Glycosylation *in vitro* of an Asparagine Sequon Catalysed by Preparations of Yeast Cell Membranes

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Preparations of yeast cell membranes can catalyse *in vitro* the *N*-acetyl- β -D-glucosaminyl-ation of the asparagine sequon at residues 34–36 of bovine pancreatic ribonuclease A. The relevant glycopeptides were isolated from tryptic hydrolysates of the glycosylated ribonuclease and analysed. The donor used was UDP-*N*-acetyl-D-glucosamine, although the mechanism of the transfer is unknown. Mn^{2+} ions at concentrations of 25 mM double the activity of the enzymic transfer.

An asparagine residue in an apoprotein may act as the site for attachment of a carbohydrate moiety (Kohn & Yamashina, 1973; Khalkhali & Marshall, 1975, 1976) through a linkage of the form 4-*N*-(2-acetamido-2-deoxy- β -D-glucopyranosyl)-L-asparagine (GlcNAc-Asn; Marshall & Neuberger, 1972). The accepting asparagine moiety must occur in a sequence of the form Asn-X-Thr or Asn-X-Ser (Marshall, 1967; Neuberger & Marshall, 1968) which has been termed a sequon (Marshall, 1974) in order that the residue may function as an acceptor for the sugar.

Glycosylation of appropriate asparagine residues of serum glycoproteins seems to occur at the polyribosomal level, at least in part (Molnar *et al.*, 1965; Lawford & Schachter, 1966; Simkin & Jamieson, 1967; Redman & Cherian, 1972), and an enzyme with the requisite substrate specificity has been shown to be present in the rough endoplasmic reticulum of rabbit liver (Khalkhali & Marshall, 1975). There is also similar enzymic activity in serum, and a reason for its presence there has been suggested (Khalkhali & Marshall, 1976).

The results described below show that there is enzymic activity in yeast cell-membrane preparations, which is able to catalyse *in vitro* the glycosylation of asparagine-34 of bovine pancreatic ribonuclease A.

Experimental

Materials

The sources of many of the materials have been described (Khalkhali & Marshall, 1976). The ribonuclease A was purchased from Boehringer Corp. (London) Ltd., Bell Lane, Lewes, East Sussex BN7 1LG, U.K. (cat. no. 15410) and was chromatographed on CM-cellulose before use (Taborsky,

1959). It was shown to be free of glucosamine (Khalkhali & Marshall, 1975) before use. The yeast mutant x 2180-1A-5mnn2 was supplied by Dr. C. E. Ballou, Department of Biochemistry, University of California, Berkeley, CA 94720, U.S.A. and tunicamycin was kindly provided by Dr. G. Tamura, Laboratory for Microbiology, Department of Agricultural Chemistry, University of Tokyo, Bunkyo-ku 113, Tokyo, Japan.

Methods

Preparation of yeast cell membranes. *Saccharomyces cerevisiae*, mutant x 2180-1A-5mnn2, was grown at 30°C in a medium similar to that described previously (Van Rijn *et al.*, 1972). Adenine (0.37 mM) was added and half the amount of $(NH_4)_2SO_4$ was replaced by the addition of 40 ml of phosphate-free casamino acid solution for each litre of medium. The casamino acid solution was prepared by dissolving 40 g of casamino acids (Difco Laboratories, Detroit, MI, U.S.A.) and 6 g of $MgSO_4 \cdot 7H_2O$ in 300 ml of water. The pH of the preparation was adjusted to 8.0 (25% v/v, satd. NH_3 in water, sp. gr. 0.88) and the volume made up to 400 ml. The mixture was kept at 0°C for 3 h and filtered. The solution contained less than 0.1 mM-phosphate.

Cells were harvested in the late-exponential phase and 1.5×10^{10} cells/20 ml of Tris/HCl buffer (0.05 M in Tris), pH 7.0, containing 1 mM-mercaptoethanol, were mechanically disrupted at 0°C in a cell homogenizer (MSK, Braun, Melsungen, German Federal Republic), by shaking them for 5 min in the presence of 25 ml of Ballotini beads (0.25–0.30 mm diameter). Cell debris was removed as a precipitate after centrifuging the homogenate (10 min, 1000g, 4°C). The supernatant was centrifuged (30 min, 40000g, 4°C), and the pellet, consisting of membranes, was used as

a suspension in the Tris/HCl buffer, pH 7.0, containing 1 mM-2-mercaptoethanol. It contained 21 mg of protein/ml, the protein being assessed by the procedure of Lowry *et al.* (1951), with bovine serum albumin as standard.

Microsomal preparation from rabbit liver. This was prepared by the procedure of Keller & Zamecnik (1956). It was finally prepared for use as a suspension in the same buffer and at the same concentration as the yeast cell-membrane preparation.

Tryptic digestion of glycosylated ribonuclease. This was carried out by procedures closely similar to those used earlier (Khalkhali & Marshall, 1975), the crystalline trypsin used (Boehringer; cat. no. 15330) having been freed of extraneous proteinases by the technique of Maroux *et al.* (1962).

All other methods used were described previously (Khalkhali & Marshall, 1976).

Results

The first series of experiments involved measuring the extent of incorporation of labelled glucosamine into bovine pancreatic ribonuclease A catalysed by preparations of yeast cell membranes. Incubations were carried out both in the presence and in the absence of added ribonuclease A, under the conditions described in Table 1, column (a), and the differences in the amounts of radioactivity incorporated were assessed (Fig. 1). There is clearly extensive incorporation of glucosamine into endogenous material in the yeast cell membranes as well as into added ribonuclease A. After incubation for 1 h under the conditions used, about 3.3 nmol of glucosamine was incorporated into endogenous glycoproteins/mg of enzyme protein, and about 7.9 nmol/mg of ribonuclease A.

Table 1. Conditions used in the incubation medium for incorporation of glucosamine into bovine pancreatic ribonuclease A

The temperature was in all cases 37°C. In (a) the enzyme was a preparation of yeast cell membranes, and in (b) it was a preparation of either yeast cell membranes or rabbit liver microsomal fraction (see under 'Methods' in all cases).

Assay conditions	(a)	(b)
Tris/HCl buffer, pH 7.2 (mM)	45	45
MnCl ₂ (mM)	25	25
UDP-GlcNAc (mM)	0.355	2.84
(c.p.m./nmol)	222	78.3
Ribonuclease A (μM)	71.4	714
(mg)	0.2	10
Triton X-100 (%)	0.4	0.4
Total volume (ml)	0.2	1
Amount of enzyme used (μl)	20	800
Incubation time (h)	0.4	1

The effects of Mn²⁺ concentration on the rate of glycosylation of ribonuclease A were studied. Incubation mixtures were set up under conditions almost identical with those described in column (a), Table 1,

Fig. 1. Rate of incorporation of *N*-acetyl-D-glucosamine from UDP-*N*-acetyl-D-glucosamine into ribonuclease A catalysed *in vitro* by yeast cell membranes

The conditions of incubation were as described in column (a) of Table 1. Incubation was effected either in the presence of added ribonuclease A (●) or in its absence (○). The incorporation into added ribonuclease A is indicated by —. Replicate values generally agreed within 6% and incorporation of radioactivity was always greater in those samples that contained added ribonuclease A.

Fig. 2. Effect of Mn²⁺ concentration on the rate of incorporation of *N*-acetyl-D-glucosamine from UDP-*N*-acetyl-D-glucosamine into ribonuclease A

The conditions of incubation were identical with those in column (a), Table 1, apart from the variation in Mn²⁺ concentration. Time of incubation was 1 h. Incubation was effected either in the presence of added ribonuclease A (●) or in its absence (○). The incorporation into added ribonuclease A is indicated by —.

Fig. 3. Chromatography on CM-cellulose columns (0.9 cm $\times$ 9 cm) of incubation mixtures prepared under the conditions described in column (b), Table 1

The mixtures were incubated with: (a) ribonuclease A (10 mg) and a rabbit liver microsomal preparation; (b) ribonuclease A (10 mg) and a preparation of yeast cell membranes; (c) yeast cell membranes and ribonuclease A (2.64 mg) was added, but only after incubation; (d) yeast cell membranes but no added ribonuclease A at all. Other details of the experiments are described in the text. $\circ$, E_{280} ; $\bullet$, radioactivity; Δ , $[\text{NaCl}]$; 3 ml fractions were collected.

apart from having Mn^{2+} concentrations over the range 0–125 mM. The results show that the optimum concentration of Mn^{2+} is of the order of 25 mM (Fig. 2).

Separation of glycosylated ribonuclease A

It was shown that the additional radioactivity incorporated into protein when ribonuclease A was in the incubation mixture did in fact represent glycosylation of the added pancreatic enzyme. The following experiments were done in order to examine this aspect.

Three incubation mixtures were prepared under the conditions described in column (b), Table 1, and each was incubated at 37°C for 1 h. The first of these contained 10 mg of added ribonuclease A and the enzyme used in this case, for comparative purposes, was a rabbit liver microsomal preparation. The second contained 10 mg of ribonuclease A and as enzyme the yeast cell-membranes preparation. The third incubation mixture was devoid of added ribonuclease A and it contained the yeast cell-membranes preparation. The mixtures were centrifuged (2000g, 10 min, 4°C) after incubation and the third was divided equally into two parts, to one of which was added 2.64 mg of ribonuclease A at that stage. The four preparations [a, with ribonuclease A (10 mg) incubated in presence of rabbit liver microsomal preparation; b, with ribonuclease A (10 mg) incubated in presence of yeast cell membranes; c, with ribonuclease A (2.64 mg) added after incubation; d, without any added ribonuclease A] were subjected to chromatography on CM-cellulose. In all cases the weights of ribonuclease A used were not corrected for either water or ash content.

The results obtained show that preparations of yeast cell membranes as well as rabbit liver microsomal preparations contain enzymic activity which can catalyse the incorporation of *N*-acetyl-D-glucosamine from UDP-*N*-acetyl-D-glucosamine into ribonuclease A. If no ribonuclease A was present in the original incubation mixture, no peak of protein was eluted from the column at a NaCl concentration of about 0.07 M (Fig. 3d), which are the conditions required for elution of ribonuclease A from the CM-cellulose column under the conditions used (Tabor-sky, 1959). In the experiment in which ribonuclease A was added to the incubation mixture, but only after incubation, the pancreatic enzyme was eluted at the expected position (Fig. 3c) in an amount of 1.8 mg, assuming $E_{280}^{1\%} = 7.2$ (Scheraga & Rupley, 1962), but no radioactive glucosamine appeared in this region.

On the other hand, when the preparation of yeast cell membrane was incubated with ribonuclease A and UDP-*N*-acetyl-D-glucosamine under the conditions described in column (b), Table 1, chromatography of the products on CM-cellulose led to the appearance of the ribonuclease A in the eluate at an

NaCl concentration of about 0.07 M (Fig. 3b), and a peak of radioactive glucosamine was also eluted in this region. The radioactive substance is almost certainly monoglycosylated ribonuclease A, in view of the results reported below. Moreover, it was shown previously that this latter substance chromatographs in that position (Khalkhali & Marshall, 1975, 1976). The amount of glucosamine incorporated is about 16.1 nmol/7.7 mg of ribonuclease A, which is equivalent to 0.029 residue/molecule. In the control experiment using a rabbit liver microsomal preparation as the enzyme source (Fig. 3a), 26.0 nmol of glucosamine was incorporated/7.9 mg of ribonuclease A, which is equivalent to 0.046 residue/molecule. The latter value is of the order found previously when rough endoplasmic reticulum, isolated from rabbit liver, was used as the enzyme source.

Identification of the position at which ribonuclease A is glycosylated

It was shown by isolation of the radioactive tryptic peptide from the glycosylated ribonuclease A that the pancreatic enzyme had undergone substitution at residue 34, where the asparagine sequon -Asn-Leu-Thr- occurs. The tryptic digest of fractions 56–65 (Fig. 3b; 1220 c.p.m. $\equiv$ 15.6 nmol of glucosamine, 3.98 mg of total ribonuclease, equivalent to 0.055 residue/molecule of ribonuclease) was subjected to gel filtration on Sephadex G-25 (1 cm $\times$ 250 cm) in 0.1 M-acetic acid. Fractions (1 ml) were collected and the only radioactive peak was in fractions 80–87 inclusive (1061 c.p.m., 87% recovery). These fractions were combined and the solution was freeze-dried. The residue was subjected to chromatography on Amberlite CG-120 under conditions identical with those described earlier (Khalkhali & Marshall, 1975). Of the three peaks that were apparent by ninhydrin assay, only one, the middle one, was radioactive (1016 c.p.m., 96% recovery). Analysis of acid hydrolysates (4 M-HCl; 100°C; 4 h) of this peak on the amino acid analyser showed the presence of aspartic acid, leucine, threonine and lysine only, in amounts of 1.0, 0.99, 1.07 and 1.03 mol respectively/0.062 mol of glucosamine. These results show that the peak analysed consisted of a mixture of about 94% of the peptide Asn-Leu-Thr-Lys and about 6% of its glycosylated derivative, and it is known from earlier studies that this peptide and the glycopeptide co-chromatograph under the conditions used (Khalkhali & Marshall, 1975). This is a relative composition almost identical with that of the mixture of ribonuclease A and its glycosylated derivative which was originally subjected to tryptic digestion. The mixture of peptide and glycopeptide must be derived from residues 34–37 of the original ribonuclease A, where the sequence is Asn-Leu-Thr-Lys and the asparagine residue is preceded by an arginine residue.

Discussion

The results of the above experiments show that there is enzymic activity in preparations of yeast cell membranes which can catalyse *in vitro* the *N*-acetyl- β -D-glucosaminylation of the single asparagine sequon in bovine pancreatic ribonuclease A, that at residues 34–36. The results demonstrate that this is the only asparagine residue in the pancreatic enzyme that was found to undergo glycosylation.

The mechanism of transfer of the sugar is unresolved because preparations of yeast cell membranes can catalyse the transfer of *N*-acetyl-D-glucosamine 1-phosphate from UDP-*N*-acetyl-D-glucosamine to dolichol phosphate (Lehle & Tanner, 1975). Dolichol pyrophosphate *N*-acetyl-D-glucosamine can be used *in vitro* as the donor for the glycosylation of endogenous glycoproteins of yeast cell-membrane preparations (C. Habets-Willems, unpublished work), but whether the sugar becomes directly linked to an asparagine residue in the protein chain(s) of the acceptor is unknown. In any case the main question is whether a sugar-lipid is an obligatory intermediate in the transfer of the sugar from UDP-*N*-acetyl-D-glucosamine to an asparagine sequon. There was found to be extensive (80%) inhibition of the formation of glycosylated ribonuclease by the preparations of yeast cell membranes in the presence of tunicamycin (12 μ M). This antibiotic has the facility to inhibit *in vitro* the enzymic transfer of the sugar from UDP-*N*-acetyl-D-glucosamine to an acceptor present in calf liver microsomal preparation with the formation of a polyisoprenyl *N*-acetyl-D-glucosaminyl pyrophosphate (Tkacz & Lampen, 1975). Further experiments are needed to establish whether the inhibition of glycosylation of ribonuclease is due to a lower rate of synthesis of a lipid intermediate.

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References

- Keller, E. B. & Zamecnik, P. C. (1956) *J. Biol. Chem.* **221**, 45–59
- Khalkhali, Z. & Marshall, R. D. (1975) *Biochem. J.* **146**, 299–307
- Khalkhali, Z. & Marshall, R. D. (1976) *Carbohydr. Res.* **49**, 455–473
- Kohno, M. & Yamashina, I. (1973) *J. Biochem. (Tokyo)* **73**, 1089–1094
- Lawford, G. R. & Schachter, H. (1966) *J. Biol. Chem.* **241**, 5408–5418
- Lehle, L. & Tanner, W. (1975) *Biochim. Biophys. Acta* **399**, 364–374
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Maroux, S., Ravery, M. & Desnuelle, P. (1962) *Biochim. Biophys. Acta* **56**, 202–206
- Marshall, R. D. (1967) *Abstr. Int. Congr. Biochem.* **7th** 3, 573–574
- Marshall, R. D. (1974) *Biochem. Soc. Symp.* **40**, 17–26
- Marshall, R. D. & Neuberger, A. (1972) in *Glycoproteins* (Gottschalk, A., ed.), 2nd edn., pp. 453–470, Elsevier Publishing Co., Amsterdam
- Molnar, J., Robinson, G. B. & Winzler, R. J. (1965) *J. Biol. Chem.* **240**, 1882–1888
- Neuberger, A. & Marshall, R. D. (1968) in *Symposium on Foods: Carbohydrates and their Roles* (Schultze, H. W., Cain, R. F. & Wrolstad, R. W., eds.), pp. 115–132, The Avi Publishing Co., Westport, CT
- Redman, C. M. & Cherian, M. G. (1972) *J. Cell Biol.* **52**, 231–245
- Scheraga, H. A. & Rupley, J. A. (1962) *Adv. Enzymol. Relat. Areas Mol. Biol.* **24**, 161–261
- Simkin, J. L. & Jamieson, J. C. (1967) *Biochem. J.* **103**, 153–164
- Taborsky, G. (1959) *J. Biol. Chem.* **234**, 2652–2656
- Tkacz, J. S. & Lampen, J. O. (1975) *Biochem. Biophys. Res. Commun.* **65**, 248–252
- Van Rijn, H. J. M., Boer, P. & Steyn-Parvé, E. P. (1972) *Biochim. Biophys. Acta* **268**, 431–441