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PHOTOLABELING OF THE α-NEURAMINIDASE/β-GALACTOSIDASE COMPLEX FROM HUMAN PLACENTA WITH A PHOTOREACTIVE NEURAMINIDASE INHIBITOR

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SUMMARY: Photolabeling of the α-neuraminidase/β-galactosidase complex in human placenta (Verheijen, F.W. et al (1987) Eur. J. Biochem. 162, 63-67) was carried out using the radioactive photoprobe, 9-S-(4-azido-3,5-^{3}H-2-nitrophenyl)-5-acetamido-2,6 anhydro-2,3,5,9- tetradeoxy-9- thio-**D**-*glycero*-**D**-*galacto*-non-2-enonic acid. Two intensely labeled bands at 61 and 46 kD were detected with autoradiography. Labeling of the 46 kD protein was blocked with the inclusion of the surfactant Triton X-100 in the photolysis mixture, indicating a nonspecific, hydrophobic interaction. The 61 kD protein was protected from labeling only when the neuraminidase inhibitor 2,3 dehydro N-acetyl neuraminic acid (1mM) was present during photolysis. These results suggest that the neuraminidase activity resides among the proteins in the 61 kD molecular weight range comigrating with the lysosomal β-galactosidase, under denaturing conditions.

INTRODUCTION: The lysosomal neuraminidase serves a primary role in the catabolism of glycoproteins and glycolipids, cleaving the terminal sialic acid residues from the oligosaccharide sidechains of both classes of glycoconjugates. In addition, the enzyme is of great importance because of its involvement in the inherited neurodegenerative storage disorder, sialidosis (for a review see reference 1). Isolation and the complete purification of the enzyme has proved to be difficult because the protein is remarkably hydrophobic, extremely heat labile, and present in relatively low abundance. As a result, this hydrolase is one of the most poorly understood of all lysosomal enzymes. A soluble neuraminidase activity, with an acid pH optimum, has been discovered in heat treated extracts of human placenta (2,3). The enzyme has been partially purified from this source as a complex with acid β-galactosidase and several other proteins . Although the complex has been well studied (4,5) and a structural model for the organization of the protein components has been developed (6), identifying the neuraminidase in the mixture has been difficult since obtaining a homogenous, catalytically

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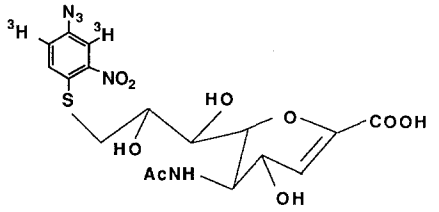


Figure 1. Structure of photoprobe 9-PANP-Neu5Ac2en.

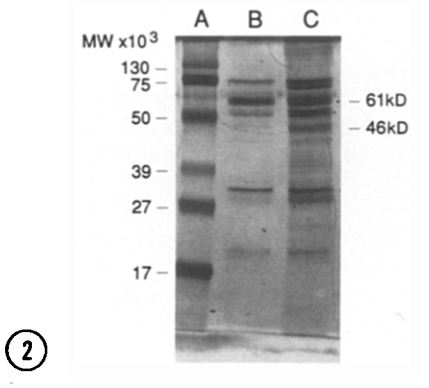
functional protein has not been possible. We have previously suggested that affinity labeling reagents which were derivatives of sialic acid might be useful for characterizing cellular neuraminidases and other sialic acid binding proteins (7). Several photolabile sialic acid compounds have been prepared (7,8) and one has been shown to be a potent inhibitor of the lysosomal neuraminidase in cultured skin fibroblasts (9-11). The latter was obtained by modifying, 2,3 dehydro-N-acetyl neuraminic acid (Neu5Ac2en), incorporating an aryl azide in thio ether linkage at the C-9 position of the molecule. We report here characterization of the placental α-neuraminidase/β-galactosidase complex using this affinity probe (Fig.1) to identify the neuraminidase among the proteins present in the complex.

MATERIALS AND METHODS

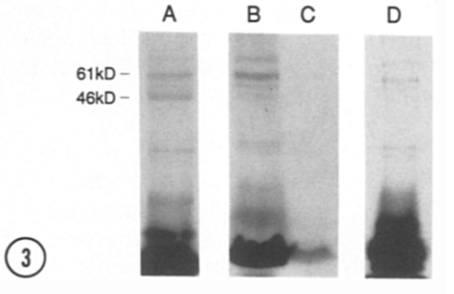
Reagents- Neu5Ac2en was prepared as previously described (8). 4-methyl-umbelliferyl-Neu5Ac was from Sigma Chemical Co. (St. Louis, Mo.). 4-fluoro-3-nitro (2,6-^{3}H) phenylazide, 52 Ci/mmol, was obtained from Amersham Corp. Arlington Heights, Ill.

Preparation of 9-S-(4-azido-3,5-^{3}H-2-nitrophenyl)-5- acetamido-2,6 anhydro-2,3,5,9-tetradeoxy-9- thio-**D**-*glycero*-**D**-*galacto*-non-2-enonic acid (^{3}H-9-PANP-Neu5Ac2en)- The radioactively labeled photoprobe was prepared using the procedure described elsewhere for unlabeled material (8), except that the reaction was carried out on a smaller scale. Methyl-5-acetamido-4,7,8-tri-O-acetyl-9-S-acetyl, 2,6, anhydro-2,3,5,9-tetradeoxy-9-thio-**D**-*glycero*-**D**-*galacto*-non-2-enonate (4.2 mg, 9.4 μmol) was combined with 4-fluoro-3-(2,6-^{3}H) nitro phenylazide (1.6 mg,8.9 μmol, at a specific activity of about 500 μCi/μmol) in 100 μl dry methanol in an amber reaction vessel. The solvent was removed with a stream of dry nitrogen and the residue dried over phosphorous pentoxide for 2 hr at room temperature. The reactants were resuspended in 100 μl of dry methanol and the solution cooled on ice. Methanolic sodium methoxide (9.4 μmol in 40 μl) was added and anhydrous conditions were maintained with a dry nitrogen purge. After 30 min, the solution was warmed to room temperature and the reaction allowed to proceed for 2 hr. The solvent was removed with nitrogen and the components dried under vacuum. Reacetylation was carried out with the addition of 150 μl pyridine/acetic anhydride, 1:1, v/v, at 4 °C. The reaction was allowed to proceed overnight at 4 °C and was subsequently terminated with the addition of 0.5 ml methanol. After 30 min at room temperature, the mixture was applied to a Dowex 50 column (0.5 cm X 2.0 cm) in methanol, eluting with methanol. Further purification was carried out using reversed phase HPLC chromatography, collecting the appropriate fractions which were previously identified using unlabeled material (8). About 2.9 mg or (49%) yield of the homogenous product was obtained. The purified peracetylated material was hydrolyzed with 0.3 N sodium hydroxide for 3 hr at room temperature. Salts and excess hydroxide were removed using a small Dowex 50 column. Removal of the solvent under vacuum gave about 1.7 mg of final product with a specific activity of about 400 μCi/μmol.

Photolysis of the complex with 9- PANP-Neu5Ac2en gave two intense radioactively labeled bands at 61 kD and 46 kD, Fig 3. When the surfactant, Triton X-100, was included in the photolysis mixture, the labeling of the 46 kD band was greatly reduced, and concomitantly the intensity of the 61 kD band was slightly increased (Fig. 3 lane B). The enhanced labeling of the 61 kD protein correlated with the observation that the neuraminidase activity increased about 25 % when assayed in the presence of the surfactant. The intensity of the 61 kD protein was greatly reduced when the competitive sialidase inhibitor, Neu5Ac2en and surfactant were included in the photolysis mixture (lane D). However, the labeling was not completely blocked at this concentration (1 mM) of inhibitor. This was not unexpected since both the photoprobe and Neu5Ac2en have similar affinities for the enzyme with nearly identical inhibitory constants of about 10 μM. Since the molar ratio of Neu5Ac2en to photoprobe is about 10 to 1, some labeling might be anticipated at about 1/10 the intensity of the control sample lacking Neu5Ac2en as the protecting reagent. No labeling was observed in a dark control, (Fig 3 lane C), which contained a complete photolysis mixture but was not irradiated prior to electrophoresis. Similarly, only background labeling was observed when the complex was heat denatured prior to photolysis (data not shown).



(2)



(3)

Figure 2. Acrylamide gel of the proteins present in the α-neuraminidase/β-galactosidase complex. Gel is stained with silver reagent as described in Methods. Lane A: Prestained molecular weight markers. Lane B: Placental preparation 1 used for photolabeling studies. Labe C: Placental preparation 2 used for sequence determinations. Shown are the location of the protein bands identified by autoradiography of the photolyzed reaction mixture.

Figure 3. Autoradiogram of the proteins in placental preparation 1 labeled with tritiated 9-PANP-Neu5Ac2en using the photolysis conditions as described in Methods. Lane A: Proteins labeled under standard conditions. Lane B: Proteins labeled in the presence of 1% Triton X-100. Lane C: Dark control, complete photolysis mixture with Triton X-100 maintained in the absence of light. Lane D: Photolysis mixture including Triton X-100 and 1 mM Neu5Ac2en. Gels which contained radioactive protein samples were not subjected to silver staining in order to increase the sensitivity during autoradiography . In these experiments the molecular weights of the protein bands which appeared on the X-ray film were determined with commercial prestained molecular weight markers that were included during electrophoresis. According to the manufacturer, determinations made with these standards can only be considered approximate (18).

Purification of the Placental Sialidase/β-Galactosidase Complex- The complex was purified according to the method of Verheijen (2) as modified by Potier (6). The preparation employed for photolabeling studies (preparation 1) had a specific activity of 160 nmol/min/mg. A second preparation (preparation 2) with lower specific activity (108 nmol/min/mg) was employed for amino acid sequence analysis of the amino terminus.

Enzyme Assays- Sialidase assays were carried out using the procedure described by Warner and O'Brien employing 4-methylumbelliferyl-Neu5Ac as substrate (12).

Photolysis- Samples of the placental complex preparation (200 μl, 0.03 units at 0.16 U/mg protein) were placed in a small polypropylene beaker containing tritiated 9-PANP-Neu5Ac2en (6 μCi,100 μM). The solution was adjusted to 20 mM acetate, pH 5.2 and allowed to equilibrate in the dark at 4 °C with mechanical stirring for 10 min. Irradiation was carried out with a medium pressure mercury arc lamp (General Electric model H85 A powered with a model MLA 85 ballast, George W. Gates, Co. Franklin Square, New York) with the sample positioned about 6 cm from the light source. After exposure for 2 min, 2 μl of 0.6 M dithiothreitol was added and the sample lyophilized.

SDS PAGE and Autoradioagraphy- The irradiated, lyophilized protein samples were suspended in 30 μl incubation buffer of Laemmli (13) and the proteins analyzed under reducing conditions with SDS PAGE on 12.5% acrylamide gels. After electrophoresis, the gels were fixed in 30% methanol-10% acetic acid for 1 hr and then soaked in Entensify (NEN, Boston, MA) according to the manufacturer's instructions. The gels were dried onto heavy paper and exposed to X-ray film (X-Omat, Eastman Kodak) for three days at -70 °C. Molecular weight determinations were made using prestained standards with a molecular weight range of 17 to 130 kD (Bio Rad Labs, Richmond, CA). Gels containing enzyme preparations which were not photolabeled were stained with commercial silver stain reagents (Sigma) for visualization of the bands.

Amino Acid Sequence Analysis- Proteins were electrophoretically transferred from 12.5 % acrylamide gels run under denaturing conditions to PVDF membranes according to the method of Matsudaria (14). After staining for protein with coomassie blue, automated Edman degradation was performed with a model 470A Applied Biosystems gas phase sequencer equipped with a 120A PTH amino acid analyzer. PTH amino acids were integrated with a Nelson Analytical model 3000 data system. Sequence interpretation was performed on a VAX computer11/785 (Digital Equipment Corp)(15).

Protein Sequence Data Banks- The protein sequence data base used at Genentech consists of proteins from the National Biomedical Research Foundations Protein Information Resource (NBRF/PIR), the SWISSPROT data base from EMBL, the Brookhaven Protein Data Bank (PDB), and a large number of protein sequences entered in-house. For the work here, the sequences of appoximately 25,000 proteins were examined.

RESULTS

Photolabeling The Placental α-neuraminidase/β-galactosidae Complex- Analysis of the complex with polyacrylamide gel electrophoresis followed by staining with silver reagent revealed a protein pattern similar to that reported by others, Fig. 2 (2,5). The major protein band observed here at about 61 kD corresponds to the acid β-galactosidase which has a reported molecular weight of about 64 kD (16). The presence of β-galactosidase was confirmed by enzyme assay with a specific activity of 10,550 nmol/min/mg protein. The protective factor protein (carboxypeptidase), which has also been identified as a component of the complex (17) and is known to give bands at 57 kD, 32 kD, and 22 kD, was present in our preparation as well.

TABLE I

Amino Terminus Sequence Of Photolabeled Proteins In the α–Neuraminidase/β–Galactosidase Complex

Protein	Amino Acid Sequence[a]
Band at 46 kD	LDNGLLNTPPMGXLAXERFR
α-N-Acetylgalactosaminidase[b]	LDNGLLQTPPMGWLAWERFR
α–Galactosidase[c]	LDNGLARTPTMGWLHWERTM
Band at 61 kD	Blocked

Amino terminus sequence of the proteins in the complex was determined as described in Methods.

[a]X=indeterminate amino acid.
[b] See reference 19.
[c] See reference 20.

Amino Acid Sequence Analysis-The proteins labeled with the photoprobe were further characterized by amino terminus microsequence analysis. Table 1 lists the first 20 residues of the 46 kD protein which shows a 85 % identity with the sequence predicted from the cloned c-DNA for human placental α-N-acetyl galactosidase (α-galactosidase B)(19) and a 68 % homology with the sequence predicted for α-galactosidase A from human lung (20). Repeated analysis of the 61 kD band gave no significant signals in the analyzer. Based on the staining intensity of the two bands on the gel, the relative amounts of the two proteins appeared to be nearly equivalent. Considering that the amount of the 46 kD protein in the gel was adequate for sequence determination, the levels of the 61 kD protein in the gel should also be sufficient to generate a significant response. Since none was observed, we conclude that the amino terminus of the protein(s) in this band is blocked and its sequence could not be determined by direct analysis.

DISCUSSION: Although the placental preparation contains only a few proteins when analyzed with acrylamide gel electrophoresis, identifying the specific protein band corresponding to the neuraminidase has been especially difficult. Antibody probes have been used to address this problem, but, in some cases, this approach has not been definitive. For example, antibodies raised against either the 76 kD or the 46 kD protein in the purified complex precipitated neuraminidase activity (2,5). On this basis both proteins were considered as attractive neuraminidase candidates . However, recent amino acid sequence analysis of the 76 kD protein has revealed a high degree of identity with immunoglobin, IgM. Consequently, it is no longer considered as a likely choice for the enzyme (4). In the photolabeling studies presented here, the 46 kD protein was one of the most intensely labeled proteins in this preparation even though

it was in relative low concentration. However, the association of the probe with the protein was apparently non-specific, probably through hydrophobic interactions, since the surfactant Triton-X 100 blocked labeling. Furthermore, amino acid sequence analysis of this protein revealed a high degree of identity with the predicted amino acid sequence of α-N-acetylgalactosidase (19). Together, these results verify that the 46 kD protein is not the neuraminidase as previously suspected.

In contrast to the effects of surfactant on the labeling of the 46 kD protein, the labeling of the 61 kD band was not adversely affected by the presence of Triton X-100, in fact it was moderately enhanced. Protection from labeling with the competitive inhibitor Neu5Ac2en demonstrated that the labeling with the photoprobe is highly specific. These results lead us to conclude that the neuraminidase is located within the 61 kD band along with the acid β-galactosidase and possibly other proteins. The photolabeling studies corroborate the recent results of Van der Horst et. al. who have identified the neuraminidase as a protein which comigrates with β-galactosidase on acrylamide gel electrophoresis. (4). This conclusion was made based on the observation that antibodies specifically directed against proteins in this region of the gel prevented the formation of an active neuraminidase complex under reconstitution conditions. The antibodies also precipitated neuraminidase activity. Up to now, this conclusion has been tentative since an equally plausible explanation of these results is that the protein identified may act as a stabilizing factor for the neuraminidase which was some other component of the complex. It might be expected that antibodies which prevent association of the stabilizing protein with the enzyme would also inhibit reconstitution of enzyme activity. This alternate explanation is no longer tenable since the photolabeling results obtained here support the contention that the neuraminidase or at least the polypeptide containing the active site lies in the 60 kD molecular weight range.

Further characterization of the labeled protein with more astringent techniques will now be possible since maintaining the catalytic integrity of the enzyme will not be necessary. These studies will be useful in establishing the relationship of the placental sialidase to the enzyme affected in sialidosis and other cellular neuraminidases.

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