FOR THE RECORD

A LexA mutant repressor with a relaxed inter-domain linker

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Abstract: The LexA protein is part of a large family of prokaryotic transcriptional repressors that contain an amino-terminal DNA binding domain and a carboxy-terminal dimerization domain. These domains are separated by a linker or hinge region, which is generally considered to be rather flexible and unconstrained. So far, no structure of any of the full-length repressors is available. Here we show that a mutant LexA repressor harboring several point mutations in the hinge region gets sensitive to trypsin and Glu-C cleavage over a segment of at least 20 amino acids, whereas the LexA wild-type hinge region is resistant to these proteases. These data are not compatible with the hypothesis of an fully flexible and/or unstructured inter-domain linker and suggest that the LexA hinge region is, in fact, constrained by contacts with the carboxy-terminal domain and/or a fairly stable local structure of the linker region.

Keywords: inter-domain linker; LexA mutant repressor

Like many other prokaryotic and eukaryotic regulatory proteins the LexA repressor contains two separable domains for DNA binding and oligomerization (for recent reviews on LexA and the SOS system see Schnarr et al., 1991; Little, 1993; Schnarr & Granger-Schnarr, 1993; Friedberg et al., 1995). These LexA domains are independent folding units that once isolated, conserve, respectively, appreciable DNA binding (Hurstel et al., 1986) and intact oligomerization capacity (Schnarr et al., 1985, 1988). The solution structure of the amino-terminal LexA DNA binding domain (DBD) has been determined by NMR (Fogh et al., 1994). The structure of the carboxy-terminal oligomerization domain may be tentatively inferred from the structure of the homologous UmuD' protein (Peat et al., 1996). The UmuD' structure contains predominantly β -sheets in agreement with the far-UV circular dichroism spectrum of the carboxy-terminal domain of LexA (Hurstel et al., 1986).

Figure 1A shows that between the last structural element of the LexA-DBD (β_2) and the first structural element of the carboxy-terminal domain (an α -helix as inferred from the UmuD' structure) there are about 30 amino acids of unknown structure that serve as a linker between the two domains. However, it should be noted that LexA contains a proline in the fourth position of the first UmuD' helix, suggesting that the LexA linker might, in fact, reach until the equivalent of the first β -strand of UmuD'.

This linker (or hinge) region contains the LexA cleavage site between amino acids Ala-84 and Gly-85. LexA cleavage is crucial for the induction of the bacterial SOS system, which is induced by DNA damaging agents. DNA damage activates the RecA protein to a form that can facilitate the specific cleavage of LexA. However, RecA seems to play an indirect role in LexA cleavage, because the proteolytic center is part of the LexA protein (i.e., amino acids Ser-119 and Lys-156). The relationship between the cleavage site (i.e., the internal "substrate") and the active site is not known, either for LexA or for any other of the LexA-related phage repressors or the full-length UmuD protein.

Shepley and Little (1996) recently put forward two opposing models. First, the cleavage site could normally bind to the binding pocket of the active site in a way that does not allow cleavage, and this reaction would occur only upon a relatively subtle realignment of groups within this complex. Second, as embodied in the "dumb-bell" structure often used to depict λ cI repressor (Ptashne, 1992), the cleavage site could be fully exposed to solvent, lying in an extended completely flexible hinge region connecting the two structural domains. In this case LexA cleavage would result when the cleavage site transiently binds to the active site.

If the linker is indeed constrained by more or less stable contacts with the carboxy-terminal domain (e.g., with the active site), it should be possible to release these contacts by mutating the amino acids within the linker that are involved in these (potential) contacts. Attractive candidates for such a mutational approach are those substitutions in the linker region that confer a LexA(Ind⁻) phenotype (Lin & Little, 1988; Oertel-Buchheit et al., 1992), because cleavage deficiency may be due to an impaired docking of the hinge region (the "substrate") to the binding pocket of the active site.

Here we show that the hinge region of a LexA variant harboring several Ind⁻ mutations close to the intrinsic cleavage site (LexA-

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Fig. 1. A: This shows the LexA primary structure, the secondary structure elements of the amino-terminal domain (as determined by Fogh et al., 1994), and the alignment and the secondary structure elements of the UmuD' protein as determined by Peat et al. (1996). **B:** This summarizes the cleavage sites observed for the LexA-5PMH variant (R67, E74, R81, and E95) and for the LexA wild-type repressor (R67) as determined by protein sequencing.

5PMH, see Fig. 1B) gets indeed sensitive to cleavage by trypsin and by Glu-C endoprotease from *Staphylococcus aureus* V8, whereas the hinge region of wild-type LexA repressor is resistant to these proteases. These data strongly suggest that the LexA linker region of the wild-type LexA protein is not entirely flexible, but constrained either by interactions with the carboxy-terminal domain and/or a stable intrinsic local structure of the linker, which would be insensitive to protease cleavage.

Trypsin and Glu-C cleavage of LexA and LexA-5PMH: Figure 2A shows the trypsin cleavage pattern of the LexA-5PMH variant compared to the pattern observed for the LexA wild-type repressor. LexA-5PMH shows two stable trypsin cleavage products on a denaturing polyacrylamide gel, whereas the LexA wild-type repressor gives rise to a single cleavage product in agreement with earlier results (Little, 1984). Upon extended incubation the lower cleavage product of LexA-5PMH gets predominant.

To determine the cleavage sites, the digests were transferred to a PVDF membrane and the individual bands were subjected to protein sequencing. The more slowly migrating cleavage product of LexA-5PMH corresponds to cleavage behind Arg-67 (R67 in Fig. 2A), whereas the more rapidly migrating species corresponds to cleavage behind Arg-81 (R81). Surprisingly, the R81 cleavage product of LexA-5PMH migrates essentially as the unique cleavage product of the LexA wild-type repressor, which has been reported to correspond to cleavage after R67 (Little, 1984), an assignment that has been confirmed in this work by protein sequencing and mass spectrometry. The LexA3 repressor (harbour-

ing a G84D mutation, which is one of the mutations introduced into the LexA-5PMH variant) is known to exhibit a slower gel migration than the LexA wild-type protein (Little, 1983), and the same holds true for the intact LexA-5PMH repressor compared to the intact wild-type repressor (see Fig. 2B). These gel migration differences are apparently amplified for the proteolytic fragments R67 of LexA and LexA-5PMH.

To make sure, that these differences do not arise from different carboxy-terminal ends of the cleavage products, the tryptic fragments were purified on a C-18 HPLC column and subjected to mass spectrometry. Table 1 shows that all the fragments contain apparently an intact carboxy-terminal extremity (extending until the ultimate amino acid 202), because the experimental molecular weights correspond well with the predicted molecular weights. Indeed, in all cases these differences are far smaller than the average molecular weight of a single amino acid.

We may conclude from these experiments that the linker mutant repressor LexA-5PMH is readily cleaved at Arg-81 by trypsin, whereas the LexA wild-type repressor is only cleaved at Arg-67, but not at Arg-81. This finding suggests that the LexA linker is protected against protease attack due to a particular structure and/or due to contacts of the linker most likely with the carboxy-terminal domain, because the wild-type LexA cleavage product R67, lacking the amino-terminal domain, is not further cleaved at Arg-81. One might argue that LexA-5PMH is cleaved at Arg-81 because some of the surrounding amino acids are also changed, making Arg-81 simply a better substrate.

Figure 2B, however, shows similar results for Glu-C, an endoprotease from *Staphylococcus aureus* V8, which cleaves mainly

Fig. 2. **A:** This shows the time course of LexA and LexA-5PMH cleavage by TPCK-treated trypsin at 25 °C (pH 7.2). **B:** This shows the corresponding Glu-C cleavage pattern at the same temperature. The gel corresponds to a 14% SDS-PAGE gel of purified proteins stained with Coomassie blue R. Digestions were conducted in storage buffer (Schnarr et al., 1985) at a LexA and LexA-5PMH concentration of about 4 $\mu\text{g}/\mu\text{L}$, with a protease/substrate weight ratio of 1:50. The two proteases were purchased from Sigma. The marker proteins correspond to 30.0, 20.1, and 14.4 kDa. R67, R81, E74, and E95 correspond to LexA-5PMH cleavage products. The LexA R67 cleavage product (indicated by an arrow) co-migrates with R81 of LexA-5PMH, and the LexA-5PMH full-length protein migrates more slowly than the LexA wild-type repressor.

at the C-terminus of glutamic acid residues. The LexA wild-type repressor is essentially resistant to Glu-C cleavage at 25 °C, whereas LexA-5PMH gives rise to two relatively stable cleavage products. Protein sequencing showed that the upper band corresponds to cleavage behind Glu-74 (fragment E74) and the lower band to cleavage at Glu-95 (fragment E95). Mass spectrometry confirmed that these fragments extend until the carboxy-terminal extremity of the full-length protein (see Table 1). We may conclude from these experiments that the LexA linker is not only protected from cleavage in the immediate neighborhood of the intrinsic LexA cleavage site (i.e., between Ala-84 and Gly-85), but rather over a segment of about 20 amino acids surrounding the intrinsic cleavage site. The wild-type LexA linker is also resistant to Glu-C cleavage at 37 °C; however, at this temperature we observe cleavage at Glu-57 within the short β_1 -strand of the LexA DNA binding domain, suggesting that the β_1/β_2 sheet is destabilized under these conditions.

The LexA-5PMH mutant repressor was initially constructed as a control for the characterization of LexA-leucine zipper fusion proteins (Schmidt-Dörr et al., 1991; Oertel-Buchheit et al., 1993). Despite its relaxed inter-domain linker the LexA-5PMH variant remains an efficient repressor in vivo, suggesting that the linker

Table 1. Summary of the mass of the trypsin (R67, R81) and Glu-C (E74, E95) cleavage products, as well as of the full-length proteins LexA-5PMH and LexA as determined by matrix-assisted laser desorption mass spectrometry

Cleavage site	Fragment	Theoretical molecular mass (Da)	Experimental molecular mass (Da)
LexA-5PMH	1–202	22,565	22,571 ($z = 1$) ^a 22,572 ($z = 2$)
R67	68–202	15,187	15,203 ($z = 1$) 15,196 ($z = 2$)
R81	82–202	13,565	13,563 ($z = 1$) 13,578 ($z = 2$)
E74	75–202	14,316	14,320 ($z = 1$) 14,323 ($z = 2$)
E95	96–202	11,959	11,963 ($z = 1$) 11,964 ($z = 2$)
LexA	1–202	22,359	22,359 ($z = 1$) 22,363 ($z = 2$)
R67	68–202	14,980	14,986 ($z = 1$) 14,980 ($z = 2$)

^a $z = 1$ and $z = 2$ stand for single- and double-charged species, respectively.

mutations do not affect the correct folding of the two subdomains. Using the indicator strain JL1436 in the absence of IPTG (Lin & Little, 1989; Oertel-Buchheit et al., 1992; Porte et al., 1995) LexA-5PMH represses *lacZ* transcription about 45-fold, whereas the LexA wild-type repressor represses only 15-fold under these conditions. However LexA-5PMH is less efficient than LexA variants harboring a single Ind[−] substitution like GD85 (1,000-fold repression) or AT84 (400-fold repression). LexA(Ind[−]) mutant repressors are known to repress transcription more tightly, presumably because they accumulate to a higher intracellular concentration (Lin & Little, 1988).

However, LexA-5PMH turned out to form a fairly unstable repressor–operator complex in vitro, because the purified protein did not form a detectable discrete protein–DNA complex in electrophoretic mobility shift assays (Oertel-Buchheit et al., 1993). At least three different explanations could account for this reduced DNA binding activity of the LexA-5PMH variant: (1) direct contacts of the LexA linker with DNA. This explanation seems unlikely because the isolated LexA DNA binding domain shows the same pattern of DNA contacts as the full-length repressor (Hurstel et al., 1986, 1988). (2) Implication of the linker in protein dimerization. This explanation seems also unlikely, because the isolated carboxy-terminal domain has been shown to have the same dimerization and tetramerization behavior as the full-length repressor (Schnarr et al., 1988). Additionally the LexA-5PMH variant migrates as the LexA wild-type repressor on a HPLC gel filtration column. (3) Modification of the spatial relationship between the DNA binding and the dimerization domain. A more extended and flexible linker may reduce DNA binding energy due to a sub-optimal positioning of two DNA-bound LexA monomers preceding dimer formation, because at low concentration LexA dimer formation occurs on the DNA (Kim & Little, 1992). An extended linker would reduce the local concentration of the carboxy-terminal domain after DNA binding of two monomers via the amino-

terminal domain. This explanation would be consistent with the enhanced cleavage susceptibility of the LexA-SPMH linker region.

The finding that the LexA wild-type linker is resistant to cleavage at positions E74, R81, and E95 (see Fig. 1B), whereas the LexA-SPMH variant gets sensitive to cleavage at these sites, is a strong argument against an unstructured and completely flexible linker between the two domains of LexA. Two-domain proteins with unstructured linkers are, in fact, rapidly cleaved by proteases within the linker region as, for example, in the case of the Lac repressor-related proteins (Schnarr & Maurizot, 1981; Schumacher et al., 1994; Lewis et al., 1996) or in the case of the α -subunit of *E. coli* RNA polymerase (Blatter et al., 1994; Negishi et al., 1995; Jeon et al., 1997).

The fact that LexA is not cleaved at positions 74, 81, and 95 may be either due to a fairly stable local structure of this linker segment and/or due to docking of the linker to the carboxy-terminal domain (docking to the amino-terminal domain seems unlikely because the LexA_{68–202} fragment is not further cleaved at Arg-81). However, a solvent-exposed structured linker without contacts to the carboxy-terminal domain would also remain fairly accessible to proteases and could serve as a substrate in the case of transient unfolding. This is apparently the case for calmodulin and troponin C, two related "dumbbell" structures, where the two domains are linked by a long central helix, which is readily cleaved by trypsin (see Strynadka & James, 1989, and references therein).

Several other experiments support the idea of a physical interaction between the linker region and the carboxy-terminal domain: (1) the carboxy-terminal fragment LexA_{85–202} is more susceptible to trypsin cleavage than the fragment LexA_{68–202}, which contains the linker region (Roland & Little, 1990). (2) The carboxy-terminal fragment LexA_{85–202} is less stable against denaturation by guanidine hydrochloride if isolated than in the context of the entire protein (Hurstel, 1989). (3) LexA is unable to accommodate one or two base pair insertions in the center of the operator, whereas a LexA_{1–87}-Jun zipper fusion protein readily does, despite the fact that this fusion protein contains only roughly half of the LexA linker (Oertel-Buchheit et al., 1993).

Together, these results suggest that the stability of the LexA linker against proteolysis is at least in part due to a physical contact of the linker with the carboxy-terminal domain of the protein. However, these results do not imply that these contacts would be necessarily formed with the binding pocket of the active site. Formation of the self-cleavage competent conformation LexA* (Little, 1993) could be restricted not only to a subtle realignment of the cleavage site within the binding pocket (Shepley & Little, 1996), but could also correspond to the disruption of a contact of the linker with another segment of the carboxy-terminal domain and a subsequent translocation into the binding pocket of the active site. Further biochemical and structural studies will be necessary to address this issue.

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