OSTI 6/25/96

FINAL TECHNICAL REPORT DOE/ER/20091--T1

PROJECT TITLE: The effect of Oligosaccharides on Glycoprotein Stability
Grant No. DE-FG03-92ER20091

PROJECT PERIOD: 7/15/92 - 7/14/95

PROJECT DIRECTOR: Charles F. Goochee, Dept. of Chemical Engineering,
Stanford University (7/15/92 - 7/14/95)
Chaitan Khosla, Dept. of Chemical Engineering,
Stanford University (7/15/94 - 7/15/95)

I. Summary of Project Tasks

This project was initiated by Charles Goochee with the goal of exploring the effects of glycosylation on protein stability. In June 1994 Dr. Goochee left Stanford to take up a position at Chiron Corporation, CA. Since this grant was the sole source of support for Peter Kramer, a fourth year PhD student working on the above problem, supervision of this project was taken over by Chaitan Khosla, whose own research activities are focused in the area of biocatalysis and protein engineering.

The original proposal (and subsequent updates) included the following goals:

1) To perform site-directed mutagenesis on the gene for the bacterial protein Staphylococcal nuclease (SNase), creating the glycosylation recognition sequence Asn-X-Ser or Asn-X-Thr at selected locations in the protein - locations that would be on the surface of the protein in the native folded state.

2) To express these SNase constructs in a lower eucaryotic expression system capable of glycosylating and secreting SNase.

3) To optimize the fermentation conditions to produce the milligram quantities of glycosylated SNase that are necessary for future studies.

4) To develop a protocol for purification of milligram quantities of glycosylated SNase.

5) To test for the presence and size of oligosaccharide at each Asn-X-Ser/Thr site.

6) To use the glycosylated SNase for a series of studies concerning the effect of oligosaccharides on glycoprotein stability.

The proposed timetable for this project was to complete goals 1-5 in years one and two, producing milligram quantities of several different glycosylated SNase species for detailed analysis in year 3.

II. Summary of Progress Prior to 7/15/94

In the first two years of the project, five mutant SNase's were constructed, expressed in *Saccharomyces cerevisiae*, and purified to homogeneity. Three of these were single glycosylation site mutants (T2N, K70S, and A145N), one contained two glycosylation sites (T2N, A145N), and one contained all three glycosylation sites. Based on Endoglycosidase H treatment analysis of the recombinant proteins, it was observed that only one of these sites (N68) appeared to be glycosylated. It was therefore proposed by Dr. Goochee that work in the final year of the project would focus on an analysis of this

glycosylated mutant, while at the same time attempting to generate four new glycosylation sites via mutagenesis (at N126, N131, N57, and N60). Dr. Goochee had projected that a publication describing the results of tasks 1-5 above would be submitted for publication in the summer of 1994.

III. Summary of Progress between 7/15/94 and 7/15/95

Shortly after Dr. Khosla took over supervision of the project, a series of detailed experiments were conducted to confirm the observation reported above, namely that Asn 68 was glycosylated in the K70S mutant of SNase. These experiments included using RNase B as a positive control for a glycosylated enzyme of similar molecular weight, using Endo H preparations from different sources, and monitoring the time-course of hydrolysis of the glycosyl groups on RNase B and K70S SNase using SDS PAGE gel-shift assays. From these results it was concluded that the original conclusion that the mutant SNase was glycosylated was erroneous. The reason for the artifactual 3 kDa smaller band that appeared upon treatment of the mutant protein with Endo H was due to the existence of low activity of a protease that specifically cleaved this mutant.

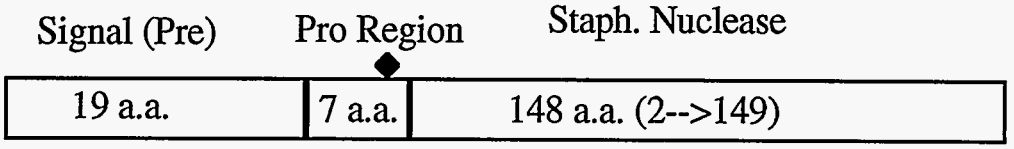
In conjunction with these studies, four additional glycosylation site mutants were constructed (at N126, N131, N57, and N60), as proposed. Analysis of these mutant proteins revealed that none of them were glycosylated.

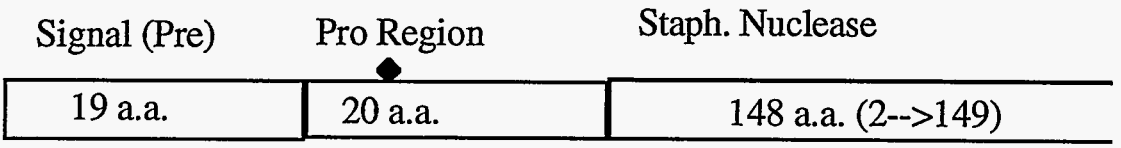
The inability to glycosylate any of the nine mutants generated in thus far was indeed surprising, for reasons discussed in earlier reports submitted by Dr. Goochee. It was argued that failure to do so could be due to three possible reasons:
1) Local conformation of the glycosylation site is important. (A series of reports from the Imperiali lab at Caltech that appeared in press around this time indicated that this was indeed the case.)
2) Gavel et al (1990) had signficantly underpredicted site occupancy (75%) based on statistical analysis of proteins that had been studied thus far.
3) The rules for the glycosylation of heterologous proteins are different from those of homologous proteins.

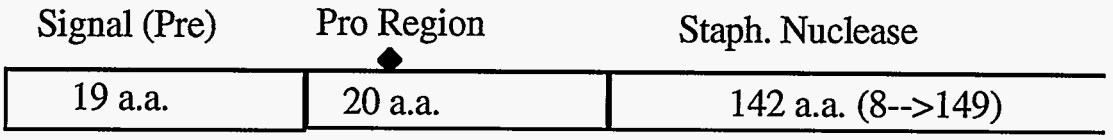
To address these issues, a new strategy was conceived which relied on the construction and analysis of fusion proteins. Specifically, it was known that two secreted yeast proteins, alpha-factor and invertase, have naturally occuring glycosylation sites at their N terminal ends. Furthermore, by constructing and analyzing fusion proteins, Nilsson et al (1993) had shown that only 14-16 wild-type amino acids were required C-terminal to the glycosylation site for these sites to be glycosylated. The following five fusion proteins were designed, constructed, purified, and analyzed for the presence of attached glycosyl moieties.

Fusion Protein Strategy

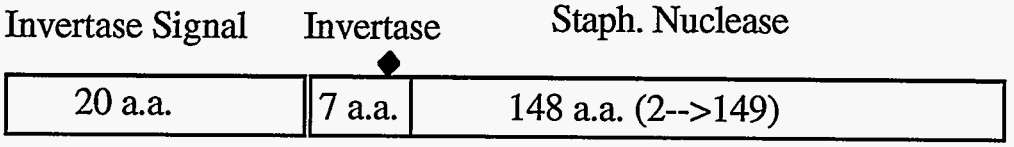
Alpha Factor Fusions

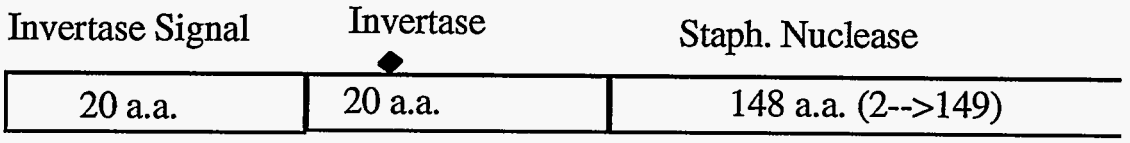






Invertase Fusions





Unfortunately, yet again, all of them turned out to be unglycosylated, even though they all had near wild-type SNase activity. Thus, after the construction, purification, and analysis of 14 mutant proteins, it was eventually concluded that the task of engineering new glycosylation sites into unglycosylated proteins is indeed very difficult, and may require a qualitatively new approach.

Given the advanced stage of Peter Kramer's tenure as a graduate student, a decision was made during the course of these studies to permit Peter Kramer to initiate a second project along-side the above studies. (This decision was made in consultation with Peter Kramer, his thesis committee comprising of Drs. Curtis Frank (Chem. Eng.) and Robert Baldwin (Biochem.), and the Chair of the Chem. Eng. dept.) It was suggested that Peter use his expertise in protein engineering to construct and analyze mutant polyketide synthases. These multifunctional enzymes, which catalyze the biosynthesis of numerous natural products called polyketides, have been subjects of intensive investigations in the

Khosla laboratory for the past four years (Fu et al., (1994) *J. Am. Chem. Soc. 116*, 4166-4170; Fu et al, (1994) *Biochemistry 33*, 9321-9326; Fu et al., (1994) *J. Am. Chem. Soc. 116*, 6443-6444; Fu et al., (1994) *Chem. & Biol. 1*, 205-210; Kao et al., (1994) *Science 265*, 509-512; Kao et al., (1994) *J. Am. Chem. Soc. 116*, 11612-11613; Kao et al., (1995) *J. Am. Chem. Soc. 117*, 9105-9106; McDaniel et al., (1993) *Science 262*, 1546-1550; McDaniel et al., (1993) *J. Am. Chem. Soc. 115*, 11671-11675; McDaniel et al., (1994) *Proc. Natl. Acad. Sci. USA 91*, 11542-11546; McDaniel et al., (1994) *J. Am. Chem. Soc. 116*, 10855-10859; McDaniel et al., (1995) *Nature 375*, 549-554; McDaniel et al., (1995) *J. Am. Chem. Soc. 117*, 6805-6810; Pieper et al., (1995) *Nature* in press; Pieper et al., (1995) *J. Am. Chem. Soc.* in press; Tsoi & Khosla, (1995) *Chem. & Biol. 2*, 355-362). Over the past six months, Peter Kramer's studies on this subject have led to several important findings, which have formed the basis for the preparation of his PhD thesis. This aspect of his studies was supported by a grant from the National Science Foundation to Chaitan Khosla. It is expected that Peter Kramer will defend his thesis in November this year.