after 3 h; −−, no activity after 24 h. Oligomers: 50, oligomers stable at 50°C; 80, oligomers stable at 80°C; wk, faint band on gel at the designated temperature; wk, very faint band on gel. Details of the assays are under “Experimental Procedures.” Selected data of evidence are shown in Figs 1–3. Data entries are in parentheses where it could not be determined whether the activity measured was due to the IASD-modified protein or residual unmodified cysteine mutant. Most often the uncertainty was for the hemolytic activity. Frequently, binding of the IASD-modified protein could still be determined despite the presence of residual unmodified protein based on the distinct electrophoretic mobility of the former. Again, the incorporation of the IASD-modified fraction into oligomers could be often be detected based on the lack of a reduction in the extent of oligomer formation, a shift in electrophoretic mobility of the oligomer or increased heat stability of the oligomer. In the few cases where both IASD-modified protein and residual unmodified cysteine mutant were present, it is likely that the oligomers that were observed were heteromers.

| Phenotype | Binding | Lysis | Oligomer | Modification |
|-----------|---------|-------|----------|--------------|
|           |         |       |          |              |
| OOOOO     | D2C     | ++    | ++       | 50           |
| OOOO      | S3C     | ++    | ++       | 50           |
| D4C       | ++      | ++    | ++       | 50           |
| K8C       | ++      | ++    | ++       | 50           |
| D13C      | ++      | ++    | ++       | 50           |
| K21C      | ++      | ++    | ++       | 50           |
| D24C      | ++      | +     | −        | 80           |
| D29C      | ++      | +     | ++       | 80           |
| D30C      | ++      | +     | ++       | 80           |
| E31C      | ++      | +     | ++       | 80           |
| H35C      | ++      | +     | −        | 80           |
| K36C      | ++      | (+)   | +        | 50 (50)      |
| K37C      | ++      | +     | ++       | 80           |
| D44C      | ++      | +     | ++       | 80           |
| D45C      | ++      | +     | ++       | 80           |
| K46C      | ++      | +     | ++       | 80           |
| H48C      | ++      | +     | ++       | 80           |
| K50C      | ++      | +     | ++       | 80           |
| K51C      | ++      | +     | ++       | 80           |
| R56C      | ++      | +     | ++       | 80           |
| K58C      | ++      | +     | ++       | 80           |
| R66C      | ++      | +     | −        | 80           |
| E70C      | ++      | +     | −        | 80           |
| E71C      | ++      | +     | −        | 80           |
| K75C      | ++      | +     | −        | 80           |
| K85C      | ++      | (+)   | +        | 50           |
| D92C      | ++      | +     | ++       | 80           |
| E94C      | ++      | +     | ++       | 80           |
| D100C     | ++      | +     | −        | 80           |
| R104C     | ++      | +     | −        | 80           |
| D108C     | ++      | +     | (+)      | 50           |
| K110C     | ++      | +     | −        | 80           |
| E111C     | ++      | +     | −        | 80           |
| D127C     | ++      | +     | −        | 80           |
| D128C     | ++      | +     | −        | 80           |
| T129C     | ++      | +     | −        | 80           |
| G130C     | ++      | +     | −        | 80           |
| K131C     | ++      | +     | −        | 80           |
| H144C     | ++      | +     | −        | 80           |
| K147C     | ++      | +     | −        | 80           |
| D152C     | ++      | +     | −        | 80           |
| K154C     | ++      | +     | ++       | 80           |
| E158C     | ++      | +     | ++       | 80           |
| D162C     | ++      | +     | ++       | 80           |
| K163C     | ++      | +     | ++       | 80           |
| K164C     | ++      | +     | ++       | 80           |
| K168C     | ++      | +     | ++       | 80           |
| D183C     | ++      | +     | ++       | 80           |
| R184C     | ++      | +     | +        | 80           |
| K198C     | ++      | +     | +        | 80           |
| K205C     | ++      | +     | +        | 80           |
| D208C     | ++      | +     | +        | 80           |
| D212C     | ++      | +     | ++       | 80           |
| K215C     | ++      | +     | ++       | 80           |
| D227C     | ++      | +     | ++       | 80           |
| D235C     | ++      | +     | ++       | 80           |
| R236C     | ++      | +     | ++       | 80           |
that tempting to conclude that these residues are buried in the H144C, D227C, R251C, R253C, and K273C (see Table I). It is modification with IASD: K36C, K51C, R56C, K58C, K85C, in SDS are not formed, which appears to be the case for H35S not examined the state of H35C-IASD on rRBC by electron IASD, failed to form SDS stable oligomers. Because we have examined here, only one that was able to bind to rRBC, H35C-dected in the screen reported here.

Table 1—continued

| IASD treatment | Binding | Lys | Oligomer | Modification |
|----------------|---------|-----|----------|-------------|
| ○○○○○ K237C   | ++      | ++  | ++       | ++          | 50          | 50          | 90% modification |
| ○○○○○ K240C   | ++      | ++  | ++       | ++          | 50          | 50          | 90% modification |
| ○○○○○ D246C   | ++      | ++  | ++       | ++          | 50          | 50          | 90% modification |
| ○○○○○ E250C   | ++      | ++  | ++       | ++          | 50          | 50          | 90% modification |
| ●○○○○ R251C   | ++      | ++  | ++       | (+ +)       | 50          | (50)        | 25-40% modification |
| ●○○○○ R253C   | ++      | ++  | ++       | (+ +)       | 50          | (50)        | 50% modification |
| ○○○○○ D254C   | ++      | ++  | ++       | --          | 50          | None        |
| ○○○○○ D255C   | ++      | ++  | ++       | --          | 50          | 50          | 80wk |
| ●○○○○ H259C   | ++      | ++  | ++       | ++          | 50          | 50          | 80wk |
| ○○○○○ K266C   | ++      | ++  | ++       | ++          | 50          | 50          | 80wk |
| ○○○○○ K271C   | ++      | ++  | ++       | ++          | 50          | 50          | 80wk |
| ○○○○○ D272C   | ++      | ++  | ++       | ++          | 50          | 50          | 80wk |
| ●○○○○ K273C   | ++      | ++  | ++       | ++          | 50          | 50          | 70% modification |
| ○○○○○ D276C   | ++      | ++  | ++       | --          | 50          | 80wk        |
| ○○○○○ R277C   | ++      | ++  | ++       | ++          | 50          | 50          | 80wk |
| ○○○○○ E280C   | ++      | ++  | ++       | ++          | 50          | 50          | 80wk |
| ○○○○○ R281C   | ++      | ++  | ++       | ++          | 50          | 50          | 80wk |
| ○○○○○ K283C   | ++      | ++  | ++       | ++          | 50          | 50          | 80wk |
| ○○○○○ D285C   | ++      | ++  | ++       | ++          | 50          | 50          | 80wk |
| ○○○○○ E287C   | ++      | ++  | ++       | ++          | 50          | 50          | 80wk |
| ○○○○○ K288C   | ++      | ++  | ++       | ++          | 50          | 50          | 80wk |
| ○○○○○ E289C   | ++      | ++  | ++       | ++          | 80          | 80          |
| ○○○○○ E290C   | ++      | ++  | ++       | ++          | 50          | 50          | 80wk |
| ○○○○○ T292C   | ++      | ++  | ++       | ++          | 50          | 50          | 80wk |

1 ADSDENKMG TTDIGEVITY KEGETYTVC KNGREVEYV SFDDEQMEK
51 KLVIRKQGE DQGQYISQ KPNKQVQGC PQPVQPGS
101 YPERNQIL KDEFLMGQG KRGPDGYG SIKSNDCYQ
151 FDYVYFLQD TGKDQGHKV PSHQDDQK YPSNDQYQT
201 NSSKRAWDD LGDQPSAL SSNTVQPAC VTTQDLRAA QQYDPVYK
251 RUVVYDDAGV ESTDGVQNY KRVKSAENV KTH

Fig. 4. Amino acid sequence of wild-type αHL (13) with minor amendments (1, 26). Most of the mutants described here were derived from αHL-K8A, a protease-resistant mutant of αHL with the replacement Lys-8 → Ala.

detected in the screen reported here.

Oligomerization on rRBC—Of the 166 altered hemolysins examined here, only one that was able to bind to rRBC, H35C-IASD, failed to form SDS stable oligomers. Because we have not examined the state of H35C-IASD on rRBC by electron microscopy, we cannot be certain that oligomers that dissociate in SDS are not formed, which appears to be the case for H35S and H35R (28).

Extent of IASD Modification—Ten cysteine mutants resisted modification with IASD: K36C, K51C, R56C, K58C, K85C, H144C, D227C, R251C, R253C, and K273C (see Table I). It is tempting to conclude that these residues are buried in the interior of the protein. Indeed, to map the structural changes that αHL undergoes during pore formation, we intend to carry out a quantitative analysis of the surface accessibility of individual cysteines in all the available αHL mutants at various stages of assembly, based on procedures outlined earlier (19). However, it is sobering to see that almost all the unreactive cysteines are replacements for positively charged residues, which may reside close to negatively charged side chains that would repel IASD and thereby reduce the rate of reaction with adjacent sulfhydryl groups. Therefore, it may be necessary to carry out the surface labeling studies with a set of reagents with a selection of net charges (31).

Gel Shifts Produced by IASD Modification—We also measured the gel shifts produced by IASD modification for both the monomeric cysteine mutants and where possible the heptamers (data not shown). In all cases, the electrophoretic mobilities of the monomers were decreased, while the mobilities of the heptamers were increased (e.g. Figs. 1 and 3). For the monomers, large shifts were seen with N-terminal cysteines in keeping with the striking effects of point mutations and small deletions in this region of the polypeptide chain (14). Large shifts were also seen at positions 104 and 110 (Fig. 1), and residues with moderate shifts were clustered at the C terminus. For the heptamer, modification at the extreme N terminus and central loop produced very small shifts, while most residues near the C terminus produced strong shifts (e.g. Fig. 3A, lanes 15 and 16). There is no simple interpretation of the gel shifts as the polypeptides are likely to be at least partly denatured, and the mobilities reflect the migration of protein-SDS complexes. Whatever their origin, the shifts produced by IASD modification have uses beyond those addressed here. For example, we used the electrophoretic separation of heteromers formed from IASD-modified and unmodified subunits to count the subunits in the assembled pore (Structure 4) (11) and in the oligomeric prepore (Structure 3) (18).

Temperature Stability of Oligomers in SDS—Oligomers formed from WT-αHL are stable to 66 °C in SDS (30, 32). We screened the temperature sensitivity of oligomers from all the altered hemolysins that assemble on rRBC at 30 °C, 50 °C (e.g. Fig. 3A), and 80 °C (e.g. Fig. 3B). None were stable at 30 °C and not at 50 °C, while several were stable at 80 °C (Table I). There is a curious parallel between stability at 80 °C and loss of hemolytic activity (Table I, Fig. 3).

DISCUSSION

The purpose of this study was to apply cysteine scanning mutagenesis to assign functions to sequences in the αHL polypeptide chain. Most of the single-cysteine mutants were highly active, as noted in other cases, e.g. the lactose permease of E. coli (33). Therefore, the value of the mutagenesis was enhanced by using targeted chemical modification of cysteine to produce a set of more drastically modified hemolysins corresponding to the single-cysteine mutants. The assignment of function after point mutagenesis is limited by the interdependence of sites that are far apart in the primary sequence of a polypeptide (an issue addressed in the accompanying paper (34)). Nevertheless, the study has provided valuable insights into the function of a pore-forming protein.

Previously, we divided the αHL chain of 293 amino acids into three functional regions: the N- and C-terminal domains and...
the glycine-rich central loop. The present findings appear to vindicate this approach, although it seems likely that the borders of the central region should be expanded and that the N-terminal domain can be further subdivided.

The Central Loop and the Regions That Flank It—The central loop was previously defined as the glycine-rich region from residue 119 to residue 143. The loop is highly sensitive to proteolytic digestion in the monomer in solution (10, 14). It is no longer attacked by proteases in the assembled pore and in assembly intermediates (10, 14, 18), although it remains accessible to chemical modification with reagents of low mass (19). Experiments in which the loop is nicked genetically (17) or with proteases (35) suggested that it might form part of the channel through the lipid bilayer. Recent work has strengthened this idea by showing that the loop moves into a hydrophobic environment during assembly (15) and that residues near the center of the loop project into the lumen of the transmembrane channel (16).

Here, we find that mutants of αHL with single cysteines located near the center of the glycine-rich loop (positions 127–131) are highly active and that all of them are largely inactivated by IASD modification. Perhaps the mass and charge of the stilbenedisulfonate prevent membrane penetration by the altered loop and subsequent transmembrane channel formation (Structure 3 → Structure 4). It is noteworthy that the central loop is flanked on one side by Lys-110 and on the other by Asp-152, two of only four residues that yield unmodified cysteine mutants in which pore formation, but not oligomerization, is prevented. The region immediately upstream from the central loop is sensitive to IASD modification throughout, suggesting that the channel forming apparatus may extend to around residue 100. IASD sensitivity is also found C-terminal of the loop in the region encompassing amino acids 147 to 168, although insensitive residues are also found here.

Interestingly, residues 169–197 have a similar character to the glycine-rich loop. An extended stretch of neutral residues is

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**Fig. 5.** Graphic representations of the phenotypes of the altered hemolysins. A, chart displaying all altered hemolysins with properties that differ greatly from WT-αHL. 83 of 293 positions in αHL were tested by individual substitution with cysteine as shown at the bottom of the chart. Defects in the cysteine mutants were noted before and after modification with IASD. Full details of the properties of the mutants can be found in Table I. For inclusion here, a defective hemolysin has the following properties (see Table I): lysis, or below; binding, or below; oligomerization, no oligomers at 50 °C. Key. Unmodified mutants, defective in: rRBC lysis, ; rRBC binding, ; oligomerization (no entry). IASD-modified mutants, defective in: rRBC lysis, ; rRBC binding, ; oligomerization, . B, functional properties of αHL assigned to regions of the primary sequence. The assignments are consistent with the present work and previous findings. Nevertheless, in the absence of structural information, they are likely to be oversimplified. The sequence in B is aligned with the chart in A and is given in single-letter code in Fig. 4.
broken at its midpoint by three charged residues (Asp-183, Arg-184, and Asp-185). Single-cysteine mutants at these positions are active and pore formation, but not oligomerization, is prevented after reaction with IASD, as observed for Asp-127, Asp-128, and Lys-131 in the central loop. Therefore, it is conceivable that residues 169–197 constitute a second membrane-penetrating region. Indeed, in the central one-third of the protein, there is an imperfect but suggestive mirror symmetry in the properties of residues on either side of the midpoint of the polypeptide chain (Fig. 5A). It should be worth examining the extended regions of uncharged sequences in αHL in more detail by the present approach and with fluorescent probes that respond to hydrophobic environments (15).

The N-terminal Region—It has been argued that the N terminus of αHL cooperates with the central loop in the final step of pore formation (Structure 3 → Structure 4). For example, small N-terminal truncations permit oligomerization but eliminate lytic activity (14). Further, proteolytic sites near the N terminus become occluded during the final step of assembly (18). These sites remain exposed when pore formation is prevented by mutation at residue 35 (18, 19, 30) or by the binding of Zn(II) to an oligohistidine motif introduced at the central loop (18).

The present findings are in keeping with the earlier studies. Near the N terminus, at positions 24 and 35, we find two residues of the four in αHL that prevent pore formation when changed to cysteine (the fifth inactive cysteine mutant, E70C, is defective in binding to rRBC). Sites that are sensitive to IASD are also scattered throughout the first 50 amino acids, although there are also many insensitive sites.

The second half of the N-terminal one-third of αHL responds quite differently to mutagenesis. When altered, most residues between 50 and 100 do not yield defective αHL molecules except for residues 66 and 70, which appear to be involved in binding to rRBC.

The C-terminal Region—The C-terminal region of αHL is the least well understood in terms of function. Mutants truncated at the C terminus bind to rRBC as monomers that slowly assemble into functional pores, suggesting that the C terminus is important for efficient oligomerization (14). The penultimate amino acid (residue 292) is more accessible to chemical modification in the membrane-bound heptamer than in the monomer in solution (19), suggesting that the C terminus may be located in the part of the pore that projects more than 5 nm from the membrane surface (9, 36). Beyond the IASD-sensitive cluster at positions 183–185, which may correspond to the center of a second membrane-penetrating loop (see above), lie only a scattering of defective mutants all of which are highly deficient in binding after IASD derivatization. The affected positions (Arg-200, Asp-254, Asp-255, and Asp-276) are surrounded by mutation sites that are associated with active hemolysins whether derivatized with IASD or not. Although, the H259C mutation and derivatization of H259C with IASD do not grossly alter membrane binding, as examined here. Jursch et al. (28) did find that H259S and H259F exhibit reduced binding in a quantitative assay, which supports the notion that this region of αHL is involved in binding to rRBC, perhaps through a direct interaction with the elusive αHL receptor.

Summary—The results of this study are summarized in Fig. 5B, in which functions have been assigned to various regions of the αHL polypeptide. While the picture is entirely consistent with current data, it is obviously a simplistic view. We cannot say that we have identified all the key residues in αHL. In particular, the scanning mutagenesis described here is biased because we focused on charged residues. An initial goal was to discover side chains that project into the transmembrane channel, and which nothing was known when we began this study. The need for an at least limited exploration of stretches of uncharged amino acids is now apparent. For example, in only one of 166 altered αHL polypeptides was oligomerization prevented. Perhaps, heptamer formation is largely determined by interactions involving neutral residues or the polypeptide backbone.

From a pragmatic viewpoint, the study has revealed residues that may be chemically modified in attempts to produce pores that have novel properties. Indeed, after this work was complete, the data were used to guide the construction of a photoactivatable αHL by targeted chemical modification of cysteine (37). Cysteine residues might also be used in the construction of hybrid proteins or for attaching the pores to surfaces. The discovery of heat stable oligomers is also interesting in the context of biotechnology (38), although in the present study they are largely associated with loss of activity.

REFERENCES
1. Bhakdi, S., and Tranum-Jensen, J. (1983) Trends Biochem. Sci. 8, 134–136
2. Bhakdi, S., and Tranum-Jensen, J. (1983) Trends Biochem. Sci. 8, 136–138
3. Bhakdi, S., Weller, U., Wale, J., Martin, E. J., Onas, D., and Bayley, H. (1991) J. Cell Biol. 113, 122–133
4. Bhakdi, S., and Tranum-Jensen, J. (1983) Trends Biochem. Sci. 8, 134–136
5. Bhakdi, S., Weller, U., Wale, J., Martin, E. J., Onas, D., and Bayley, H. (1991) J. Cell Biol. 113, 122–133
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