His-859 Is an Essential Residue for the Activity and pH Dependence of Escherichia coli Toxin α-Hemolysin*

Aitziber L. Cortajarena‡, Félix M. Goñi, and Helena Ostolaza§

From the Unidad de Biofísica (CSIC-UPV/EHU), and Departamento de Bioquímica, Universidad del País Vasco, Aptdo. 644, 48080 Bilbao, Spain

Escherichia coli α-hemolysin (HlyA) is a toxin protein that, in common with other members of the RTX family, contains a calcium-binding domain consisting of a number of Gly- and Asp-rich nonapeptides (17 in this case) repeated in tandem. Amino acid number 6 in these nonapeptides is almost invariably Asp, and occasionally Asn, but HlyA contains a His residue (number 859 in the chain) in position 6 of the last-but-one nonapeptide. HlyA mutants have been prepared, by site-directed mutagenesis, in which His-859 has been replaced by an Asn (H859N) or by Asp (H859D). HlyA exists in aqueous media in an aggregate-monomer equilibrium, but only the monomer containing bound Ca$^{2+}$ (HlyA-Ca$^+$) appears to be competent to achieve target membrane insertion and subsequent lysis. In mutant H859N, equilibrium appears to be shifted toward the aggregate, therefore the protein does not exchange Ca$^{2+}$ with the aqueous environment, no HlyA-Ca$^+$ monomers are detected, and the protein lacks any membrane lytic activity. Mutant H859D in turn is almost indistinguishable from the wild-type regarding its calcium binding and membrane lytic activity, however, it differs significantly in its pH dependence. Wild-type HlyA activity decreases sigmoidally with pH, following rather closely the protonation curve of a His residue (apparent $pK_a$ ∼ 6.5). With mutant H859D activity decreases almost linearly with pH and to a smaller extent. It can be concluded that His-859 plays a critical role in several aspects of HlyA activity, namely self-aggregation properties, calcium binding, hemolysis, and pH dependence.

Escherichia coli α-hemolysin (HlyA)$^1$ is a cytolytic exotoxin protein (mass ∼107 kDa) secreted by pathogenic strains of this bacterium. The toxin, that is involved in several extraintestinal infections in humans, is a member of a family of widely disseminated toxins and non-toxin proteins known as "RTX family," for a series of Gly- and Asp-rich nonapeptide repeats in the carboxyl end of their structural proteins. They also share other structural features derived from their high homology: a NH$_2$-terminal hydrophobic domain containing hydrophobic and/or amphipathic helices, and a COOH-terminal uncleavable export sequence (for reviews, see Refs. 1–5).

Perhaps because of the difference in hydrophobies between the NH$_2$-terminal membrane-binding domain and the COOH-terminal calcium-binding domain, E. coli α-hemolysin shows a remarkable tendency for self-aggregation (6, 7). The protein exists in aqueous solution in a monomer-aggregate equilibrium. In the presence of a cell membrane, the monomeric form binds the membrane and eventually becomes inserted. In the absence of a lipid bilayer, the system evolves into an irreversibly aggregated form. Only in the presence of 6 M urea can the protein be stored, apparently because under those conditions the protein is reversibly denatured and does not become irreversibly aggregated (8). When urea is removed, by dilution or dialysis, the active monomer-aggregate equilibrium is restored and the pathway to irreversible aggregation/inactivation is reopened (7).

Calcium ($\sim$10$^{-4}$ M) is essential for the lytic activity of α-hemolysin. The repeat region in its COOH-terminal end is also the calcium-binding domain (9, 10). Previous work from this laboratory has shown that HlyA can reversibly "bind," i.e. adsorb onto, the membrane lipid bilayer in the absence of calcium, but only in its presence does the toxin undergo a conformational change, and become irreversibly inserted into the host membrane, thereby inducing membrane disruption (11–13). The calcium-binding domain of HlyA extends approximately between residues 720 and 870. It is characterized by the presence of 11–17 (depending on how much deviation from the optimal consensus sequence is allowed) nonapeptide repeats of the consensus sequence GGXXDXXUX, where X is an arbitrary amino acid residue, and U is a bulky hydrophobic residue. The three-dimensional structure of a non-toxin member of the RTX family, the alkaline protease from Pseudomonas aeruginosa, is known at a 0.164-nm resolution (14). Its COOH-terminal domain consists of a 21-strand sandwich, in which successive β-strands are wound in a right-handed spiral. Ca$^{2+}$ are bound within the turns between strands formed by the repeated nonapeptide motif. It can be reasonably assumed that a similar structure exists in the repeat domain of HlyA. In a previous study (15), we had pointed out that the last-but-one of the 17 putative repeats in HlyA contained a His residue (His-859) in the same position occupied in all other 16 repeats by the Ca$^{2+}$ ligand Asp or (in one case) by Asn. To explore the role of this particular His residue, we have prepared HlyA mutants by site-directed mutagenesis, in which His-859 has been replaced by Asn (H859N) or Asp (H859D). The Asn mutant is totally inactive, because it cannot bind Ca$^{2+}$ and remains in the aggregate state under all conditions, while H859D is active, but displays a pH dependence different from that of wild-type HlyA.

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§ To whom correspondence should be addressed. E-mail: gbzoseth@g.ehu.es.

The abbreviations used are: HlyA, α-hemolysin; ANTS, 8-amino-4-naphthalene-1,2,3-trisulfonyl acid; ANS, 1-anilinonaphthalene-8-sulfonate; DPX, p-xilene bis(pyridinium bromide); FPLC, fast protein liquid chromatography; LUV, large unilamellar vesicles.

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**His-859 Is Essential for RTX Toxin α-Hemolysin Activity**

**EXPERIMENTAL PROCEDURES**

**Materials**—Egg phosphatidylcholine was obtained from Lipid Products (South Nutfield, England). Horse erythrocytes were purchased from Microlab (Madrid, Spain). ANTS (8-aminonaphthalene-1,2,3-trisulfonic acid), ANS (1-anilinonaphthalene-8-sulfonate), and DPX (p-xylene bis(pyridinium bromide)) were from Molecular Probes (Eugene, OR). N-Rhodamine phosphatidylethanolamine was supplied by Avanti Polar Lipids (Alabaster, AL). 45Ca in the form of CaCl2 was obtained from Amersham Biosciences (Uppsala, Sweden).

**Bacterial Strains, Growth Conditions, and Plasmids**—The E. coli D1210 strain was used throughout this work for DNA manipulation and HlyA expression. The recombinant plasmid pSU124, containing the hlyA gene (16), was used to introduce the mutations into the hlyA gene by sequential PCR steps. In the first step, fragments upstream and downstream of the point mutation were PCR-amplified, in two separate reactions, using the flanking primers and oligonucleotides containing the point mutation to be introduced. The resulting plasmid was digested with the same restriction enzymes. The resulting plasmid was transformed into E. coli expression strains. Mutagenic primers were H859N, 5'-5GATACTCCATGGGCATGGG-3' and 5'-GATCATTAAATTGATGATTTGCGGCACTAATGATG-3'. Mutagenic primers were H859N, 5'-CATCATAATGTATGGCATTACATTATTTGATG-3'; H859D, 5'-CATCATAATGTATGGCATTACATTATTTGATG-3'; H859N, 5'-CATCATAATGTATGGCATTACATTATTTGATG-3'.

Expression and Purification of Wild-type and Mutant HlyA—WT and H859D were purified as described previously for the wild type (6). H859N, that was expressed in smaller amounts, was purified by FPLC using a Superdex HR-200 column instead of the Sephacryl S-500 gel-filtration column. The purified proteins were stored at −20 °C in 150 mM NaCl, 6 mM urea, 20 mM Tris-HCl, pH 7.0.

Urea Removal—Urea used in protein storage was removed before any protein assay. This was usually accomplished by dialution, when HlyA was assayed at such concentrations that, upon dilution of the stock protein in the assay buffer, urea concentration fell below 0.25 mM, a concentration that did not interfere with our assays. When required, urea was removed by dialysis against 150 mM NaCl, 20 mM Tris-HCl, pH 7.0, at 4 °C, for 6 h.

**Hemolysis Assay**—A standard red blood cell suspension was used, obtained by diluting the erythrocytes with saline so that 37.5 µl of the mixture in 3 ml of distilled water gave an absorbance of 0.6 at 412 nm. Cubic volumes of the standard suspension of washed horse erythrocytes were added to serial 2-fold dilutions of hemolysin in buffer (150 mM NaCl, 10 mM CaCl2, 20 mM Tris-HCl, pH 7.0). The assay was carried out either in microtiter plates or in test tubes. In the first case, the cell + toxin mixtures were incubated at room temperature for a few hours, so that erythrocyte sedimentation occurred. In the test tube assay, the tubes were left for 1 h at 37 °C with gentle shaking, then centrifuged in an Eppendorf centrifuge. The absorbance of the supernatants, appropriately diluted with distilled water, was read at 412 nm. The blank (zero hemolysis) consisted of a mixture of equal volumes of buffer and erythrocytes.

**Large Unilamellar Vesicles**—Large unilamellar vesicles (LUV) made of egg phosphatidylcholine were prepared by extrusion through polycarbonate filters (Nuclepore, Pleasanton, CA), pore size 0.1 µm. Buffer was 150 mM NaCl, 20 mM Tris-HCl, pH 7.0. The diameter of the resulting vesicles was about 100 nm, according to quasi-elastic light scattering measurements. More details on the preparation of these vesicles can be found in Ref. 17.

**Release of Liposomal Contents**—Leakage of vesicular aqueous contents was assayed according to Ref. 18 with ANTS and DPX entrapped in the liposomes. LUV were prepared in 70 mM NaCl, 12.5 mM ANTS, 45 mM DPX, 20 mM Tris-HCl, pH 7.0. Non-entrapped probes were removed passing the LUV through a Sephadex G-75 column, eluted with 150 mM NaCl, 20 mM Tris-HCl, pH 7.0. Assays were performed at 100 µM lipid in a total volume of 1 ml, with continuous stirring, at 25 °C. Buffer was as above, with the addition of 10 mM CaCl2. The assay was started by adding 100 mM calcium-free dialyzed HlyA. ANTS fluorescence was recorded continuously (λem = 355 nm; λex = 520 nm). When leakage reached equilibrium, Triton X-100 was added (final concentration 0.1% w/v) to induce 100% release. Percent release was computed as follows,

\[
\% \text{ Release} = \frac{(F_o - F_{\text{em}})}{(F_{\text{em}} - F_{\text{triton}})} \times 100
\]  
(Eq. 1)
Expression and Purification of HlyA Mutants—The H859D and H859N mutants were expressed and purified in the same way as the wild-type (WT) protein, except that for the H859N mutant, a Superdex HR-200 chromatography was used instead of a TLA 120.2 Beckman rotor (627,000×g).

RESULTS

Expression and Purification of HlyA Mutants—The H859D and H859N mutants were expressed and purified in the same way as the wild-type (WT) protein, except that for the H859N mutant, a Superdex HR-200 chromatography was used instead.
of the Sephacryl S-500 column, as detailed under "Experimental Procedures." The yield of the proteins was about 2.0 mg/liter culture filtrate for WT and H859D, and about 0.5 mg/liter culture filtrate for H859N. For each of the mutants, a single band with identical electrophoretic mobility as that of WT was seen in SDS-PAGE (not shown). In Western blots, the mutant bands were stained with the polyclonal anti-HlyA antibodies used in previous work (20).

**Membrane Lytic Activity**—HlyA lytic activity was typically assayed on erythrocytes. A dose-response curve of hemolysis is shown in Fig. 1A for the native and mutant forms. H859D behaved virtually like wild-type HlyA, while H859N was totally inactive in producing hemolysis. HlyA can also disrupt the permeability barrier of pure lipid large unilamellar vesicles (liposomes) (20, 21). HlyA induced release of fluorescent probes entrapped in liposomes can be seen in Fig. 1B. Again the H859D mutant behaved like the wild-type protein, while the H859N mutant was totally inactive.

**Binding to Lipid Bilayers**—Binding (adsorption) of wild-type and mutant HlyA to liposomes was assayed by flotation, as indicated under "Experimental Procedures." The results are summarized in Table I. Slightly less than one-half of the protein remained bound to the lipid bilayer, at both protein:lipid ratios tested. No significant differences were found between the WT and mutant proteins. Binding measured under these conditions represents protein adsorption onto the membrane, that may or may not be accompanied/followed by insertion of the toxin into the lipid bilayer (11, 12).

**Calcium Dependence**—The calcium dependence of fluorescent probe efflux from liposomes is shown in Fig. 2 for the wild-type and mutant proteins. HlyA reached a maximum of activity with about 100 μM Ca²⁺, in agreement with previous
data (15), and H859D behaved in a similar way. In contrast, H859N was inactive even in the presence of 250 μM Ca2+.

High-affinity calcium binding was measured next for the three proteins under study, using 45Ca. It should be noted that only a small fraction of all Ca2+ ions bound to the β-roll are easily exchangeable with Ca2+ in solution, corresponding probably to those bound to the COOH-terminal (outermost) repeats (14, 15). The data in Fig. 3 show that wild-type HlyA bound 3–4 calcium ions/protein molecule at 100 μM Ca2+, and this was enough to allow maximum activity. Mutant H859D bound on average less than 1 Ca2+/protein molecule at 100 μM Ca2+, and this also induced maximum activity, as seen in Fig. 2 (this will be discussed below). Finally, H859N could not bind Ca2+ even at 500 μM CaCl2 (Fig. 3). In our previous study (15) we had shown that the intrinsic fluorescence of HlyA tryptophanyl residues was increased, and the maximum emission wavelength shifted to lower wavelengths when HlyA bound Ca2+. The same phenomenon was observed here for H859D for Ca2+ ≥200 μM, while the fluorescence spectrum of H859N remained unchanged even at 1 mM Ca2+ (spectra not shown).

Protein Aggregation—Calcium binding to HlyA in the absence of target membranes leads to irreversible protein aggregation, presumably through exposure of hydrophobic surfaces following a Ca2+-dependent conformational change (13). Aggregation can be conveniently monitored through changes in light scattering of the protein suspension. Fig. 4 depicts the Ca2+-dependent changes in light scattering of WT and mutant HlyA. For WT, increasing Ca2+ concentrations led to increased aggregation, particularly in the 0–250 μM Ca2+ concentration range. A similar behavior, although to a smaller extent, and at higher Ca2+ concentrations was displayed by H859D (Fig. 4B). H859N departed again from WT and H859D behavior by showing, even in the absence of Ca2+, a high degree of aggregation, that was not modified by Ca2+ concentrations up to 1 mM (Fig. 4A). The increased aggregation of H859N might be a clue for its lack of activity, since the active form of HlyA is the monomer (7), thus this aspect was examined in more detail.

HlyA aggregation also occurs, in the absence of Ca2+, when urea is removed from a HlyA suspension that had been stored in 6 M urea. In fact, the protective effect of 6 M urea appears to consist of a displacement of the aggregate-monomer equilibrium toward the monomer side (7). Fig. 5 shows the increase in light scattering of WT and H859N suspensions, originally in 6 M urea, when urea was removed by dialysis. In both cases, aggregation increased with time, but the mutant aggregated faster, and to a larger extent than the WT.

The hypothesis that H859N is more aggregated than WT under all conditions was further confirmed by the studies of ANS fluorescence. The fluorophore ANS partitions in non-polar with preference to polar media. When transferred from aqueous to hydrophobic environments its fluorescent emission is enhanced, and shifted toward lower wavelengths (22). When HlyA was added to ANS in aqueous solution, the fluorescence intensity was indeed increased and blue-shifted, but the H859N mutant had a clearly larger effect on both parameters of ANS fluorescence (Fig. 6). The increase in ANS fluorescence in the presence versus absence of protein (F/F0) was of 8.2 and 10.6 for the WT and mutant, respectively. The maximum wavelengths of emission (λmax) were 490 and 482 nm, respectively. This suggests that, even in the absence of Ca2+, H859N has more hydrophobic patches that are accessible to ANS than WT, and this would explain the higher tendency of the mutant to aggregate.

Additional evidence of the unusual displacement of the aggregate-monomer equilibrium toward the aggregate in the case of H859N came from FPLC. Fig. 7, A, C, and E, show typical chromatograms of, respectively, WT, H859N, and H859D HlyA. The chromatograms were dominated by a large peak that came out with or near the void volume, and corresponded, according to blue native gels (not shown), to some kind of aggregate (note, however, that the largest aggregates were removed by filtration prior to sample application to the column). In the presence of Ca2+ (Fig. 7, B and F) both WT and H859D gave rise to a prominent peak at higher elution volumes (about 18 ml) that appeared as monomeric protein in blue native gels. This peak had the same electrophoretic mobility as HlyA in SDS-PAGE,
and had hemolytic activity. Mutant H859N, however, did not give rise to the monomer peak in the presence or absence of Ca\(^{2+}\), the aggregate being the only species that was observed in the chromatograms (Fig. 7, C and D).

The aggregation behavior of H859N can also explain some experimental results with red blood cells treated with both this mutant and WT HlyA. In the experiment in Fig. 8A, erythrocytes were incubated for 30 min at 37 °C with increasing amounts of H859N, then washed three times by centrifugation and treated with 10 ng (≈0.4 nM) of WT protein. Preincubation with the mutant had virtually no effect. This is probably indicating that the H859N mutant does not bind the glycoporphin receptors of HlyA. Alternatively, binding may be so weak that the toxin is removed in the washing steps. In a different experiment, H859N and WT protein are mixed at different ratios, keeping constant the WT concentration at 0.4 nM, and incubated for 10 min at room temperature before being added to the cell suspension. The result was that HlyA mixtures containing mutant:WT ratios 5 or higher were totally inactive in hemolysis, even if the amount of WT alone could induce over 80% cell suspension. The liposome system allows the study of HlyA lytic activity over a wide pH range. Release of liposomal contents by WT and H859D HlyA in the pH range 4–8 is depicted in Fig. 9. WT HlyA displayed a sigmoidal pH dependence, with a maximum change at about pH 6.5, while H859D activity decreased steadily and slowly with decreasing pH in the 4–8 range. This is an indication that the pH dependence of WT HlyA is largely due to His-859. In most proteins the His side chain is protonated with \(pK_a\) values in the pH region 6–7. The results in Fig. 9 suggest that His-859 in HlyA has a \(pK_a\) of about 6.5, and that its protonation impairs severely the protein lytic activity. Aspartate, in turn, has no \(pK_a\) in the 4–8 pH range, and consequently the activity of H859D decreases smoothly and regularly with pH, remaining clearly above that of WT at pH < 6.5.

**DISCUSSION**

**Rationale Behind the Mutant Constructions**—The observation that His-859 occupies position 6 in a nonapeptide of the RTX repeat region where aspartate is usually found (15) prompted us to perform a comparative study on other RTX toxins. Table II summarizes data from the RTX repeat regions of 10 bacterial toxins, including HlyA. A total of 141 repeats were found, of which position 6 was occupied by Asp in 127 cases, Asn was found in 10 cases, and His in 4. No other amino acid was found in position 6 of the nonapeptide in any of the 141 repeats. Asn (codons AAU, AAC) or His (codons CAU, CAC) may have arisen as point mutations from an original Asp (codons GAU, GAC). Asn is never found in position 6 in the last four nonapeptides. Conversely, when His occurs in position 6 of a nonapeptide, it is always in the last, or last but one, of the repeats. Note that the nonapeptides closer to the COOH-terminal may be more accessible from the aqueous phase, and they are supposed to bind the exchangeable Ca\(^{2+}\) (14). In view of these observations, two possible His-859 mutants were predicted to have a particular interest, one in which His was replaced by Asp, by far the most common residue in that position, and another one in which His was replaced by Asn, a residue that never occurs in position 6 in the last repeats. It is interesting that in fact H859D behaved very much like the wild type, while H859N was very different from almost every point of view tested.

**Aggregate-monomer Equilibria**—The fact that HlyA contains an N-terminal hydrophobic and a C-terminal hydrophilic domain explains probably its tendency to self-aggregation. However, only the monomeric form appears to be active from the point of view of membrane insertion and eventual lysis. Solóaga et al. (7) have described how urea and calcium ions can modify the aggregate-monomer equilibrium. From those studies, and from those by Bakás et al. (12, 13) it can be concluded that the lytic form of HlyA is a monomer that contains a number of exchangeable calcium ions bound to its repeat region.

Data in the present paper illuminate from a different point of view the relevance of the aggregate-monomer equilibrium. Light scattering (Figs. 4 and 5), ANS fluorescence (Fig. 6),

| Toxin              | Organism                      | Accession No. | No. of repeats* | Amino acid in position 6 |
|--------------------|-------------------------------|---------------|-----------------|-------------------------|
| α-Hemolysin (HlyA) | *Escherichia coli*            | P08715        | 17 (615–872)    | Asp 15 1 1 0            |
| Hemolysin (Rlx A) | *Escherichia coli*            | O85101        | 16 (619–854)    | Asn 14 1 1 0            |
| Leukotoxin        | *Actinobacillus actinomycetemcomitans* | P16462        | 12 (740–858)    | His 11 0 1 0            |
| RTX-I             | *Actinobacillus pleuropneumoniae* | I39643        | 13 (739–867)    | Asp 10 2 1 0            |
| RTX-II            | *Actinobacillus pleuropneumoniae* | P15377        | 13 (614–810)    | Asn 12 1 0 0            |
| RTX-III           | *Actinobacillus pleuropneumoniae* | P55130        | 12 (753–861)    | Asp 10 2 0 0            |
| Adenylate cyclase toxin | *Bordetella pertussis* | P15318        | 30 (919–1612)   | Asn 28 2 0 0            |
| Leukotoxin        | *Pasteurella haemolytica*     | P55118        | 8 (629–798)     | Asp 8 0 0 0             |
| Hemolysin         | *Actinobacillus suis*         | Q00961        | 10 (719–810)    | Asp 9 1 0 0             |
| RTX A             | *Vibrio cholerae*             | Q9X4W2        | 10 (4194–4402)  | Asn 10 0 0 0             |
| **Total**         |                               |               | 141             | 127 10 4 0              |

* A minimally stringent criterion has been followed to detect the nonapeptides, other criteria may lead to smaller numbers of repeats in a given protein. The figures in parentheses indicate, respectively, the positions within the protein of the initial residue of the first repeat, and of the last residue of the last repeat.
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**FPLC (Fig. 7), and WT-mutant competition experiments (Fig. 8) concur in presenting mutant H859N as a protein in which the aggregate-monomer equilibrium is clearly shifted toward the aggregate form, so that the concentration of free monomer appears to be negligible. The H859N aggregates do not take up Ca$^{2+}$ from the aqueous medium (Fig. 3) and are totally inactive (Figs. 1 and 2). There is no direct proof that only monomeric WT HlyA may bind Ca$^{2+}$, but the fact that the highly aggregated H859N does not bind the cation speaks in favor of that hypothesis. In this case the scheme proposed by Soloaga et al. (7) for the equilibria involving HlyA and calcium may be given in more detail in Scheme 1, where $h$ represents a denatured form of HlyA.

Mutant H859D represents a different situation. Judging from its activity (Figs. 1 and 2), similar to WT, the aggregate-monomer equilibrium in the absence of Ca$^{2+}$ must also be comparable in H859D and WT. However, the low tendency of H859D to aggregate in the presence of Ca$^{2+}$ (Fig. 4B) suggests that, in terms of the above scheme, the rate of conversion of $H_{mon}$·Ca into $h$·Ca is lower in the mutant than in the WT. In other words, the lifetime of $H_{mon}$·Ca is longer for H859D than for WT. In turn, this explains that with a lower average Ca$^{2+}$:protein ratio, at e.g. 150 μM total Ca$^{2+}$, the mutant is as active as the wild type (Figs. 2 and 3).

**pH Dependence**—Ostolaza et al. (23) had observed that HlyA activity was much reduced at pH ≤ 5. However, the comparison of the pH dependence of WT and the H859D mutant reveals some important details. First, the pH dependence of WT follows closely the His protonation curve in many proteins. Second, substitution of His-859 by Asp changes the profile of the pH dependence curve, and makes the mutant clearly more active at low pH (Fig. 9). It is remarkable that a single amino acid substitution changes the pH profile of such a large and complex protein. The data in Fig. 9 alone are enough to identify His-859 as a very important residue in HlyA, and point to a physiological adaptive advantage for His over Asp in that position. The His side chain protonable group has a $pK_a$ at 6–7, so that protonation (i.e. gain of a positive charge) appears to “switch off” the protein. Asp has a net negative charge in all the 4 – 8 pH range, and the lytic activity remains all along. In the absence of a crystal structure, it is difficult to explain why either a negative charge or no charge in residue 859 are compatible with HlyA activity, while a positive charge is not. However, the pH dependence data of WT and H859D, together with those on the lack of activity of H859N, concur in pointing to His-859 as an essential residue in E. coli α-hemolysin activity.

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