Biosynthesis of Human Sialophorins and Analysis of the Polypeptide Core†

Eileen Remold-O'Donnell,*,‡,§,⊥ Dianne Kenney,‡ and Fred S. Rosen‡,§,∥

The Center for Blood Research and Division of Immunology, The Children's Hospital, and Departments of Pediatrics and Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT: Biosynthesis was examined of sialophorin (formerly called gpL115) which is altered in the inherited immunodeficiency Wiskott–Aldrich syndrome. Sialophorin is >50% carbohydrate, primarily O-linked units of sialic acid, galactose, and galactosamine. Pulse-labeling with [^{35}S]methionine and chase incubation established that sialophorin is synthesized in CEM lymphoblastoid cells as an M_r 62 000 precursor which is converted within 45 min to mature glycosylated sialophorin, a long-lived molecule. Experiments with tunicamycin and endoglycosidase H demonstrated that sialophorin contains N-linked carbohydrate (approximately two units per molecule) and is therefore an *N,O*-glycoprotein. Pulse-labeling of tunicamycin-treated CEM cells together with immunoprecipitation provided the means to isolate the [^{35}S]-methionine-labeled polypeptide core of sialophorin and determine its molecular weight (58 000). This datum allowed us to express the previously established composition on a "per molecule" basis and determine that sialophorin molecules contain ~520 amino acid residues and ≥100 O-linked carbohydrate units. A recent study showed that various blood cells express sialophorin and that there are two molecular forms: lymphocyte/monocyte sialophorin and platelet/neutrophil sialophorin. Biosynthesis of the two forms was compared by using sialophorin of CEM cells and sialophorin of MOLT-4 cells (another lymphoblastoid line) as models for lymphocyte/monocyte sialophorin and platelet/neutrophil sialophorin, respectively. The time course of biosynthesis and the content of N units were found to be identical for the two sialophorin species. [^{35}S]Methionine-labeled polypeptide cores of CEM sialophorin and MOLT sialophorin were isolated and compared by electrophoresis, isoelectrofocusing, and a newly developed peptide mapping technique. The polypeptides were indistinguishable, strongly indicating that the two sialophorin species contain identical polypeptide cores and suggesting that their differences arise through the action of Golgi region enzymes.

Sialophorin is a major surface glycoprotein of human T lymphocytes that is altered in patients with the X-linked disease Wiskott–Aldrich syndrome (Parkman et al., 1981) (WAS).[1] These patients display severe dysfunctions of T lymphocytes and platelets (Wiskott, 1937; Cooper et al., 1968). Sialophorin was undetectable, or reduced in quantity, and/or found in an altered form on lymphocytes of eight WAS patients (Remold-O'Donnell et al., 1984). A molecule indistinguishable from sialophorin of normal lymphocytes has been identified on the lymphoblastoid cell line CEM, it was purified to homogeneity, and its composition has been determined (Remold-O'Donnell et al., 1986). Sialophorin of CEM cells has very high content of serine, threonine, and proline. Greater than 50% of the molecule is carbohydrate, primarily sialic acid, galactose, and galactosamine in O-glycosidic linkage.

Recent studies showed further that sialophorin is not exclusively a lymphoid molecule but is also expressed on other blood cells, including platelets, but excluding erythrocytes (Remold-O'Donnell et al., 1987). Two forms of sialophorin were defined on normal blood cells; these are lymphocyte/monocyte sialophorin (apparent M_r 115 000) and neutrophil/platelet sialophorin (apparent M_r 135 000).

In the current study, we use pulse-labeling of CEM cells with [^{35}S]methionine to investigate the pathway of sialophorin biosynthesis, i.e., the biosynthetic precursor, the time course of synthesis, and the stability of the molecule. The polypeptide core of sialophorin is also examined. The biosynthesis of the second naturally occurring sialophorin species (neutrophil/platelet sialophorin) was examined by using the cell line MOLT-4 as a model. Electrofocusing, electrophoresis, and peptide mapping are used to compare the polypeptide cores of the two molecular forms of sialophorin.

Experimental Procedures

Cell Lines. The T lymphoblastoid cell lines CEM (Foley et al., 1965) and MOLT-4 (Minowada et al., 1972; ATCC) were grown in Dulbecco's minimum essential medium with 4.5 mg/mL glucose, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum and washed twice before use in Hanks' balanced salt solution.

Incorporation of [^{35}S]Methionine into Lymphoblastoid Cell Proteins. Cells were preincubated for 15 min in methionine-free Eagle's minimum essential medium with 100 units/mL penicillin, 100 μg/mL streptomycin, and 5% dialyzed fetal bovine serum; they were pelleted and cultured at 10^7/mL in the same medium with 200–1000 μCi/mL [^{35}S]methionine (900–1200 Ci/mmol) for 10 min. [^{35}S]Methionine incorporation was terminated with 4 volumes of medium with 30 mg/L nonradiolabeled methionine, and portions of the cell suspensions were "chase" cultured. Incorporated [^{35}S]-

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* Correspondence should be addressed to this author at The Center for Blood Research, The Children's Hospital, Boston, MA 02115.

‡ The Center for Blood Research, The Children's Hospital.

§ Division of Immunology, The Children's Hospital.

∥ Department of Pediatrics, Harvard Medical School.

⊥ Department of Biological Chemistry, Harvard Medical School.

[1] Abbreviations: WAS, Wiskott–Aldrich syndrome; NP-40, detergent Nonidet P-40; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PNGase F, peptide:*N*-glycosidase F; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Endo H, endo-β-*N*-glycosidase H.

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methionine was quantified (Roberts & Paterson, 1973).

Tunicamycin and Monensin Treatment of Cells. Cells were pretreated with 1.0 μg/mL tunicamycin (Calbiochem) for 4 h. Tunicamycin at 1.0 μg/mL and monensin at 1.0 μM (Calbiochem) were present during the methionine-free 15-min preculture and the pulse-labeling and chase cultures. Neither agent affected cell viability (≥90% by trypan blue exclusion) or [^{35}S]methionine incorporation.

Radioiodination, Sialidase Treatment, and Cell Lysis. Cells were labeled with ^{125}I by lactoperoxidase and H_2O_2 and were treated with sialidase as described (Remold-O'Donnell et al., 1984). Cells [(2–3) × 10^7] were lysed with 1 mL of 0.5% NP-40, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM diisopropyl fluorophosphate, and 3 mM iodoacetamide by pipetting for 3 min at 22 ± 2 °C and for 8 min at 4 °C. Insoluble material was removed at 12000*g* for 13 min.

Antibodies. L10 is a mouse monoclonal IgG_1 antibody which immunoprecipitates native and sialidase-treated sialophorin (Remold-O'Donnell et al., 1984) but does not immunoprecipitate the biosynthetic precursor (not shown).

A polyclonal rabbit anti-sialophorin antiserum was generated by injecting a New Zealand female rabbit subcutaneously with homogeneous asialosialophorin [100 μg of protein; purification in Remold-O'Donnell et al. (1986)] emulsified with complete Freund's adjuvant and boosted after 2 and 4 weeks by subcutaneous injection of 25 μg in incomplete Freund's adjuvant. Serum, collected 1–4 weeks thereafter, was found to immunoprecipitate precursor, native sialophorin, and asialosialophorin.

Immunoprecipitation. Protein A–agarose (Pierce Chemical Co.) was incubated for 1 h at 22 ± 2 °C with rabbit anti-sialophorin antiserum or normal rabbit serum (5 μL of serum/10 μL of resin); the resulting complexes were washed once in buffer B (10 mM Tris-HCl, pH 8.6, 0.1% SDS, 0.05% NP-40, and 300 mM NaCl) and once in buffer A (12 mM sodium phosphate buffer, pH 7.4, and 200 mM NaCl) with 1 mg/mL albumin. The complexes in 10 volumes of buffer A with albumin were combined with 40–100 μL of [^{35}S]-methionine-labeled cell extracts for 1–3 h at 22 ± 2 °C, and the resulting resin–antibody–antigen complexes were washed twice with buffer B. For SDS electrophoresis, the complexes were extracted at 100 °C for 2 min with 1% SDS in 60 mM Tris–glycine buffer, pH 6.8. For isoelectrofocusing, the complexes were extracted twice with 9.5 M urea, 0.5% NP-40, and 60 mM dithiothreitol at 50 °C for 20 min. For glycosidase treatment, the complexes were washed with 0.15 M NaCl and extracted with 1% SDS at 100 °C for 2 min. ^{125}I-Labeled cell extracts were immunoprecipitated as described with L10 antibody, fixed *Staphylococcus aureus*, and rabbit anti-mouse IgG antiserum (Remold-O'Donnell et al., 1984).

Glycosidase Treatments. Extracts of immunoprecipitates (1% SDS) were diluted 10-fold with 25 mM sodium citrate buffer, pH 5.5, and incubated at 37 °C for 3 h with 5–50 milliunits/mL endo-β-*N*-glycosidase H (Endo H, *Streptomyces plicatus* from plasmid pkCE3-transformed *Escherichia coli*; Miles Laboratories). The reaction was terminated at 100 °C after addition of Tris–glycine buffer, pH 6.8, and SDS to 1%. The extracts (1% SDS) were diluted 5-fold with 1.9% NP-40 and Tris-HCl, pH 8.6, and incubated with 6–4000 milliunits/mL peptide:*N*-glycosidase F (PNGase F; *Flavobacterium meningosepticum*; Genzyme Corp.) at 37 °C for 3 or 16 h. The reaction was terminated as above with SDS at 3%.

Gel Electrophoresis. SDS electrophoresis conditions were as described (Laemmli, 1970; Remold-O'Donnell, 1985) with 2.2-mm gels of 7.5% polyacrylamide or 1.5-mm gels of 14% polyacrylamide (peptide mapping). Gels containing ^{35}S-labeled proteins were impregnated for fluorography with 10% w/v 2,5-diphenyloxazole in dimethyl sulfoxide (Bonner & Laskey, 1974).

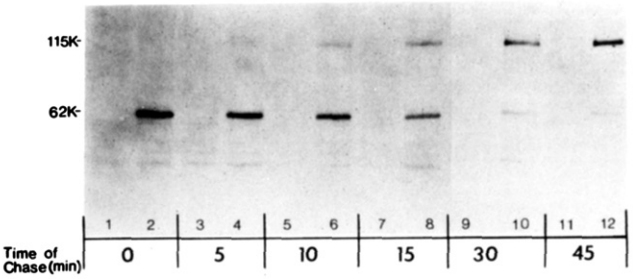

FIGURE 1: Components of CEM cells labeled with [^{35}S]methionine and immunoprecipitated with anti-sialophorin antiserum. CEM cells were pulse-labeled (10 min) and "chase" incubated for the indicated times. Shown is a fluorograph of an SDS electrophoresis gel of material immunoprecipitated from cell extracts by normal rabbit serum (lanes 1, 3, 5, 7, 9, and 11) or rabbit anti-sialophorin antiserum (lanes 2, 4, 6, 8, 10, and 12). The molecular weights of the precipitated ^{35}S-labeled components are indicated on the left. Note the M_r 62000 component which is present after pulse-labeling and disappears with time of chase incubation as the M_r 115000 component appears.

Two-Dimensional Electrophoresis. In a modified procedure (O'Farrell, 1975), the sample was applied to the basic end of a 2.4-mm tube gel containing 3.3% polyacrylamide, 9.2 M urea, 3.2% Triton X-100, 0.3% ampholytes of range pH 4–6, 4.2% ampholytes of range pH 5–7, and 0.5% ampholytes of range pH 3.5–10 and focused for 20 h at 400 V and for 2 h at 1000 V. The pH values are averages of water extracts of 5-mm slices of duplicate gels. The focused gels were incubated in 80 mM Tris-HCl, pH 6.8, 2% SDS, and 5% mercaptoethanol for 50 min at 22 ± 2 °C and separated on SDS electrophoresis.

Peptide Mapping. Particulate immunoprecipitates consisting of protein A–agarose, rabbit antiserum, and ^{35}S-labeled sialophorin polypeptides were washed with 0.15 M NaCl and incubated at 37 °C for 20 min with agitation in 10 volumes of 20 mM Tris-HCl, pH 7.4, and 75 mM NaCl with 0, 0.1, 0.3, or 1.0 μg/mL trypsin (bovine, TPCK treated, 225 units/mL, Cooper Biomedical) or 2, 6, or 20 μg/mL *S. aureus* V-8 protease (ICN Biomedicals). The reaction was terminated with 0.1 volume of 20 mM diisopropyl fluorophosphate; the complexes were extracted at 100 °C for 2 min with 0.1 volume of 20% SDS. The extracted peptides were analyzed by SDS electrophoresis (14% polyacrylamide gels) and fluorography.

RESULTS

[^{35}S]Methionine Incorporation into Sialophorin. When cells of the lymphoblastoid line CEM were pulse-labeled with [^{35}S]methionine for 10 min, an apparent sialophorin precursor, i.e., a molecule precipitated by rabbit anti-sialophorin antiserum, but not by normal serum, was detected (Figure 1, lanes 1 and 2). The apparent molecular weight of the sialophorin precursor is 62000. On "chase" incubation, the precursor disappeared over 45 min concomitant with the appearance of a ^{35}S-labeled molecule which comigrates with sialophorin. The labeled mature molecule was verified as sialophorin by precipitation by L10 monoclonal antibody and sensitivity to sialidase (not shown). The precursor was unaffected by these treatments but was verified as a sialophorin species since homogeneous sialophorin (18 μg/mL) inhibited its precipitation by rabbit antiserum (not shown). Thus, sialophorin is synthesized in CEM cells as an M_r 62000 precursor which is rapidly converted to the mature glycosylated protein of apparent M_r 115000. The large difference in ap-

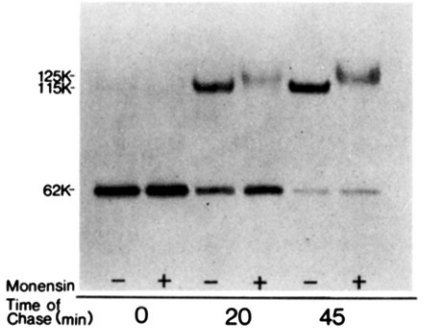

FIGURE 2: [^{35}S]Methionine-labeled components of monensin-treated and untreated CEM cells immunoprecipitated with anti-sialophorin antiserum. The cells were pulse-labeled with [^{35}S]methionine for 10 min and chase incubated as indicated. Shown is a fluorograph of an SDS electrophoresis gel with the molecular weight of the precipitated ^{35}S-labeled components indicated.

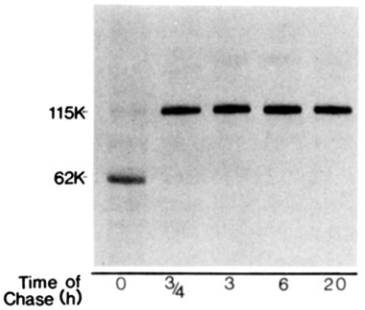

FIGURE 3: Components of CEM cells pulse-labeled (10 min) with [^{35}S]methionine, "chase" incubated, and immunoprecipitated with anti-sialophorin antiserum. Details as in Figures 1 and 2.

parent molecular weight (SDS mobility) of the precursor and mature species is not surprising because the earliest detectable precursor is expected to lack the multiple O-linked carbohydrate units added by Golgi region enzymes (Roth, 1984). No intermediate biosynthesis species were detected (Figure 1).

Monensin Treatment. When CEM cells were treated with monensin which blocks glycoprotein transport through middle Golgi regions (Griffiths et al., 1983), synthesis of the M_r 62000 precursor and its time-dependent disappearance were unaffected, but mature M_r 115000 sialophorin did not form. In its place, a species of M_r ~125000 accumulated (Figure 2), which might be a short-lived biosynthesis intermediate not detectable under normal conditions.

Turnover of Sialophorin. When [^{35}S]methionine-labeled CEM cells were chase incubated for periods up to 20 h, no degradation of mature sialophorin was detected (Figure 3), indicating that sialophorin is a long-lived molecule in these cells.

Tunicamycin Treatment. The bulk of sialophorin carbohydrates are O-linked units containing sialic acid, galactose, and *N*-acetylgalactosamine (Remold-O'Donnell et al., 1984, 1986). In order to determine whether sialophorin also has N-linked units, biosynthesis of the molecule was examined in cells treated with tunicamycin, an inhibitor of N-glycosylation (Tkacz & Lampen, 1975). In tunicamycin-treated CEM cells, an M_r 58000 species was detected instead of the normal M_r 62000 precursor (Figure 4). The altered molecular weight of the precursor indicates that sialophorin contains N-linked carbohydrate; the difference in molecular weight indicates that the normal precursor contains approximately 4000 daltons of N-linked carbohydrate.

It should be noted that the molecular weights determined by comparative SDS electrophoretic mobility for the normal precursor and the "tunicamycin precursor", unlike those of

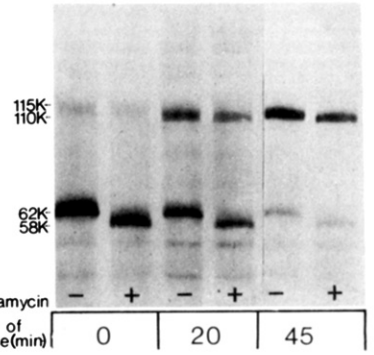

FIGURE 4: Components of tunicamycin-treated and untreated CEM cells pulse-labeled (10 min) with [^{35}S]methionine, chase incubated, and immunoprecipitated with anti-sialophorin antiserum. Details as in Figures 1 and 2.

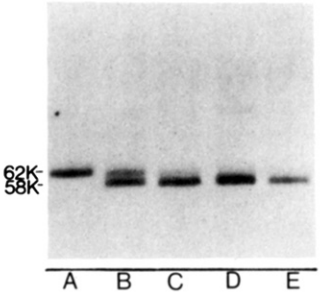

FIGURE 5: Effect of Endo H and PNGase F on the sialophorin precursor isolated from CEM cells pulse-labeled with [^{35}S]methionine. Shown is a fluorograph of an SDS electrophoresis gel of the precursor after incubation at 37 °C for 3 h with (A) no additive, (B) Endo H (6 milliunits/mL), (C) Endo H (12 milliunits/mL), (D) Endo H (24 milliunits/mL), and (E) PNGase F (250 milliunits/mL).

mature sialophorins, are expected to be close to their true molecular weights since these species contain little or no carbohydrate, respectively (Weber & Osborn, 1969).

The M_r 58000 "tunicamycin precursor" was converted on chase incubation to apparent M_r 110000 (Figure 4; molecular weight determination based on four experiments), suggesting that N units are not required for sialophorin maturation.

Treatment with Endo H and PNGase F. N-Glycosylation was also examined by the use of endoglycosidase H (Endo H) which cleaves N-linked "high-mannose" units (Tarentino et al., 1974) and peptide:*N*-glycosidase F (PNGase F) which cleaves all types of N units (Tarentino et al., 1985). Both glycosidases converted the M_r 62000 precursor to M_r 58000 (Figure 5), thus verifying the presence and amount of N-linked units. Their sensitivity to Endo H demonstrates that the N units on the precursor are high-mannose units. Mature sialophorin was not detectably affected by Endo H at levels 5-fold sufficient to affect the precursor (three experiments, not shown). Surprisingly, mature sialophorin was also unaffected by PNGase F at levels 8-fold and 20-fold sufficient to affect the precursor (two experiments; not shown).

Different Sialophorin Species on CEM and MOLT-4 Cells. In order to compare the biosynthesis of the two naturally occurring blood cell forms of sialophorin, cell lines were sought which could serve as models for the two species. CEM sialophorin was used as the model for lymphocyte/monocyte sialophorin. Whereas ^{125}I-labeled sialophorin of CEM cells, isolated by L10 immunoprecipitation, displays apparent M_r 115000 and is converted by sialidase to apparent M_r 150000, sialophorin of MOLT-4 cells (another lymphoblastoid line) is of apparent M_r 135000 and is converted by sialidase to apparent M_r 170000 (Figure 6). On the basis of these shared properties (Remold-O'Donnell et al., 1987), sialophorin of

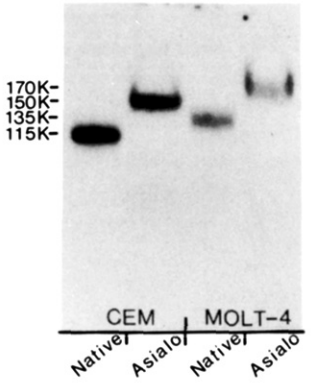

FIGURE 6: Immunoprecipitates (L10 antibody) of extracts of CEM and MOLT-4 cells which have been labeled in surface molecules with ^{125}I and treated without (native) or with (asialo) sialidase. Shown is an autoradiograph of an SDS electrophoresis gel.

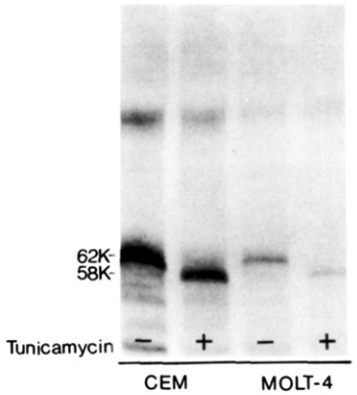

FIGURE 7: SDS electrophoresis of immunoprecipitates (anti-sialophorin antiserum) of tunicamycin-treated and untreated CEM and MOLT-4 cells which have been pulse-labeled with [^{35}S]methionine.

MOLT-4 cells was chosen as a model for platelet/neutrophil sialophorin.

Comparison of Biosynthesis of CEM Sialophorin and MOLT Sialophorin. The sialophorin precursor isolated from MOLT-4 cells which had been pulse-labeled with [^{35}S]-methionine was found to comigrate with the sialophorin precursor of CEM cells (M_r 62 000; Figure 7), suggesting that the precursors are identical. Further, the sialophorin precursor of tunicamycin-treated MOLT-4 cells was found to comigrate with the sialophorin precursor of tunicamycin-treated CEM cells (M_r 58 000; Figure 7), suggesting that the "tunicamycin precursors" are identical. These findings also demonstrate that MOLT sialophorin and CEM sialophorin contain the same number of N-linked carbohydrate units. Since tunicamycin precursors lack *N*-carbohydrate and *O*-carbohydrate (added by Golgi enzymes), these species are equivalent to the polypeptide portion (polypeptide core) of mature molecules. Comigration on SDS electrophoresis of the tunicamycin precursors suggests that the polypeptide cores of CEM sialophorin and MOLT sialophorin are identical.

On chase incubation, the sialophorin precursor of MOLT-4 cells is converted to mature MOLT sialophorin (M_r 135 000) within 45 min (not shown), suggesting that the time course of maturation is similar for MOLT sialophorin and CEM sialophorin.

Isoelectrofocusing Used To Compare CEM Sialophorin–Polypeptide and MOLT Sialophorin–Polypeptide. The polypeptide portion of CEM sialophorin and the polypeptide of MOLT sialophorin were examined by isoelectrofocusing. Radiolabeled CEM sialophorin–polypeptide generated by [^{35}S]methionine pulse-labeling of tunicamycin-treated cells was found to focus on two-dimensional gels (isoelectrofocusing–electrophoresis) as a defined spot at pH 6.8 (Figure 8). Radiolabeled MOLT sialophorin–polypeptide was also found to focus as a defined spot at pH 6.8, providing further evidence to suggest the identity of the two polypeptides.

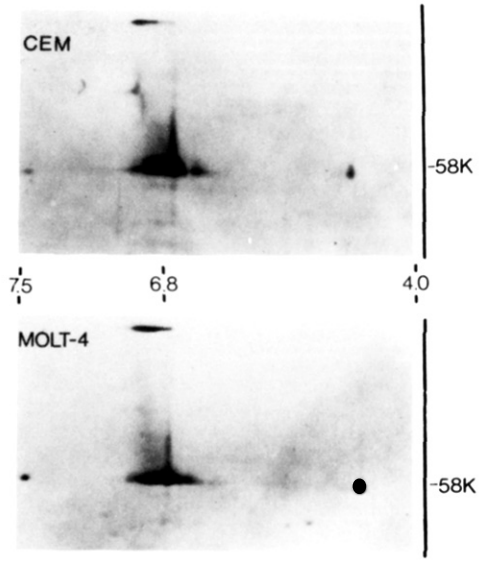

FIGURE 8: Isoelectrofocusing–electrophoresis of CEM sialophorin–polypeptide (upper panel) and MOLT sialophorin–polypeptide (lower panel). The polypeptides were immunoprecipitated from tunicamycin-treated cells pulse-labeled with [^{35}S]methionine. Shown is a fluorograph with isoelectrofocusing on the horizontal axis (pH gradient is indicated) and SDS electrophoresis on the vertical axis. Note that CEM sialophorin–polypeptide and MOLT sialophorin–polypeptide both focus at pH 6.8. A minor species in both preparations focused at pH 5.0 in three of three experiments.

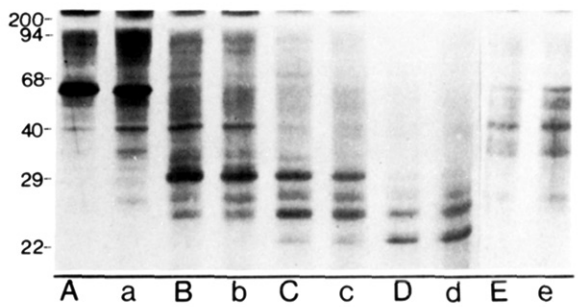

FIGURE 9: Peptide fragments of CEM sialophorin–polypeptide (lanes A, B, C, D, and E) and MOLT sialophorin–polypeptide (lanes a, b, c, d, and e). The [^{35}S]methionine-labeled polypeptides were prepared as in Figure 8. Shown is a fluorograph of an SDS electrophoresis gel (14% polyacrylamide) of peptide fragments generated with (A, a) no additive, (B, b) 2 μg/mL V-8 protease, (C, c) 6 μg/mL V-8 protease, (D, d) 20 μg/mL V-8 protease, and (E, e) 0.1 μg/mL trypsin.

Peptide Mapping Used To Compare CEM Sialophorin–Polypeptide and MOLT Sialophorin–Polypeptide. Peptide mapping was used to probe for differences in CEM sialophorin–polypeptide and MOLT sialophorin–polypeptide. The [^{35}S]methionine-labeled polypeptides were treated with varying concentrations of two proteases, and the resultant spectra of small peptides were compared by SDS electrophoresis. All tryptic peptides generated from the MOLT sialophorin–polypeptide were found to comigrate with the tryptic peptides generated from the CEM sialophorin–polypeptide (Figure 9). All *S. aureus* V-8 peptides generated from MOLT sialophorin–polypeptide comigrated with the V-8 peptides generated from CEM sialophorin–polypeptide (Figure 9). The identity of the four pairs of peptide maps provides further evidence for the identity of the polypeptide cores of CEM

Table I: Amino Acid and Carbohydrate Composition of Sialophorin of CEM Cells[a]

residue	no. of residues/ 100 amino acids	no. of residues/ molecule
Asx	6.5	34
Glx	9.1	48
His	1.9	10
Lys	3.3	17
Arg	3.9	20
Ser	12.5	66
Thr	12.5	66
Pro	9.9	52
Ala	7.1	37
Cys	ND[b]	ND[b]
Gly	9.3	49
Tyr	1.4	7
Val	7.0	37
Ile	3.5	18
Leu	7.8	41
Phe	1.8	9
Met	1.8	9
Trp	ND[b]	ND[b]
GalNAc	22.5	117
galactose	24.6	128
GlcNAc	6.2	32
mannose	1.8	9
fucose	1.3	7
sialic acid	ND[c]	ND[c]

[a] Residues per 100 amino acids for sialophorin from CEM cells are from Remold-O'Donnell et al. (1986). Residues per molecule were calculated by setting the sum of the amino acids at 58 000, the molecular weight of the polypeptide core. [b] ND, not determined. [c] Asialosialophorin was analyzed.

sialophorin and MOLT-4 sialophorin.

Discussion

The biosynthesis was examined of sialophorin, the heavily O-glycosylated protein (>50% carbohydrate) which is altered in lymphocytes of patients with the inherited immunodeficiency Wiskott–Aldrich syndrome (Parkman et al., 1981; Remold-O'Donnell et al., 1984). Pulse-labeling with [^{35}S]methionine and chase incubation established that sialophorin is synthesized as an M_r 62 000 precursor in CEM lymphoblastoid cells; the precursor is rapidly (45 min) converted to mature sialophorin which was found to be a long-lived component of CEM cells; i.e., no degradation was detected after 20-h culture.

Tunicamycin and Endo H experiments demonstrated that the sialophorin precursor contains N-linked carbohydrate; there are ~4000 daltons of high-mannose-type N-linked units on the precursor or approximately two N units per molecule, assuming ~2400 daltons per unit ($Glc_3Man_9GlcNAc_2$; Robbins et al., 1977). The resistance of mature sialophorin to Endo H which cleaves only high-mannose units (Tarentino et al., 1974) cannot be interpreted because the mature molecule is also resistant to PNGase F which cleaves all classes of N-linked units (Tarentino et al., 1985). The resistance to both enzymes may be due to inaccessibility of the sites in the heavily glycosylated mature molecule.

The current study represents an effective approach with general applicability for studying the polypeptide portion of *N,O*-glycoproteins. Such studies are hampered by the lack of convenient agents to inhibit O-glycosylation and to remove O units. In the case of sialophorin, attempts to remove O-linked chains by chemical means (the β-elimination reaction and treatment with trifluoromethanesulfonic acid) led to degradation of the polypeptide (not shown). Although precursor molecules free of O-linked carbohydrate added in the Golgi may be short-lived trace cellular components, they can be obtained as the sole radiolabeled species by a combination of [^{35}S]methionine pulse-labeling followed by immunoprecipitation with an appropriate antibody. The normal precursor contains N-linked carbohydrate added concomitant with polypeptide elongation, but N-glycosylation can be inhibited with tunicamycin, or the units can be removed by Endo H or PNGase F. In the current study of sialophorin, the tunicamycin technique and the Endo H/PNGase F treatments were both used to generate the carbohydrate-free polypeptide (polypeptide core). The molecular weight of the sialophorin polypeptide core (58 000) was determined by SDS electrophoretic mobility; the reliability of this technique for proteins (without carbohydrate) has been well documented (Weber & Osborn, 1969).

The amino acid and carbohydrate composition of sialophorin isolated from CEM cells has been previously established (Remold-O'Donnell et al., 1986); the asialo molecule is 52% carbohydrate, primarily galactose–*N*-acetylgalactosamine units, and 48% peptide with a very high content of serine, threonine, and proline. Knowledge of the molecular weight of the polypeptide core made it possible to assemble the compositional information established in previous studies in the form of an overall quantitative description of the molecule (Table I). These data indicate that sialophorin contains 520 amino acid residues per molecule, including 132 serine and threonine residues, 128 residues of galactose, and 117 residues of *N*-acetylgalactosamine. These data, together with studies of the carbohydrate (Remold-O'Donnell et al., 1984), indicate that there are >100 O-linked sialylated galactose–*N*-acetylgalactosamine units per molecule.

Two molecular forms of sialophorin were recently found on blood cells, lymphocyte/monocyte sialophorin of apparent M_r 115 000 and platelet/neutrophil sialophorin of apparent M_r 135 000 (Remold-O'Donnell et al., 1987). The equal shift in electrophoretic mobility on removal of sialic acid (Remold-O'Donnell et al., 1987; Figure 6) indicated that the two sialophorin species have approximately the same number of sialylated O-linked carbohydrate units. In the present study, the two sialophorin species were found to contain the same number of N-linked carbohydrate units. The polypeptide cores of the two sialophorin species were found to be indistinguishable by three criteria, including peptide mapping. This constitutes very strong evidence that the polypeptide regions of the two sialophorin species are identical and suggests that the difference in the two species arises due to the action of Golgi region enzymes. Possibilities to explain the higher apparent molecular weight of platelet/neutrophil/MOLT sialophorin include additional neutral saccharide residues, probably on O-linked units since there are only two N-linked units, and/or sulfate, phosphate, lipid, or other moieties.

The description of sialophorin synthesis and stability in cell lines will facilitate its analysis in normal and WAS lymphocytes. The characteristics of the polypeptide core (Figures 8 and 9) will serve as standards in the search for sialophorin defects in WAS lymphocytes. The combined techniques of biosynthetic pulse-labeling in the presence of tunicamycin together with peptide mapping as used in this study provide a generally applicable approach for analysis of the polypeptide core of *N,O*-glycoproteins.

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Continuous Monitoring of Adenosine 5′-Triphosphate in the Microenvironment of Immobilized Enzymes by Firefly Luciferase†

Claude Aflalo and Marlene DeLuca*
Department of Chemistry, University of California, San Diego, La Jolla, California 92093
Received December 9, 1986; Revised Manuscript Received February 25, 1987

ABSTRACT: The study of enzymes sequestered in artificial or biological systems is generally conducted by indirect methodology with macroscopic measurements of reactants in the bulk medium. This paper describes a new approach with firefly luciferase to monitor ATP concentration directly in the microenvironment of enzymes producing or consuming ATP. Upon addition of ATP to immobilized firefly luciferase, the onset of light production is slower than that observed with the soluble enzyme, due to a slower diffusion of ATP to the immobilized enzyme. With immobilized pyruvate kinase, a relative accumulation of ATP inside the beads is demonstrated, as measured with coimmobilized firefly luciferase. The accumulation of product (ATP) is enhanced when the bead suspension is not stirred. This ATP in the beads is relatively inaccessible to soluble hexokinase added to the bulk medium. Similarly, a rapid ATP depletion in the microenvironment of immobilized hexokinase is demonstrated. This microscopic event is kinetically distinguishable from the slower macroscopic depletion of substrate in the bulk medium. The rate of depletion in the microenvironment depends on the local activity of the immobilized enzyme but not on the total amount of enzyme in suspension, as does the macroscopic phenomenon. The theoretical principles for the interaction of diffusion and catalysis in these systems are briefly summarized and discussed. These results are relevant to various molecular mechanisms proposed for membrane-bound enzyme action and regulation, derived from macroscopic kinetic measurements assuming a negligible diffusion control.

Enzymes and enzymic systems are frequently associated with membranous structures of definite topology (De Pierre & Ernster, 1977) and thus are associated with, or segregated into, different compartments within the cell. Consequently, in addition to enzymic steps, metabolic processes in situ include physical phenomena such as transport, partition, or diffusion of metabolites between the *loci* of the catalytic reactions (Katchalski et al., 1971; Engasser & Horvath, 1976; Goldstein, 1976; Laidler & Bunting, 1980). Unfortunately, due to great experimental difficulties, little is known at present about diffusional resistances inside the cell. Most metabolic studies are done through macroscopic measurements of the concentration of reactants in the bulk medium surrounding solubilized or particulate systems. These measurements reflect both local catalytic events and the transfer of reactants and intermediates between the individual catalytic units or complexes. The results of such studies are often interpreted in terms of steady-state enzyme kinetics, implicitly assuming a large excess of reactants over the enzymes and a homogeneous distribution of freely diffusible intermediates and products. This situation does not apply generally to cellular metabolism, and the physical phenomena are likely to be of physiological importance in view of the intricate structure of the cell and the large concentration of enzymes in vivo (Ottaway & Mowbray, 1977; Weber & Bernhard, 1982; Srere, 1984).

Immobilization of enzymes on solid supports allows the design of artificial, relatively simple and defined systems of

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* Author to whom correspondence should be addressed.

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