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Structural analysis and comparison of the C-terminal transport signal domains of hemolysin A and leukotoxin A

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Abstract NMR spectroscopy was used to study the structure of the C-terminal signal sequences of the bacterial toxins, hemolysin A(HlyA) and leukotoxin A (LktA). The two signals share little sequence homology; however, both can direct toxin transport with equal efficiency. We report here that in a membrane mimetic environment both peptides form two short non-interacting α-helices separated by a short loop. This higher order structure may be a common feature of C-terminal signals and may be required for interaction with the membrane associated transporter complex.

Key words: ABC transporters; NMR spectroscopy; Hemolysin A C-terminal signal; Leukotoxin A C-terminal signal

1. Introduction

The RTX toxins make use of a dedicated secretion pathway to cross both inner and outer membranes of Gram negative bacteria [1]. The 107 kDa *E. coli* α-hemolysin (HlyA) is secreted by such a pathway which depends on the presence of the two inner membrane proteins hemolysin B and D (HlyB/D) [2,3], and the outer membrane protein TolC [4]. The transport signal of HlyA resides in the C-terminal 50–60 amino acids of the toxin [5–7]. Similar systems are responsible for the secretion of other RTX toxins such as *Pasteurella haemolytica* leukotoxin A (LtkA) [8,9], *Bordetella pertussis* adenylate-cyclase toxin [10,11], and *Erwinia chrysanthemi* metalloproteases [12]. The C-terminal signal sequences of these toxins contain little sequence similarity. However, the hemolysin transporter complex is able to transport these toxins, indicating a possible common recognition feature [13].

Previously we have shown that the C-terminal sequence of LktA can functionally replace the HlyA signal with equal transport efficiency [14]. A comparative study of these two signals may provide a good model system for understanding structure and function of the RTX toxin C-terminal signals. We have purified small peptides containing either the HlyA or LktA signal sequences and analyzed their biophysical behaviour by circular dichroism spectroscopy. Both signal peptides are unstructured in aqueous solution but form helical structures in membrane mimetic environments such as TFE, SDS and negatively charged lipid vesicles [15]. In this report, we use NMR spectroscopy to further analyze the higher order structure of these two signal peptides. In SDS micelles both peptides contain two helical regions separated by a short loop, but no overall tetriary or globular fold. The secondary structures identified in these peptides may be important features of RTX toxin C-terminal signal sequences necessary for their recognition and transport. These results also serve as a starting point for future structure-based mutational analysis to test the role of specific residues and secondary structural elements in recognition and transport.

2. Materials and methods

The C-terminal 61 amino acids of HlyA and the C-terminal 70 amino acids of LktA were expressed as His-tag fusion proteins as reported by Zhang et al. [15]. Signal peptides for NMR analysis were purified by Ni affinity chromatography (Qiagen Inc., Chatsworth, CA), and eluted with a 10 mM solution of perdeuterated sodium-dodecylsulfate (SDS-d_{25}), pH 7.0. The peptide samples were concentrated by ultrafiltration with a 3,000 molecular weight cutoff membrane which retained both the signal peptides and the SDS micelles. Final sample conditions were 1-1.5 mM protein, 1 mM DTT, 200 mM SDS-d_{25}, 250 mM NaCl, 25 mM sodium phosphate, pH 5.6.

The following NMR spectra of uniformly ^{15}N-labelled samples of HlyA and LktA were taken at 25°C on either Varian Unity 600 or Unity + 500 spectrometers, with the nitrogen carrier set to 119.5 ppm and the proton carrier at 4.773 ppm: ^{15}N HSQC using pulsed field gradients and the enhanced sensitivity method of data collection [16]; ^{15}N-HMQC-J [17]; 3D ^{15}N-NOESY-HMQC [18,19] with a mixing time of 150 ms; two 3D ^{15}N-TOCSY-HSQC spectra [20] with mixing times of 30 and 45 ms for HlyA and 45 and 51 ms for LktA using the DIPSI-2 sequence [21]. Both the 3D NOESY and TOCSY experiments were performed at 500 MHz using gradient enhanced techniques [16]. 2D ^{15}N filtered NOESY were acquired for HlyA and LktA as described by Ikura and Bax [22]. All other parameters for the NMR experiments were similar to those of the original references.

Data processing of 2D spectra was performed using NMRZ (New Methods Research Inc., Syracuse, NY) using deconvolution of the time domain data [23] to improve solvent suppression experiment. The HMQC-J experiment was processed with a 90° shifted sinebell applied in t2 and Lorentzian-to-Gaussian filtering applied in t1 using NMRZ parameters G1 = 14.0 Hz (Lorentzian line width to remove), G2 = 8 Hz (Gaussian line width to create), and G3 = 0 Hz (corresponding to a maximum at the first point in the FID). Three-dimensional data were processed with NMRPipe and NMRDraw software [24] using a 60° phase shifted sinebell apodization function and single zero filling in each dimension. The programs CAPP and PIPP [25] were used for peak picking and spectral analysis.

3. Results

Previous CD studies [15] have shown that the HlyA and LktA signal peptides are unstructured in aqueous solution but take on helical content in membrane mimetic environments such as TFE, SDS micelles, in negatively charged lipid vesicles but not in uncharged lipid vesicles. We postulated that the

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Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; HSQC, heteronuclear single quantum correlation; TOCSY, total correlation spectroscopy; SDS, sodium dodecyl sulfate; TFE, trifluoroethanol; CD, circular dichroism.

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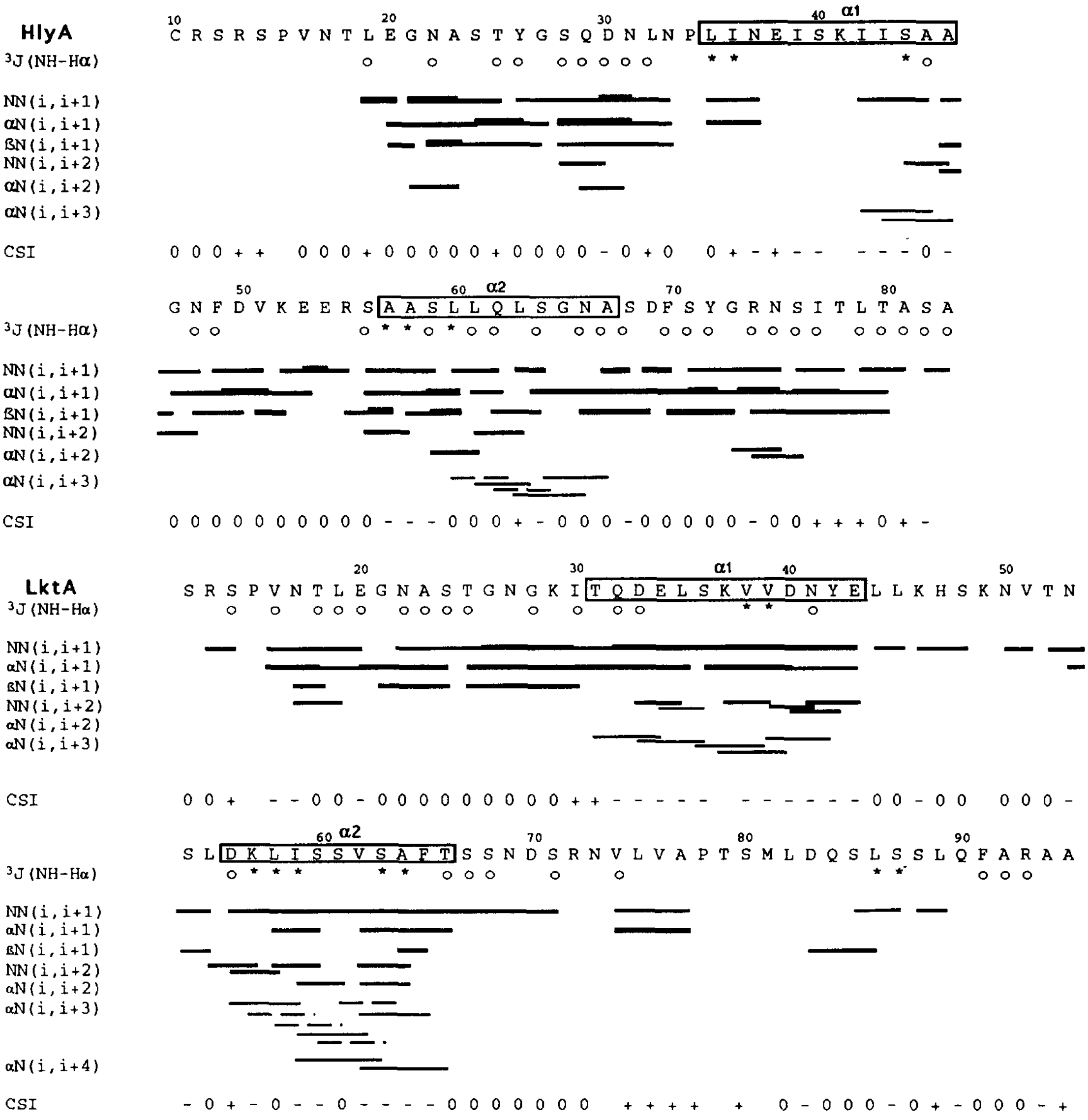

Fig. 1. Summary of NMR data which provide information on secondary structure for HlyA and LktA signal sequences in SDS micelles at 25°C, The primary sequence is displayed across the top. The three-bond coupling constant J(NH-Hα) was classified as either > 8 Hz (●), < 5 Hz (*), or between 8 and 5 Hz (○). NOE connectivities are displayed as lines between the participating residues in the primary sequence and involve the protons indicated on the left of the diagram. A question mark or dashed line indicates a connectivity that could not be unambiguously identified due to overlap. Results of analysis of the α proton chemical shift using the chemical shift index (CSI) of Wishart et al. [37] are summarized as corresponding to a helical (–), random (0) or β structure (+). Suggested helical regions deduced from this data are indicated with cylinders.

conformational change observed in these peptides may be an important feature of RTX toxin signal sequences. The purpose of the present study was to determine in greater detail the nature of this conformational change. Since peptides in lipid vesicles would have rotational correlation times too slow to enable analysis by high resolution state NMR, we tried to use lysophospholipid micelles, however, we found that te peptides were insoluble at millimolar concentration in lysophosphatidylglycerol micelles. Consequently, we chose SDS micelles as the environment in which to study the HlyA and LktA signal domains. Recent results comparing membrane peptide conformations in both SDS micelles and lipid bilayers have shown that peptides most likely exist in the same conformation in SDS as in the more physiological lipid environments [26]. Signal peptides were labelled with ^{15}N to facilitate the NMR analysis. A striking feature of the NMR spectra of both is the poor dispersion in the ^{1}H nuclei, possibly indicating the presence of a significant amount of random-coil or unfolded protein conformation [27]. The ^{15}N-nuclei, on the other hand, have reasonable chemical shift dispersion as has been shown previously for this nucleus in unfolded proteins [28,29]. Side chain assignments and residue types were identified from intra-residue correlations from side chain protons to each N-H in the 3D ^{15}N-HSQC-TOCSY spectra. The amino acid-type was determined from the chemical shifts of the side chain protons associated with each NH and comined with sequential connectivities from the 3D ^{15}N-NOESY-HSQC spectra to make a sequence-specific identification of most residues. (Assignments are available from

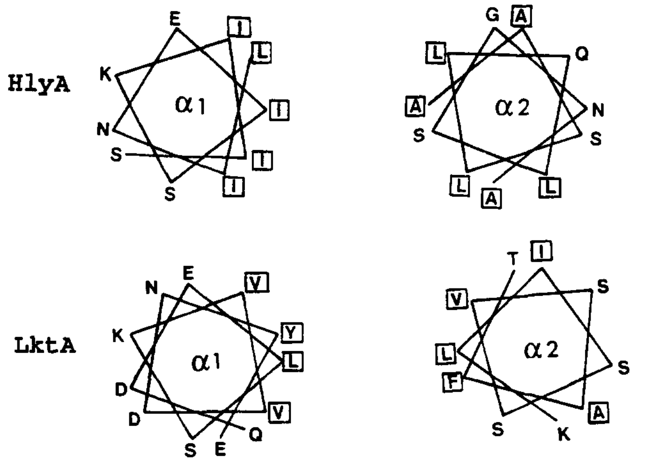

Fig. 2. 'Helical wheel' representation of the helices in HlyA and LktA. Hydrophobic residues are boxed.

the authors upon request.) The majority of the side chain resonances occurred at 'random coil' values [27], facilitating the assignment process.

Both peptides contained two regions with NMR parameters that are characteristic of α-helices (Fig. 1). Typical helical NOEs were observed for residues 31–43 and 56–66 of LktA. These same regions of LktA have ^{1}Hα chemical shifts that are shifted upfield from random coil values, another characteristic of α-helices. Further evidence for the existence of an α-helix in these regions of LktA are the small value of the $^{1}H_{N}$–^{1}Hα coupling constants for many of these residues. Residues 74–79 may possibly be in an extended conformation based on their strong $H\alpha^{i}$–HN^{i+1} NOEs and downfield chemical shifts of their Hα resonances. However, no partner strand for a β-sheet could be identified. The remaining residues of LktA appear to be in a random-coil type of conformation, based on few NOE connectivities, random coil Hα chemical shifts and ^{3}J(NH-Hα) values between 6–8 Hz characteristic of backbone dihedral angles intermediate between an extended and helical conformation, or possibly averaging between conformations. Thus, approximately 30% of LktA signal peptide forms stable α-helices under our NMR solution conditions consistent with previous CD measurements [15].

In SDS micelles, the CD signal at 220 nm, indicative of α-helix, is weaker for HlyA than for LktA, although the HlyA signal peptide still clearly contains helix [15]. The NMR evidence for the helices in HlyA is not as strong as for LktA, presumably due to instability of the helices. Residues 1–34, 47–56 and 68–83 have for the most part random coil Hα chemical shifts, ^{3}J(NH-Hα) values between 6–8 Hz and only short-range sequential NOEs (i,i+1 and i,i+2). The remaining residues, 35–46 and 57–67 contain some of the characteristics of helices, but not enough to clearly define a helix. One reason for this is that these residues of HlyA have broader linewidths and weaker NOEs than residues 1–34, 47–56, and 68–83 of HlyA as well as those of LktA. This poor lineshape and sensitivity in this region of HlyA together with chemical shift degeneracies in this region prevented us from identifying unambiguous helical connectivities. Furthermore, spectral overlap in the region E38–S40 prevented the unambiguous identification of NOEs in this region. However, since the CD clearly indicates the presence of a helix in the HlyA peptide [15] and the remainder of the protein is clearly not helical, we propose that residues 35–46 and 57–67 form two unstable helices which may be undergoing some form of conformational averaging with other, possibly unfolded, conformations. A recent study of the distantly related C-terminal secretion signal of *E. chrysanthemi* PrtG in TFE solution also found regions of helix that were in equilibrium with alternate, possibly unfolded states [30]. In the PrtG study, as in ours, the intensity of $H\alpha^{i}$–HN^{i+3} and $H\alpha^{i}$–HN^{i+4} NOEs were also weak and suffered from overlap. NMR and CD studies of other small peptides in solution have also demonstrated the 'unstable' nature of isolated peptides, especially at temperatures above 5°C [31–33]. Although the helices of HlyA appear to be less stable than those of LktA we observe no correlation between transport efficiency and helix stability in SDS or other membrane-like solutions tested [14].

Both helices of each signal sequence are amphipathic (Fig. 2). In aqueous solution one would expect the hydrophobic surfaces of the amphipathic helices to associate with one another. However, in SDS solutions there is no evidence of long-range tertiary interactions between the two helices in either peptide. In particular, ^{15}N-filtered NOESY spectra of LktA showed no long-range NOEs from the three aromatic rings (Y42, F65 and F91), two of which are in the helices. It is more likely that the helices are associated with the micelle since it is the presence of the detergent that induces the helices.

4. Discussion

We have previously shown that the signal peptides used in this study are necessary and sufficient for transport by the HlyB/D/TolC transporter complex and that GST-fusion proteins containing these C-terminal signals can compete with the intact HlyA toxin for transport by HlyB/D/TolC [15]. This study also demonstrated that in a membrane mimetic environment the signal peptides of HlyA and LktA behave similarly despite their apparent unrelated sequences. Thus, the major features that these two signal peptides appeared to have in common was their biophysical behaviour and similar secondary

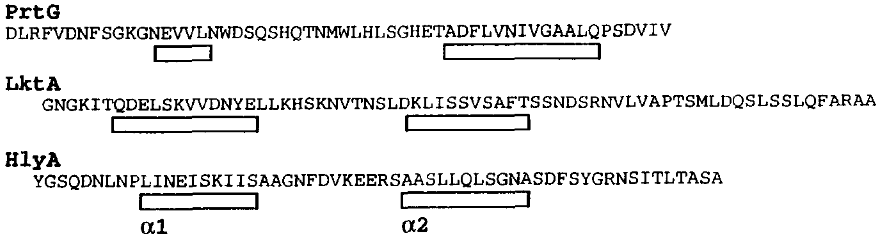

Fig. 3. Alignment of elements of α helical secondary structure (boxes) of PrtG (30), HlyA and LktA C-terminal signal sequences.

structural elements as predicted by Zhang et al. [14]. We now demonstrate further secondary structural similarities, in that both signal peptides contain two amphipathic α-helices, albeit with differential stability. We are also able to localize these helices within each sequence so that the specific residues in each helix can be examined for their role, if any, in transport.

Several previous studies have tried to correlate the transport activity of HlyA signal sequences containing point mutations and deletions with the *predicted* secondary structure of the peptides [6,7,14]. However, in all three cases the prediction algorithms were based on soluble cytosolic proteins, and therefore may not be applicable to proteins in a membrane-like environment [34]. Based on the data presented here, we can now re-evaluate these mutational data in light of the current knowledge of the regions of secondary structure. The putative helices, α1 and α2 of HlyA and α1 of LktA, correspond to regions of predicted helical structure. However, α2 of LktA extends well into a region of predicted β-structure. As shown by Kenny et al. [7] and Stanley et al. [6], the signal sequence of HlyA is, in general, insensitive to point mutations including those which are *predicted* to disrupt the helices identified here. Furthermore, several point mutations that reduced protein secretion by >50% have been identified [6,7], most of them outside the helical regions identified here. Thus, the helices alone can not be the sole factor necessary for secretion. On the other hand, all C-terminal deletions that extend into tha α1 or α2 regions of HlyA result in significant defects in transport [6,14,35], suggesting a role for these regions in transport. It is possible that the helical structure associated with the membrane is required for presenting the critical amino acids in an appropriate position for recognition by the transporter.

Mutational data are not available for the LktA signal sequence, so a similar analysis of the requirements for the helices in this signal domain can not be made. However, the main feature that HlyA and LktA have in common are the presence of two amphipathic helices and similar biophysical behaviour in different membrane-like environments. Thus, the presence of the helices in LktA may also be required for transport.

A recent NMR study of the signal sequence of protease G (PrtG) from *Erwinia chrysanthemi*, also a substrate for HlyB/D/TolC, found similar biophysical traits for this peptide compared to HlyA and LktA [30]. The PrtG signal sequence is also largely unfolded in aqueous solution and forms α-helices in membrane mimetic environments such as aqueous TFE mixtures and dodecyl-β-D-maltoside micelles. NMR data for the 56 C-terminal residues of PrtG in 50% TFE solution showed the majority of residues to be in a helical conformation while at the same time in equilibrium with other less structured conformations. Two smaller sections were considered to be stable helices based on their selective stability at 40°C (Fig. 3). The helices identified for PrtG were not clearly amphipathic, however, as they are for HlyA and LktA. Since the NMR study of PrtG was not performed in the same solvent as we have used here, a direct comparison is not possible. However, it is likely that the two regions of PrtG with the most stable helices in TFE correspond to regions which are helical in other more lipid-like environments. Fig. 3 shows the C-terminal signal sequences of PrtG, LktA and HlyA arranged for maximum alignment of the pair of helices between each sequence.

The NMR data for PrtG toghether with the current study of HlyA and LktA represent the first structural data for this class of C-terminal secretory signal peptides. The biophysical similarity between these three signal peptides is striking despite an apparent lack of sequence homology. All three C-terminal sequences are unfolded in aqueous solution but in membrane mimetic solvents they form (at least to some degree) two α-helices separated by a short unstructured region. All three peptides appear to have no higher order tertiary structure. This type of biophysical behavior found for the C-terminal signal peptides parallels that of the well characterized N-terminal signal peptides of the general secretory pathway [36]. Both N-and C-terminal signal peptides are unstructured in aqueous solution, but take on helical content in membrame mimetic environments. Neither type of signal sequence requires a specific primary or tertiary structure; rather, it is the presence of a common secondary structure and a similar stability of that secondary structure in various environments that is the common feature of both types of signal peptides. These common features for two very different secretory mechanisms suggest a fundamental common denominator for the transport of proteins and peptides across biological membranes in bacteria.

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