Functional Molecular Mass of *Escherichia coli* K92 Polysialyltransferase As Determined by Radiation Target Analysis

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ABSTRACT: The polysialyltransferase of *Escherichia coli* K92 catalyzes the transfer of sialic acid from CMP-sialic acid to a growing chain of polysialic acid at the nonreducing end. The enzyme encoded by the *neuS* gene is membrane-associated and has been suggested to be organized within a complex of several proteins encoded by the K92 gene cluster. Attempts to prepare a soluble active NeuS enzyme have been unsuccessful. Recent results suggest that de novo synthesis of polysialic acid requires coexpression of four genes from the cluster: *neuS*, *neuE*, *kpsC*, and *kpsS*. However, elongation of preexisting polysialic acid chains only requires expression of *neuS*. The molecular organization of the catalytic unit of bacterial polysialyltransferases has not been described. We used radiation inactivation to measure the size of the minimum functional unit catalyzing the polysialyltransferase chain extension and *de novo* reactions. Membranes harboring NeuS in the presence and absence of other products of the K92 gene cluster were exposed to high-energy electrons. The rate of loss of polysialyltransferase activity reveals the mass of the molecules essential for catalytic activity. We observed that the transfer of neuNAc from CMP-neuNAc to a polysialic acid acceptor is catalyzed by a complex with a target size larger than that of monomeric NeuS. The target size of the unit catalyzing the extension of existing polysialic acid chains does not differ significantly from the size of the unit catalyzing transfer of sialic acid to the endogenous acceptor. Parallel samples of membranes containing NeuS and a green fluorescent protein (GFP) chimera were compared by target analysis. The target size of this structural unit was estimated by analysis of the rate of decay of the GFP-NeuS chimera band migrating in the immunoblots. The target size of the structural unit is larger than expected for a monomer. The results of these experiments show that while the target size of the catalytic activity for K92 polysialyltransferase is larger than a monomer of NeuS, a large complex is not required for catalysis.

Capsular polysaccharides are essential virulence factors in invasive diseases caused by many pathogenic bacteria. The structures of these polysaccharides are diverse and include but are not limited to such polymers as hyaluronic acid of streptococci, ribose ribitol phosphate of *Haemophilus*, and the polysialic acids (PSA). *Escherichia coli* K1 and K92 and certain serotypes of *Neisseria meningitidis* are encapsulated with PSA. Although gene clusters encoding production of several capsular polysaccharides have been identified (*1*), many details of the biochemical mechanism of capsular polysaccharide synthesis in these pathogenic bacteria have not been elucidated. The biosynthesis of PSA in Gram-negative bacteria is thought to take place on the inner surface of the cytoplasmic membrane through the addition of Neu5Ac residues from the donor, CMP-Neu5Ac, to the nonreducing ends of nascent (acceptor) PSA chains (*2*). The functions of most of the proteins required for biosynthesis (encoded by the *neu* genes) have been identified, including those needed for Neu5Ac synthesis, activation, and polymerization. Polymerization consists of at least two reactions:

$$\text{CMP-neuNAc} + \text{acceptor} \rightarrow \text{neuNAc-acceptor} + \text{CMP}$$

$$n\text{CMP-neuNAc} + \text{neuNAc-acceptor} \rightarrow (\text{neuNAc})_n\text{-neuNAc-acceptor} + n\text{CMP}$$

The polysialyltransferase catalyzing these reactions is encoded by *neuS* (*3*, *4*). This enzyme can transfer sialic acid to the nonreducing end of oligosaccharides, yet by itself, it is unable to initiate chain synthesis without exogenous PSA (*4*, *5*). We have recently shown that NeuS can catalyze *de novo* synthesis of PSA from CMP-sialic acid when it is coexpressed with NeuE, KpsC, and KpsS (*6*).

The membrane-bound polysialyltransferase is suggested to be organized as a catalytic complex involving several proteins encoded by the K92 gene cluster (*7*). Our biochemical data limit the number of different proteins that would be directly involved in the transfer of sialic acid to an acceptor. The eligible proteins are NeuE, NeuS, KpsS, and KpsC whose polypeptides have molecular masses of 45, 48, 44, and 75 kDa, respectively (*6*). Thus, a minimum size of a

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Table 1: Bacterial Strains and Plasmids Used in This Study

	relevant genotype or description	source or ref
strain		
DH5α		Invitrogen
EV239	*-nanA4, -neuB25, -neuS*:Tn10	*3*
plasmid		
pWN603	K92 *neuS* in pENTR	this study
pWV213	K92 *neuS*	*5*
pWV218	K92 *neuS*-GFP chimera	this study
pWV230	K92 *neuS*-biotinylated peptide chimera	this study

catalytic complex containing each of these proteins is 212 kDa. We used radiation target analysis to determine the size of the catalytic complex. This technique works well for unfractionated membrane fragments since the measurements rely only on the ability to assess a catalytic reaction (*8*). Membrane fragments are irradiated at low temperatures with varying doses of radiation and then assayed for residual transferase activity. We used this technique to determine the target size of polysialyltransferase in the presence and absence of other capsule gene products encoded by the gene cluster. Since we are using catalytic activity as our assay, the target size that is shown represents the maximum mass of the unit that is essential for catalytic function.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* K1 hybrid strain EV239 (*neuS*::Tn10 *nanA4*, *neuB25*) was obtained from E. Vimr (University of Illinois, Urbana, IL). The pWV213 plasmid was described previously (*5*). The pWV218 plasmid was constructed by using primers *pK92for* (ATG ATA TTT GAT GCT AGT TTA AAG) and *pK92rev* (CTC CCC CAA GAA AAT CCT TTT) with chromosomal K92 DNA as a template. The resulting fragment containing the complete *neuS* gene was ligated into the pTrc-HisII-TOPO vector (Invitrogen). The fragment encoding green fluorescent protein from the pEGFP-C1 plasmid (Clontech) (*9*) was cloned into the EcoRI site to produce a *neuS*-GFP chimera.

Plasmid pWV230 expresses a chimera of NeuS and a biotinylated amino-terminal peptide. This plasmid was constructed as follows. K92 neuS was amplified using primers *Fr wfv neuS* and *Rv wfv neuS* (*6*) and plasmid pGB20 as a template (*10*). The DNA fragment containing K92 neuS was directionally TOPO-cloned into the Gateway pENTR/D vector (Invitrogen). The resulting plasmid was designated pWN603.

The K92 *neuS* gene in pWN603 was transferred to the pET104 biotin expression plasmid (Invitrogen) by in vitro recombination using the Invitrogen Gateway system. The resulting plasmid was designated pWV230.

Preparation and Irradiation of Membranes. Bacterial cultures of DH5α:pWV218, DH5α:pWV213, and EV239:pWV213 in some experiments were grown on 1.5 L of LB medium. However, because LB is rich in sialic acid, in other experiments bacterial cultures were grown on minimal medium, which does not contain sialic acid, to eliminate interference in our assays by low levels of this sugar. Minimal medium consists of a solution of M9 salts (Sigma Chemical Co., catalog no. M-6030) to which is added 2 g per liter of casamino acids (Difco) prior to autoclaving. The medium is completed by the addition of 1 mL of 1 M $MgSO_4$, 0.1 mL of 1 M $CaCl_2$, 10 mL of glycerol, and 10 mL of low-molecular weight yeast extract dialysate per liter (*11*). Both LB and minimal media were supplemented with appropriate antibiotics. The cultures were grown at 37 °C until the A_{600} reached 0.6 and induced for NeuS expression with 1 mM isopropyl thiogalactopyranoside (IPTG) for 2 h. Membranes were isolated as described previously (*5*). The membranes (6−12 mg of protein/mL) were resuspended in cryoprotectant buffer (*8*) that contained 14% (v/v) glycerol, 1.4% (w/v) D-sorbitol, 150 mM KCl, 50 mM Tris-HCl, and 25 mM $MgCl_2$ (pH 8).

Membrane preparations were frozen in 2 mL glass vials (Kimble #4361) and sealed with an oxygen-gas torch. Vials were held at −80 °C except during irradiation at −135 °C. Irradiations were performed with 13 MeV electrons produced by a linear accelerator, either at the Armed Forces Radiobiology Research Institute (Bethesda, MD) or at the National Institute of Standards and Technology (Gaithersburg, MD). Details of radiation treatments were described previously (*12*).

After being exposed to radiation, samples were held at −80 °C for several days. Sample vials were opened and the vials purged with argon to remove any ozone prior to the samples being thawed. Aliquots were removed for enzymatic activity assay and analysis by electrophoresis as described below.

Polysialyltransferase Activity Assay. The enzymatic assay was performed as described previously (*5*). The ability of the polysialyltransferase to initiate synthesis de novo is assessed by incubating the enzyme in the presence of only sugar nucleotide substrate. Elongation activity is measured by addition of an exogenous acceptor to the incubation mixture. Substrate cytidine 5′-monophosphate [4,5,6,7,8,9-^{14}C]sialic acid ([^{14}C]CMP-NeuNAc) was obtained from American Radiolabeled Chemicals, Inc. The exogeneous acceptor, K92 PSA (K92 PS), was isolated and purified from *E. coli* Bos 12 (*11*). Each 50 μL reaction mixture contained 25 μL of membrane preparation, 13.5 nmol of [^{14}C]CMP-NeuNAc (specific activity of 29.6 μCi/μmol), and, as needed, 10 μg of exogenous acceptor dissolved in buffer A. The reaction mixture was then incubated at 37 °C for 30 min. A parallel reaction mixture with 25 μL of boiled membrane preparation, acceptor, and labeled substrate was prepared as a negative control. The reaction was quenched by spotting 30 μL onto Whatman 3M chromatography paper. The chromatogram was developed overnight with a 7.5:3 ethanol/1 M ammonium acetate (pH 7.0) solvent system. The paper was dried, and the radiolabel incorporated into PSA that remains at the origin was quantitated by liquid scintillation counting.

Determination of Molecular Masses. The fraction or amount of enzyme activity remaining after irradiation or the intensity of a protein band on an immunoblot remaining after irradiation was analyzed by target theory (*8*, *12*, *13*) using the following equation to determine molecular mass:

$$A = A_0 e^{-qmD}$$

where m is the mass (in kilodaltons) associated with the affected property, the constant $q = 1792$ at the irradiation

temperature (−135 °C), and *D* is the irradiation dose in megarads (*13*). The data were plotted on a semilog graph and fit with a straight line whose slope, *k*, was used to estimate mass using the equation mass = 1792*k*.

The radiation-sensitive mass associated with membranes of several *neuS*-containing strain constructs was determined. In every case, enzymatic activity initially decreased exponentially but then showed a very small amount of residual activity that remained after exposure to large amounts of radiation. The radiation-insensitive fractions were 0.2% in NeuS (DH5α:pWV213), 0.6% in the NeuS-GFP fusion (DH5α:pWV218), and 0.05% in EV239:pWV213. Similar observations have been reported in many other irradiated enzymes. This residual activity can be due to a nonenzymatic reaction with the substrate, while in other cases, it can be due to a limitation in the enzymatic assay (*12*). It has always been found that subtraction of this very small amount of residual activity from every irradiated sample and control resulted in a simple exponential decrease in activity, as observed for neuS in this study (data not shown).

Electrophoresis and Immunoblots. Discontinuous sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis (PAGE) was performed using NuPAGE Novex Bis-Tris Gels (Invitrogen). Aliquots of irradiated and nonirradiated DH5α:pWV218 were diluted with 50 mM Tris-HCl and 25 mM $MgCl_2$ and electrophoresed at 160 V with NuPAGE MOPS SDS running buffer. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) by electrophoresis at 280 mA using a Bio-Rad Mini Trans-Blot cell. Immunoblotting was performed with GFP mouse monoclonal IgG_{2a} antibody diluted 1:1000 in TBS-T (Santa Cruz Biotechnology, catalog no. sc-9996) followed by anti-Mouse IgG, horseradish peroxidase-linked antibody diluted 1:5000 in TBS-T (Amersham Biosciences, catalog no. NA931V). Detection was performed using the ECL Plus Western Blotting Detection System (Amersham Biosciences), the Kodak Image Station 440 CF, and ImageQuant (Amersham Biosciences). Each SDS−PAGE gel contained control samples at several concentrations to calibrate the antibody reaction on that gel and to facilitate comparison among different gels.

RESULTS AND DISCUSSION

Target Size of Polysialyltransferase Chain Elongation Activity. It has been reported that the NeuS protein functions as part of a polysialyltransferase complex that includes several gene products of the K1/K92 gene cluster (*1*, *7*). Our recently published results (*6*) show that de novo synthesis required *neuE* and *kpsC* in addition to *neuS*. Our objective was to determine the minimum functional size of the enzyme or enzyme complex catalyzing the polysialyltransferase reactions. Since NeuS exhibits only polysialyltransferase activity when present in a membrane, we chose radiation target analysis to estimate the molecular size associated with this activity.

The *neuS* gene of K92 encodes a polypeptide of 409 amino acids which yields a protein of 48.3 kDa (*3*, *14*). We irradiated membrane preparations of *E. coli* strain DH5α possessing the *neuS* gene but lacking other capsule genes. We measured the ability of surviving enzyme molecules to add neuNAc to existing PSA chains to determine the functional size for this activity. In all experiments, the target size for elongation activity was much larger than that expected for a monomer of 48.3 kDa (Figure 1). Membrane-bound *E. coli* lactate dehydrogenase, which has a polypeptide mass of 65 kDa (*15*), was used as an internal standard (*16*) (Figure 1). As shown in Table 2, the average target size for NeuS elongation activity is 126 kDa; the LDH activity gave a size of 59 kDa similar to the previously reported value of 58 kDa (*16*).

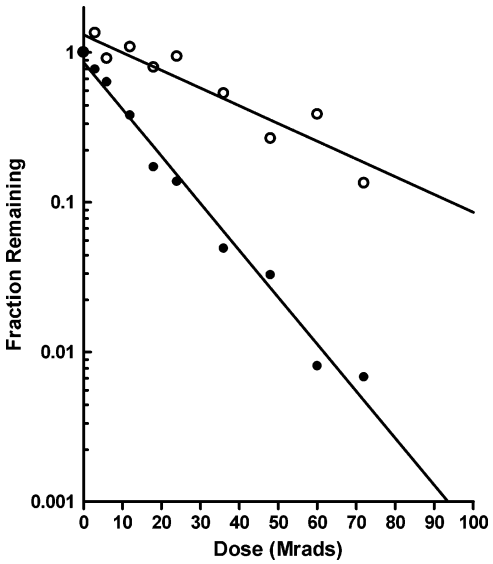

FIGURE 1: Target size analysis of K92 polysialyltransferase. Membranes isolated from *E. coli* strain DH5α:pWV213 were irradiated as described in Materials and Methods. Residual polysialyltransferase activity was determined in the presence of K92 polysaccharide (●). The same membrane samples were assayed for lactate dehydrogenase activity (○).

Table 2: Radiation Target Sizes of Various Forms of Polysialyltransferases[a]

strain	target size: activity ± standard error	target size: SDS−PAGE
DH5α:pWV213 (*neuS*)	126 ± 13	
DH5α:pWV218 (GFP-*neuS*)	122 ± 19	107 ± 10
EV239:pWV213 (no K92 polysaccharide)	147 ± 10	
EV239:pWV213 (with K92 polysaccharide)	147 ± 10	

[a] The target size of the catalytic unit was calculated on the basis of residual polysialyltransferase activity. The target size of the structural unit was determined from the band intensity of the immunoblot after SDS−PAGE as described in Materials and Methods. These results were derived from four independent experiments. The standard errors were calculated from the target sizes determined in these experiments.

Radiation Sensitivity of the Enzyme Structure. The target size of polysialyltransferase suggests that the minimum size required for activity was equivalent to a dimer of the NeuS protein or a monomer plus the additional radiation-sensitive mass. To explore this possibility, we estimated the target size of a green fluorescent protein (GFP) chimera of NeuS by both gel electrophoresis and activity measurements.

Irradiated membranes harboring the NeuS-GFP chimera were subjected to SDS−PAGE, and the chimera was detected via immunoblot analysis by reaction with a monoclonal antibody reactive with GFP. The NeuS-GFP mobility on SDS−PAGE corresponded to an M_r of ∼75500 (Figure 2),

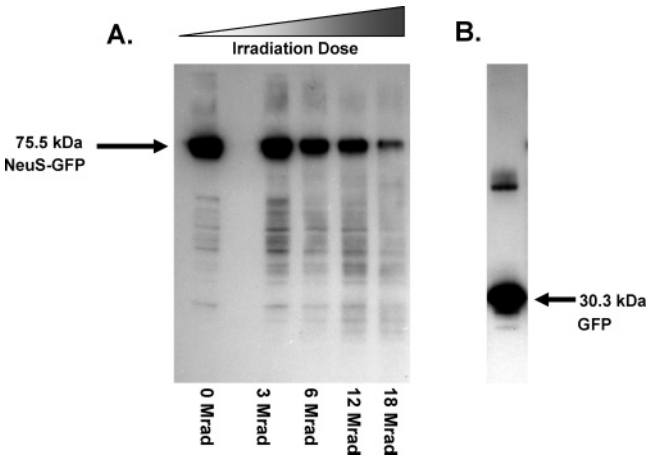

FIGURE 2: Western blot of the irradiated NeuS-GFP chimera. Membranes isolated from *E. coli* DH5α:pWV218 were irradiated and then analyzed via SDS−PAGE and immunoblotting. (A) The residual chimera in samples irradiated with varying doses of radiation and (B) a purified GFP reference were detected with the anti-GFP monoclonal antibody. Band intensity in immunoblot was quantitated and used to calculate target size as described in Materials and Methods.

similar to the expected value of 74 kDa. A plot of band intensity versus radiation dose yielded a single-exponential decay. The radiation sensitivity of the material in the SDS gel band was found to correspond to a mass of 107 kDa (Table 2). Thus, the NeuS-GFP protein migrates in SDS at its expected monomer molecular mass; however, it decays after irradiation at a rate expected for a larger structure. Presumably, some of the radiation energy deposited in these other molecules was transferred to the NeuS-GFP molecule, resulting in cleavage of the polypeptide chain (*8*, *17*). Such energy transfer in radiation inactivation experiments has been observed with several membrane-associated activities such as glucose transport, cytochromes, and ion pumps and has been reviewed by Kempner and Fleischer (*8*).

The possibility of energy transfer via a disulfide bond between NeuS and a second protein was ruled out by irradiating membranes harboring either NeuS or the NeuS-GFP chimera in the presence and absence of the reducing agents DTT and 2-mercaptoethanol. The presence of a reducing agent had no effect on the target size determined neither by the decay of activity nor by the decay of the 75.5 kDa band via SDS−PAGE.

The effect of radiation on enzymatic activity is not related to changes in the K_m values. We irradiated membranes prepared from DH5a:pWV213 and EV239:pWV213 cultures at 0, 18, and 24 Mrad and compared the K_m values for CMP-neuNAc. K_m values obtained from irradiated membranes did not differ significantly from that obtained with unirradiated membranes.

If the polysialyltransferase is active only as a dimer of two NeuS molecules, then the estimated target size of the active chimera is expected to be 148 kDa. The GFP-polysialyltransferase chimera lost enzymatic activity after irradiation as a simple exponential function of radiation dose (data not shown). The calculated target size from this plot of activity was 122 kDa (Table 2). The expected target size for a monomer is (48 + 26) 74 kDa, and that for a dimer is 148 kDa. Pummill et al. (*18*) used the difference between the GFP fusion protein and the hyaluronic acid synthase protein to conclude that the target size of the hyaluronic acid synthase-GFP chimera contained the mass of a single GFP. Although the target size obtained for the NeuS-GFP chimera is smaller than that expected for a dimer, such an analysis is not valid in our experiments since the target size for activities of NeuS and the NeuS-GFP chimera are not statistically different. Therefore, the structure required for activity cannot be larger than a dimer and consists of either a dimer or only one 48 kDa NeuS monomer associated with additional material. This additional material should have a mass between (126 − 48) 78 kDa and (122 − 74) 48 kDa.

Blue Native Gel Electrophoresis. The experiments described above predict that NeuS exists in a complex, resulting in a molecular mass larger than that of the NeuS monomer. To test this theory, we extracted membranes containing two different chimera of NeuS and subjected them to blue native gel and SDS−PAGE. We used the GFP chimera described above and a chimera having a biotinylated peptide at the amino terminus that was then detected in immunoblots with a specific antibody. Both chimeras migrated in blue native gels as high-molecular weight aggregates. Monomers of GFP-NeuS and biotinyl-NeuS chimeras were not detected. However, the immunoblot of SDS−PAGE gels detected proteins migrating at the expected monomer size. These results support the claim that NeuS target size is greater than its monomer molecular mass.

Target Size of de novo Polysialyltransferase Activity. The EV239 strain lacks an active polysialyltransferase and active sialic acid synthase, but EV239 possesses all of the other genes of the K1 gene cluster. We expressed the K92 *neuS* gene (pWV213) in this background to compare the effect of radiation on *de novo* synthesis and polysialyltransferase elongation activity. Since several proteins other than NeuS are required for *de novo* synthesis, one might expect the target sizes for *de novo* and elongation activities to differ. Extensive experiments were performed with the EV239:pWV213 membrane preparations assayed with and without the exogenous K92 PS acceptor. In all experiments, the activity decay due to irradiation was the same for de novo and elongation activity (Figure 3A). The target size required for polymerization is the same for both activities, which suggests that one complex catalyzes both de novo and elongation reactions. In both cases, the activity was much greater than that observed in both the DH5α:pWV213 and DH5α:pWV218 constructs and the inactivation curves showed a "shoulder" at small doses, followed by a single exponential over 4 orders of magnitude. Control experiments proved that the shoulder was an artifact of the assay conditions. Reassay with one-tenth the amount of enzyme removed the shoulder, indicating that the substrate was limiting at small radiation doses. The exponential portion of the inactivation curve was fit with a straight line (Figure 3B), yielding a target size of 147 kDa for de novo activity and 147 kDa for elongation activity. The fact that these values are the same implies that both elongation and initiation reactions require the same proteins for catalysis. Thus, although NeuE and KpsCS are needed for de novo synthesis of PSA, the transfer of sialic acid to either the endogenous acceptor or the growing chain is catalyzed by NeuS alone (Figure 4).

These target sizes are the same within experimental error, but in each of four different irradiation experiments, the target

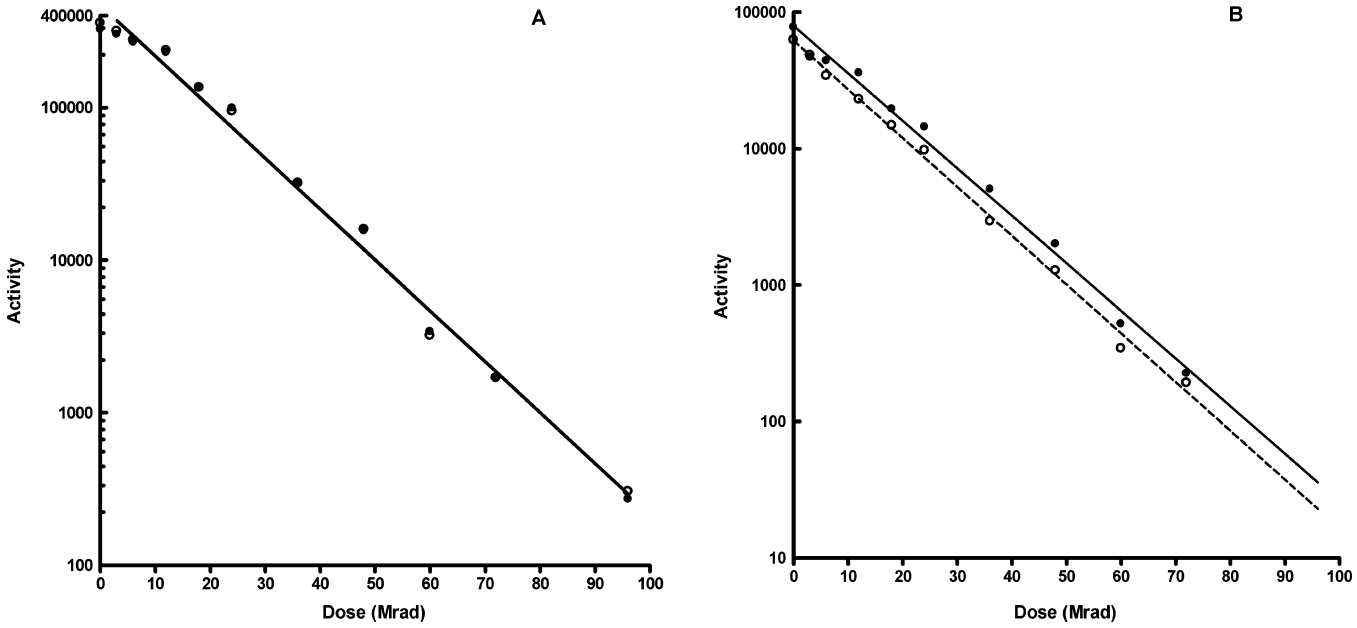

FIGURE 3: Target size analysis of de novo polysialyltransferase activity. Membranes isolated from *E. coli* strain EV239:pWV213 were irradiated as described in Materials and Methods. (A) Residual polysialyltransferase activity was determined in the presence (●) and absence (○) of K92 polysaccharide. (B) Irradiated membrane preparations were diluted 1:10 in cyroprotectant buffer and assayed in the presence (●) and absence (○) of K92 polysaccharide.

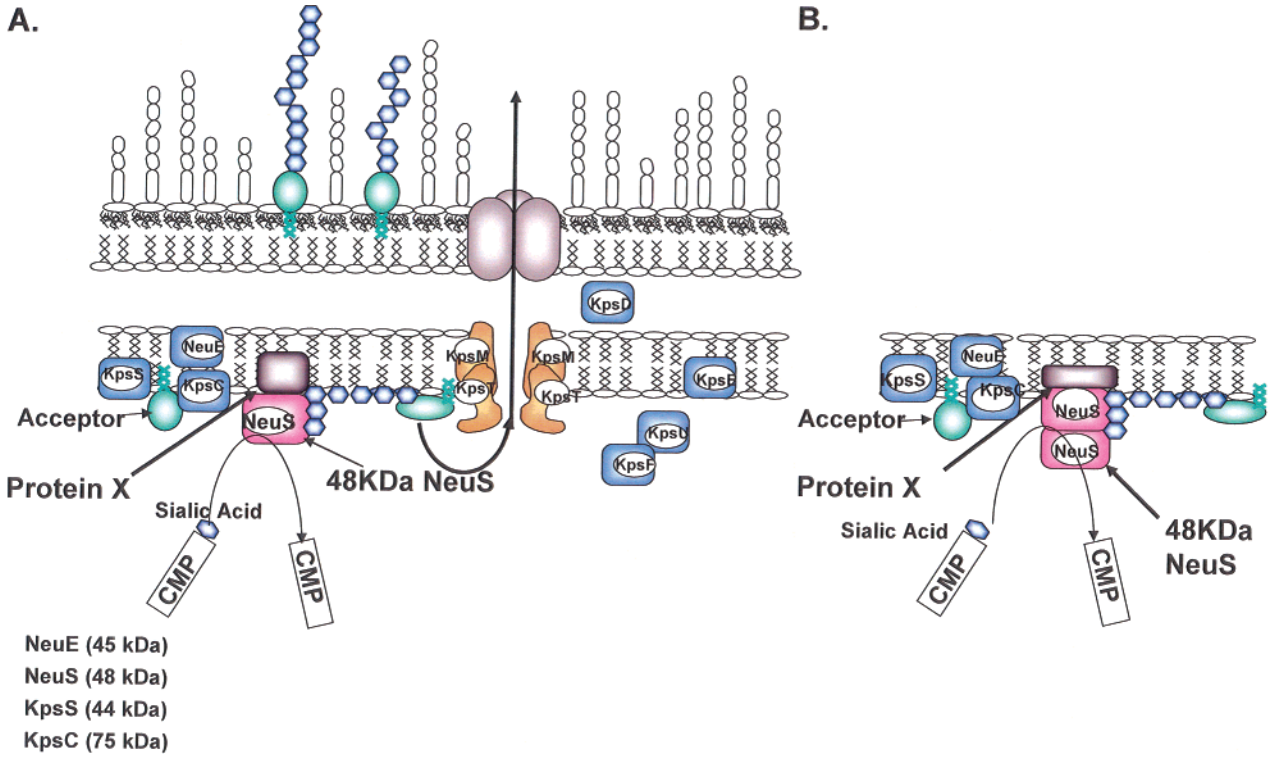

FIGURE 4: Proposed model for the K92 polysialyltransferase functional unit. The NeuS protein may occur as (A) a monomer or (B) a dimer associated with a membrane protein(s) with a mass ranging from 48 to 78 kDa. NeuS can then transfer sialic acid from CMP-sialic acid to either an endogenous acceptor or the growing chain. While other gene cluster proteins may be important for formation of capsular PSA in vivo, they do not participate directly in the catalytic transfer of sialic acid to the acceptor.

size observed for NeuS in a DH5α background was always smaller than that observed for NeuS in EV239. We do not have experimental data to explain this difference. Nevertheless, one can speculate that in strain EV239 the NeuS protein is associated with a radiation-sensitive mass different from or in addition to that observed in strain DH5α. Perhaps in its native state other products of the gene cluster are associated with NeuS in a radiation-sensitive fashion. The target sizes of NeuS and the NeuS-GFP chimera were determined in DH5α which excludes other products of the K1 gene cluster. These results taken together with the observation of energy transfer are consistent with NeuS being associated with a membrane component in both DH5α and EV239 backgrounds (Figure 4).

The experiments described herein show that the target size of the K92 polysialyltransferase is larger than its monomer

size but does not require the large complex previously proposed for catalytic activity (*1*, *7*). Since energy transfer was observed, it is possible that the size of the catalytic unit is smaller than the molecular mass of the structural unit suggested by the observed target size (*8*). Even if this were the case, these experiments establish an upper limit for the size of the polysialyltransferase complex necessary for catalytic activity. Furthermore, most of the other protein products of the K1/K92 gene cluster do not have a direct role in catalysis since the target size for the polysialyltransferase in a DH5α background differs only slightly from its target size in a background containing all of the K1/K92 gene products.

The fact that other gene products do not play a direct role in catalysis of polymer formation has greater implications for K92 polysaccharide assembly. Membranes prepared from strain EV239:pWV213 can catalyze de novo synthesis of PSA when incubated with CMP-neuNAc, suggesting that this strain is capable of synthesizing an endogenous acceptor. If the other K1/K92 gene cluster products do not participate in catalysis, then perhaps they are involved in the synthesis of this endogenous acceptor molecule. This would imply that EV239 could produce an acceptor molecule in the absence of an active NeuS. In preliminary experiments, we showed that extracts of EV239 membranes can reconstitute polysaccharide synthesis in DH5α:pWV213 (NeuS containing) membranes. Addition of the membrane extracts to a reaction mixture containing only CMP-neuNAc and NeuS-containing membranes resulted in the formation of a polymer. This result was not observed with extracts of membranes from background strain DH5α, suggesting that indeed K1 gene products present in EV239 do participate in the synthesis of an endogenous acceptor. Work to characterize these extracts in an effort to identify the native acceptor molecule is currently underway.

Few detailed biochemical studies have examined the role of the host proteins in the activity of unfractionated membrane-associated glycosyltransferases. We observed at least two effects of proteins on in vitro polysialyltransferase activity. Proteins normally present in nonencapsulated *E. coli* contribute to the target size of the polysialyltransferase. In addition, the relative amount of enzymatic activity is greater in an EV239 strain than that in a DH5α strain. While only NeuS may participate in catalysis, other proteins may associate with the polysialyltransferase and play an important role in its stability or proper orientation in the membrane. Extraction and isolation of an active polysialyltransferase from *E. coli* K1 and K92 membranes has proven to be difficult. As shown here, this problem may be related to the critical association of the polysialyltransferase with a membrane protein(s).

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