The UDP-*N*-acetylglucosamine–Asparagine-Sequon *N*-Acetyl- β -D-glucosaminyltransferase Activity in Preparations of Rough Endoplasmic Reticulum from Regenerating Rat Liver

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The UDP-*N*-acetylglucosamine – asparagine-sequon *N*-acetylglucosaminyltransferase activity in preparations of rough endoplasmic reticulum from rat liver is less than 1 % of that in preparations from rabbit liver. The activity of the enzyme is increased about 20-fold in preparations from regenerating rat liver within 48 h after partial hepatectomy. A smaller, but still marked, increase (8–10-fold) occurs in preparations of rough endoplasmic reticulum from sham-operated rats.

Synthesis of the glycoproteins of serum and of the structural glycoproteins of the hepatocyte must involve the enzymic formation of the carbohydrate–protein linking moiety, 4-*N*-(2-acetamido-2-deoxy- β -D-glucopyranosyl)-L-asparagine. The asparagine residue to which the sugar becomes attached must occur in a sequence of the form ...Asn-X-Thr... or ...Asn-X-Ser... (Marshall, 1967; Neuberger & Marshall, 1968), which is termed an asparagine sequon (Marshall, 1974). There is known to be enzymic activity, in preparations of rough endoplasmic reticulum from rabbit liver, which can catalyse formation of the 4-*N*-(2-acetamido-2-deoxy- β -D-glucopyranosyl)-L-asparagine linkage (Khalkhali & Marshall, 1975). Attempts (Z. Khalkhali, F. Serafini-Cessi & R. D. Marshall, unpublished work) to demonstrate such an enzymic activity in similar preparations from rat liver yielded equivocal results, although glycoproteins are known to be synthesized by rat liver (Molnar, 1975).

There is an increased rate of synthesis, per unit wet wt. of liver, of serum glycoproteins by regenerating rat liver (Mutschler & Gordon, 1966; Akamatsu *et al.*, 1976), as well as an increased activity of glucosamine phosphate isomerase (glutamine-forming) (EC 5.3.1.19; Akamatsu & Maeda, 1971). The latter enzyme may be involved in the regulation of glycoprotein biosynthesis. It seemed possible that the increased rate of production of glycoproteins by regenerating liver might require higher activities than normal of that sugar transferase involved in the formation of 4-*N*-(2-acetamido-2-deoxy- β -D-glucopyranosyl)-L-asparagine linkages. It was decided therefore to examine the activity of UDP-*N*-acetylglucosamine – asparagine-sequon *N*-acetylglucos-

aminyltransferase in preparations of rough endoplasmic reticulum isolated from regenerating rat liver.

Experimental

Materials

The sources of most of the materials used were described previously (Khalkhali & Marshall, 1976). Wheat-germ agglutinin bound to Sepharose was from Miles Laboratories (Stoke Poges, Bucks., U.K.).

Female rats

Female rats (200–250 g) of the Wistar strain, fed on laboratory chow and water *ad lib.*, were used. Partial hepatectomy (Higgins & Anderson, 1931; Waynforth, 1969) was performed under diethyl ether anaesthesia. Sham operation consisted of laparotomy and palpation of the liver.

Methods

Preparations of rough endoplasmic reticulum. These were isolated from the livers of control animals, as well as from sham-operated and partially hepatectomized rats 24, 48 and 72 h after operation. The technique of Dallner (1963), as described by Lawford & Schachter (1966), was used. The subcellular fractions were prepared for use by washing them with 0.9% KCl, and finally suspending them in 50 mM-Tris/maleate buffer (50 mM final concentrations of both Tris and maleate, adjusted to pH 7.1 with NaOH).

Protein. This was determined by the procedure of Lowry *et al.* (1951), with bovine serum albumin as standard.

Measurements of enzyme activity. Incubation mixtures of 100 μ l volume contained 50 mM-Tris/maleate, pH 7.1, 50 mM-MnCl₂, 0.506 mM-UDP-*N*-acetyl[U-¹⁴C]glucosamine (1096 d.p.m./nmol), 0.4% Triton X-100, 100 μ g of bovine pancreatic ribonuclease A and appropriate amounts of the various preparations of rough endoplasmic reticulum. In all cases control incubation mixtures were also set up, and these did not contain added ribonuclease A. Incubations were done for measured periods of time at fixed temperatures, and reactions were stopped by the addition of 2 ml of a cold solution containing 1% phosphotungstic acid in 0.5 M-HCl. The precipitates formed were collected on glass-fibre filters (Whatman GF/A, 2.1 cm diam.), carefully washed with the precipitating acid (10 $\times$ 5 ml), then with 5 ml of 5% (w/v) trichloroacetic acid and air-dried. The incorporation of *N*-acetyl-D-glucosamine into the precipitates was determined by radioactivity counting.

Radioactivity counting. This was done by placing the glass-fibre filters in 6 ml of methylCellosolve and adding 10 ml of scintillator [2 vol. of toluene, containing 0.5% 2,5-diphenyloxazole and 0.02% 1,4-bis-(5-phenyloxazol-2-yl)benzene, and 1 vol. of Triton X-100]. A Packard Tri-Carb scintillation counter was used and the efficiency of ¹⁴C counting in the solvent used was 62%. Corrections were applied for quenching by protein.

Identification of the product formed by enzymic glycosylation. Ribonuclease A (1 mg) was incubated at 37°C for 1 h in presence of 50 mM-Tris/maleate, pH 7.1, 50 mM-MnCl₂, 0.506 mM-UDP-*N*-acetyl[U-¹⁴C]glucosamine (1096 d.p.m./nmol) and a preparation of rough endoplasmic reticulum (1 mg of protein) isolated from rat liver 48 h after partial hepatectomy, all in a final volume of 1 ml. A control experiment was also done in which ribonuclease A was omitted from the incubation mixture.

The reaction was stopped by cooling and the mixture was centrifuged (3000g, 10 min, 2°C). The supernatant was applied to a column (9 cm $\times$ 0.9 cm) of CM-cellulose and chromatographed as described before (Khalkhali & Marshall, 1976). The fractions containing both ribonuclease A and the putative monoglycosylated ribonuclease were combined, dialysed against water and freeze-dried. The residue was dissolved in 1 ml of Tris/HCl, pH 7.0 (10 mM in Tris), and the solution was applied to an affinity column (2.0 cm $\times$ 0.3 cm) of wheat-germ agglutinin-Sepharose and chromatography was performed as described previously (Khalkhali & Marshall, 1976).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. This was carried out by the procedure of Marshall & Zamecnik (1969) (see also Khalkhali & Marshall, 1976).

Ribonuclease activity. This was assayed by the method of Kalnitsky *et al.* (1959).

Results

Preparations of rough endoplasmic reticulum isolated from regenerating rat liver 48 h after partial hepatectomy were found to contain higher activities of UDP-*N*-acetylglucosamine-asparagine-sequon *N*-acetylglucosaminyltransferase than did those from rats which had been subjected to laparotomy and palpation of the liver. There was no direct proportionality between the extent of incorporation of *N*-acetylglucosamine into the acceptor substrate, ribonuclease A, and the amount of rough endoplasmic reticulum used (Fig. 1). Comparative estimates of activity may nevertheless be made by considering the results obtained in the presence of relatively small amounts of enzyme protein (for example 20 μ g), and it is suggested that the rough endoplasmic reticulum from the hepatectomized animal was about 4–5 times as active as that from the laparotomized rat. There

Fig. 1. Incorporation of *N*-acetylglucosamine into ribonuclease A catalysed by preparations of rough endoplasmic reticulum: ○, from rabbit liver; ■, from regenerating rat liver, 48 h after partial hepatectomy; ●, from sham-operated rat liver, 48 h after operation.

Incubations (see under 'Methods') were for 1 h at 37°C, and the UDP-*N*-acetylglucosamine concentration in the experiments in which preparations of rabbit liver were used was 0.28 mM. Control experiments were always done, where ribonuclease A was omitted from the incubation mixtures, and corrections were applied for glycosylation of endogenous glycoproteins. Replicate values with a given preparation of rough endoplasmic reticulum generally agreed within 6%.

was even higher enzymic activity in preparations of rough endoplasmic reticulum from rabbit liver (Fig. 1), which was tested at a lower concentration (0.28 mM) of UDP-*N*-acetylglucosamine than was used in the experiments with preparations from rat liver. Activities of rabbit liver preparations in the presence of 100 μ g of rough-endoplasmic-reticulum protein at concentrations of UDP-*N*-acetylglucosamine of 0.1, 0.2, 0.28, 0.4 and 0.5 mM were in the proportions 0.54, 0.84, 1.0, 1.16 and 1.26 respectively.

Preparations of rough endoplasmic reticulum from normal rat liver appeared to contain extremely low

4-*N*-(2-acetamido-2-deoxy- β -D-glucopyranosyl)-L-asparagine synthetase activity, which increased to a maximum value about 20-fold greater in regenerating liver within 48 h after partial hepatectomy (Fig. 2).

In the experiments described so far, it has been assumed that the differences obtained in incorporation of *N*-acetylglucosamine into acid-precipitable protein in the presence or absence of added ribonuclease A could be interpreted as an indication that the latter was indeed the acceptor of the sugar. This reasonable assumption was confirmed by isolation and identification of the glycosylated ribonuclease.

The products obtained when 1 mg of bovine pancreatic ribonuclease A was glycosylated, with a preparation of rough endoplasmic reticulum made from regenerating liver 48 h after partial hepatectomy as the enzyme source (see under 'Methods'), were chromatographed on CM-cellulose (Fig. 3). Ribonuclease that had undergone glycosylation (fractions 52–60) is known to be eluted, under the conditions of chromatography used, immediately before and overlapping with ribonuclease A (Khalkhali & Marshall, 1975, 1976). Fractions 50–70 (Fig. 3; 1752 pmol of *N*-acetylglucosamine, equivalent to 2.5% glycosylation of the ribonuclease A used) were combined and subjected to chromatography (see under 'Methods') on wheat-germ agglutinin-Sepharose. The radioactive compound was eluted from the affinity column at a relatively low concentration (0.15 mM) of the *N*-acetylglucosamine eluting agent. This concentration is near to that found earlier to be needed to release monoglycosylated ribonuclease from an affinity column (Khalkhali & Marshall

Time after partial hepatectomy or sham operation (h)

Fig. 2. *N*-Acetylglucosaminyltransferase activities of preparations of rough endoplasmic reticulum made from regenerating rat liver (■) or from the livers of sham-operated animals (●)

▲, Results with control animals. Assays were done as described in the legend to Fig. 1, except that the results are in all cases for 100 μ g of rough-endoplasmic-reticulum protein.

Fig. 3. Chromatography on a column (9 cm $\times$ 0.9 cm) of CM-cellulose of a mixture incubated as described under 'Methods'

About 2.5% conversion of ribonuclease A (eluted from the column at 0.07 M-NaCl) into *N*-acetylglucosaminyl-ribonuclease (eluted at 0.067 M-NaCl) had occurred. ○, *A*₂₂₀ (protein); ■, *N*-acetylglucosamine incorporated into ribonuclease (i.e. monoglycosylated ribonuclease); ----, concn. of NaCl in the eluent. Fractions of volume 3 ml were collected.

Fig. 4. Separation by chromatography on a column (2.0 cm $\times$ 0.3 cm) of wheat-germ agglutinin-Sephadex of ribonuclease A and N-acetylglucosaminyl-ribonuclease

For details see under 'Methods'. $\circ$, A_{220} ; $\blacksquare$, N-acetylglucosamine incorporated into ribonuclease; $\square$, ribonuclease activity (A_{260} ; Kalnitsky *et al.*, 1959) of monoglycosylated ribonuclease; ----, concentration of sugar (N-acetylglucosamine) in the eluent. Fractions of volume 1.4 ml were collected. There was no radioactive bound glucosamine in fractions 1–25 or in fractions 37–45. Only fractions 26–37 were tested for ribonuclease activity (Kalnitsky *et al.*, 1959), to assess whether the radioactivity-labelled protein eluted at that position had ribonuclease activity.

1976). Moreover, this peak is associated with ribonuclease activity (Fig. 4). This peak of ribonuclease contained 1475 pmol of bound N-acetylglucosamine, which corresponds to glycosylation of 2.1% of the ribonuclease A originally used.

A portion (0.30 ml) of the radioactive fraction 31 from the wheat-germ agglutinin-Sephadex column (Fig. 4) was subjected to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Marshall & Zamecnik, 1969). The gel was cut laterally into 3 mm slices and these were dissolved in 2 ml of aq. 30% (v/v) H_2O_2 in scintillation vials (Goodman & Matzura, 1971). The solutions were evaporated to dryness and the residues were counted for radioactivity. In only one of them, that corresponding to the position of ribonuclease A run as a control, was there any radioactivity (70 pmol; 94% recovery).

All these results show that preparations of rough endoplasmic reticulum from regenerating rat liver or from the liver of injured rats can catalyse the incorporation of glucosamine into ribonuclease A, with UDP-N-acetylglucosamine as the donor. It is likely from our previous studies (Khalkhali & Marshall, 1975, 1976; Khalkhali *et al.*, 1976) that asparagine-34 is the glycosylated residue.

Discussion

Preparations of rough endoplasmic reticulum, made from rat liver by the procedure of Dallner (1963), appear to have extremely low UDP-N-acetylglucosamine-asparagine-sequon N-acetylglucosaminyltransferase activity. The results obtained by our standard assay procedure suggest that preparations from rabbit liver have transferase activity that is about 100 times that in normal rat liver. The reasons for the observations are unknown and the results serve solely to explain why we were unable, in earlier experiments, to isolate monoglycosylated ribonuclease from appropriate incubation mixtures containing rat liver rough endoplasmic reticulum as a source of enzyme, whereas the product could be isolated in a reasonable yield when rabbit liver rough endoplasmic reticulum was used (Khalkhali & Marshall, 1975). It is possible that the low proportion of carbohydrate (23%) in orosomucoid in rat serum (Charlwood *et al.*, 1976) compared with that in rabbit serum (44%) is due, in part at least, to the lower activity of the asparagine-sequon-glycosylating enzyme in rat liver, but there may be other explanations.

There is an increased transferase activity in the rough endoplasmic reticulum of the livers of rats which have undergone laparotomy and palpation of the liver, over the course of a few days after the operation. There are also higher activities of the enzyme in regenerating rat liver, with an apparent peak of activity about 48 h after partial hepatectomy. The implications of our findings with respect to the increases in the contents of serum glycoproteins seen in a number of pathological states cannot be usefully speculated on until much more knowledge is available about the mechanism of action of the transferase in question.

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