Biosynthesis of the Polysialic Acid Capsule in *Escherichia coli* K1

THE ENDOGENOUS ACCEPTOR OF POLYSIALIC ACID IS A MEMBRANE PROTEIN OF 20 kDa*

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The nature of endogenous acceptor molecules implicated in the membrane-directed synthesis of the polysialic acid (polySia) capsule in *Escherichia coli* K1 serotypes is not known. The capsule contains at least 200 sialic acid (Sia) residues that are elongated by the addition of new Sia residues to the nonreducing termini of growing nascent chains (Rohr, T. E., and Troy, F. A. (1980) *J. Biol. Chem.* 255, 2332-2342). Presumably, chain growth starts when activated Sia residues are transferred to acceptors that are not already sialylated. In the present study, we used an capsular mutant defective in synthesis of CMP-NeuAc to label acceptors with [14C]NeuAc and an anti-polysia-specific antibody (H.46) to identify the molecules to which the polySia was attached. [14C]Sia-labeled acceptors were solubilized with 2% Triton X-100, immunoprecipitated with H.46, and partially depolymerized with poly-α-2,8-endo-N-acetylneuraminidase. Approximately 5% of the [14C]Sia incorporated remained attached to endogenous acceptors. Double-labeling experiments were used to show that the non-Sia moiety of the acceptor was labeled in *vivo* with [14C]leucine and elongated in *vitro* with CMP-[3H]NeuAc. Concomitant with desialylation of the [3H]polySia-[14C]Leu acceptor was the appearance of a new [14C]Leu-labeled protein at 20 kDa. After strong acid hydrolysis, the 20-kDa labeled protein was shown to contain [14C]Leu. The acceptor molecules were not labeled metabolically with D-[3H]GlCN, 35SO₄, or 32PO₄, indicating that they do not appear to contain lipopolysaccharide, peptidoglycan, phosphatidic acid, or phospholipid. Based on these results, we conclude that the endogenous acceptor molecule is a membrane protein of about 20 kDa. The nature of attachment of polySia to acceptor is unknown.

There are only 400-500 acceptor molecules/cell, which is about 100-fold fewer than the 50,000 polySia chains/cell. This suggests that each acceptor molecule may participate in the shuttling of about 100 polySia chains/cell. We hypothesize that the acceptor protein may function to translocate polySia chains from their site of synthesis on the cytoplasmic surface of the inner membrane to the periplasm.

Capsular polysaccharides represent the outermost layer of the bacterial cell surface and as such can serve as virus receptors and as mediators of cell-cell interactions. Capsules are involved in virulence, bacteriophage infection, invasion, and colonization (1). A well characterized capsule is the polysialic acid (polySia)† antigen found in certain pathogenic strains of *Escherichia coli* K1. In *E. coli* K-35 (01:K1:H1), the K1 capsule is composed of at least 200 sialic acid (Sia, NeuAc) residues joined internally through α-2,8-ketosidic linkages (2). A structurally identical capsule is present in *Neisseria meningitidis* serogroup B (3). Interest in polySia capsules stems from their association with neonatal meningitis. The potential reducing termini of several capsular antigens have been shown to be attached to 1,2-diacylglycerol through a phosphodiester linkage (4, 5). Not all of the capsular polysaccharide chains in a culture are lipidated, however (6). It has been proposed that the phospholipid may play a role in linking some capsular chains to the membrane (4, 5), although no direct biochemical evidence has been presented to support this supposition.

Studies to delineate the biosynthesis of the polySia capsule in *E. coli* K1 have shown that a membranous CMP-NeuAc:poly-α-2,8-sialosyl sialyltransferase (poly-ST) complex catalyzes the synthesis of linear polySia chains containing α-2,8-linked sialyl residues (2, 6-11). Sialylmonophosphorylunde-caprenol (NeuAc-P-unde-caprenol) is an intermediate in the formation of these chains, and polySia synthesis requires *endo-siaceptor* molecules present within the enzyme complex (7, 8). Reactions 1-3 summarize the postulated mechanism of synthesis of the polySia capsule in *E. coli* K1 catalyzed by the poly-ST complex (7, 9, 11).

\[
\text{CMP-NeuAc} + P\text{-unde-caprenol} \rightleftharpoons \text{NeuAc-P-unde-caprenol} + \text{CMP}
\]

(1)

\[
\frac{n(\text{NeuAc-P-unde-caprenol})}{(\text{NeuAc})_n\text{-P-unde-caprenol}} + (\text{NeuAc})_n\text{-acceptor} + n(\text{P-unde-caprenol})
\]

(2)

\[
\frac{\text{NeuAc-P-unde-caprenol}}{(\text{NeuAc})_n\text{-P-unde-caprenol}} \rightleftharpoons \text{endogenous acceptor}
\]

(3)

Whether the putative phospholipid at the reducing terminus (4, 5) is itself an endogenous acceptor or whether the polySia chains are lipidated at a later stage in the biosynthetic pathway is not known.

The objective of the present study was to establish the nature of the endogenous acceptor molecule which serves as

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1 The abbreviations used are: polySia, poly-α-2,8-linked polysialic acid; Sia, sialic acid; DP, degree of polymerization; Endo-N, poly-α-2,8-endo-N-acetylneuraminidase; poly-ST, CMP-NeuAc-poly-α-2,8-sialosyl sialyltransferase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s).
the attachment site for polySia. Several lines of evidence have suggested that the polySia chains are attached to a membrane protein (7, 9, 10, 12-14). Further, synthesis of a select number of membrane proteins is required for initiation of polySia synthesis both in vitro and in vivo (10, 12, 13). Studies to identify the acceptor have been hampered by the apparent low copy number of acceptors/cell and the difficulty in their isolation. In SDS-PAGE, the in vitro and in vivo synthesized sialyl polymers show considerable polydispersity (7, 14-16), resulting from variation in the degree of polymerization (DP) of the polySia chains (2). Because the number of Sia residues in these chains is large (DP > 200), less than 1 in 200 is linked directly to an acceptor. Further, only about 10% of the endogenous acceptor chains in the poly ST complex are elongated in vitro (2). Thus, it has not been possible to efficiently label the acceptor in vitro because only about 1 in 2000 of the [14C]Sia residues would be attached directly to an unoccupied acceptor. 

To circumvent these experimental limitations, we have taken advantage of a mutant of E. coli Kl, designated EV5, that fails to synthesize CMP-NeuAc (17). EV5 provides a source of acceptor that has not already been sialylated. These acceptors were then labeled with [14C]NeuAc. The labeled polymers were solubilized, immunoprecipitated with an anti-polySia antibody, and depolymerized with poly-α-2,8-endonuclease (Endo-N). Endo-N depolymerization reduced the Sia to protein ratio and thereby facilitated isolation of the endogenous acceptor-Sia region. Endo-N is specific for hydrolyzing poly-α-2,8-sialosyl chains containing at least 5 Sia residues (18, 19) and was expected to generate endogenous acceptors with four or fewer [14C]Sia residues attached. Using this approach we now demonstrate that the polySia chains are attached to a membrane protein of 20 kDa. The possible function of this protein in the translocation of polySia chains is discussed.

**Experimental Procedures**

**RESULTS**

**Kinetics of [14C]PolySia Synthesis in the CMP-NeuAc Synthetase-defective Mutant, EV5, and Direct Labeling of the Endogenous Acceptor**—When the membraneous poly-ST complex is isolated from wild type E. coli K1 serotypes, e.g. K-235, most of the endogenous acceptors are already sialylated. In contrast, all of the acceptors should be unoccupied in EV5, a K1 derivative with a defect in CMP-NeuAc synthetase activity. Results supporting this supposition were first provided by comparing the kinetics of [14C]polySia synthesis in EV5 and E. coli K-235.

The incorporation of [14C]NeuAc from CMP-[14C]NeuAc into polysia in EV5 membranes showed biphasic kinetics similar to those reported for membranes from E. coli K-235 (Ref. 2; results not shown). These kinetics were characterized by an initial rapid incorporation of [14C]NeuAc followed by a slower constant rate of synthesis (see Fig. 1 in Ref. 2). The initial rate of incorporation of [14C]NeuAc in EV5 membranes was 4-5-fold greater than in E. coli K-235 (180 nmol of NeuAc incorporated per mg protein h⁻¹ in EV5 versus 40 nmol in E. coli K-235), indicating a substantially greater extent of chain initiation.

After maximal incorporation of [14C]NeuAc into polySia in EV5 membranes (2-4 h), the membrane fraction was recovered by centrifugation, washed with TMD buffer, and isolated by sedimentation. The supernatant and membrane fractions contained nearly equivalent amounts of [14C]polySia, as assessed by the chromatographic assay (results not shown). The material remaining in the supernatant fraction after high speed centrifugation (200,000 × gavg, 4 h) was designated “soluble” polySia, and its partial characterization will be described below. The membrane-associated [14C]polySia was solubilized by incubating membranes in 2% Triton X-100 (25 °C, 20 min), as described previously (2). When polySia synthesis in E. coli K-235 membranes was carried out under identical conditions, both soluble and membrane-bound [14C]polySia were also observed in nearly equal amounts (results not shown). This was unexpected because we previously reported that most of the polySia synthesized in vitro in E. coli K-235 remained membrane-bound unless treated with NaCl (2, 7). These new results suggest, therefore, that differences in the growth conditions, the freeze-thaw method used to prepare the sialyltransferase complex, or washing of the membranes in TMD buffer containing dithiothreitol may be responsible for the partial release of polySia from the membranes.

**Properties of the [14C]PolySia Synthesized in Vitro by EV5**—The polymeric nature of the in vitro-synthesized [14C]polySia was confirmed by gel permeation chromatography and SDS-PAGE (Fig. 1). Both the polySia solubilized from EV5 membranes with detergent and the soluble polySia were excluded from Ultragel AcA34. As shown in Fig. 1, these polymers were polydisperse and migrated in SDS-PAGE with apparent molecular masses of about 45–150 kDa (C, membrane-bound form) and 60–180 kDa (◇, soluble form). Thus, the in vitro-synthesized polySia of EV5 has size characteristics.

**Fig. 1. Characterization of the membrane-associated and soluble [14C]polySia from E. coli K1 (EV5) by SDS-PAGE.** The in vitro-synthesized membrane-associated [14C]polySia polymers (◇—◇) were first solubilized with 1% SDS (1 h at 37 °C), then resuspended in Laemmli sample buffer (1:1), incubated at 28 °C for 2 h, and electrophoresed in a 5–15% polyacrylamide gradient gel. [14C]NeuAc incorporated in the sialyl polymers was determined by slicing the gel into 0.5 cm sections and quantitated by scintillation counting, as described (24, 26). The “soluble” [14C]polySia polymers (◇—◇, see text) were resuspended directly in Laemmli sample buffer and electrophoresed in SDS-polyacrylamide gels as described above. BPB designates the tracking dye, bromphenol blue.
Proof of the α-2,8-Ketosidic Linkage in PolySia Synthesized in EV5 and Isolation of the Sia-Acceptor Linker Region—To establish the nature of the ketosidic linkage in polySia synthesized in vitro by EV5 membranes, the polymers were tested for their sensitivity to depolymerization by Endo-N, an enzyme specific for catalyzing the hydrolysis of α-2,8-linkages in polySia (18, 19). Membrane-bound polySia was solubilized with 2% Triton X-100 and freed of detergent as described under “Experimental Procedures.” The 14C-labeled polymers were depolymerized with Endo-N (18). Fig. 2 shows the 14C elution profile on Bio-Gel P-10 of the Endo-N-treated polySia from the detergent-solubilized membranes (left panel). Approximately 95% of the [14C]Sia was recovered in sialyl oligomers with the expected mobility of DP4–8 (fractions 40–62) accounted for most of the radioactivity. Approximately 1% of the [14C]Sia incorporated eluted slightly ahead of the column V₀ and was designated “peak I” (see text).

Properties of the [14C]Sia-Acceptor—The [14C]Sia that remained linked to acceptor molecules that eluted in the V₀ fraction from the Bio-Gel P-10 column (Fig. 2, left panel) was designated “Sia-Acceptor” and showed the following properties: 1) resistant to further treatment with Endo-N, indicating that it contained 4 or fewer Sia residues (18); 2) sensitive to digestion with exosialidase, which quantitatively converted the radioactivity to [14C]Sia; 3) contained an average of 2 Sia residues, as determined by sequential periodate oxidation, borohydride reduction, and exoneuraminidase digestion (Ref. 2; Fig. 3, panel B). This material had a blocked reducing terminus since it could not be reduced by KBH₄. Fig. 4, panel A) under conditions where authentic sialyl oligomers were reduced (Fig. 4, panel B). Sia-acceptor was immobile after paper chromatography in Solvent System I under conditions where sialyl oligomers with a DP10 were mobile. This result also showed that the [14C]Sia in Sia-acceptor was attached to a higher M₉ component; 4) insoluble in 1-butanol (50%) or CHCl₃-MeOH (2:1), implying that the disialosyl residues were not linked to a lipid. NeuAc-P-undecaprenol and higher sialyl oligomers (DP2–8) linked to undecaprenol are soluble under these conditions and are also mobile on Whatman No. 3MM paper in Solvent System I (8, 29); 5) migrated with an apparent molecular mass on SDS-PAGE of about 21.5 kDa (Fig. 5, ○), implying that the disialosyl units remaining after Endo-N digestion were linked to an acceptor of approximately 20–21 kDa; 6) the 20–21-kDa protein was resistant to treatment with Pronase or protease K under conditions where bovine serum albumin was degraded. Susceptibility of Sia-acceptor to proteolysis was monitored by looking for changes in M₉ on SDS-PAGE and by mobility on paper chromatography. Protease resistance is a property relatively common to a variety of bacterial membrane proteins including several of the major outer membrane proteins in E. coli (30); 7) resistant to β-elimination under alkaline borohydride conditions, as described under “Experimental Procedures.” These data implied that the disialosyl units were not linked to the acceptor protein through a conventional O-glycosidic linkage.

Fig. 2. Endo-N characterization of the α-2,8-ketosidic linkage in [14C]polySia synthesized in vitro by E. coli K1 (EV5) and isolation of the [14C]Sia-acceptor linker region. The membrane-bound [14C]polySia was solubilized with 1% SDS (37 °C, 1 h), chromatographed on Ultrogel AcA34 in the presence of 2% Triton X-100, and rechromatographed on Extracti-Gel D. The labeled polymers were then digested with Endo-N, as described under “Experimental Procedures,” before fractionation on Bio-Gel P 10. On Bio-Gel P 10, approximately 95% of the [14C]Sia appeared in sialyl oligomers with DPs 4–8 (left panel, fractions 43–65) with a trace of smaller oligomers (fractions 67–90). The remaining 5% of the [14C]Sia eluted in the column void volume (V₀; fractions 31–38) and was designated “Sia-Acceptor.” The soluble [14C]polySia fraction, isolated as described under “Experimental Procedures,” was also treated with Endo-N and fractionated on Bio-Gel P 10 (right panel). Sialyl oligomers with the expected mobility of DP4–8 (fractions 40–62) accounted for most of the radioactivity. Approximately 1% of the [14C]Sia incorporated eluted slightly ahead of the column V₀ and was designated “peak I” (see text).
Identification of the Non-Sia Moiety in Sia-Acceptor as a Protein by Double-labeling Techniques—To characterize the non-Sia moiety of Sia-acceptor, we attempted to label metabolically in vivo the endogenous acceptor in EV5 with [3H]GlcN, [35S]SO4, [32P]04, or [14C]leucine. [14C]Leucine was used because EV5 is auxotrophic for leucine (see Ref. 17, Table 1). For these experiments, EV5 was grown separately in the presence of [14C]leucine as described under "Experimental Procedures." The radiolabeled membranes containing the endogenous acceptors were isolated, washed, and incubated with either CMP-[3H]NeuAc or CMP-[14C]NeuAc to label acceptors with Sia. Membrane-associated polySia was then solubilized with Triton X-100, chromatographed over Bio-Gel P-100, and the double-labeled radioactive profiles determined. PolySia has an unusually high apparent molecular mass in gel permeation chromatography (2, 7) and as a consequence is excluded by Bio-Gel P-100 at a flow rate of 1 cm/min.

These data imply that endogenous acceptors lack sulfate and hexosamines and thus rule out lipopolysaccharide and peptidoglycan as acceptors. A relatively simple procedure to ascertain if the [3H]polySia in the V0 fractions was linked directly to polymeric material containing [14C]leucine was to determine if the double-labeled V0 material was sensitive to digestion with Endo-N. Accordingly, the V0 equivalent material (Fig. 6A) was isolated, treated with Endo-N, and the resultant radioactive profile compared by SDS-PAGE to an undigested control. Since Endo-N will depolymerize the [3H]polySia, a change in the SDS-PAGE mobility of the [14C]Leu-labeled protein or the [32P]labeled component would be consistent with covalent attachment of polySia to that moiety. When this procedure was carried out on the [32P]labeled material (Fig. 6B), no change in the [32P] labeled profile of the SDS gel was seen. In contrast, Endo-N depolymerization of the [14C]Leu-labeled material (Fig. 6A) resulted in a lowering of the [14C]Leu profile to a molecular mass of about 20 kDa (see below). On the basis of these results, we conclude that the [3H]polySia appears to be linked to a protein of approximately 20 kDa. The high M, polySia material (Fig. 6B) appeared to be nucleic acids or lipopolysaccharide. Further proof that [3H]polySia was attached to a Leu-labeled protein was obtained by showing that [3H]polySia-[14C]Leu-labeled polymers were immunoprecipitated by the anti polySia specific antibody, H46, as described below.

The molecular mass of the [14C]Leu-labeled moiety of the [3H]polySia-[14C]Leu-labeled acceptor was determined directly by SDS-PAGE after immunoprecipitation of the Bio-Gel P-100 V0 fraction with H46 antibodies. For the experiment shown in Fig. 7, the [3H]Sia residues attached to the
protein. That the label remained in leucine and was not an apparent molecular mass of 20 kDa. Evidence that the new band contained \[^{14}C\]Leu was obtained by cutting the band out of the gel, subjecting it to strong acid hydrolysis, two-dimensional thin layer chromatography, and autoradiography, as described in the legend to Fig. 8. As shown in Fig. 8, a single radioactive band that migrated with the mobility of \[^{14}C\]Leu was observed, thus confirming that the acceptor was protein. That the label remained in leucine and was not detected in other amino acids was expected for three reasons. First, there is no protein turnover in growing E. coli (31), in contrast to many eukaryotic organisms. Second, leucine is only poorly used as a source of carbon in E. coli (32). Third, derepressed mutants of the leucine biosynthetic pathway in E. coli do not degrade leucine but rather excrete branched chain amino acids (33). Thus, on the basis of these results, we conclude that the \[^{14}C\]polySia in EV5 is linked to a \[^{14}C\]-labeled membrane protein with an apparent molecular mass of about 20 kDa.

Properties of the Soluble PolySia—The soluble polySia (Fig. 1, ♦) remained in the supernatant after the \textit{in vitro}-synthesized membrane-associated polySia was sedimented by ultracentrifugation (200,000 × g$_{av}$ for 4 h). Fig. 2 (right panel) shows the elution profile on Bio-Gel P10 of the soluble polySia after Endo-N digestion. Similar to the detergent-solubilized polySia, most (about 99%) of the radioactivity in the soluble polySia was sensitive to Endo-N depolymerization and eluted as sialyl oligomers (Fig. 2, right panel), thus confirming the presence of polySia in α-2,8-linkage. The remaining 1% of radioactivity eluted near the column V$_0$ (fractions 32–37) and was designated “peak I.” Further studies were carried out to partially characterize peak I. End group analysis employing potassium borohydride reduction before hydrolysis did not reveal any sialitol (Fig. 9), thus establishing that the polySia had a blocked reducing terminus. Sequential periodate oxidation and borohydride reduction (2) was used to show that peak I contained an average of three sialyl residues yet remained at the origin during high voltage electrophoresis on DEAE paper in solvent system IV (data not shown). Under these conditions, an authentic sialyl trimer (DP3) migrated 14 cm, thus establishing that the Sia in peak 1, like Sia-acceptor, was attached to a non-Sia moiety through its potential reducing terminus. Because the potential reducing ter-

FIG. 6. Gel exclusion chromatography of \[^{3}H\]polySia synthesized \textit{in vitro} on \[^{14}C\]Leu or \[^{32}P\]-labeled membranes. \[^{3}H\] PolySia was synthesized \textit{in vitro} using either \[^{14}C\]Leu or \[^{32}P\]-labeled membranes. The \[^{3}H\]polySia was solubilized with 2% Triton X-100, fractionated on Bio-Gel P-100, and the radioactivity determined, as described under “Experimental Procedures.” \[^{3}H\]PolySia was excluded from Bio-Gel P-100, indicating an apparent molecular mass of >100 kDa. The excluded V$_0$ fractions also contained \[^{14}C\]Leu (panel A) and \[^{32}P\] (panel B). The radiolabeled polymers in the V$_0$ fractions were isolated, digested with Endo-N, and subjected to SDS-PAGE. Endo-N depolymerization reduced the apparent molecular mass of the \[^{14}C\]Leu-labeled polymers to about 20 kDa (see Fig. 7), indicating attachment of \[^{3}H\]polySia to a protein of 20 kDa. In contrast, Endo-N digestion did not alter the high $M_r$ profile of the \[^{32}P\]-labeled material, indicating that the \[^{32}P\] label in the V$_0$ fraction (panel B) was not attached to \[^{3}H\]polySia.

\[^{14}C\]-labeled acceptor were released by heating at 95 °C for 10 min in Laemmli sample buffer. In this experiment, desialylation was carried out by heating instead of using Endo-N, because it quantitatively releases polySia residues from the \textit{in vitro} product of the poly-ST complex (see Ref. 7, Fig. 9). In addition, heating in Laemmli sample buffer dissociates the antigen-antibody complex in the immunoprecipitate, thus improving the subsequent resolution of proteins on SDS-polyacrylamide gels.

\textit{Flanes 1 and 3 are controls that are described in the legend to Fig. 7. Lane 2 shows that concomitant desialylation of \[^{3}H\]polySia-[\[^{14}C\]Leu-acceptor was the appearance of a new \[^{14}C\]Leu-labeled protein band with an apparent molecular mass of 20 kDa. Evidence that the new band contained \[^{14}C\]Leu was obtained by cutting the band out of the gel, subjecting it to strong acid hydrolysis, two-dimensional thin layer chromatography, and autoradiography, as described in the legend to Fig. 8. As shown in Fig. 8, a single radioactive band that migrated with the mobility of \[^{14}C\]Leu was observed, thus confirming that the acceptor was protein. That the label remained in leucine and was not detected in other amino acids was expected for three reasons. First, there is no protein turnover in growing E. coli (31), in contrast to many eukaryotic organisms. Second, leucine is only poorly used as a source of carbon in E. coli (32). Third, derepressed mutants of the leucine biosynthetic pathway in E. coli do not degrade leucine but rather excrete branched chain amino acids (33). Thus, on the basis of these results, we conclude that the \[^{3}H\]polySia in EV5 is linked to a \[^{14}C\]-labeled membrane protein with an apparent molecular mass of about 20 kDa.

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FIG. 7. Identification of the non-Sia moiety in Sia-acceptor as a 20-kDa membrane protein by double labeling. Confirmation that \[^{3}H\]polySia was linked to a membrane protein of approximately 20 kDa was obtained by characterizing the non-Sia moiety in the \[^{3}H\]polySia-[\[^{14}C\]Leu acceptor. The double-labeled polymers were isolated after detergent solubilization by immunoprecipitation of the Bio-Gel P-100 V$_0$ fraction (Fig. 6) with anti-polySia antibodies (H.46), as described under “Experimental Procedures.” The \[^{3}H\]polySia was quantitatively released by heating at 95 °C/10 min in Laemmli sample buffer, as previously described (7). After desialylation, SDS-PAGE, and fluorography, a new \[^{14}C\]Leu-labeled protein with an apparent molecular mass of about 20 kDa appeared (lane 2). Lanes 1 and 3 are controls. Lane 1 shows the fluorogram of authentic \[^{3}H\]polySia (185,000 cpm). Lane 3 shows the high $M_r$ profile characteristic of the \[^{3}H\]polySia-[\[^{14}C\]Leu acceptor before desialylation by heating at 95 °C/10 min in Laemmli sample buffer. Lanes 2 and 3 both contained 30,000 cpm of the \[^{3}H\]Leu-labeled material. DF, tracking dye front.
Since the radioactivity could not be electroeluted from the gel, described in Fig. 7 (lane 2) was cut out and rehydrated in distilled water. The portion of the gel containing about 10^{9} cpm was cut into small pieces and hydrolyzed in the presence of 0.1 mg of bovine serum albumin in 1.5 ml of constant boiling HCl for 26 h at 110 °C. The HCl was removed by evaporation and the residue extracted with 70 ul of 1-butanol. The butanol extract contained about 650 cpm. Approximately 430 cpm of this material was spotted on a cellulose glass plate (Macherey & Nagel, Duren, Federal Republic of Germany) and developed in the first dimension in sec-butyl alcohol-formic acid:H_{2}O (70:15:15) and in the second dimension in methyl ethyl ketone-pyridine:H_{2}O (70:18:18). The chromatography was repeated four times. The amino acids from the internal control were detected by spraying with ninhydrin and developed by heating at 90 °C. The plate was autoradiographed for 2 weeks prior to developing the film. The mobility of a reference sample of ['%'Leu chromatographed under similar conditions on a companion TLC plate is indicated by the dashed marks.

Since sialyl undecaprenol-bound oligosaccharides with DPs up to about 10 sialyl residues are extracted into organic solvents (29), the radiolabeled soluble polymers were degraded with Endo-N and subjected to butanol extraction. No radioactivity appeared in the butanol phase, leading us to conclude that the soluble polySia did not appear to be linked to a lipid. Further studies will be required to characterize this molecule and to determine its structural and functional relationship to the membrane-associated polySia.

**FIG. 8.** Confirmation that the 20-kDa endogenous acceptor is actually a protein. The region corresponding to the 20-kDa band described in Fig. 7 (lane 2) was cut out and rehydrated in distilled water. Since the radioactivity could not be electroeluted from the gel, a portion of the gel containing about 10^{9} cpm was cut into small pieces and hydrolyzed in the presence of 0.1 mg of bovine serum albumin in 1.5 ml of constant boiling HCl for 26 h at 110 °C. The HCl was removed by evaporation and the residue extracted with 70 ul of 1-butanol. The butanol extract contained about 650 cpm. Approximately 430 cpm of this material was spotted on a cellulose glass plate (Macherey & Nagel, Duren, Federal Republic of Germany) and developed in the first dimension in sec-butyl alcohol-formic acid:H_{2}O (70:15:15) and in the second dimension in methyl ethyl ketone-pyridine:H_{2}O (70:18:18). The chromatography was repeated four times. The amino acids from the internal control were detected by spraying with ninhydrin and developed by heating at 90 °C. The plate was autoradiographed for 2 weeks prior to developing the film. The mobility of a reference sample of ['%'Leu chromatographed under similar conditions on a companion TLC plate is indicated by the dashed marks.

**FIG. 9.** End group analysis of "Peak I" shows a blocked reducing terminus. The soluble ['^{14}C]polySia (Fig. 1) was isolated, digested with Endo-N, and chromatographed on Bio-Gel P-10. "Peak I," which eluted near the column Vo (Fig. 2), was subjected to end group analysis with KB_{3}H_{4}, and then to radiolabeling as described in the Fig. 4 legend. The absence of any ['^{14}C]NeuAc-OH (top panel) confirms that the soluble polySia also has a blocked reducing terminus. The lower panel is a control showing that a reference sample of ['^{14}C]sialyl oligomer was reduced by KB_{3}H_{4} under the same experimental conditions. The electrophoretic migrations of NeuAc and NeuAc-OH are indicated.

**DISCUSSION**

The endogenous acceptor to which Sia residues are attached during the synthesis of polySia in *E. coli* K1 has been identified as a membrane protein of approximately 20 kDa. While this is not the first report of protein-bound Sia in *E. coli* K1, it is the first direct evidence that the acceptor is a protein and the first documentation of the size of the protein moiety. A number of experimental findings has suggested that polySia is associated with a membrane protein. First, correlated with desialylation of the product of the *E. coli* K-235 poly-ST complex was the appearance of a new protein of about 20 kDa, as revealed by Coomassie Blue staining (7). When *E. coli* K1 cells were grown at 15 °C they did not express the polySia capsule, and a polypeptide of 20 kDa was found to be one of the proteins missing in 15 °C membranes (7, 9). Second, ['^{14}C]polySia, purified 70-fold after Triton X-100 solubilization, eluted from DEAE-Sephadex as a single symmetrical peak, coincident with absorbance at 280 nm (7). Third, activation of polySia synthesis in 15 °C membranes required protein synthesis (10, 12, 13). Four membrane proteins whose synthesis was temporally correlated with the initiation of polySia synthesis were identified in temperature-upshift experiments, and one of these proteins had an apparent molecular mass of 18–21 kDa (9, 13). Although no function was ascribed to this protein, it is possible that it is the protein identified in this study as the endogenous acceptor. Finally, recent studies by Rodrigues-Aparicio et al. (14) also suggested the involvment of a Sia-protein acceptor in polySia synthesis in *E. coli* K-235. Using our membranous sialyltransferase assay (8), these investigators confirmed the presence of ['^{14}C]polySia in SDS-PAGE with an apparent molecular mass of about 100 kDa. While no attempt was made to identify the molecular size of the nonsialylated moiety, time course studies showed that ['^{14}C]sialyl oligomers with molecular masses of 10 kDa or less were released from the 100-kDa complex. Soluble ['^{14}C]polySia has been described that is of higher molecular weight (Fig. 1; see also Fig. 9 of Ref. 7). Because polySia is labile to even slightly acidic conditions (7), it seems likely that the 10-kDa ['^{14}C]polySia observed by Rodrigues-Aparicio et al. (14) may have been derived from the 100-kDa species or from oligoSia-P-undecaprenol by acid hydrolysis, since 10% trichloroacetic acid was used in their isolation.

Our finding that the Sia acceptor is a 20-kDa protein adds new information which relates to the possible role of gene products encoded by the polySia gene cluster in *E. coli* K1. These genes are designated the *kps* cluster (34–39). The *kps* genes are encoded in 17 kilobases (kb) of DNA that is divided into three functional regions (36–39). Region 1 (about 9 kb) encodes for five proteins (80, 77, 60, 40, and 37 kDa) that have been implicated in capsular polysaccharide assembly and possibly export to the cell surface (38, 39). These proteins are common for the translocation of different capsular antigens in *E. coli* (37). A central region of about 5–6 kb, Region 2, encodes for proteins required for sugar biosynthesis and polymerization. In *E. coli* K1, this includes the enzymes for *Sia* synthesis, activation, and polymerization. An unknown number of proteins is contained in Region 2. A flanking region of about 2.5 kb, Region 3, encodes for functions that are not well defined but may relate to postpolymerization modification or polymer translocation and assembly. A total of 12 proteins involved in polySia biosynthesis was identified by derivatives of plasmid pSR23 when expressed in the *E. coli* minicell system by Silver and colleagues (38). Proteins of 18 and 19

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*Polysialic Acid Synthesis in E. coli K1*
kDa were two of the proteins identified. Since expression of both of these proteins was eliminated by Tn5 insertions in pSR47 and subclones pSR35, 28, 26, 41, and 43 (36), this provides additional evidence for the importance of proteins of this size in K1 expression.

Future studies will be required to establish the nature of the Sia linkage to the 20-kDa protein. Because only small quantities of the Sia-acceptor were available for analyses, we have been unable to determine whether the Sia is attached directly to the protein, a linkage which has yet to be described, or is linked to another substituent that is joined to the protein. Based on the results of our in vivo labeling experiments, however, such a substituent would appear to lack hexosamine, phosphate, and sulfate. It is always possible, of course, that such labeling experiments are not sensitive enough to detect minor components. Direct studies bearing on the nature of the linkage region have also been hampered because of the small number of acceptors/cell, estimated to be approximately 400-500, and the difficulty in isolating sufficient mass of the disialyl-acceptor for detailed structural analyses. Frevert and Bailleu (40) encountered similar difficulties in their studies on a unique structural cell wall mannoprotein in Saccharomyces cerevisiae. Like Sia-acceptor, the mannoprotein was also resistant to proteolytic enzymes, and they failed in their attempts to isolate any protein from their deglycosylated material. Similarly, identification of the glucosyl-tyrosine linkage in the glycosylated glycogenin molecule in rabbit skeletal muscle (41) has taken years of effort to demonstrate, since it was first suggested as a possible intermediate in glycogen synthesis in 1972 (42). Analysis of this glycosylated protein was also made difficult by the large sugar to protein ratio.

The function of the 20-kDa acceptor protein in the synthesis, translocation, or assembly pathways for polysia is unknown. The 400-500 Sia-acceptors/cell are about 100-fold fewer acceptors than the number of polysia chains/cell, estimated to be about 50,000. This suggests that each acceptor molecule may participate in the shutting of at least 100 polysia chains/cell, and implies, therefore, that the acceptor protein is not a final anchor for polysia chains within the membrane. Rather, the acceptor may facilitate the export or translocation of polysia from their site of synthesis on the cytoplasmic surface of the inner membrane to the periplasm (43). Studies have been carried out in pursuance of this possibility and our recent observations to determine the topological orientation of the poly-ST complex are pertinent. To determine the transmembrane organization of the poly-ST complex, membrane vesicles of defined orientation were used to assay for poly-ST activity. Using sealed right-side-out vesicles, inside-out vesicles, and trypsinolysis we have shown that the poly-ST complex is asymmetrically oriented on the cytoplasmic surface of the inner membrane (43). This requires that the polysia chains on the inner surface of the inner membrane must somehow transverse the membrane to the periplasmic side, subsequent to being transported to the outer membrane. Because the 20 kDa acceptor protein is sialylated early during biosynthesis and appears to encounter or shuttle about 100 polysia chains/cell, we suggest that it may be a translocator protein that assists in the transit of polysia chains across the inner membrane (43). It is also possible, of course, that the 20-kDa protein is a sialyltransferase that initiates its own sialylation, which could be of importance in forming a sialyl primer for subsequent polysialylation. Such an autosialylation would be analogous to the self-glycosylation of glycogenin, which then serves as a template for de novo glycogen biosynthesis (41, 44, 45). While attractive, this latter possibility appears less likely at this time for two reasons. First, we see no poly-ST activity in right-side-out vesicles above background and only a slight decrease in activity when right-side-out vesicles were treated with trypsin and then inverted (43). These data imply that the active site of the poly-ST does not appear to exist on both the cytoplasmic and periplasmic surfaces of the inner membrane. Second, preliminary evidence from two-dimensional SDS-PAGE analyses of 3H-labeled proteins from poly-ST competent and deficient strains indicated that the putative sialyltransferase proteins were of higher molecular weights.

The isolation and biochemical characterization of additional mutants defective in polysia expression and the construction of appropriate E. coli K-12/K1 hybrids amenable to recombinant DNA methods are current approaches that hold promise to make studies on the nature and function of the endogenous acceptor protein tractable.

Acknowledgments—We thank Becky Greer for her extreme patience and expert help in preparation of this manuscript. We also wish to express our thanks to Drs. R. I. Merker and E. R. Vimar for their helpful comments and suggestions during the course of this work. We are indebted to Dr. P. W. Jungblut for advice and help concerning the two-dimensional TLC separation of amino acids.

REFERENCES
9. L. D. Benning, E. R. Vimar, and F. A. Troy, unpublished results. These studies showed that proteins with apparent molecular masses of about 32–34 and 57 kDa were absent in a mutant strain defective in polysia capsule expression. The phenotype of this mutant was consistent with these proteins being functionally related to the poly-ST (50).
Biosynthesis of the Poly saccharide in E. coli K1.

The Endogenous Acceptor of Polysialic Acid is a Membrane Proteins

Experimental Procedures

Bacterial Strains: E. coli K12 (12) hybrid strains able to express the polysialic acid (poly sialyl or K1) capsular antigen were constructed earlier in this laboratory by conjugation of appropriate F' recipients with the 11 r K1+ strains, 610.85, as previously described (17). Strain 610.85 of the prototype E. coli K12 (11) hybrid, designated EV1, which were able to express the polsialyl capsule were isolated by screening conjugants for sensitivity to a K1-specific bacteriophage, designated PAP, and for their ability to form immune precipitates with antiserum specific to polysialoside (15-17). Susceptibility to killing in expression of the polysialic capsule was induced from EV1 by selecting resistance to the K1 phage. KAP requires surface expression of the polylosialic capsule for binding and infectivity. The stepwise flow reaction in these two assays was then determined by assaying enzyme activities specific to polysialic assay, and determining the content of expressed polylsialic bond by the method of Siegel et al. (17). The relative avidity for various molecules was determined in activation of the ICAP-40-Hepes synthase, CTP:polysialic acid:cytidine transferase, E. C. 3.2.1.40. The polysialic acid

Growth Conditions - EV5 was grown in LB-broth to late log phase (OD595 = 1.0-1.3). Cells were chilled on ice, harvested by centrifugation, washed in TMD buffer (50 mM Tris-HCl, pH 7.0, containing 0.01M magnesium chloride and 2 mM manganese) and stored as a cell pellet at 20°C overnight. For large scale preparative cultures, EV5 was grown in a 500 ml conical shake-flask in 2 liters of low density medium as described (18). A non-biuret method was added to 5 liters of medium and grown at 37°C to an OD600 of 0.7 to 1.0 dilution. Aeration was at 12 B0 to 1 min. Mid-to-late log phase cells were chilled quickly by the addition of 1:2 volumes of ice. Cells were harvested by centrifugation, washed with 400 ml TMD buffer and frozen. The yield was 60-70% by weight of the cells. Membrane fractions containing the transglycosylase complex were prepared as described below, and incubated with CMP-4-C14-N-acetyl-D-lactosamine (CLN, 0.5 mg) at the usual final concentration. The membrane-bound, [14C]-labeled polylsialic acid was subjected to 3% Triton X-100, as described.
Preparation of the Membraneous Sialyltransferase Complex - Membrane-associated sialyltransferase was prepared by combining both inner membrane (50 mg) and outer membrane (5 mg) fractions. The washed membranes were prepared from E. coli by modification of our previously described procedures (2). Briefly, cells were thawed in tap water, resuspended in TMD (2 g wet weight/ml 150 mM NaCl) and denaturated by heating and sonication (20 s, 300 W, 4°C). The sialyltransferase complex was recovered on Econo-gel G 5 to 10 detergent. Radioactive fractions were pooled, mixed with 0.5 mg celletose as a cold carrier of sialyl slimmer, and incubated in the presence of 200 μM CMP-[14C]NeuAc, 10 mM EDTA, 100 μM D-GlcN at 37°C for 18 hr. After digestion with proteinase K (1 μg/ml) and ethanol precipitation, the sample was resuspended in TMD buffer to a final concentration of 20 mg protein/ml.

In Vivo Synthesis of [14C]-Labeled Polyosialic Acid and Substitution of Membrane-Bound Polysialic Acid - In vitro synthesis of NeuAc was carried out at 37°C in a 25 ml reaction mixture containing 1% Triton X-100, pH 8.0, 20 mM magnesium chloride, 25 mM disodiumEDTA, 1 mM and CMP-[14C]NeuAc 560 μM (3 μCi/mmol). Polyosialic acid synthesis was measured by sucrose ultracentrifugation from the reaction mixture on Whatman No. 3MM paper, followed by descending chromatography in Solvent System I, as described previously (2). [14C]-labeled polyosialic acid, which was chromatographically identical, remained at the origin and was quantitated by scintillation counting. For detergent solubilization, membrane fractions were first washed on ice, sedimented by centrifugation at 27,000 g for 60 minutes; the brownish, translucent pellet was then reuspended in TMD buffer to a final concentration of 20 mg protein/ml.

Depolymerization of [14C]-Polyosialic Acid with Endo-N-acetylmuramidase (Endo-N) - Solubilized polyosialic acid that had been freed of detergent by chromatography over Extrait-Gel D (Pharmacia) was used in the presence of 50 mM Tris-Cl, pH 8.6 and 100 μM EDTA to test the efficiency of the Endo-N enzymes. One unit of Endo-N catalyzes the release of 1 nmol of NeuAc per minute at 37°C from labeled polyosialic acid (5). Endo-N digestion was monitored by comparison of the rates of digestion of labeled polyosialic acid on Whatman 3MM paper and chromatography in Solvent System I. Radioactivity that remained at the origin was determined by scintillation counting. Under these conditions, 14C-stained/stained membranes were digested with 20% SDS, 50 mM Tris-Cl, pH 8.0, 0.5% Triton X-100 in the presence of Endo-N treated sialyl polymers were passed over a Bio-Gel P10 column (250 ml, Bio-Rad). Approximately 5% of the [14C]NeuAc appeared as sialylmucins. The remaining unidentified material eluted in the void volume and was designated 'Sia acceptor.' The [14C]NeuAc in Sia-acceptor was presumed to be linked to endogenous acceptors and subsequent studies were carried out on this material.

Deamination of Sia residues or deamination by sequential proteinase enzyme was removed by SDS-polyacrylamide gel electrophoresis. A control sample not treated with Endo-N was also run. Radioactivity in the gel was determined by slicing the gel into 1 cm strips, transferring to Whatman 3MM paper, and dual channel scintillation counting in a Packard Model 9600 Liquid Scintillation Counter.

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Protein, Starch Acid and Ketodeoxyoctonate Determinations: Protein was determined using the modified Lowry assay (BCA, Pierce). Starch acid was quantitated according to the procedure of Skaar and Mohse (27). Ketodeoxyoctonate was determined according to Kerkhoven et al. (28).

Chromatography: Paper chromatograms were developed according to Whatman No. 3MM paper. The following solvent systems were used: (a) Solvent system I: ethanol/1M ammonium acetate pH 7.5 (5:3), Solvent system II butyric acid acetate-acetic acid/H2O (3:1:0.5). Solvent system I was used to separate CMP-NeuAc (80 0.5%) or oligosaccharide acid from polysialic acid or Sia-acceptor, which were chromatographically immobile. Solvent system II was used to separate NeuAc from NeuAc2, which had an Rf NeuAc of 1.7.

Chromatography on Biogel P-100 (1 x 40 cm) was carried out in 50 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol and 0.05% SDS as elution buffer. Chromatography on Ultragel AcA 4B (1 x 20 cm) was run in 50 mM Tris-HCl, pH 8.0, 0.1 M sodium chloride and 0.2% Triton X-100. Chromatography on Biogel P-100 (1 x 60 cm) was carried out in 50 mM Tris-HCl, pH 8.0, 0.2 M sodium chloride. Chromatography on Sephadex G-100 (100 cm) was run in 50 mM Tris-HCl, pH 7.6, 100 mM NaCl.  Chromatography on Dextran G-100 (100 cm) was run in 50 mM Tris-HCl, pH 7.6, 100 mM NaCl.

Electrophoresis: Starch acid and its reduced alcohol, stialyl [DIAc-3OH] were separated by thin-layer electrophoresis on Whatman No. 3 MM paper saturated with 0.12 M sodium tetraborate, pH 9.2 as previously described (7). Oligosaccharides of NeuAc Gp1 and Gp2 were separated by electrophoresis on DE52 paper (Whatman) in Solvent System IV (pyridine-acetic acid-water, 200:10:1800, pH 6.0). Electrophoresis was carried out on a Raven flat plate electrophoresis apparatus at 40V/cm for approximately 3 hours.
Biosynthesis of the polysialic acid capsule in Escherichia coli K1. The endogenous acceptor of polysialic acid is a membrane protein of 20 kDa.
C Weisgerber and F A Troy