Reversible Denaturation, Self-Aggregation, and Membrane Activity of *Escherichia coli* α-Hemolysin, a Protein Stable in 6 M Urea†

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ABSTRACT: *Escherichia coli* α-hemolysin (HlyA) is an extracellular protein toxin (107 kDa) whose cell lytic activity may be preserved for months at −20 °C in the presence of 6 M urea, although it decays rapidly in urea-free buffers. This paper describes experiments addressed to unravel the role of urea in HlyA stabilization. Urea up to 8 M inhibits the Ca^{2+}-binding and hemolytic activities of the protein, alters its secondary and tertiary structures, and reduces its tendency to self-aggregation. All these changes are largely reversed upon urea removal by dilution or dialysis, suggesting that they are interrelated. Furthermore, the extent of recovery of the native activities and structural features of α-hemolysin that follows urea removal increases with the concentration of urea during the previous phase. Thus, it seems that urea elicits the reversible transition of HlyA to a less active but more stable state whose structure differs significantly from that of the native protein. Moreover dialysis equilibration of the protein with buffers containing 3 M urea induces the formation of a molecular form of HlyA 5−10 times more active than the native protein in the absence of urea. This hyperactive intermediate appears to keep the native secondary structure of HlyA, but with a less compact tertiary structure, that increases the number of exchangeable Ca^{2+} ions under these conditions. Changes in the intrinsic fluorescence of HlyA also support the notion of a conformational change in the high-activity intermediate. The intermediate is only detected when assayed in the presence of Ca^{2+} and 3 M urea and can bind a large number of calcium ions (≈12 vs ≈3 for the native protein); it shows a large tendency to self-aggregation and presumably, in the presence of membranes, a similar tendency to irreversible insertion, which may be the reason for its high lytic activity.

The bacterial toxin α-hemolysin (HlyA) is secreted by *Escherichia coli* to the extracellular medium, and is responsible for some of the pathogenic activities of the bacterium (*1*). The toxin consists essentially of a single 107 kDa polypeptide (Figure 1); it does not contain any Cys residues and belongs to the so-called RTX toxin family, a group of proteins secreted by Gram-negative bacteria, having in common a repeated nonapeptide sequence that contains a number of calcium-binding sites (*2*−*4*). Calcium (or strontium, or barium) is essential for the cell lytic activity of HlyA (*5*). Binding of calcium ions appears to induce changes in the protein tertiary structure, such that hydrophobic regions emerge at the protein surface, thus favoring its irreversible insertion in membranes (*6*). HlyA must contain bound Ca^{2+} prior to interacting with membranes for the lytic activity to occur (*7*).

A number of peculiarities have hindered in the past, and still constitute a serious problem for the study of α-hemolysin. Chief among these are (a) the tendency of HlyA to exist in aqueous solution in the form of aggregates (*6*−*10*), (b) the surprising observation that the otherwise unstable HlyA is stabilized, and its activity preserved, in the presence of 6 M urea (*11*), although glycerol has also been used with the same purpose (*12*), and (c) the large size of the single α-hemolysin polypeptide (≈107 kDa), whose various domains appear to be functionally interconnected. For example Ca^{2+} binding to a polar domain facilitates the membrane insertion of another, relatively hydrophobic region (*2, 6, 13*). Also the lack of fatty acylation of Lys residues 564 and 690 (*14*) impairs Ca^{2+} exchange at the Ca^{2+}-binding domain (*8*).

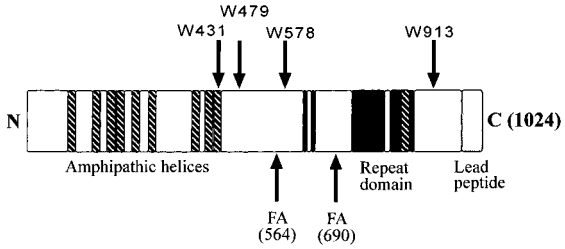

FIGURE 1: Schematic representation of the primary structure of *E. coli* α-hemolysin. The positions of the four tryptophan residues (W) and of the two fatty acids (FA) are indicated. From data by Felmlee et al. (1985), Stanley et al. (1994), Ostolaza et al. (1995), and R. Brasseur (unpublished). The repeat nonapeptides are represented by black vertical bars, while the striped bars correspond to predicted amphipathic helices.

The present work is directed to the understanding of the complex relationship between urea, calcium, α-hemolysin

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activity, and α-hemolysin stability. Urea is found to reversibly denature HlyA, thus preserving it from irreversible aggregation and inactivation. In addition, a Ca^{2+}-dependent, urea-dependent hyperactive HlyA intermediate has been detected.

MATERIALS AND METHODS

α-Hemolysin. The plasmid-encoded protein was purified as described by Ostolaza et al. (*9*). Protein preparations were dialyzed against a 20 mM Tris-HCl, 150 mM NaCl, 6 M urea, 1 mM EGTA, pH 7 buffer ("buffer A"), in order to remove Ca^{2+}, and then dialyzed against the same buffer, but without EGTA ("buffer B"), to remove this chelating agent. The protein can be stored in buffer B, containing 6 M urea, for several months at −20 °C, without major loss in hemolytic activity. The toxin is not altered by proteolysis along the various urea and/or dialysis treatments, when performed, as shown by SDS−PAGE of the protein before and after such treatments.

Protein Assays. Protein was assayed according to Bradford (*15*) with bovine serum albumin as standard.

Hemolysis Assays. Sterile, defibrinated horse blood was supplied by Biomedics (Bilbao, Spain). A stock solution was prepared by diluting 100 mL of horse blood in 700 mL of sterile Alsever buffer (4.2 g of NaCl, 8.0 g of trisodium citrate·$2H_2O$, 0.55 g of citric acid·H_2O, 20.5 g of glucose, made up to 1 L with water). Shortly before the assays, the blood was washed in 0.9% NaCl and standarized, so that 37 μL erythrocytes in 3 mL of distilled water would give a reading of 0.6 absorbance unit at 412 nm.

Hemolysis was assayed occasionally in microtiter plates (*9*) but most frequently in test tubes. The hemolysis buffer was 20 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$, pH 7 ("buffer C"). Five hundred microliters of buffer C and 500 μL of the standard red blood cell suspension were mixed in each tube. The toxin (always less than 50 μL) was then added, and the mixtures were incubated at 37 °C for 30 min with constant shaking. The tubes were then briefly centrifuged, and the absorbance of the supernatants was read at 412 nm. Percent hemolysis was defined as $\% L = [(A - A_o)/(A_{100} - A_o)] \times 100$, where A is the absorbance in the presence of a given toxin concentration, A_o is the lysis in the absence of toxin, and A_{100} is the total lysis as caused by distilled water. A hemolytic unit (HU) is defined as the amount of protein required for producing, under the above conditions, 50% lysis. A typical HlyA preparation had ≈400 HU/mg of protein.

Intrinsic Fluorescence Measurements. The intrinsic tryptophanyl fluorescence of HlyA was measured in a Perkin-Elmer LS50 spectrofluorometer. The excitation wavelength was 295 nm while emission was scanned between 310 and 400 nm. Slits were of 5 nm; 1 mL cuvettes with constant stirring were used. Protein concentration was in the 10−25 μg/mL range.

Circular Dichroism Spectra. Far-ultraviolet circular dichroism spectra were recorded in a Jasco J-720 spectropolarimeter. Spectra were obtained by taking measurements every 0.5 nm, and averaging 5 measurements for each spectral point. Protein concentration was 80 μg/mL. A demountable quartz cuvette, 0.5 mm path length, was used. Spectra were normalized to a constant protein concentration. The α-helix content of the protein was estimated from the CD spectra using the MS-DOS version of the program of Deléage and Geourjon (*16*), and the reference data of Bolotina et al. (*17*).

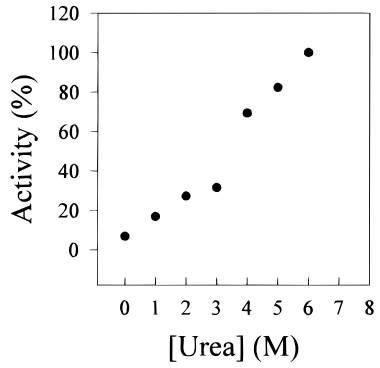


FIGURE 2: Effect of urea in storage buffers on the activity of α-hemolysin when assayed in urea-free media. Hemolytic activity is plotted as a function of urea concentration in the storage buffer. Protein concentration in the hemolysis assay: 1 μg/mL.

Ca^{2+} Binding. Exchangeable Ca^{2+} binding to HlyA was measured with $^{45}Ca^{2+}$ as described by Ostolaza et al. (*5*). Free and bound radioligands were separated using a Millipore GSWP 02500 filter. Nonspecific binding was estimated by adding an excess (100-fold) of nonradioactive Ca^{2+}. Prior to the measurement, the protein was extensively dialyzed against buffer A, and then against buffer B (or modified buffers A and B containing 0 or 3 M urea), all prepared with MilliQ water. Protein was then concentrated by embedding the dialysis bag in solid poly(ethylene glycol) 20000.

RESULTS

Urea in Storage and Assay Media. A series of three experiments was designed in order to test separately the influence of urea in the storage and assay of α-hemolysin. The first one consisted of studying the stability of the protein in various urea concentrations. A stock solution of HlyA in 6 M urea was dialyzed for 24 h at 4 °C against buffers similar to buffer B but containing between 0 and 6 M urea. Then the hemolytic activity was tested in the urea-free assay medium. Figure 2 shows an almost linear decrease in activity with decreasing urea concentrations, thus confirming the results of González-Carreró et al. (*11*) on the stabilizing power of urea. Similar results had also been obtained in a preliminary study from this laboratory, using either urea or guanidinium chloride (*18*).

The second experiment intends to test the effect of urea in the hemolysis assay medium. Again a stock solution of HlyA in 6 M urea was used; the protein is always added in a small volume, so that the urea concentration in each assay is virtually the same as in the assay buffer. Assay buffers containing between 0 and 8 M urea were used in the experiment shown in Figure 3. It is clear that urea has an inhibitory effect on the cell-lytic effect of HlyA; hemolysis is negligible above 7 M urea. In this experiment, the results have been corrected for small hemolytic effects of urea on the red blood cells. Inhibition of hemolysis is not due to a direct effect of urea on the membrane proteins or the lipid bilayer of red blood cells. Control experiments in which pure phospholipid vesicles containing fluorescent solutes (*19*) were used instead of erythrocytes show the same inhibitory effect of urea on hemolysis (not shown). Liposomes

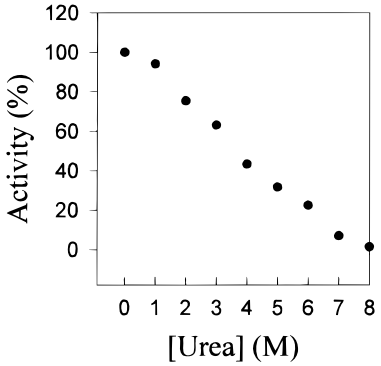


FIGURE 3: Effect of urea on the hemolytic activity of α-hemolysin. Activity is plotted versus urea concentration in the hemolysis buffer. Protein concentration in the hemolysis assay: 1 μg/mL.

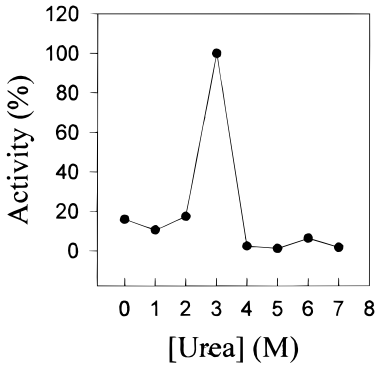


FIGURE 4: Effect of urea on the storage and hemolytic activity of α-hemolysin. The abscissa represents the urea concentration in both the storage and assay buffers. Initial protein concentration was 80 μg/mL. Protein concentration in the hemolysis assay: 1 μg/mL.

containing 6 M urea, in addition to the fluorescent solutes, in an assay medium also containing 6 M urea, were unaltered by α-hemolysin. The same liposomes equilibrated in urea-free assay buffer (apart from suffering some spontaneous leakage of fluorescent contents) were lysed by HlyA. Urea did not act as an osmoprotectant for red blood cells under our conditions, since hemolysis caused by Triton X-100 was not perturbed in the presence of urea.

In the next experiment, both the stabilizing and the inhibitory effects of urea were tested simultaneously. For that purpose, a stock solution of HlyA was dialyzed for 24 h at 4 °C against buffers similar to "buffer B" but with urea concentrations ranging between 0 and 7 M. Then each protein aliquot was tested in an assay buffer containing the same urea concentration as the corresponding dialysis buffer. A somewhat inexpected result can be seen in Figure 4: a peak of activity is observed at an intermediate urea concentration, that varied around 3 ± 0.5 M in individual experiments. The high activity was only detected when the protein had been equilibrated for a long time (in this case by dialysis) with 3 M urea, but not after rapid equilibration (e.g., by dilution, as in the results shown in Figure 3). Detection of high activity requires as well that the assay is carried out in the presence of ≈3 M urea, as occurs in Figure 4, but not in Figure 2.

The behavior of HlyA in each of the above experiments was further explored. Specifically, the stabilizing properties of 6 M urea were examined in detail. Ostolaza et al. (*9*) mentioned that HlyA instability is probably related to protein aggregation. This hypothesis was tested by diluting HlyA

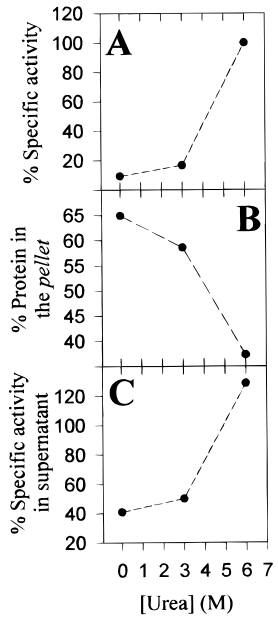


FIGURE 5: Effect of urea on α-hemolysin self-aggregation properties. In this experiment, a protein preparation was equilibrated in buffer B with 0, 3, or 6 M urea and its activity tested in the absence of urea (A). The protein suspensions were then centrifuged, and the proportion of protein in the pellet was determined (B). Finally the hemolytic activity of the supernatants was assayed (C). Initial protein concentration was 20 μg/mL. 100% specific activity corresponds both in (A) and in (C) to the activity of α-hemolysin equilibrated in buffer B with 6 M urea, and then assayed in the absence of urea, as in Figure 2.

samples in urea (0−6 M) and then centrifuging the suspensions (Beckman TLA-45 rotor, 109000*g*, 4 °C, 2 h). The fraction of protein in the pellet and the specific activities (measured in the absence of urea) before and after centrifugation are plotted in Figure 5. In the absence of urea, the average specific activity of the protein is rather low (Figure 5A); however, centrifugations show that 66% of the protein can be sedimented under these conditions (Figure 5B), leaving in the supernatant a small fraction of HlyA 4.3 times more active than the original suspension (Figure 5C). The situation is different in the presence of 6 M urea; only 37% of the protein is sedimented, and the specific activity of the supernatant increases by just 22% with respect to the original suspension. Essentially similar results were obtained with protein concentrations in the starting solutions ranging from 20 to 400 μg/mL. These results show that the more stable, reversibly inactivated state induced by urea has a lower tendency to aggregate than the native state.

To explore in some detail the effect of urea on HlyA, circular dichroism spectra were recorded in the presence and absence of urea (Figure 6). Inspection of the spectra shows that 3 M urea has little effect on the protein helical contents, while 6 M urea appears to be more active in this respect. The α-helix contents of HlyA in 0, 3, and 6 M urea are respectively 36%, 39%, and 27%. Thus, urea concentrations that stabilize the protein, preventing its aggregation, also induce significant changes in the secondary structure. In fact, the substantial decrease in α-helix contents in the presence of 6 M urea would be enough to explain the lack of activity under those conditions.

Additional information concerning urea-induced conformational changes of HlyA is obtained from measurements

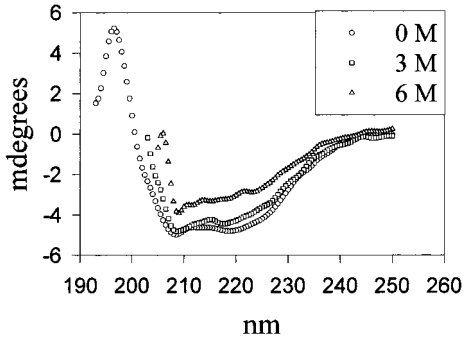


FIGURE 6: Far-UV circular dichroism spectra of α-hemolysin in buffer B containing (○) 0 M, (□) 3 M, and (△) 6 M urea.

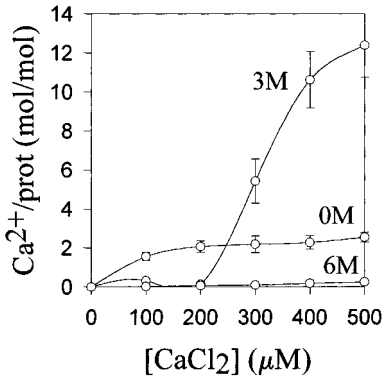


FIGURE 7: Calcium ion binding to α-hemolysin. The number of Ca^{2+} bound per protein molecule are plotted as a function of Ca^{2+} concentration in the buffer, in the presence of 0, 3, or 6 M urea as indicated by each curve. Experimental details as in Ostolaza et al. (1995).

of bound Ca^{2+} (*5*). The data are shown in Figure 7. For these experiments, HlyA was previously dialyzed against buffers containing 0, 3, or 6 M urea so that, in the case of 3 M urea, the conditions were such that formation of the highly active form of hemolysin should be expected. In the absence of urea, HlyA binds about three calcium ions per protein molecule, as shown previously (*5*). In the presence of 6 M urea, however, virtually no Ca^{2+} is bound, while in the presence of 3 M urea about 12 calcium ions can be exchanged per protein molecule. The latter is close to the expected total number of Ca^{2+} that HlyA can bind (*5*). Thus, the Ca^{2+}-binding measurements indicate that urea has a significant effect on the conformation of the whole or part of the HlyA molecule. The fact that 3 M urea changes drastically the number of bound calcium ions per protein molecule without modifying significantly its CD spectrum is compatible with a urea-dependent change in tertiary structure, without modifying the secondary structure. The inability of HlyA to exchange Ca^{2+} in the presence of 6 M urea is consistent with the expected requirement of structured protein sites for Ca^{2+} binding.

The Highly Active Intermediate. A number of experiments were designed in order to characterize the highly active form of HlyA observed in the presence of ≈3 M urea (Figure 4). Initially, the protein equilibrated in 6 M urea was dialyzed against urea-free buffer and the activity assayed at various intervals. The results are shown in Figure 8. The general tendency is toward a decrease in activity, as expected from the results in Figures 2 and 5, but an intermediate situation, occurring after 1−2 h of dialysis depending on the experimental conditions, is detected characterized by a very high

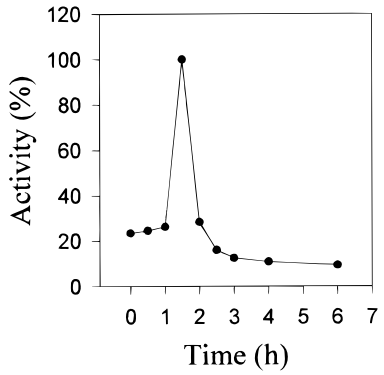


FIGURE 8: Effect of urea removal by dialysis on the lytic activity of α-hemolysin. The protein was equilibrated with buffer B containing 6 M urea, and then dialyzed against urea-free buffer. Aliquots were removed from the dialysis bag at regular time intervals and their activities assayed. Initial protein concentration was 200 μg/mL. Protein concentration in the assay was 1 μg/mL.

activity. Urea concentration in the dialysis bag is 2−3 M at this stage. Probably the experiments in Figures 4 and 8 correspond to the same phenomenon; a critical urea concentration is reached, in one case through dialysis against that urea concentration, i.e., 3 M, in the other through dialysis against a urea-free buffer. These correspond, respectively, to equilibrium and kinetic methods for reaching a similar condition. Urea concentrations leading to high lytic activity appear to induce a significant change in the tertiary structure of HlyA (Figure 7) without modifying greatly its secondary structure (Figure 6).

The activity data in Figure 8 were obtained in the presence of calcium ions, since these are essential for HlyA-induced cell and liposome lysis (*5*). Since it has also been shown that for lysis to occur Ca^{2+} must bind the protein before the latter binds the membrane (*7*), some experiments using fluorescence spectroscopy were undertaken with the aim of detecting spectral changes that could in turn be related to the highly active state of HlyA in the absence of membranes. Emission spectra corresponding to the intrinsic Trp fluorescence of the protein were recorded at different urea concentrations (conditions similar to the measurements in Figure 4) in the presence and absence of Ca^{2+}. The spectral parameters F/F_o (intensity of fluorescence emission relative to the emission in 0 M urea, both measured at their respective λ_{max}) and λ_{max} are plotted versus urea concentration in Figure 9. The emission band shifts gradually toward higher wavelengths and their intensity decreases as the urea concentration increases. In the absence of Ca^{2+}, both F/F_o and λ_{max} change linearly with urea concentration, but if Ca^{2+} is added before the fluorescence measurement an increase in fluorescence intensity is detected in the presence of 2−3 M urea, while λ_{max} appears to resist the red shift. The decrease in fluorescence intensity together with the red shift is compatible with a urea-dependent gradual unfolding of the protein (or part of it) so that the average microenvironment of the Trp residues becomes more polar, in principle because of an increased water accessibility [see (*20*) for a review]. The Ca^{2+}-elicited modifications of the urea influence on intrinsic HlyA fluorescence strongly suggest that the HlyA−Ca^{2+} complex is more resistant than the free protein to the loosening effect of urea on the protein structure. Note, however, that changes in fluorescence cannot be

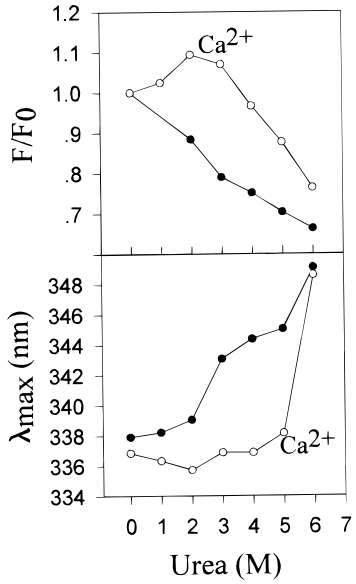


FIGURE 9: Changes in the intrinsic Trp fluorescence of α-hemolysin as a function of urea concentration. The protein was equilibrated in buffer B containing various urea concentrations, in the presence (○) or absence (●) of Ca^{2+}. Upper panel: Fluorescence intensities at the maximum wavelength of emission relative to the fluorescence in the absence of urea. Lower panel: Maximum wavelengths of emission.

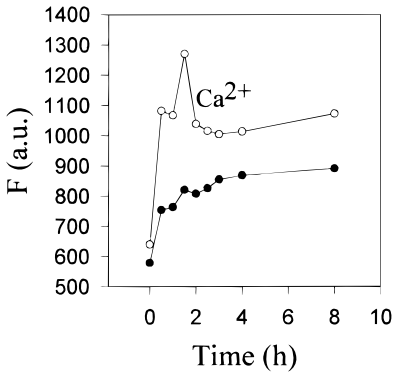


FIGURE 10: Changes in the intrinsic Trp fluorescence of α-hemolysin as urea is removed by dialysis. The protein equilibrated in buffer B with 6 M urea was dialyzed against urea-free buffer. Aliquots were removed from the dialysis bag at regular time intervals and the fluorescence intensities recorded. The experiment was performed in the absence (●) and in the presence (○) of Ca^{2+}.

directly linked to the observed changes in Ca^{2+} binding, among other reasons because there are no Trp residues in the Ca^{2+}-binding repeat domain (Figure 1).

Hemolysin fluorescence was also measured as a function of time during dialysis of the protein, initially in 6 M urea, against a urea-free buffer (conditions similar to Figure 8). As shown in Figure 10, in the absence of Ca^{2+}, HlyA fluorescence intensity increases hyperbolically with decreasing urea concentrations. The presence of Ca^{2+} induces a larger increase in fluorescence intensity along the whole dialysis process, and a transient further increase at about 2 h after the onset of urea removal. Thus, under both equilibrium and dynamic conditions Ca^{2+} appears to induce changes in HlyA fluorescence intensity indicative of a decreased conformational flexibility precisely under the conditions in which the highly active state is detected. These observations suggest that calcium ions, even in the absence of membranes, can induce the high activity state.

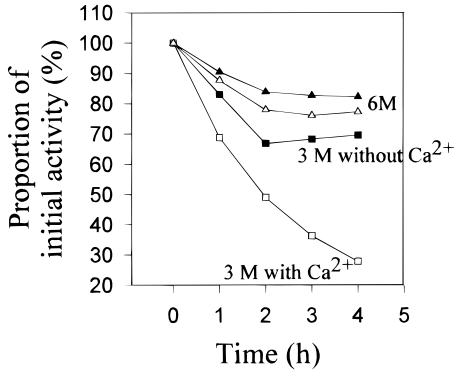


FIGURE 11: Loss of α-hemolysin lytic activity as a function of time. The protein (100 μg/mL) was equilibrated by dialysis against buffer B containing 3 M (□, ■) or 6 M (△, ▲) urea, in the presence (open symbols) or absence (filled symbols) of Ca^{2+}, and then assayed in hemolysis buffer in the absence of urea.

The properties of the highly active state were also tested by measuring the loss of HlyA lytic activity with time in the presence and absence of Ca^{2+}, and at various urea concentrations. The results corresponding to 3 and 6 M urea are shown in Figure 11. For 6 M urea, a moderate decrease in activity is observed, that is stabilized after about 2 h; inactivation is somewhat more pronounced in the absence of Ca^{2+}. The preparation containing 3 M urea is even less stable, but in this case addition of Ca^{2+} leads to a very steep and pronounced decrease in activity, that becomes virtually undetectable after 4 h. In the absence of urea, both in the absence and in the presence of Ca^{2+}, the decrease is similar and intermediate between the cases of 3 M urea with and without calcium (data not shown). Bakás et al. (*6*) have shown that Ca^{2+} binding by α-hemolysin brings to the protein surface hydrophobic patches that favor its insertion in bilayers or, alternatively, lead to protein aggregation. The extensive inactivation seen in Figure 11 for HlyA in 3 M urea in the presence of Ca^{2+} can be explained by the large amount of calcium bound under these conditions (Figure 7) and the subsequent large increase in hydrophobic surface, leading to aggregation and inactivation.

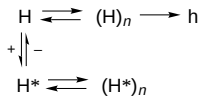
DISCUSSION

The three-dimensional structure of proteins is the result of numerous interactions of various types, of the polypeptide chain with the solvent, and of the chain (and side-chain) components among themselves. For soluble, globular proteins, the protein core appears to be hydrophobic, while the surface of the molecule is polar in nature. In turn, intrinsic membrane proteins display a hydrophobic surface that makes them soluble in the bilayer lipids.

E. coli α-hemolysin, like other amphitropic proteins, represents an intermediate situation: in some cases, it exists as a soluble protein while it can also be found in a membrane-bound form. However, at variance with the truly soluble proteins, HlyA does not exist in the aqueous medium as a monomer, or with a fixed quaternary structure, but in the form of polydisperse aggregates (*9*). Such aggregation suggests a certain amphipathic character for the protein molecule, that is also suggested by its primary structure, rich in hydrophobic residues at its N-terminal end and with a large polar Ca^{2+}-binding domain near the C-terminal end (*21, 22*) (Figure 1).

An additional relevant difference between HlyA and most soluble proteins is the effect of urea. Soluble proteins are usually stable in urea-free buffers, while at and above 6 M urea they usually become denatured, their secondary and tertiary structures being highly perturbed. For HlyA, however, 6 M urea prevents the spontaneous irreversible inactivation of the protein that takes place in its absence (*11*).

The results in this paper contribute to explain the paradoxical effect of urea on α-hemolysin. Urea *denatures* HlyA since it causes a conformational change (Figures 6, 7) and makes it inactive (Figure 3). However, such a denaturation easily *reverts* (Figure 2), at variance with the nonreversible inactivation observed in the absence of urea. The protective effect of 6 M urea appears to consist of a displacement of the aggregate−monomer equilibrium toward the monomer side (Figure 5). The monomer is probably the active form of the protein (*9, 12, 19*), and, besides, aggregation in the absence of urea favors the formation of irreversibly inactivated aggregates (Figure 5). The following multiple equilibrium may be proposed:



where H* and h mean respectively a reversibly and an irreversibly denatured form of HlyA, H is the active form, + and − indicating respectively the presence or absence of 6 M urea. De Young et al. (*23*) have studied a somewhat similar system, namely, the aggregation and denaturation of apomyoglobin in aqueous urea solutions. They propose a 2-fold effect of urea, that would (a) cause protein denaturation but also (b) make the solvent favorable for both the native and the denatured state. The latter would explain the observed increase in the fraction of monomeric (soluble) HlyA in the presence of 6 M urea.

The above results on α-hemolysin aggregation and inactivation may have a physiological significance. The toxin, once secreted by the bacterium, either binds a target plasma membrane or, alternatively, aggregates and becomes inactive. Although HlyA concentrations in physiological conditions must be usually too low to allow significant aggregation, high concentrations could occur in localized infected points. The self-aggregation and inactivation of α-hemolysin may constitute an evolutionary advantage for the bacterium, by limiting the host damage, thus prolonging the availability of a suitable environment for the microorganism.

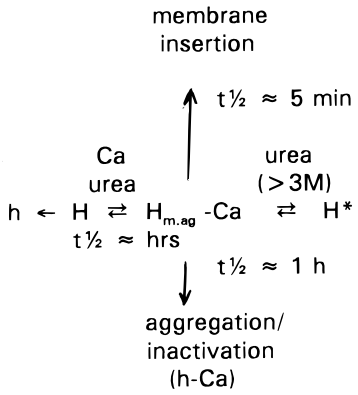
Studies on the action of urea have led to the observation of a form of α-hemolysin that is particularly active in the lysis of red blood cells (Figures 4 and 8). The hyperactive intermediate is observed within a given range (i.e., 2−3 M) of urea concentrations, and although it is detected following a purely functional criterion (cell lytic activity) the presence of Ca^{2+}, even in the absence of membranes, is enough to produce some changes in the protein intrinsic fluorescence under the same urea conditions inducing the activated state, that could correspond to changes in protein conformation. Folding−unfolding intermediates have been frequently described for soluble proteins (*24*−*27*) and their role in the physiological mechanism of protein folding interpreted in terms of a "nucleation-condensation" theory (*28*). The HlyA highly active intermediate is characterized by keeping its native secondary structure, as shown by the circular dichroism spectra (Figure 6), while the tertiary structure appears to be less compact than in the absence of urea, judging from the high number of exchangeable Ca^{2+} ions under the activation conditions (Figure 7) that suggests an increased accessibility of the solvent to the calcium-binding domain. Previous studies (*5, 7*) had demonstrated that Ca^{2+} was essential for the lytic activity, and that the protein had to contain bound Ca^{2+} before binding the membrane in order to produce lysis. The observed fluorescence changes (Figures 9 and 10) are reflecting probably the structural correlation of this calcium effect (*20*). Szpikowska and Mas (*26*), studying the urea-induced unfolding of yeast phosphoglycerate kinase, also describe the presence of a stable intermediate characterized by a high fluorescence intensity, at a wavelength of maximum emission that lies between those of the native and urea-denatured states. Note that those authors, as well as De Young et al. (*23*) and others, find λ_{max} shifts of 20 nm and even larger accompanying urea denaturation of proteins. The smaller shift observed in our case (Figure 9) agrees with our interpretation of inactivation with only partial loss of secondary structure.

Bakás et al. (*6*) have shown that Ca^{2+} binding has the effect of modifying the tertiary structure of HlyA, so that hydrophobic regions emerge to the surface, and this leads to protein self-aggregation (in the absence of membrane) or to irreversible bilayer insertion (in their presence). The hyperactive intermediate detected may arise from a urea concentration allowing a high number of Ca^{2+} ions bound, which would in turn lead to an extensive surfacing of hydrophobic side chains. These observations may also be interpreted in light of the results of De Young et al. (1993) with apomyoglobin that were mentioned above. α-Hemolysin unfolding and aggregation is more complex because there are at least two domains: an amphipathic helical region which presumably leads to slow irreversible aggregation, and a water-soluble Ca^{2+}-binding domain. When Ca^{2+} binds, a tertiary structural change occurs, unmasking hydrophobic sites that lead to a greatly increased rate of aggregation and membrane insertion. Following the ideas of De Young et al. (*23*), urea could have two competing effects: (a) between 0 and 3 M, it exposes the calcium-binding sites by inducing a tertiary structural change; (b) at all concentrations, urea makes the solvent more favorable for exposed hydrophobic sites, thereby inhibiting aggregation and membrane insertion. At urea concentrations below ≈3 M, the tertiary structural change is most important, whereas at higher urea concentrations solvent stabilization of hydrophobic residues predominates. Urea concentrations above 3 M also denature the Ca^{2+}-binding domain.

These ideas can be summarized in Scheme 1 where H is the monomer in the absence of urea or calcium and h is the irreversibly formed aggregate, as above. Addition of calcium produces $H_{m.ag}$-Ca, a calcium-bound form that is competent to rapidly aggregate (h-Ca) or bind to membranes. H* is a denatured form of the enzyme, in which the amphipathic helices are unfolded. The half-times ($t_{1/2}$) are only estimates derived from data in Figures 8−11 and in Ostolaza et al. (*19*).

Structural intermediates are known for other proteins that, under certain conditions, change mainly their tertiary structures, thus becoming "competent" for membrane insertion

Scheme 1



(*29*, *30*). Whether the HlyA active intermediate described above occurs only and precisely under the conditions mentioned (≈3 M urea) or else a varying fraction of active molecules occurs under any conditions, with a maximum near ≈3 M urea, cannot be ascertained at present, and its elucidation will require further experimentation.

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