Affinity labelling of the G_{M2}-activator protein

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In contrast to ganglioside G_{M2} (G_{M2}), its fatty acid free derivative, lyso-G_{M2}, and *N*-acetyllyso-G_{M2} are hydrolyzed by purified human hexosaminidase A (Hex A). The addition of G_{M2}-activator stimulates the Hex A-catalyzed hydrolysis of G_{M2} and that of *N*-acetyllyso-G_{M2} much more than that of lyso-G_{M2}. Based on this observation *N*-bromoacetyllyso-G_{M2} was synthesized as a potential affinity ligand of the G_{M2}-activator protein. *N*-Bromo[^{3}H]acetyllyso-G_{M2} inactivated the G_{M2}-activator selectively and irreversibly by covalent modification.

Ganglioside *Hexosaminidase* *Lysoganglioside G_{M2}* *Bromoacetyllysoganglioside G_{M2}*

1. INTRODUCTION

Two proteins, Hex A and the G_{M2}-activator protein, are involved in the lysosomal degradation of ganglioside G_{M2} [1]. Inherited deficiencies of each of these proteins result in fatal ganglioside storage disorders [2]. The G_{M2}-activator is an enzymatically inactive glycolipid-binding protein which stimulates the glycolipid transfer from donor to acceptor liposomes [3]. Binding studies indicate that it forms a water-soluble, stochiometric complex with ganglioside G_{M2} which is the putative substrate of Hex A as suggested by kinetic analysis [4]. Here we present direct support for the complex formation by affinity labelling of the G_{M2}-activator protein using an analogue of ganglioside G_{M2}, *N*-bromo[^{3}H]acetyllyso-G_{M2}.

Abbreviations: G_{M2}, ganglioside G_{M2}, II^{3}Neu5Ac-GgOse$_3$Cer, monosialogangliotriaosylceramide; lyso-G_{M2}, II^{3}Neu5Ac-GgOse$_3$Sph, monosialogangliotriaosylsphingosine; G_{M3}, ganglioside G_{M3}, II^3 Neu5Ac-LacCer, monosialolactosylceramide; lyso-G_{M3}, II^{3}Neu5AcLac Sph, monosialolactosylsphingosine; NAc-lyso-$G_{M2(3)}$, *N*-acetyllyso-$G_{M2(3)}$; NBrAc-lyso-$G_{M2(3)}$, *N*-bromoacetyllyso-$G_{M2(3)}$; MUG1cNac; 4-methylumbelliferyl-β-D-*N*-acetylglucosaminid; MUGa1Nac-6-SO$_4$, 4-methylumbelliferyl-β-D-*N*-acetylgalactosaminid-6-sulfate; Hex A, hexosaminidase A, β-2-acetamido-2-desoxy-D-glucosid: acetamidodesoxyglucohydrolase, EC 3.2.1.30; *tlc*, thin-layer chromatography

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2. EXPERIMENTAL

2.1. *Synthesis of [^{3}H]lyso-G_{M2}, [^{3}H]NAc-lyso-G_{M2} and [^{3}H]NBrAc-lyso-G_{M2}*

Ganglioside G_{M2} was deacylated and the amino group of its sphingosine moiety selectively blocked as described [5] using fluorenylmethoxycarbonyl chloride as protecting reagent in a two-phase system. Acetylation of the free amino group of the neuraminic acid residue with [^{3}H]acetic anhydride (18 500 GBq/mol) followed by acetic anhydride and the removal of the protecting group in liquid ammonia yielded 30% [^{3}H]lyso-G_{M2} (1100 GBq/mol). Acylation of [^{3}H]lyso-G_{M2} with either acetic anhydride or bromacetic anhydride in 5% $NaHCO_3$ solution at 0°C, followed by dialysis against water yielded 90% [^{3}H]NAc-lyso-G_{M2} (1100 GBq/mol) and 90% [^{3}H]NBrAc-lyso-G_{M2} (1100 GBq/mol), respectively.

2.2. *Incubation with Hex A*

The incubation volume of 40 μl contained 10 nmol glycolipid as substrate, 10 mM citrate buffer, pH 4.0, 60 nmol NaN_3, 10 μg bovine serum albumine, 220 mU Hex A [6] and, when indicated,

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1 unit [4] of activator protein. After incubation at 37°C the mixtures were analyzed by TLC using silica gel 60 plates and chloroform:methanol:15 mM $CaCl_2$ (60:35:8, by vol.) as solvent system. Distribution of radioactivity on the dried plates was analyzed employing a radioscanner (Berthold LB 2723).

2.3. *Preparation of G_{M2}-activator protein*

G_{M2}-activator was prepared from postmortem human kidneys as described [4]. A crude preparation was obtained after extraction, heat- and acid precipitation, ion-exchange and gel chromatography. A purified preparation was obtained after fractionation by further steps: hydrophobic chromatography on octyl-Sepharose and HPLC (silica gel TSK G 2000 SW; solvent 100 mM phosphate buffer pH 6.9).

Proteins of the crude G_{M2}-activator preparation were radiolabelled: the protein mixture was dissolved in 5% $NaHCO_3$ solution and tritium-labelled by reaction with 2 μmol [^{3}H]acetic anhydride (18 500 GBq/mol in toluol, 3.7 GBq/ml) and then dialyzed against water.

2.4. *Affinity labelling*

G_{M2}-activator (purified or crude preparation, containing 2.5 nmol G_{M2}-activator each) was dissolved in 200 μl of 20 mM citrate buffer, pH 4.0, 10 mM NaN_3. [^{3}H]NBrAc-lyso-G_{M2} (3 nmol, 1100 GBq/mol), dissolved in 6 μl water, was added and the mixture incubated for 23 h at 37°C, dialyzed against water (20 h) and lyophilized. Aliquots of 20% were subjected to SDS electrophoresis in a 12.5% acrylamide gel according to Laemmli [7]. Radioactive bands were visualized by fluorography [8,9].

3. RESULTS AND DISCUSSION

In the absence of detergents or G_{M2}-activator purified Hex A has no detectable activity against the ganglioside G_{M2} but hydrolyzes lyso-G_{M2} and NAc-lyso-G_{M2} (fig.1). Ganglioside G_{M2} forms rather stable micelles in aqueous solution with a CMC at or below 10^{-9} M [10]. Ganglioside micelles are not recognized as substrate by Hex A [11]. Lyso derivatives have only one hydrophobic chain, the sphingosine moiety, and probably a much higher CMC. In this case monomers might

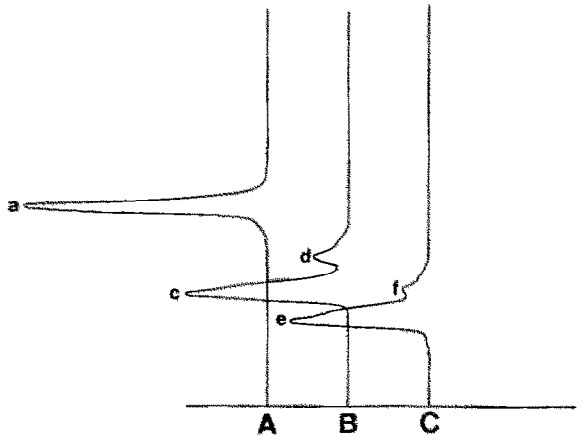

Fig.1. Hydrolysis of [^{3}H]G_{M2} (A), [^{3}H]NAc-lyso-G_{M2} (B) and [^{3}H]lyso-G_{M2} (C) by Hex A in the absence of G_{M2}-activator. Incubation mixtures were run for 10 h at 37°C and analyzed by TLC (as described in section 2). Distribution of radioactivity on the TLC plate was analyzed with the help of a radioscanner. (a) [^{3}H]G_{M3}, (c) [^{3}H]NAc-lyso-G_{M2}, (d) [^{3}H]NAc-lyso-G_{M3}, (e) [^{3}H]lyso-G_{M2}, (f) [^{3}H]lyso-G_{M3}.

be available for interaction with Hex A. Addition of G_{M2}-activator to the incubation mixtures stimulates the degradation of G_{M2} and NAc-lyso-G_{M2} much stronger than that of lyso-G_{M2} (fig.2). In the incubation mixture at pH 4 a positive charge on the free amino group of sphingosine

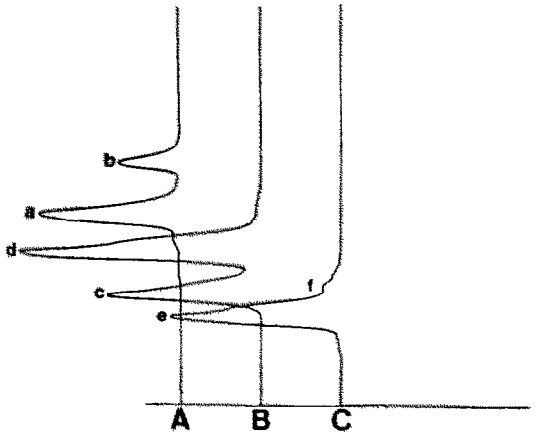

Fig.2. Hydrolysis of [^{3}H]G_{M2} (A), [^{3}H]NAc-lyso-G_{M2} (B), and [^{3}H]lyso-G_{M2} (C) by Hex A in the presence of G_{M2}-activator protein. The assay mixtures were incubated for 2 h at 37°C and analyzed by TLC and radioscanning as described in section 2. (a) [^{3}H]G_{M2}, (b) [^{3}H]G_{M3}, (c) [^{3}H]NAc-lyso-G_{M2}, (d) [^{3}H]NAc-lyso-G_{M3}, (e) [^{3}H]lyso-G_{M2}, (f) [^{3}H]lyso-G_{M3}.

presumably inhibits complex formation between the lyso-G_{M2} and the G_{M2}-activator protein. This suggests a positive group, such as lysine residue, in the lipid binding site of the G_{M2}-activator. Therefore an analogue of ganglioside G_{M2}, NBrAc-lyso-G_{M2}, was synthesized as a potential affinity ligand for labelling. This analogue is rather similar to NAc-lyso-G_{M2} and G_{M2}, contains as a reactive group a properly positioned bromoacetyl residue, which may react with a nucleophile of the protein binding region, but is rather inert to unspecific hydrolysis. In contrast to NAc-lyso-G_{M2}, NBrAc-lyso-G_{M2} inhibits irreversibly the degradation of [^{3}H]G_{M2} by Hex A in the presence of G_{M2}-activator (not shown). On the other hand, it inhibits less than NAc-lyso-G_{M2} the hydrolysis of MUGalNAc-6-SO_4 and MUGlcNAc by either Hex A or Hex B (not shown). Incubation of [^{3}H]NBrAc-lyso-G_{M2} with either pure or crude G_{M2}-activator preparations resulted in radioactive labelling of the activator protein (fig.3). Under the conditions used the G_{M2}-activator was specifically labelled among all the proteins present in the crude activator preparation (fig.3). If, however, the crude G_{M2}-activator preparation is incubated with increasing amounts of [^{3}H]NBrAc-lyso-G_{M2} other protein bands are also labelled. In fig.3 the G_{M2}-activator protein shows up as a double band like the purified activator protein [12]. This is probably due to the heterogeneity of its carbohydrate portion (unpublished). The affinity labelling, shown in fig.3 directly supports the postulated ganglioside G_{M2}- G_{M2}-activator protein complex [4]. The derivatized activator protein should be helpful in the elucidation of its lipid-binding site.

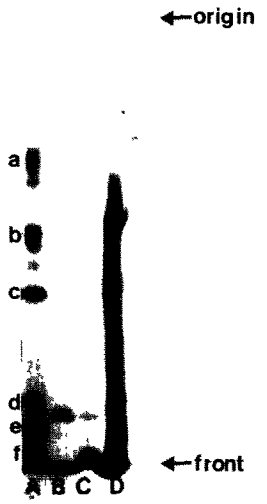

Fig.3. SDS gel electrophoresis of G_{M2}-activator preparations after affinity labelling (as described in section 2). The exposure time of the fluorogram was 113 h. Lanes: (A) standards: (a) bovine serum albumin (69 kDa), (b) ovalbumin (46 kDa), (c) carbonic anhydrase (30 kDa), (d) soybean trypsin inhibitor (21.5 kDa), (e) lactoglobulin A (18.37 kDa), (f) cytochrome *c* (12.3 kDa). (B) Purified G_{M2}-activator protein which yields a double band after incubation with [^{3}H]NBrAc-lyso-G_{M2}, (C) crude G_{M2}-activator preparation after incubation with [^{3}H]NBrAc-lyso-G_{M2}, (D) labelling of all protein bands present in the crude G_{M2}-activator preparation by [^{3}H]acetic anhydride.

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REFERENCES

[1] Sandhoff, K. (1984) in: The Molecular Basis of Storage Disorders (Brady, R.O. and Barranger, J.A. eds) pp. 19–49, Academic Press, Washington.
[2] Sandhoff, K. and Conzelmann, E. (1984) Neuropediatrics 15, Suppl. pp. 85–92.
[3] Conzelmann, E., Burg, J., Stephan, G. and Sandhoff, K. (1982) Eur. J. Biochem. 123, 455–464.
[4] Conzelmann, E. and Sandhoff, K. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 1837–1849.
[5] Neuenhofer, S., Schwarzmann, G., Egge, H. and Sandhoff, K. (1985) Biochemistry 24, 525–532.
[6] Sandhoff, K., Conzelmann, E. and Nehrkorn, H. (1977) Hoppe-Seyler's Z. Physiol. Chem. 358, 779–787.
[7] Laemmli, U.K. (1970) Nature 227, 680–685.
[8] Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83–88.
[9] Laskey, R.A. and Mills, A.D. (1975) Eur. J. Biochem. 56, 335–341.
[10] Mraz, W., Schwarzmann, G., Sattler, J., Momoi, T., Seemann, B. and Wiegandt, H. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 177–185.
[11] Kytzia, H.-J. and Sandhoff, K. (1985) J. Biol. Chem., in press.
[12] Burg, J. and Sandhoff, K. (1985) in preparation.