

Short Communication

The paper was presented at the symposium "Glycosylation and glycoconjugates in physiology and pathology"

Synthesis of 5-IASA-UDP-GlcNAc and its use for the photoaffinity labeling of a novel UDP-GlcNAc pyrophosphorylase

Halina Szumiło^a, Tadeusz Szumiło^b and Alan D. Elbein^c

^aDepartment of Inorganic and Analytical Chemistry, ^bDepartment of Biochemistry, Medical School, S. Staszica 6, 20-081 Lublin, Poland

^cDepartment of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, U.S.A.

Key words: 5-[3-(*p*-iodoazidosalicylamide)allyl]-UDP-*N*-acetylglucosamine, UDP-*N*-acetylglucosamine pyrophosphorylase

Photoreactivable 5-[3-(*p*-iodoazidosalicylamide)allyl]-UDP-GlcNAc (5-IASA-UDP-GlcNAc) was synthesized by a four-step procedure and used for photoaffinity labeling of UDP-GlcNAc-dependent enzymes. Upon iodination with ¹²⁵I, the compound was successfully applied to probe a purified UDP-GlcNAc pyrophosphorylase from pig liver. The enzyme was photoinactivated by the probe in the concentration-dependent manner, and was protected by UDP-GlcNAc and, to a lesser extent, by UTP and UDP-GlcCOOH.

Azido-substituted nucleotide photoaffinity analogues have been found to be very effective tools in biochemical studies on nucleotide binding and/or utilizing enzymes [1]. Two aspects of the application of these compounds are worth emphasise: 1, as an aid in purification and identification of "difficult" enzymes (examples are labile membrane associated glycosyltransferases), and 2, for mapping of active sites of the nucleotide binding enzymes. Several photoactivable compounds with the incorporated ³²P label have been synthesized and successfully tested, such as: 5-N₃UTP [2], 5-N₃UTP-glucose [3], 5-N₃UTP-glucuronate [4], UDP-pyridoxal [5] or 8-N₃ATP [6]. In others, e.g. 5-IASA-UDP-glucose [7] or GDP-hexanolaminyl-4-IASA [8] ¹²⁵I label for a longer preservation has been inserted. In this report, we have described a protocol for the synthesis of a photosensitive 5-[3-(*p*-iodoazidosalicyl-

amide)allyl]-UDP-Glc-NAc (5-IASA-UDP-GlcNAc) to be used for photoaffinity labeling of various GlcNAc transferases engaged in glycoprotein biosynthesis. The compound has been found to react with UDP-GlcNAc pyrophosphorylase from pig liver extracts [9].

MATERIALS AND METHODS

Materials. Reagents were from the following sources: Na¹²⁵I, Amersham (England); UDP-[2-³H]GlcNAc (6.1 Ci/mmol), ICN (U.S.A.); UDP-GlcNAc and other nucleotides, Sigma Chemical Co. (U.S.A.); allylamine and K₂PdCl₄, Aldrich Chemical Co. (U.S.A.); *N*-hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA), Pierce Chemical Co. (U.S.A.); PEI cellulose plates, EM Science (U.S.A.); cellulose

¹Abbreviations: AL, aminoallyl; GlcNAc, *N*-acetylglucosamine; IASA, iodoazidosalicylamide; NHS-ASA, *N*-hydroxysuccinimidyl-4-azidosalicylic acid; t.l.c., thin-layer chromatography.

plates, Kodak (U.S.A.); silica gel plates, Merck (Germany); PAGE reagents, Bio-Rad (U.S.A.).

Enzyme preparations. UDP-GlcNAc pyrophosphorylase preparations were isolated from pig liver as described previously [9].

Enzyme assay. The enzyme activity was measured in reverse direction as described by Szumilo *et al.* [10]. The incubation mixture contained in 50 μ l: 5 mM sodium pyrophosphate, 250 μ M UDP-[3 H]GlcNAc (2.2 Ci/mmol), 100 mM Tris/HCl, pH 8.0, 2 mM MgCl₂ and enzyme. Occasionally, the appearance of UTP was measured spectrophotometrically [10].

Photoaffinity labeling. Eppendorf tubes (1.5 ml) contained a reaction mixture (50 μ l) with the following constituents: 25 μ M 5-[125 I]ASA-UDP-GlcNAc, 1 mM MgCl₂, 50 mM Tris/HCl, pH 7.5, and enzyme. The reaction was initiated by adding the probe and the reaction mixture was maintained at room temperature for 30 s, followed by 2 min irradiation with a hand-held UV-lamp (Mineral Light UVSL-58, San Gabriel, U.S.A.) at 365 nm from a distance of 1 cm. Then, 0.2 ml of 10% trichloroacetic acid was added and the mixture was centrifuged. The proteins were solubilized with the SDS-PAGE sample buffer for 10 min at 80°C. The acid treatment was omitted when homogeneous enzyme was labeled. The SDS-PAGE on 12% gels was performed according to Laemmli [11]. The gels were developed with Coomassie R-250, destained, dried and radioactive polypeptides were detected by autoradiography (Kodak X-Omat film with a Cronex intensifier screen).

Photoinactivation. The mixture (50 μ l) containing 1 mM MgCl₂, 20 mM Tris/HCl, pH 7.5, various amounts of the probe and enzyme, was placed in a quartz cuvette, incubated in the dark for 3 min, irradiated with the UV-lamp (365 nm) for the next 3 min and the remaining enzyme activity was determined. In protection experiments, the mixture was supplemented with UDP-GlcNAc.

Synthesis of 5-IASA-UDP-GlcNAc

Synthesis of 5-Hg-UDP-GlcNAc. Mercuric acetate (500 mg, 3.75 mmol), UDP-GlcNAc (500 mg, 0.77 mmol) and 25 μ Ci of UDP-[3 H]GlcNAc (a tracer) were dissolved in 75 ml of 0.5 M acetate buffer, pH 7.0, and the solution was incubated at 50°C for 5 h. The chilled solution was treated with LiCl and ethyl acetate to remove Hg²⁺ ions and the synthesized com-

pound was precipitated with ethanol [7, 12]. The extent of mercuration was monitored by t.l.c. as described in a following section (t.l.c. system). The yield was 78%. The analysis of UV-spectrum showed a shift of the maximum from 260 nm to 267 nm. The compound can be purified on a DEAE-cellulose column as described in the next section, or can be used directly for further studies.

Synthesis of 5-(3-aminoallyl)UDP-GlcNAc (5-AL-UDP-GlcNAc). Hg-UDP-GlcNAc solution (15 ml, 0.3 mmol) was mixed with allylamine acetate solution (0.2 M, pH 6.0, 1.8 ml) and K₂PdCl₄ (99 mg, 0.3 mmol) and the mixture incubated at room temperature for 16 h. The filtered yellow solution was diluted with 10 vol. of water and purified on a DEAE-cellulose column (2.3 cm \times 23 cm), previously equilibrated with 10 mM NH₄HCO₃. AL-UDP-GlcNAc was eluted with 50 mM NH₄HCO₃ and the contaminating UDP-GlcNAc and Hg-UDP-GlcNAc, with 300 mM NH₄HCO₃. The yield was 53%. The compound showed maxima at 241 nm and 280 nm and a minimum at 263 nm (Fig. 1A), it reacted with ninhydrin and migrated slowly on PEI cellulose.

Synthesis of 5-ASA-UDP-GlcNAc. 5-AL-UDP-GlcNAc (110 mg, 0.16 mmol) and NHS-ASA (54 mg, 0.2 mmol) was dissolved in 2 ml of dimethylformamide and incubated with stirring in the dark for 2 days. The product was purified on DEAE-cellulose, using a linear gradient of NH₄HCO₃ (1000 ml) from 50 to 1000 mM. The last peak (273 nm) was collected, concentrated, desalted with triethylamine and stored in 70% methanol in the dark. The yield was 56%. The UV-spectrum showed maxima at 223 nm and 273 nm and a minimum at 255 nm. The compound was photoreactivable as shown in Fig. 1B.

Synthesis of 5-IASA-UDP-GlcNAc. A solution of 5-ASA-UDP-GlcNAc (20 μ mol) and NaI (40 μ mol) in 0.2 ml of 0.5 M phosphate buffer, pH 7.4, was mixed with 0.1 ml chloramine T (40 μ mol) in the buffer. After 2 min, the reaction was quenched with 0.1 ml of 5% sodium metabisulfite. Further purification of the product was performed according to Meikle *et al.* [7]. The yield was 60%. The spectrum of the photoreactivable compound is shown on Fig. 1C. The radioiodinated product was obtained in the same way, except that 0.5 μ mol of 5-ASA-UDP-GlcNAc and 1 mCi of Na¹²⁵I were used

in a volume of 100 μ l. The radioactive compound was purified by t.l.c. on PEI cellulose plates in solvent B (t.l.c. system). Specific activity of a 25 μ M probe solution was 1.5 mCi/ μ mol (0.1 μ Ci/ μ l).

NMR analysis. ^1H NMR spectra of UDP-GlcNAc (A), 5-AL-UDP-GlcNAc (B) and 5-ASA-UDP-GlcNAc (C) were obtained in $^2\text{H}_2\text{O}$ at 300.520 MHz on a General Electric GN-300WB FT-NMR spectrometer using sodium 3-trimethylsilylpropionate-2,2,3,3- d_4 as inter-

nal shift reference ($\delta = 0.000$ ppm). The uridyl H-6 doublet (7.97 ppm, $J = 8$ Hz) in A collapsed to a singlet in C (7.92 ppm), and the H-5 doublet (5.97 ppm, $J = 8$ Hz) was replaced by a pair of coupled *trans* ($J = 16$ Hz) vinyl proton signals (d, 6.39 ppm and dt, 6.57 ppm). Homonuclear decoupling experiments confirmed that the latter vinyl proton was also coupled to an amido methylene group (d, 4.12 ppm, $J = 5$ Hz), which was shifted by 0.4 ppm downfield relative to the *N*-methylene signal (d, 3.73 ppm, $J = 5$ Hz)

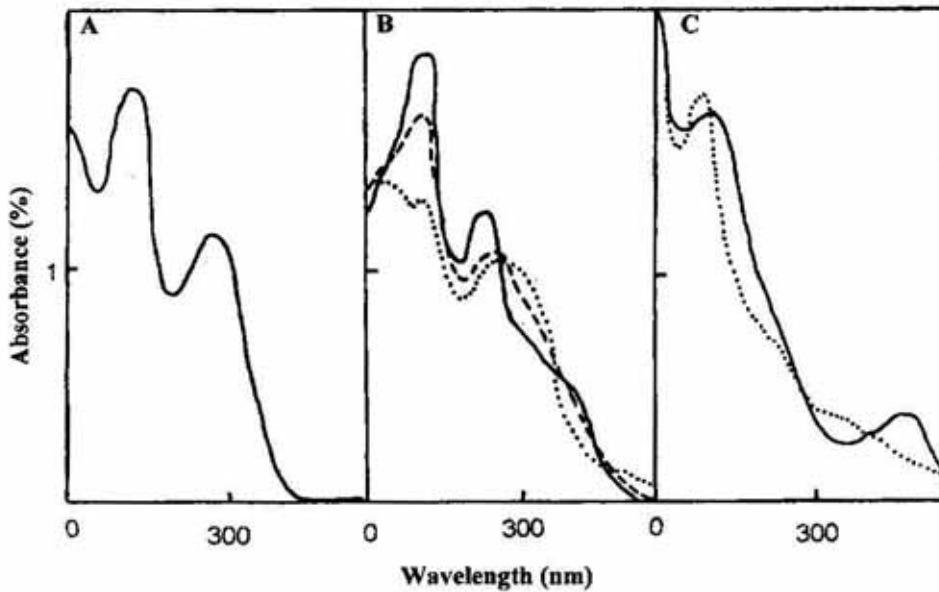


Fig. 1. UV-spectra of 5-AL-UDP-GlcNAc (A), 5-ASA-UDP-GlcNAc (B) and 5-IASA-UDP-GlcNAc (C) in 50 mM Tris/HCl, pH 7.5.

Irradiation at 365 nm of compounds B and C lasted for 15 s (---) and 120 s (...).

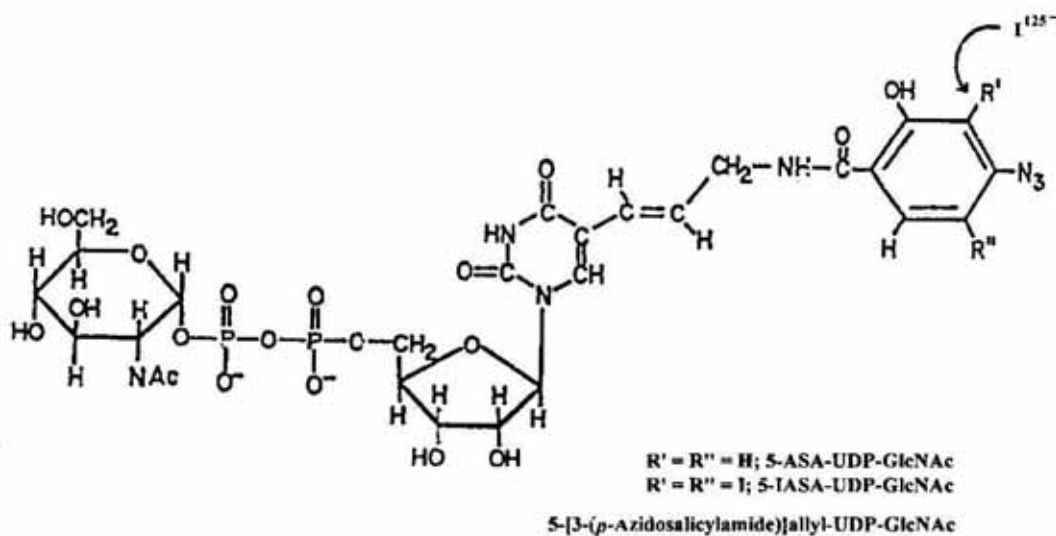


Fig. 2. Proposed structures of 5-ASA-UDP-GlcNAc and 5-IASA-UDP-GlcNAc.

in B. The *p*-azidosalicyloyl H-3, H-5 and H-6 signals were found at 6.64 (d, $J = 2$ Hz), 6.72 (dd, $J = 2.9$ Hz) and 7.78 (d, $J = 9$ Hz) ppm, respectively. Other assignments for C include the ribosyl H-1 (d, 5.98 ppm, $J = 5$ Hz), glucosyl H-1 (dd, 5.50 ppm, $J = 3.7$ Hz) and 2-NCOCH₃ (s, 2.04 ppm) and other glucosyl protons (3.6–4.4 ppm). Thus, the structure of C proved to be as shown in Fig. 2.

T.l.c. systems. A) PEI cellulose, 0.2 M LiCl as solvent; B) PEI cellulose, 0.4 M LiCl; C) cellulose, ethanol:1 M ammonium acetate, pH 7.3 (6:4, v/v); D) silica gel, ethanol:1 M ammonium acetate, pH 5.0 (6:4, v/v). R_F values of particular compounds in respective systems were as follows. UDP-GlcNAc: 0.57, 0.79, 0.52, 0.74; 5-Hg-UDP-GlcNAc: 0.10, 0.32, 0.17, 0.37; 5-AL-UDP-GlcNAc: 0.74, 0.87, 0.39, 0.67; 5-ASA-UDP-GlcNAc: 0.15, 0.36, 0.79, 0.88; 5-IASA-UDP-GlcNAc: 0.03, 0.09, 0.83, 0.90.

RESULTS AND DISCUSSION

To facilitate photoaffinity labeling of enzymes dependent on UDP-GlcNAc, its photoreactive radioiodinated analogue [¹²⁵I]ASA-UDP-GlcNAc has been synthesized with the aid of heterobifunctional NHS-ASA originally developed by Ji & Ji [13] as well as taking advantage of the previously described methods [7, 12, 14, 15]. The suggested structure of the basic compound, 5-ASA-UDP-GlcNAc, shown in Fig. 2, has been confirmed by UV-spectra (Fig. 1) and ¹H-NMR analysis. Upon irradiation at 365 nm, the compound was able to inactivate UDP-GlcNAc pyrophosphorylase in a concentration-dependent manner (Fig. 3A). The activity dropped by 60% at about 30 μM concentration of the probe. If the enzyme was not

exposed to the irradiation, the activity remained unchanged. UDP-GlcNAc protected the enzyme against photoinactivation (Fig. 3B).

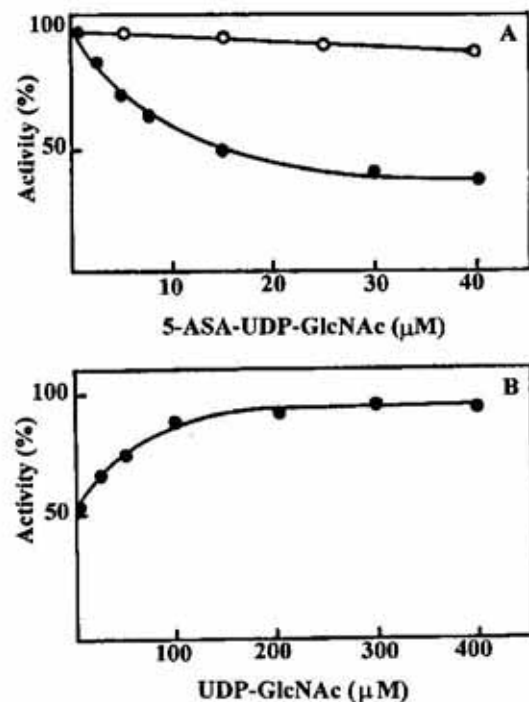


Fig. 3. Inactivation of UDP-GlcNAc pyrophosphorylase by 5-ASA-UDP-GlcNAc (A) and its protection (B).

A. Inactivation. The reaction mixtures contained 1 mM MgCl₂, 20 mM Tris/HCl, pH 7.5, enzyme (5 μg protein) and the indicated final concentrations of 5-ASA-UDP-GlcNAc in a total volume of 50 μl. After pre-incubation in the dark for 3 min, the samples were irradiated at 365 nm for 3 min from a distance of 1 cm and remaining activity was determined as described in Methods (●). Duplicate samples were not irradiated (O). B. Protection. The reaction mixtures contained 1 mM MgCl₂, 20 mM Tris/HCl, pH 7.5, 0.1 mM 5-ASA-UDP-GlcNAc, enzyme (5 μg protein) and the indicated final concentrations of UDP-GlcNAc. The other conditions of the enzyme inactivation as shown above.

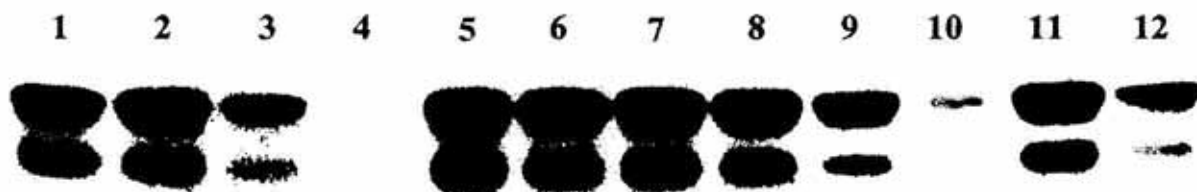


Fig. 4. SDS-PAGE of the [¹²⁵I]ASA-UDP-GlcNAc photolabeled UDP-GlcNAc pyrophosphorylase and effect of protection.

1, No protection; + UDP-GlcNAc: 2, 0.01 mM; 3, 0.1 mM; 4, 1.0 mM; + UDP-GalNAc: 5, 0.1 mM; 6, 1.0 mM; + UDP-Glc: 7, 0.1 mM; 8, 1.0 mM; + UTP: 9, 0.1 mM; 10, 1.0 mM; 11, + 1.0 mM UDP-Gal; 12, + 1.0 mM UDP-GlcCOOH.

Both subunits of the purified pyrophosphorylase (64 and 55 kDa) underwent affinity labeling (Fig. 4). The labeling could be partially prevented in the presence of 0.1 mM UDP-GlcNAc, and completely abolished by 1 mM concentration of this nucleotide. However, UDP-Gal, UDP-Glc and UDP-GalNAc had no effect (Fig. 4). These observations confirm that [¹²⁵I]ASA-UDP-GlcNAc can function as the active site-directed photoaffinity analogue of UDP-GlcNAc.

REFERENCES

1. Drake, R.R. & Elbein, A.D. (1992) *Glycobiology* **2**, 279–284.
2. Evans, R.K., Johnson, J.D. & Haley, B. E. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5382–5386.
3. Drake, R.R., Evans, R.K., Wolf, M.J. & Haley, B.E. (1989) *J. Biol. Chem.* **264**, 11928–11933.
4. Drake, R.R., Zimniak, P., Haley, B.E., Lester, R., Elbein, A.D. & Radomińska, A. (1991) *J. Biol. Chem.* **266**, 23257–23260.
5. Tagaya, M., Nakano, K. & Fukui, T. (1985) *J. Biol. Chem.* **260**, 6670–6676.
6. Rotter, R. & Haley, B.E. (1983) *Methods Enzymol.* **91**, 613–633.
7. Meikle, P.J., Ng, K.F., Johnson, E., Hoogeneraad, E. & Stone, B.A. (1991) *J. Biol. Chem.* **266**, 22569–22581.
8. Holmes, E.H. (1990) *J. Biol. Chem.* **265**, 13150–13156.
9. Szumiło, T., Szumiło, H. & Elbein, A.D. (1993) *Proc. XII-th International Symposium on Glycoconjugates, Kraków, Poland.*
10. Szumiło, T., Drake, R.R., York, J.L. & Elbein, A.D. (1993) *J. Biol. Chem.* **268**, 17943–17950.
11. Laemmli, K.K. (1970) *Nature (London)* **227**, 680–685.
12. Langer, P.N., Waldrop, A.A. & Ward, D.E. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6633–6637.
13. Ji, T.H. & Ji, I. (1982) *Anal. Biochem.* **121**, 286–289.
14. Shanahan, M.F., Wadziński, B.E., Lowndes, J.M. & Rudeo, A.E. (1985) *J. Biol. Chem.* **260**, 10887–10900.
15. Bendiak, B. & Schachter, H. (1987) *J. Biol. Chem.* **262**, 5775–5783.