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Multiple *exo*-glycosidases in human serum as detected with the substrate DNP-α-GalNAc. I. A new assay for lysosomal α-*N*-acetylgalactosaminidase

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ABSTRACT

This paper presents a new assay to determine the activity of the lysosomal enzyme α-*N*-acetylgalactosaminidase (Naga, EC 3.2.1.49) in human serum. It is based on the use of a new chromogenic substrate, DNP-α-GalNAc (2,4-dinitrophenyl-*N*-acetyl-α-D-galactosaminide) and is performed at pH 4.3 and 37 °C. This allows continuous monitoring of the absorbance of the released DNP. The assay can be performed with a standard spectrophotometer. Compared to established methods using an endpoint assay with MU-α-GalNAc (4-methylumbelliferyl-GalNAc), the present method gives a ca. 3-fold higher specific activity, while only one tenth of the serum concentration in the assay is required. Hence, the assay is at least 30-fold more sensitive than that with MU-α-GalNAc. The pH dependence of the reaction with DNP-α-GalNAc in the pH 3.5 to 6.5 region, while using 4% serum in the assay, shows only one peak around pH 4. This pH optimum is similar to that reported with MU-α-GalNAc. In the accompanying paper (Albracht and Van Pelt (2017) Multiple *exo*-glycosidases in human serum as detected with the substrate DNP-α-GalNAc. II. Three α-*N*-acetylgalactosaminidase-like activities in the pH 5 to 8 region. Biochim. Biophys. Acta 159 (2017) Part I and II), the method is used to show that, under special assay conditions, three more Naga-like activities can be uncovered in human serum.

1. Introduction

The lysosomal enzyme α-*N*-acetylgalactosaminidase (Naga, EC 3.2.1.49) is an *exo*-glycosidase which can hydrolyse R-α-GalNAc molecules, where R can be a polypeptide, a polysaccharide or an artificial organic compound, and α-GalNAc is *N*-acetyl-α-D-galactosaminide. Naga is one of the many lysosomal glycosidases involved in the removal of sugar units from glycoproteins, to enable further degradation by peptidases. Its occurrence in mammals has first been described in 1966 [1], using phenyl-α-GalNAc as substrate. Reports on partial purification of the enzyme from pig and beef liver soon followed, and experimental evidence suggested that it was a lysosomal enzyme [2].

The catalytic properties of the partly purified enzyme from human liver were first characterized in 1973, using 4-nitrophenyl-α-GalNAc (*para*-nitrophenyl-α-GalNAc, pNP-α-GalNAc) as substrate. The pH optimum of this enzyme was determined as 4.3 [3].

During a study of two apparent isoenzymes of human α-galactosidase (at that time termed α-galactosidase A and B) Schram et al. discovered that in the liver from patients with Fabry disease, a lysosomal storage disease caused by the absence of intact lysosomal α-galactosidase A (Gla, EC 3.2.1.22), the residual activity, until then ascribed to α-galactosidase B, was a distinct protein and not an isoenzyme [4]. Its substrate specificity characterized it as an α-*N*-acetylgalactosaminidase rather than Gla [5]. It had an apparent molecular mass of 110 ± 5 kDa [6]. Independently, Dean et al. had purified the α-galactosidase B from human liver and demonstrated its high specificity for *ortho*-nitrophenyl-α-GalNAc and 4-methylumbelliferyl-α-GalNAc (MU-α-GalNAc) [7].

The first patients, deficient in Naga activity, were described in the late 1980s [8,9]. Later, the phenotype of this deficiency became known as Schindler disease [10].

The amino-acid sequence of Naga was published in 1990 and showed a remarkable homology with that of Gla, but not with that of any other protein. It was suggested that both enzymes have evolved from a common ancestral gene [11].

Comparison of the X-ray structures of the human α-galactosidase

Abbreviations: A_{380}, optical absorbance at 380 nm; α-GalNAc, *N*-acetyl-α-D-galactosaminide; DMSO, dimethylsulphoxide; DMF, dimethylformamide; DNP^-, 2,4-dinitrophenolate; DNPH, 2,4-dinitrophenol; DNP-α-GalNAc, 2,4-dinitrophenyl-*N*-acetyl-α-D-galactosaminide; Gla, α-galactosidase A; Naga, α-*N*-acetylgalactosaminidase; MU, 4-methylumbelliferone; pNP-α-GalNAc, *para*-nitrophenyl-α-GalNAc; RT, room temperature; S.A., specific activity in nmol substrate per min per mL serum ($nmol \cdot min^{-1} \cdot mL^{-1}$), using 2 mM DNP-α-GalNAc

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[12] and the human α-*N*-acetylgalactosaminidase [13] revealed that the active sites of these enzymes were amazingly similar; 11 of the 13 amino acids forming the active-site pocket in both enzymes were identical. In Gla a glutamate (E) and a leucine (L) residue are positioned close to the 2-OH group of the substrate. In Naga these residues are serine (S) and alanine (A), respectively. These smaller residues allow the substrate with the extra *N*-acetyl group to enter the active-site pocket. This was elegantly demonstrated by mutation studies. The Gla mutant protein (E203S, L206A) now preferred pNP-α-GalNAc as substrate. Conversely, the Naga mutant protein (S188E, A191L) could no longer react with pNP-α-GalNAc, but only with pNP-α-Gal (pNP-α-D-galactose). The knowledge of the X-ray structures also enabled a better understanding of the many phenotypes described for patients with defective Gla (Fabry disease) or defective Naga (Schindler disease) [12–14].

At pH 4.3 the yellow colour of *p*-nitrophenol (pK_a = 7.15 [15]) released from pNP-α-GalNAc by Naga, cannot be detected. Hence, to evoke the full colour of the chromophore, the pH is raised to 10.6. This high pH also stops the reaction. The absorbance of the formed chromophore is then measured at 400 nm (ε_{400} = 10,300 $M^{-1}{\cdot}cm^{-1}$ [16]).

A more sensitive assay has been described with the fluorescent compound 4-methylumbelliferone (MU) as the leaving group in the substrate MU-α-GalNAc [7,17]. Also with this substrate, optimal fluorescence (λ_{ex} 348 nm, λ_{em} 460 nm) is only obtained after adjusting the pH to 10.7. In 1996 this substrate was first used to determine the Naga activity in human serum and plasma at pH 4.5 [18]. The disadvantage of these indirect assays may be overcome by using a substrate with the more appropriate leaving group 2,4-dinitrophenol (DNP, pK_a = 4.06 [15]), enabling continuous monitoring of the catalytic reaction at pH 4.5. Glycosides with DNP as leaving group have been introduced as substrates for glycosidases already in 1979 [19]. However, the synthesis of DNP-α-*N*-acetylhexosaminides appeared unsuccessful [20]. Only in 2007, a convenient synthesis of a number of 2,4-dinitrophenyl α-D-glycopyranosides, including DNP-α-GalNAc, has been described [21]. DNP-α-GalNAc has already been of use in a study on *endo*-α-*N*-acetylgalactosaminidase from *Streptococcus pneumoniae* R6 [22]. To our knowledge, this substrate has not yet been used to quantify Naga activity in human serum.

The objective at the start of this investigation was, to develop a direct assay method for Naga, that can also be used to verify reports on a Naga-like activity at pH 6 in human serum. The latter activity was claimed to be elevated in serum from patients with a wide range of cancers [23–25]. The present paper describes some essential properties of the new substrate DNP-α-GalNAc and its use to directly determine the lysosomal Naga activity at pH 4.3 in human serum. In the accompanying paper [26] the new substrate has been used to show that, under special assay conditions, three more Naga-like activities can be revealed in human serum.

2. Materials and methods

2.1. Substrate

DNP-α-GalNAc (2,4-dinitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside, MW = 387.30) was not commercially available at the start of this research. Therefore we requested Dr. J. van Wiltenburg (Syncom, Groningen, The Netherlands) to synthesise the substrate according to the method of Chen and Withers [21]. The delivered solid was 99.1% pure. DNP (2,4-dinitrophenol) was from Aldrich. Other chemicals were from Merck.

2.2. Serum and buffer

Serum was obtained after clotting of blood (in BD vacutainers, SST II Advance, 3.5 mL, red-brown cap) and centrifugation (BD vacutainers instructions). Most experiments were performed with pooled serum samples obtained from capped tubes stored at 4 °C for 7 to 10 days. This serum is here referred to 'routine serum' and had earlier been used for a variety of routine analyses in the laboratory. It originated from anonymous individuals (females, males, all ages) who had given their consent that after use for the intended analyses, the serum could be used for other purposes, like scientific research. Serum from apparently healthy male blood donors (here referred to as 'donor serum'), kept at 4 °C in capped tubes for one day, was obtained from Sanquin, Blood Transfusion Department Northern Holland, Amsterdam, The Netherlands. All serum originating from the stored, capped tubes was acidified by addition of 11 μL 5 M acetic acid per mL (final pH ca. 5.2, final acetate concentration 54 mM) and was either used directly or stored at − 20 °C [18,27]. Before use, the acidified serum samples were centrifuged at room temperature (RT) for 10 min at 16,000 x*g* (Eppendorf Microcentrifuge, type 5424). Unless specified otherwise, activity assays were carried out at 37 °C in Na-citrate buffer (50 mM citric acid adjusted at RT to pH 4.3 with 5 M NaOH) plus 100 mM NaCl.

2.3. Instruments and data analysis

Optical measurements were performed with a Jasco V-650 spectrophotometer, equipped with a Jasco PAC-743 thermostatted sample changer for six cuvettes. Disposable polystyrene cuvettes with a 2.5 mL assay volume and a path length of 1 cm were used. Cuvettes with an assay volume of 1.5 mL were not suited; their shape hindered a proper thermal equilibrium between the cuvette contents and the thermostatted sample changer. The absorbance was recorded every minute and the data were processed with Microsoft Excel. Measurements of the pH were performed with a MetrOhm 691 pH meter equipped with a 6.0234.100 probe head.

2.4. Choice of the monitoring wavelength for the assay

In enzyme assays with pNP or DNP as leaving groups, an observing wavelength in the 400 to 420 nm region has commonly been used. The anion of DNP has an absorbance peak at 360 and a shoulder at 400 nm (Fig. S1). The extinction coefficient at 360 nm for a solution of DNP prepared with analytical precision, is 14,800 $M^{-1}{\cdot}cm^{-1}$ (deduced from Fig. 1 in [28]). The acidic form (DNPH) does not absorb above 400 nm (Fig. S1).

Serum has a faint yellow-brown colour with a prominent absorption peak at 419 nm (the Soret band of heme, Fig. S2). At lower pH the peak broadened considerably and shifted to 407 to 410 nm. In view of this, and considering the spectra in Fig. S1, it was decided to choose 380 nm as the observing wavelength in the present paper, unless specified otherwise. At 380 nm, DNPH has an absorbance of only 5% of that of the anion; at lower wavelengths this contribution increases (isosbestic point at 324 nm). The minor (pH dependent) correction for the contribution of DNPH at 380 nm has been neglected in this paper.

2.5. Serum treatments

The optimum pH of the Naga activity in human plasma has been reported as pH 4.5 [18]. As anticipated, it was found that when acidified, routine serum was added to some conventional buffers used in established endpoint assays (citrate-phosphate, citrate or acetate) of pH 4 to 5.5, a steadily-increasing turbidity appeared due to proteins that are no longer soluble at low pH. For an optically monitored assay, any change in turbidity is undesirable. With donor serum, which was acidified, frozen and stored at − 20 °C, only a tiny yellow-brown precipitate appeared upon thawing and centrifugation (10 min at 16,000 x*g*). Thus, hardly any protein was removed and the supernatant was still a concentrated protein solution (serum contains ca. 62 to 84 mg protein per mL). Addition of this serum to buffers of pH 4.0 again resulted in an increasing turbidity. Addition of 100 mM NaCl to a 50 mM Na-citrate buffer (pH 4.0) greatly diminished or even abolished