SPECIFIC IRREVERSIBLE INHIBITION OF HUMAN AND BOAR N-ACETYL-β-D-HEXOSAMINIDASE BY 2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL ISOTHIOCYANATE

M.L.Shulman, O.E.Lakhtina and A.Ya.Khorlin

Shemyakin Institute of Bioorganic Chemistry,
USSR Academy of Sciences, Moscow V-312, USSR

Received November 12, 1976

SUMMARY

2-Acetamido-2-deoxy-β-D-glucopyranosyl isothiocyanate (I) was obtained by the action of thiophosgene on 2-acetamido-2-deoxy-β-D-glucopyranosylamine. Compound I irreversibly inhibits the human and boar N-acetyl-β-D-hexosaminidase; the dialysis does not restore the enzyme activity. N-Acetyl-D-glucosamine, the competitive inhibitor of N-acetyl-β-D-hexosaminidase, protects the enzyme from inactivation, that testifies to the binding of isothiocyanate I in the active site of the enzyme.

INTRODUCTION

The method of specific irreversible inhibition widely used to clarify structure of active sites of the enzymes has been recently applied for the study of a number of glycosidases of various origin: β-glucosidase[1], β-galactosidase[2,3] and others[4,5]. Hitherto, irreversible inhibition of N-acetyl-β-D-hexosaminidase (hexosaminidase; EC 3.2.1.52) has not been described; meanwhile, recently this enzyme attracted attention for it appeared that the genetically determined absence of one or more isozymes of hexosaminidase is directly responsible for such lethal diseases as Tay-Sachs disease and its variant forms[6,7].

Earlier we described the active-site-directed irreversible inhibition of sweet-almond β-glucosidase by β-D-glycopyranosylepoxyalkanes and β-D-glucopyranosyl isothiocyanate[8]; it was

assumed that similar compounds may be used to block active sites of other glycosidases.

This note deals with the specific irreversible inhibition of human and boar N-acetyl-β-D-hexosaminidase by 2-acetamido-2-deoxy-β-D-glucopyranosyl isothiocyanate (I).

MATERIALS AND METHODS

Synthesis of 2-acetamido-2-deoxy-β-D-glucopyranosyl isothiocyanate (I). - A solution of 2-acetamido-3,4,6-tri-O-acetyl-α-D-glucopyranosyl chloride (1 g) and NaN_3 (0.8 g) in acetone (4 ml) was boiled for 4 hours, evaporated to dryness, chromatographed on silica gel LS (La Chema, ČSSR)(100 - 150 μm, 3 x 15 cm) with chloroform - methanol mixture (19 : 1) and crystallized from ether - light petroleum to give 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosylazide (II) in a yield of 0.84 g (82%), m.p. 167 - 168°, $I \propto I_D^{20}$ -45°(c1,$CHCl_3$) (cf. ref. 9); PMR data (signal of H-1 as a doublet at δ 4.75, $J_{1,2}$ = 9 Hz) confirm β-D configuration of a glycosyl bond. Compound II was deacetylated by the action of sodium methoxide in dry methanol, followed by hydrogenation in ethanol in the presence of 20% Pd/C for 3.5 hours to give amorphous 2-acetamido-2-deoxy-β-D-glucopyranosylamine (III) in a yield of 85%, $I \propto I_D^{20}$ -5°(c1,EtOH)(cf. ref. 10). A solution of III (220 mg) in water (2.2 ml) was added to a stirred suspension of $CaCO_3$ (250 mg) in acetone (1.6 ml) containing freshly distilled thiophosgene (0.4 ml) at 10 - 15° C. The suspension was stirred for 2 hours, filtered and evaporated to dryness. The residue was chromatographed on silica gel (100 - 150 μm, 2.5 x 12 cm) with chloroform - methanol mixture (3 : 2) to give hygroscopic syrupy I (150 mg), $I \propto I_D^{20}$ +25°(c1,MeOH); t.l.c.: R_f 0.4 ($CHCl_3$: MeOH = 3 : 1); IR data: ν_{max} 2040 (N = C = S), 1650, 1560 cm^{-1} (NHAc). Compound I, if well dried, is stable for 2 - 3 weeks at -10°C. I was acetylated (Ac_2O/Py), chromatographed on silica gel with ether - acetone mixture (19 : 1) and crystallized from chloroform - ether to yield 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl isothiocyanate (IV), m.p. 156 - 157°, $I \propto I_D^{20}$ +11°(c1,$CHCl_3$); t.l.c.: R_f 0.5 (Et_2O : Me_2CO = 19 : 1); IR data: ν_{max} 2080 (N = C = S), 1750 (OAc), 1660, 1560 cm^{-1}

(NHAc). Anal. Calc. for $C_{15}H_{20}N_2O_8S$ (MW 388.4): C 46.4, H 5.2, N 7.2, S 8.25. Found: C 46.3, H 5.2, N 7.1, S 8.4.

Compound IV was identical to the sample prepared by a counter synthesis according to the method of ref. 11.

N-Acetyl-β-D-hexosaminidase B from human placenta with specific activity* of 310 units per mg of protein was prepared according to the method of ref. 12.

N-Acetyl-β-D-hexosaminidase B from boar epididymis with specific activity of 400 units per mg of protein was prepared according to the method of ref. 13.

Incubation of the enzyme with isothiocyanate I and determination of residual enzymatic activity. - A solution (1 ml) containing the enzyme (0.02 - 1 mg), bovine serum albumin (100 μg) and isothiocyanate I (50 μmoles), a solution (1 ml) containing the same components and N-acetyl-D-glucosamine (150 μmoles) in addition, and a solution containing only the enzyme and albumin (control) in citrate - phosphate buffer (pH 4.5, μ 0.1) were incubated at 37° C. Throughout the indicated time intervals aliquotes (0.05 ml) were added to 3 mM solution of substrate V containing 0.01% of albumin in the same buffer (2 ml). The mixtures were incubated for 10 minutes at 37° C, the hydrolysis was ceased by adding 1 M Na_2CO_3 solution (1 ml) and the liberated p-nitrophenol determined spectrophotometrically at 400 nm.

Dialysis was carried out against citrate - phophate buffer (pH 6.0, μ 0.05) at 4° C for 4 hours (4 x 1 l).

RESULTS AND DISCUSSION

It has been shown that isothiocyanate I irreversibly inhibits N-acetyl-β-D-hexosaminidase of various degree of purity. This refers both to the sulphate - ammonium fractions from human placenta[12] and boar epididymis[13] containing components A and B and to the highly purified components B of the same origin.

*1 unit of enzymatic activity is expressed as 1 μmole of p-nitrophenol liberated for 1 minute as result of enzymatic hydrolysis of p-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (V) at 37° C and pH 4.5 in the presence of 0.01% bovine serum albumin.

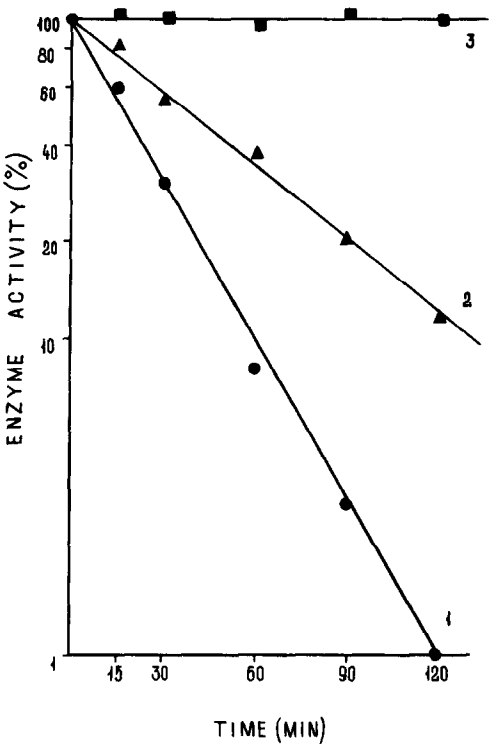

Figure 1. Irreversible inhibition of human hexosaminidase B by isothiocyanate I at pH 4.5 and 37° C in the presence of 0.01% bovine serum albumin. 1) (E) = 0.02 mg/ml, (I) = 50 mM; 2) (E) = 0.02 mg/ml, (I) = 50 mM, (GlcNAc) = 150 mM; 3) (E) = 0.02 mg/ml (control).

The inactivation of human hexosaminidase B under the action of I is shown in Fig.1. The inactivation of boar hexosaminidase B develops similarly. It is irreversible for the dialysis of the incubation mixtures does not restore the enzymatic activity; the activity of the control mixture (without I) is maintained.

N-Acetyl-D-glucosamine (GlcNAc), the competitive inhibitor of N-acetyl-β-D-hexosaminidase[13-15], preserves the enzyme from inactivation (Fig. 1). Thus, it points to the binding of isothiocyanate I in the active site of the enzyme.

Hence, I is a specific irreversible inhibitor of N-acetyl-β-D-hexosaminidase. The structural analogy of the inhibitor I to the substrate V allows us to assume that in the enzyme - inhibitor complex isothiocyanate group spaced at C-1 atom of the residue of N-acetyl-D-glucosamine would interact with one of the catalytic groupings of the enzyme active site.

REFERENCES

1. Bause, E. and Legler, G. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 438 - 442, and literature cited therein.
2. Naider, F., Bohak, Z., and Yariv, J. (1972) Biochemistry 11, 3202 - 3208.
3. Brockhaus, M. and Lehmann, J. (1976) FEBS Lett. 62, 154 - 156.
4. Saman, E., Claeyssens, M., Kersters-Hilderson, H., and De Bruyne, C. (1976) FEBS Lett. 63, 211 - 214.
5. Quaroni, A., Gershon, E., and Semenza, G. (1974) J. Biol. Chem. 249, 6424 - 6433.
6. Okada, S. and O'Brien, J. S. (1969) Science 165, 698 - 700.
7. Sandhoff, K. (1969) FEBS Lett. 4, 351 - 354.
8. Shulman, M. L., Shiyan, S. D., and Khorlin, A. Ya. (1976) Biochim. Biophys. Acta 445, 169 - 181, and literature cited therein.
9. Marshall, A. D. and Neuberger, A. (1964) Biochemistry 3, 1596 - 1600.
10. Makino, M., Kojima, T., Ohgushi, T., and Yamashina, I. (1968) J. Biochem. 63, 186 - 192.
11. Micheel, F. and Lengsfeld, W. (1956) Chem. Ber. 89, 1246 - 1253.
12. Tallman, J. F., Brady, R. O., Quirk, J. M., Villalba, M., and Gal, A. E. (1974) J. Biol. Chem. 249, 3489 - 3499.
13. Vikha, G. V., Kaverzneva, E. D., and Khorlin, A. Ya. (1971) Biokhimiya 36, 33 - 42.
14. Pugh, D., Leaback, D. H., and Walker, P. G. (1957) Biochem. J. 65, 464 - 469.
15. Robinson, D. and Stirling, J. L. (1968) Biochem. J. 107, 321 - 327.