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ENZYMATIC TRANSFER OF OLIGOSACCHARIDE FROM OLIGOSACCHARIDE-LIPIDS TO AN ASN-ALA-THR CONTAINING HEPTAPEPTIDE

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SUMMARY. Thyroid rough microsomes catalyzed the transfer of oligosaccharide from previously labeled thyroid [Man-^{14}C] oligosaccharide-lipids to a synthetic dinitrophenylated heptapeptide containing a sequence Asn-Ala-Thr. The resulting product revealed an N-glycosidic attachment, probably to the asparaginyl residue in this sequence. The reaction which was time-dependent up to 1 h and exhibited an apparent Km of 94 µM for the DNP-heptapeptide was favoured by dimethylsulfoxide and inhibited by EDTA.

Several lines of evidence have led to the realization that polyprenol phosphosugar derivatives contribute in eukaryotic membranes to the glycosylation of proteins from nucleotide sugars (reviewed in refer 1). According to this pathway lipid pyrophospho oligosaccharides would function as donors to newly made proteins of pre-assembled units containing N-acetylglucosamine and mannose. We have recently shown, with thyroid rough microsomes incubated with GDP-[^{14}C]Man, that lipid-released and protein-released [Man-^{14}C] oligosaccharide moieties exhibited several common properties (2), a result substantiating such a possibility, also suggested from other approaches in the case of thyroid (3,4). The identities of the endogenous proteins glycosylated in this way were nevertheless difficult to ascertain, in thyroid as well as in other tissues.

Two papers have reported that oligosaccharide-lipids could act in the presence of membrane enzymes as donors for the glycosylation of exogenous well-defined proteins : these acceptors were a carbohydrate-free kappa-type immunoglobulin light chain (5) or several sulfitolyzed proteins containing at least one vacant Asn-X-$\begin{matrix}\text{Ser}\\\text{Thr}\end{matrix}$ (6). It has been anticipated from structural examination of numerous glycoproteins that this tripeptide is a necessary but not sufficient requirement for N-glycosylation (7). In the work we wish to report, a synthetic dinitrophenylated heptapeptide has been used as an efficient oligosaccharide acceptor in a rough microsomal system from pig thyroid, supplemented with [Man-^{14}C] oligosaccharide lipids. Its structure is based on a sequence that was determined

Abbreviation : DNP-peptide, dinitrophenylated peptide.

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for a tryptic glycopeptide from human thyroglobulin (8) :

DNP-Ala-Leu-Glu-Asn-Ala-Thr-Arg-NH_2

EXPERIMENTAL

Materials : GDP-[U-^{14}C] mannose (179 Ci/mol) and 1-fluoro-2,4-dinitro-[3,5-^{3}H] benzene (30 Ci/mmol) were from the Radiochem. Centre. Bio-Gels P-2 and P-4 were from Bio-Rad Sephadex G-15 from Pharmacia and carboxymethyl-cellulose (CM-52) from Whatman. The resin and the protected amino acids used for the solid phase synthesis of the peptide were from Beckman.

Synthesis of the heptapeptide H-Ala-Leu-Glu-Asn-Ala-Thr-Arg-NH_2 : This sequence of amino acids has been assembled on 0.8 g of the benzhydrylamine derivative of 1 % cross-linked polystyrene (0.5 mequiv. NH_2/g) by the solid phase method (10) as described for the synthesis of apamin (11). After HF cleavage, the synthetic peptide was purified on a Sephadex G-15 column developed with 0.1 M ammonium acetate. Ion exchange chromatography on a carboxymethyl-cellulose column, equilibrated in 0.012 M ammonium acetate pH 6.0, revealed a nice well-retarded peak. After lyophilization the purity of this material was assessed by amino acid analysis after acid hydrolysis ($Asn_{1.0}$, $Thr_{1.0}$, $Glu_{1.1}$, $Ala_{2.0}$, $Leu_{1.0}$, $Arg_{1.0}$) and total enzymatic digestion (Asn and Thr co-elute, $Glu_{1.0}$, $Ala_{2.0}$, $leu_{1.0}$, $Arg_{1.0}$). The yield of purified peptide over starting NH_2 has been 53 %.

Preparation of the DNP-, and [^{3}H] DNP-heptapeptide(s) : 3 µmoles of the heptapeptide were dissolved in 120 µl of 1 % triethylamine in water and reacted with 30 µmoles of fluorodinitrobenzene dissolved in 240 µl of ethanol, for 90 min under vigorous stirring. The DNP-peptide was purified on a Bio-Gel P_2 column developed with 0.1 M acetic acid. Amino acid analysis after acid hydrolysis showed the disappearance of one alanine.

A similar dinitrophenylation was carried out with 0.8 µmol of heptapeptide, but in the presence of fluorodinitro -[^{3}H] benzene (250 µCi). The specific activity of the resulting purified [^{3}H] DNP-heptapeptide was 8 µCi/µmol.

Preparation of [Man-^{14}C] oligosaccharide-lipids : They were labeled by incubating thyroid rough microsomes (40 mg of protein) with 14 µM GDP-[^{14}C] Man (10 µCi) in a final volume of 4 ml for 10 min at 37°C, as previously described (2). The delipidation procedure was slightly modified according to Lucas et al. (9) in order to minimize the contamination by dolichol-P-Man in the final $CHCl_3/CH_3OH/H_2O$ extract : in these conditions a DEAE-cellulose chromatography revealed 5 % of contamination and the radioactivity obtained was 200 - 250 000 cpm.

Standard assay for transfer of [Man-^{14}C] oligosaccharide from [Man-^{14}C] oligosaccharide-lipids to the DNP-peptide (or to the peptide) : incubation mixtures contained thyroid rough microsomes (240 µg of protein), [Man-^{14}C] saccharide-lipids (1 to 10 x 10^3 cpm, supplied in 15 µl of 25 % dimethylsulfoxide), 530 µM DNP-heptapeptide, 20 mM Tris-HCl pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 12 mM $MnCl_2$, 1.25 mM dithiothreitol and 0.25 M sucrose, in a final volume of 75 µl. Reactions were started with microsomes and stopped after 60 min at 37°C by adding 125 µl of ethanol. After staying for 16h at -17°C the supernatants were spotted on Whatman 3 MM paper and submitted to electrophoresis in 1.5 N formic acid at 34 V/cm for 135 min. Radioactivity was counted in 1 cm wide strips of paper after solubilization in 1 ml of water.

RESULTS AND DISCUSSION

Assay of the DNP-heptapeptide as a potential acceptor of [Man-^{14}C] oligosaccharide from lipid derivatives

Exogenous [Man-^{14}C] oligosaccharide-lipids were incubated with thyroid rough

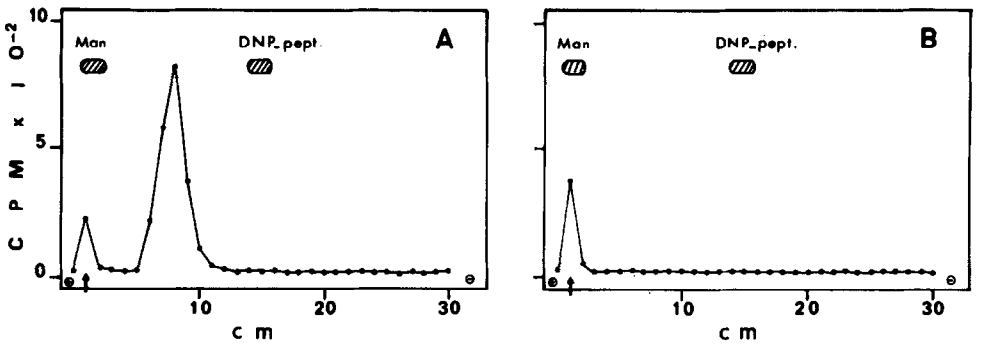

Fig. 1 Electrophoretic comparison of ethanol/water-soluble labels issued from a standard incubation assay of rough microsomes with [Man-^{14}C] oligosaccharide-lipid in the presence (A) or absence (B) of exogenous DNP-heptapeptide. Markers : [^{3}H] DNP-heptapeptide and [^{14}C] mannose. Arrow = origin.

microsomes in the presence or in the absence of unlabeled DNP-heptapeptide, and the supernatants of ethanol precipitation were examined by paper electrophoresis (Fig. 1). In the presence of DNP-peptide, a labeled material was observed at about 8 cm from the origin which was absent in the control assay. The mobility of this compound differs from that of the DNP-peptide (approx. 15 cm, localized from its yellow color, and from the radioactivity of the [^{3}H] DNP-heptapeptide) and from that of oligosaccharides, known to stay at the origin (2), which were present in both assays indicating some cleavage from the labeled donor. The intermediate mobility of the new compound suggests a glycopeptide. In the standard incubation with 530 µM DNP-peptide the label in this peak has repeatedly represented 30 % of the radioactivity in the total incubation (checked by spotting an entire reaction mixture on a paper strip without electrophoretic migration).

As shown in Fig. 2 the formation of the labeled compound migrating at 8 cm was dependent on time, reaching a plateau for 60 min. The effect of increasing the concentration of exogenous DNP-peptide is shown in Fig. 3. An apparent Km of 94 µM has been deduced from these data.

Isolation and further characterization of the glycosylated DNP-peptide

The supernatant of an ethanol precipitation issued from a preparative scale incubation was concentrated and chromatographed on Bio-Gel P-4 (Fig. 4). The major labeled material behaved as a much larger species than the DNP-heptapeptide (detected from its absorbance at 360 nm). This would be expected for a glycosylated form of the latter : we have previously reported on an aver Mr of 1500 for the [Man-^{14}C] saccharide moiety cleaved by mild acid from endogenous saccharide-lipids in thyroid rough microsomes (2), and our DNP-heptapeptide has a Mr of 940.

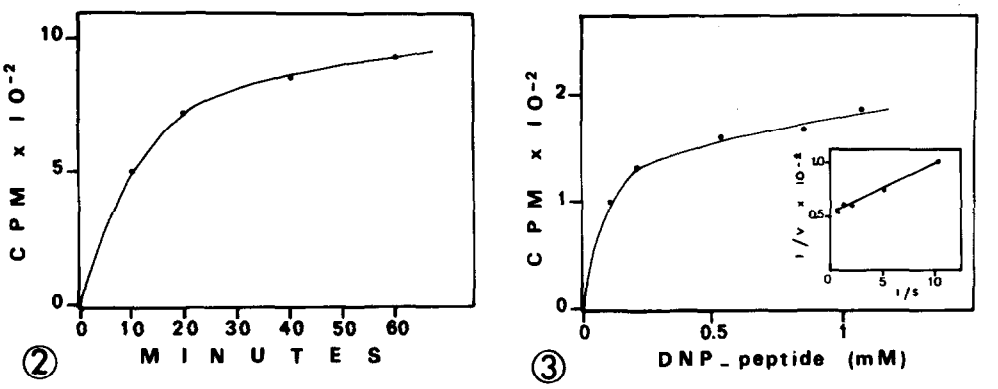

Fig. 2 Time-course of labeling of the material migrating at 8 cm by paper electrophoresis in formic acid. Conditions of a standard assay.

Fig. 3 Effect of varying the concentration of DNP-heptapeptide on the labeling of the material migrating at 8 cm by paper electrophoresis in formic acid. The inset shows the Lineweaver-Burk plot of the data.

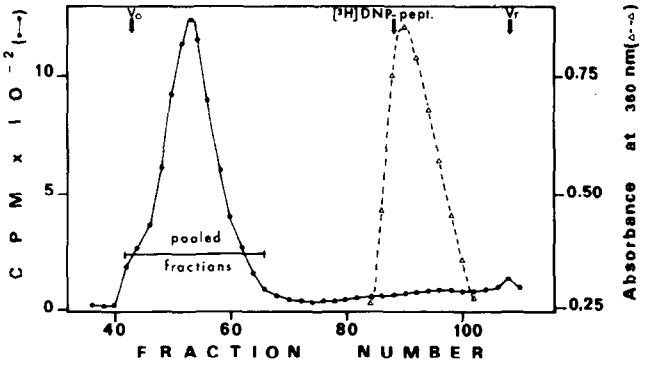

Fig. 4 Gel filtration on Bio-Gel P_{-4} of the [^{14}C] labeled ethanol/water supernatant issued from a 2 ml incubation in standard conditions (except for a 20 % final concentration in dimethylsulfoxide). Running buffer : pyridine-acetate, pH 5.1 (1 M in pyridine). Column size : 0.8 x 75 cm. Fractions of 0.6 ml. Markers : Blue Dextran , [^{3}H] DNP-heptapeptide and [^{14}C] mannose.

Pooled radioactive fractions were lyophilized. The pale yellow colored material was submitted to paper electrophoresis in formic acid and found to migrate at a distance of 8 cm from origin (data not shown).

In order to assess the nature of the linkage between the [^{14}C] mannosylated moiety and the peptide, aliquots of this lyophilized material were submitted to reductive alkaline treatments generally used for selective cleavage of either O-glycosidic or N-glycosidic linkages, as previously reported (2). It was found

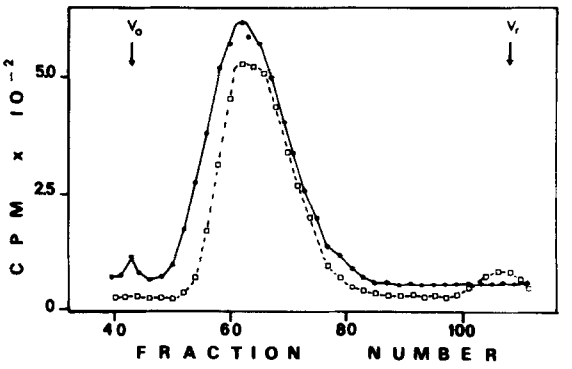

Fig. 5 Gel filtration on Bio-Gel P_{-4} of the [Man-^{14}C] oligosaccharide moiety cleaved by strong reductive alkaline hydrolysis from the purified [Man-^{14}C] glycosylated DNP-peptide, and comparatively (dotted line) of the [Man-^{14}C] oligosaccharide moiety released by mild acid from the saccharide-lipid donor. Column size and experimental conditions as in Fig. 4.

by gel filtration on Bio-Gel P-4 that a treatment for β-elimination resulted in no detectable change (data not shown). On the contrary after a treatment by 2 N NaOH, 2 M Na BH_4, at 80°C for 16h, an essentially complete cleavage of the labeled moiety was observed (Fig. 5) : the radioactive peak now emerged from the column at a position close to that determined in a parallel run for [Man-^{14}C] oligosaccharide moieties that were cleaved by mild acid from the endogenously labeled donor. Since deacylation is known to occur in strong reductive alkaline conditions the material released from the [^{14}C] glycosylated DNP-peptide was treated for N-acetylation of its putative glucosamine residues (2), and examined by paper electrophoresis : it behaved as a neutral compound and stayed entirely at the origin, as does the acid-released moiety issued from [Man-^{14}C] saccharide-lipids (2).

Altogether these results provide reasonable evidence that within the rough membranes an enzyme is present which transfers an oligosaccharide unit from exogenous oligosaccharide-lipids to the asparaginyl residue of the synthetic DNP-heptapeptide.

Other properties of the system

Increasing the final concentration of dimethyl sulfoxide, used as a dispersing agent, from 5 to 25 % was found to have a slightly stimulatory effect on the extent of transfer. When, instead, we used Triton X-100 at a final concentration of 0.02 % the results were not satisfactorily reproducible. The reaction was totally inhibited by 10 mM EDTA. These observations suggest that the enzyme is dependent on a relative integrity of the membrane and on a metal ion.

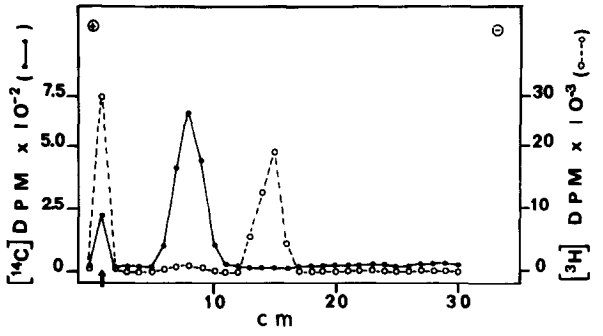

Fig. 6 Electrophoretic profile of the ethanol/water soluble labels issued from an incubation of [Man-^{14}C] oligosaccharide-lipids with [^{3}H] DNP-heptapeptide. The incubation was as described in Experimental except for being 700 µM in DNP-peptide. Double isotope counting using adequately quenched standards.

When the synthetic heptapeptide was used in a standard assay instead of its DNP-derivative it exhibited about half the acceptor activity of the latter, as deduced from the proportion of label localized by paper electrophoresis in a new species, migrating beyond the glycosylated DNP-peptide. It is probable that the DNP-group facilitates the peptide to penetrate the lipophilic environment of the enzyme. As regards the usefulness of this hydrophobic group a similar observation has been reported by Adamany and Spiro when assaying various mannose terminating acceptors in a search for α-mannose transferase activity (12).

We have nevertheless noticed that an extensive cleavage of the dinitrophenylated moiety occurs in our system. This cleavage probably competes with the reaction of glycosylation.

When the [^{3}H] DNP-heptapeptide was used as acceptor in a microsomal incubation with [Man-^{14}C] oligosaccharide-lipids, and the supernatant from an ethanol precipitation analyzed by paper electrophoresis, it was observed (Fig. 6) that, in spite of this important cleavage (resulting in a [^{3}H] radioactivity, peak remaining at origin), there was a definite [^{3}H] label associated with the material migrating at 8 cm. From the [^{3}H] radioactivity associated with this double-labeled peak, it has been evaluated that 0.25 nmoles of DNP-heptapeptide have been glycosylated (of the 49 nmoles initially present).

In conclusion our data demonstrate the N-glycosylation of a synthetic well-defined peptide catalyzed by a microsomal oligosaccharide-lipid : peptide oligosaccharide transferase. Other synthetic analogs might help to find out if some conformational requirements are necessary in addition to the basic sequence Asn-X-Thr (or Ser). Another important problem will be to assess the specificity of this

transferase as regards the length and composition of the oligosaccharide moiety in lipid donors. Finally, the ability of thyroid rough microsomes to glycosylate an exogenous peptide indicates that this transfer might not be dependent on a concomitant protein synthesis, as recently concluded by Vargas and Caminatti from a different approach (13) ; alternatively the proposal made by Kiely et al. (14) of a lipid-mediated glycosylation occuring on the hydrophobic extremities of ribosome attached nascent peptides might correspond to the process occuring in vivo at the level of the rough E.R.

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