Nucleic Acids Research, Vol. 20, No. 19 ***4971–4974***

Disulfide bonds are required for *Serratia marcescens* nuclease activity

Timothy K.Ball[+], Yousin Suh and Michael J.Benedik*
Department of Biochemical and Biophysical Sciences, University of Houston, Houston, TX 77204-5934, USA

Received August 12, 1992; Accepted September 9, 1992

ABSTRACT

The role of the two disulfide bonds found in the *Serratia marcescens* nuclease were tested by site directed mutagenesis and were found essential for nuclease activity, although slight residual activity remained. The requirement for disulfide bond formation may play a role in preventing the lethal action of nuclease while in the bacterial cytoplasm.

INTRODUCTION

Many extracellular proteins whose activity might be toxic to the cell are co-expressed with a specific inhibitor such as colicins (13), the extracellular proteases of *Serratia marcescens* and *Erwinia caratovorocin* (11,15), the *S. marcescens* extracellular phospholipase (M. Givskov and S. Molin, personal communication) and the periplasmic ribonuclease of *Escherichia coli* (14,19). However some potentially lethal proteins such as the extracellular nuclease of *S. marcescens* do not have known inhibitor proteins. This nuclease is an extremely active enzyme which non-specifically degrades RNA and DNA yet it can be over-expressed in *E.coli* without lethality (2). In *E.coli* the enzyme is not efficiently released to the medium but is primarily localized to the periplasm. However both *E.coli* and *S. marcescens* strains overexpressing nuclease are slightly SOS induced (2,3) suggesting that there is some nuclease activity in the cytoplasm of those cells. Nevertheless the vast majority of nuclease protein must exit the cytoplasm before becoming active or must somehow be sequestered without reaching intracellular DNA and RNA.

Many bacteria which secrete proteins include a protease among the extracellular proteins, creating an additional problem for the other extracellular proteins. This suggests that for a protein to survive in the extracellular milieu it must be unusually stable both from a perspective of protease stability and also insult by other environmental factors. *S. marcescens* for example secretes at least 2 proteases (12,18) along with its other extracellular proteins.

Disulfide bonds play an important role in the folding and stability of many exported proteins including some extracellular proteins. But their role in specifying the proper folding pathway may be equally important by ensuring proper folding occurs only in the correct milieu. Disulfide bonds are not formed efficiently in the reducing environment of the bacterial cytoplasm but can form in the periplasm where the folding and oligomeric assembly of exported proteins takes place. In vitro, disulfide bonds are often formed inefficiently. There are proteins which serve to catalyze disulfide bond formation and one of these, DsbA, is found in the periplasm of *E.coli* (4). Therefore many proteins whose folding depends upon proper disulfide bond formation are expected to be inactive in the bacterial cytoplasm. This may provide one regulatory mechanism, in addition to efficient export, to ensure that these proteins are not active in the bacterial cytoplasm. Alkaline phosphatase is one well characterized example of an enzyme which is only active in the periplasm due to the requirement for disulfide bond formation (7).

There are four cysteine residues found in the nuclease of *S. marcescens* which form two disulfide bonds, on between C9 and C13 and a second between C201 and C243 (5). Agents which disrupt disulfide bonds such as β-mercaptoethanol cause partial inactivation of nuclease at high concentrations (600mM) (9).

It is quite reasonable to hypothesize that nuclease only becomes active after secretion to the periplasm or extracellularly as a survival mechanism to ensure that active nuclease is not found in the cytoplasm and that disulfide bond formation plays a role. In this work we use a genetic approach and mutate these cysteine residues to ask whether these disulfides are crucial to nuclease stability and activity.

MATERIALS AND METHODS

Strains and plasmids

The plasmid pNuc5 contains the nuclease gene (Genbank M19495) on a 900 nt fragment cloned into the vector pUC18, extending from the *Rsa*I site at position 1 to the Δ6 deletion immediately downstream of the nuclease coding region (1,2). The nuclease gene is oriented such that it is expressed from the *lac* promoter of the plasmid; there is little expression from the nuclease promoter (6). The plasmids were normally propagated in *E.coli* strain JM101 (17) or in *S. marcescens* strain TT392

* To whom correspondence should be addressed

[+] Present address: Monsanto Co., 700 Chesterfield Village Parkway, St Louis, MO 63198, USA

(F'*pro-lac*) which carries a mutation in the chromosomal nuclease gene (16). Bacteria were routinely grown in LB media. Ampicillin was added to all media at 100 μg/ml for plasmid selection.

The mutations were created by site directed mutagenesis using the procedure of Kunkel (10). Multiple mutants were constructed by multiple rounds of mutagenesis or by combining appropriate restriction fragments from mutants. All final plasmids were verified by DNA sequencing.

Nuclease assays

Nuclease indicator plates (DNase Test Agar, Gibco, supplemented with 80 μg/ml methyl green) provide a simple and very sensitive qualitative assay. The relative halo size is an accurate measure of extracellular enzyme activity, released either by secretion or cell lysis. Fresh colonies were always gridded onto the same plate to accurately measure relative halo sizes.

For more quantitative determination of nuclease activity cultures were grown in LB with ampicillin to a density of about A_{600}= 0.5 when IPTG was added to 10^{-3} M for an additional 2 hours. Cells were harvested by centrifugation and the supernatant collected. The periplasmic fraction was selectively released by osmotic shock and the cellular fraction released by sonication in the presence of 0.1% Triton X-100 (1). Although nuclease is normally an extracellular protein in *S. marcescens*, it remains periplasmic in *E.coli* under these conditions. Nuclease activity was measured using the microtiter dish assay described previously (3). This assay is based on the dilution factor required to show no loss of fluorescence caused by DNA degradation in an ethidium bromide and DNA solution.

For western blotting protein samples were fractionated by electrophoresis on a discontinuous 12% SDS-polyacrylamide gel. The gels were blotted to Immobilon P membranes (Millipore), probed with rabbit polyclonal anti-nuclease serum and developed with alkaline phosphatase conjugated goat anti-rabbit second antibody (1).

RESULTS

Mutagenesis of cysteine residues in nuclease

Each cysteine codon (2), diagrammed in Figure 1, was mutagenized individually by oligonucleotide site directed mutagenesis (10) and changed from TGC to an AGC serine codon. The mutations were confirmed by DNA sequencing and the remainder of the nuclease gene was either sequenced or replaced by subcloning to ensure that no secondary mutations were present after mutagenesis. Double mutants were constructed by consecutive rounds of mutagenesis to create *nuc*C9S,C13S and *nuc*C201S,C243S. The quadruple mutant was created by subcloning each appropriate half of the double mutants to create *nuc*C9S,C13S,C201S,C243S.

Behavior of cysteine mutants

The single cysteine mutants were all deficient in expression of nuclease activity as determined from DNase Test Agar plates. No nuclease activity could be observed from the C13S, and C201S single mutants (Table 1) however C9S and C243S did have very low but detectable levels of nuclease activity. On indicator plates a small halo could be seen after 24 hours with C9S, but not with C243S, however by 36 hours C243S had a visible halo which had nearly caught up in size with C9S. It seems that active nuclease appeared extracellularly in this mutant only after prolonged incubation in this mutant, possibly due to cell lysis. However the level of nuclease activity of both mutants, even after 48−60 hours, was dramatically less than that observed from wild type nuclease.

Nuclease activities from the above mutants were measured from cellular fractions using the microtiter dish and the results, shown in Figure 2, are similar to that observed from the indicator plates. After incubation of the assay mix for about 16 hours (normally the reactions go for 10 minutes) a very low level of nuclease activity can be observed. There was more nuclease found in the C9S mutant than in the others. In all cases activity of the mutant nuclease is found predominantly in the cellular fraction, which represents both cytoplasmic contents and the membrane fraction, whereas the wild type nuclease was periplasmic. Probably the mutant nuclease is membrane bound, but we have no direct evidence for this. Note that only the wild type control was diluted 1000-fold for these assays, which is about the difference in activity between wild type and mutant nucleases ($10^{-3}-10^{-4}$).

The cysteine mutants are obviously very defective for nuclease activity but was this defect due to loss of a disulfide or to the presence of the remaining free sulfhydryl. To test this the respective double mutant pairs were constructed so that no free sulfhydryls remain. For both double mutants there was no nuclease observed in either assay system (Table 1). The quadruple mutant was also generated where all four cysteines were altered. This mutant was also inactive.

Is stability of mutant nuclease altered?

Was the loss of nuclease activity in these mutants due to the creation of an inactive enzyme or due to a change in the stability of nuclease such that it was rapidly turned over in the cell? Proteins samples from the periplasmic contents (Figure 3) were separated by SDS-polyacrylamide gel electrophoresis and visualized by western blotting from all the single mutants. As can be seen there is a weaker nuclease signal from the mutant

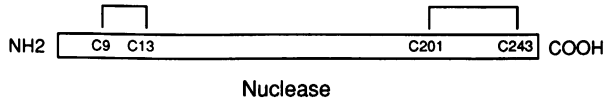

Figure 1. Representation of the *S. marcescens* nuclease showing the location of the cysteine residues and the disulfide bonds.

Table 1. Halo size of nuclease alleles.

Plasmid	Halo size JM101	Halo size TT392
pUC18Nuc5	++++	++++
pUC18Nuc5C9S	+	+
pUC18Nuc5C13S	−	−
pUC18Nuc5C201S	−	−
pUC18Nuc5C243S	+	+
pUC18Nuc5C9S,C13S	−	−
pUC18Nuc5C201S,C243S	−	−
pUC18Nuc5C9S,C13S,C201S,C243S	−	−
pUC18Nuc5C246+	+++	+++
pUC18	−	−

Halo sizes were compared by picking freshly grown colonies on DNase Test Agar plates with 100 μg/ml ampicillin and 10^{-3} M IPTG and incubating for 48 hours. The ++++ designates a large and +++ a medium halo which was visible in less than 8 hours of incubation, + designates a small halo visible after prolonged incubation, a−designates no halo was visible after prolonged incubation.

samples relative to the wild type, C9S has the strongest signal among the mutants. However a nuclease band was visible in all cases. Therefore, although nuclease is rendered less stable due to loss of a disulfide bond, this can not completely account for the absence of activity. Nuclease protein must itself be essentially inactive in these mutants. Western blots of the double and quadruple mutants (not shown) show that the double mutants have similar levels of protein as the single mutants but the quadruple mutant has noticeably less nuclease. This is not surprising in that the double mutants still have lost only a single disulfide bond but the quadruple mutant has lost both, presumably leading to greater instability and protease susceptibility.

Plasmids carrying the cysteine mutants were also introduced into *S. marcescens* strain TT392 F'*prolac* which is a nuclease deficient mutant of *S. marcescens* strain Sr41 (16). The mutants behaved similarly in this strain as compared to *E.coli* strain JM101 (Table 1) on indicator plates and western blot analysis (not shown).

Can a free sulfhydryl poison nuclease activity?

We have shown that loss of a disulfide inactivates nuclease presumably because of its role in stability and folding of the active protein. This defect is not suppressed by removal of the free sulfhydryl in the double mutants. Nevertheless the presence of a free sulfhydryl may still act to poison the protein and render it inactive. To test this another mutant was isolated by site directed mutagenesis which converted the translational stop codon to a cysteine (TGA → TGT) to generate Nuc5C246+. Additional amino acids after the cysteine are encoded by the plasmid polylinker such that the following sequence is appended to normal nuclease C-R-P-A-G-G-E-L-V-G-M-E-D-L. This construct is slightly reduced for nuclease activity on DNase Test Agar, but has much greater activity than the cys mutants. Therefore a free sulfhydryl does not have nearly as significant a deleterious effect on nuclease activity as does loss of a cysteine.

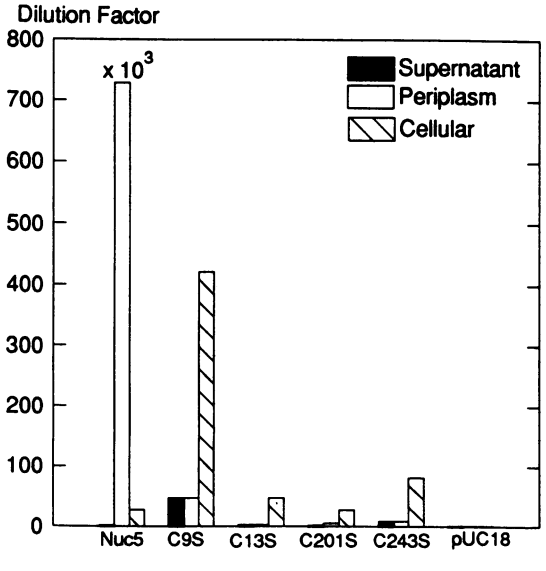

Figure 2. Activity of nuclease alleles. Cellular fractions from JM101 expressing nuclease plasmids were assayed by the microtiter dish assay and results are displayed as the dilution factor required to show no loss of fluorescence. The assays were incubated for 16 hours. The wild type control, pNuc5, was diluted 1000-fold before applying to assay plate.

DISCUSSION

The presence of two disulfide bonds is a clear requirement for activity of the *S. marcescens* nuclease. The enzyme is quite inactive in three of the single cysteine mutants and has less than 10^{-3} of wild type activity in the mutant C9S. One role for the disulfides is to increase stability of the protein. All the mutants are significantly less stable than the parent, based on accumulation of the protein in the cell. However the reduced level of nuclease protein accumulated in cells expressing the mutant nuclease alleles is not sufficient to explain the loss of nuclease activity. The most active mutant, C9S is reduced by more than 1000-fold in terms of nuclease activity accumulated in the cell whereas the level of protein accumulated is reduced perhaps 5−10 fold.

This theme of disulfides increasing the stability of proteins is not new for nuclease and is true for many other proteins (7,8). It certainly is not surprising to find that disulfides are important in this example where nuclease is an extracellularly secreted protein of a bacterium which also secretes abundant extracellular proteases. However it is somewhat surprising to find that mutants of each cysteine which form a disulfide bond do not have identical phenotypes. The C9S mutant for example is more active than C13S yet these two are partners in a disulfide bond. Possibly the remaining cysteine residue in these mutant proteins have differing effects on folding or interfere with formation of the remaining disulfide bond to differing extents.

In addition to stability the disulfides clearly play a second role, presumably in promoting the correct folding of nuclease to allow full activity, but only after it has reached an extracytoplasmic environment. This becomes a *de facto* regulatory role to ensure that active nuclease is not found in the cytoplasm. The *S. marcescens* nuclease is extremely active and would almost certainly be lethal were much of it found within the cytosol. By rapidly secreting nuclease out of the cytoplasm and by ensuring that any nuclease which is in the cytoplasm is not active, the cell is able to remain viable. The normal cellular secretion systems appear capable of dealing with the level of nuclease normally expressed in *S. marcescens* (6).

Alkaline phosphatase is similar to nuclease in that disulfides are required for activity (7), again perhaps playing an important regulatory role. Other potentially lethal proteins use a variety of mechanisms to ensure their inactivity in the cytoplasm. Some proteins are co-expressed with a specific inhibitor protein, such as the colicins (13). The periplasmic RNase I similarly has a

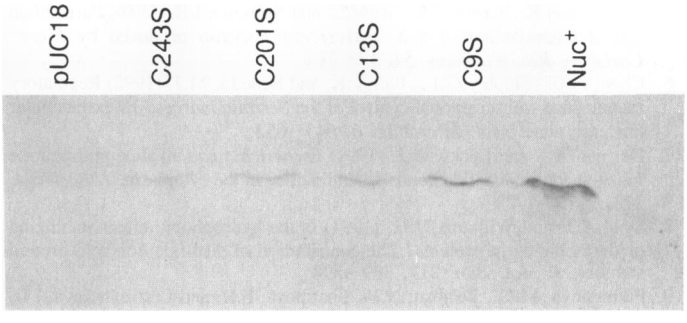

Figure 3. Western blot of nuclease protein produced by cys mutants. Lanes 1−5 are periplasmic fraction from pUC18, C243S, C201S, C13S, C9S and Nuc$^+$ respectively prepared by osmotic shock of IPTG induced JM101 carrying the respective plasmids.

specific inhibitor to maintain inactivity in the cytoplasm (14,19). Other proteins, notably proteases, require precise activation steps to convert the preprotein to active protease. In some cases this is dependent on the secretion step itself (15).

Nevertheless, despite mechanisms to ensure no nuclease is active in the cytoplasm, some residual nuclease activity does occur in the cytoplasm under conditions of overexpression. Both in *E.coli* and in *S. marcescens* nuclease overexpression leads to a partial derepression of SOS regulated genes, implying that some DNA damage is occurring (2,3). This suggests that nuclease is able to become active in the cytoplasm, although inefficiently. Disulfides may be able to form slowly in the cytoplasmic environment; alternatively the non-mutant nuclease protein has a low level activity in the absence of disulfide bonds. Our mutants suggest a very low specific activity in the absence of one or more disulfides. How many units of nuclease are required to induce the SOS system to this level is not known. We should point out an alternative but unlikely explanation; that secreted nuclease which is fully active is able to re-enter the cell and cause the observed SOS induction. This might be similar to but less efficient than import of proteins into the cell, such as the import of colicins. Adding nuclease exogenously to cells does not cause SOS induction, however this may not be the same as having nuclease in the periplasm. But under conditions of normal nuclease expression in *S. marcescens*, no SOS induction was observed so this low residual cytoplasmic activity does not present any problem to the bacterium under normal conditions.

ACKNOWLEDGMENTS

This work was supported by grant GM36891 from the National Institutes of Health and awards 3652-117 and 3652-233 through the Texas Advanced Research Program. Some equipment used in this work were purchased through equipment grant DIR-9109294 from the National Science Foundation.

REFERENCES

1. Ball,T.K. (1990) Regulation of expression and excretion of a *Serratia marcescens* nuclease. Ph.D Dissertation. Texas A & M University.
2. Ball,T.K., Saurugger,P.N. and Benedik,M.J. (1987) The extracellular nuclease gene of *Serratia marcescens* and its secretion from *Escherichia coli*. *Gene* **57**, 183–192.
3. Ball,T.K., Wasmuth,C.R., Braunagel,S.C. and Benedik,M.J. (1990) Expression of *Serratia marcescens* extracellular proteins requires *recA*. *J. Bacteriol.* **172**, 342–349.
4. Bardwell,J.C.A, McGovern,K. and Beckwith,J. (1991) Identification of a protein required for disulfide bond formation in vivo. *Cell* **67**, 581–589.
5. Biedermann,K., Jepsen,P.K., Riise,E. and Svendsen,I.B. (1989) Purification and characterization of a *S. marcescens* nuclease produced by *E.coli*. *Carlsberg Res. Commun.* **54**, 17–27.
6. Chen,Y.-C., Shipley,G.L., Ball,T.K. and Benedik,M.J. (1992) Regulatory mutants and transcriptional control of the *Serratia marcescens* extracellular nuclease gene. *Mol. Microbiol.* **6**, 643–652.
7. Derman,A.I. and Beckwith,J. (1991) *Escherichia coli* alkaline phosphatase fails to acquire disulfide bonds when retained in the cytoplasm. *J. Bacteriol.* **173**, 7719–7722.
8. Doig,A.J. and Williams,D.H. (1991) Is the hydrophobic effect stabilizing or destabilizing in proteins? The contribution of disulfide bonds to protein stability. *J. Mol. Biol.* **217**, 389–398.
9. Filimonova, M.N., Balaban, N.P., Sharipova, F.R. and Leshchinskaya, I.B. (1980) Isolation and physico-chemical properties of homogenous nuclease from *Serratia marcescens*. Biokhimiya 45:2096–2103.
10. Kunkel,T., Roberts,J. and Zakour,R. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**, 367–382.
11. Létoffé,S., Delepelaire,P. and Wandersman,C. (1989) Characterization of a protein inhibitor of extracellular proteases produced by *Erwinia chrysanthemi*. *Mol. Microbiol.* **3**, 79–86.
12. Nakahama,K., Yoshimura,K., Marumoto,R., Kikuchi,M., Lee,I.S., Hase,T. and Matsubara,H. (1986) Cloning and sequencing of *Serratia* protease gene. *Nucleic Acids Res.* **14**, 5843–5855.
13. Pugsley,A.P. (1984) The ins and outs of colicins. Part II. Lethal action, immunity and ecological implications. *Microbiol. Sci.* **1**, 203–205.
14. Spahr,P.F. and Hollingworth,B.R. (1961) Purification and mechanism of action of ribonuclease from *Escherichia coli* ribosomes. *J. Biol. Chem.* **236**, 823–831.
15. Suh,Y. and Benedik,M.J. (1992) Production of active *Serratia marcescens* metalloprotease from *E.coli* by α-hemolysin HlyB and HlyD. *J. Bacteriol.* **174**, 2361–2366.
16. Takagi,T. and Kisumi,M. (1985) Isolation of a versatile *Serratia marcescens* mutant as a host and molecular cloning of the aspartase gene. *J. Bacteriol.* **161**, 1–6.
17. Vieira,J. and Messing,J. (1982) The pUC plasmids, an M13mp7 derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259–268.
18. Yanagida,N., Uozumi,T. and Beppu,T. (1986) Specific excretion of *Serratia marcescens* protease through the outer membrane of *Escherichia coli*. *J. Bacteriol.* **166**, 937–944.
19. Zhu,L.Q., Gangopadhyay,T., Padmanabha,K.P. and Deutscher,M.P. (1990) *Escherichia coli rna* gene encoding RNase I: cloning, overexpression, subcellular distribution of the enzyme, and use of an rna deletion to identify additional RNases. *J. Bacteriol.* **172**, 3146–3151.