Vol. 82, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 14, 1978 Pages 800-804

THE EFFECT OF BACITRACIN ON THE FORMATION OF POLYPRENOL DERIVATIVES IN YEAST MEMBRANE VESICLES

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Received April 11,1978

SUMMARY: Addition of the antibiotic bacitracin to a membrane preparation of Saccharomyces cerevisiae enriched in plasma membrane and incubated *in vitro* with UDP-[^{3}H]GlcNAc, leads to an inhibition of the formation of polyprenyl diphosphate di-N-acetylchitobiose, with a concomitant accumulation of label in polyprenyl diphosphate N-acetylglucosamine. Bacitracin also prevents to a large extent the incorporation of N-acetylglucosamine into a protein fraction.

In bacterial systems the action of the antibiotic bacitracin has been clearly established: by binding to C_{55}-polyprenyl diphosphate it inhibits the hydrolysis of this compound, thus preventing the monophosphate form to participate as a sugar carrier in the biosynthesis of peptidoglucan (1). Little is known about the action of bacitracin in eukaryotic systems. Recently two papers have been published reporting different effects of bacitracin upon the formation of polyprenoid glycolipids. In an oviduct membrane system it causes a preferential accumulation of a mannose(GlcNAc)$_2$-polyprenoid lipid (2); Herscovics et al. (3), on the other hand, have reported that in calf pancreas microsomes bacitracin has the same effect as tunicamycin: it inhibits the transfer of GlcNAc-1-phosphate from UDP-GlcNAc to polyprenylphosphate (4,5).

In the present paper we show that a purified yeast membrane preparation, enriched in plasma membrane, forms GlcNAc-P-P-Dol and (GlcNAc)$_2$-P-P-Dol from UDP-GlcNAc. In this system addition of bacitracin has an effect that is strikingly different from the above-mentioned actions observed in other eukaryotic membranes (2,3): it inhibits the transfer of GlcNAc from UDP-GlcNAc to GlcNAc-P-P-Dol.

GlcNAc-P-P-Dol, polyprenyl diphosphate N-acetylglucosamine
(GlcNAc)$_2$-P-P-Dol, polyprenyl diphosphate di-N-acetylchitobiose

0006-291X/78/0823-0800$01.00/0

MATERIALS AND METHODS

Materials. UDP-[^{3}H]GlcNAc (spec. radioact. 6.6 Ci/mmole) was purchased from the Radiochemical Centre, Amersham (U.K.). Yeast (C_{65-85})-polyprenols were isolated from baker's yeast as described by Burgos et al. (6). The purification procedure was modified slightly (7). Phosphorylation of these polyprenols was performed according to Popják et al. (8). Bacitracin (B-0125) was from Sigma Chemical Co. Saccharomyces cerevisiae X2180-1A wild type, a gift from Dr. C.E. Ballou, was grown at 30°C in a continuous culture (Bioflow Model C30, New Brunswick Scientific Co.) with an air-flow of 100 ml/min in a medium described earlier (9). Insta-Gel from Packard-Becker B.V., Groningen, The Netherlands, was used as scintillation solution.

Preparation of membrane vesicles from yeast. Membrane vesicles were isolated according to a modification of the method of Fuhrmann et al. for preparing plasma membranes (10,11). Cultivated yeast (15 g) was suspended in 20 ml of an osmotic stabilizer (450 mM KCl, 20 mM triethanolamine, 10 mM $MgCl_2$, pH 6) and mechanically disrupted in a Cell Homogenizer (Braun, Melsungen, G.F.R.) with 35 ml Ballotini beads (0.25-0.30 mm Ø) for 1 min (0°C). Whole cells and cell debris were removed by centrifugation (2 000xg, 4°C, 5 min) and the supernatant recentrifuged (5 000xg, 4°C, 10 min). The pellet was washed 3 times with osmotic stabilizer, suspended in 10%(w/w) sucrose in osmotic stabilizer and applied to a discontinuous sucrose gradient (2.2 ml of 60%, 50%, 40%, 30%, and 20%(w/w) sucrose in osmotic stabilizer). After centrifuging (94 500xg, 4°C, 1 h), the material upon the 50% layer (d: 1.21-1.26 g/ml) was removed and washed with osmotic stabilizer (23 500xg, 4°C, 15 min). The resulting pellet was suspended in 2 ml of 50 mM Tris.HCl (pH 7) containing 450 mM KCl. The membrane preparation (protein: 3-5 mg/ml (12)) was used for experiments within a few hours.

Assay procedure. To measure the transfer of GlcNAc from UDP-[^{3}H]GlcNAc to endogenous lipids and proteins the same standard reaction mixture was used as mentioned earlier (13), except for adding 450 mM KCl and omitting the detergent. The reaction mixture contained 0.3-0.5 mg protein. After incubation (30°C, 30 min) the reaction was stopped with $CHCl_3/CH_3OH$ (2:1, v/v). To obtain the glycolipid and protein fractions, the mixture was treated in almost the same way as reported earlier (13). The only change in the procedure was the addition of about 1 mg soluble yeast protein (100 000xg supernatant of a yeast homogenate) to the mixture after extraction with $CHCl_3/CH_3OH$ (2:1) and just before treatment with $CHCl_3/CH_3OH/H_2O$ (10:10:3, v/v), to increase the amount of denatured protein.

Other procedures. Alkaline and acid hydrolysis of glycolipids and the methods of TLC and column chromatography were performed as described previously (13). Proteolysis of the protein fraction was carried out in 50 mM Tris.HCl (pH 7.8) containing 10 mM $CaCl_2$ and 1 mg Pronase for 48 h at 37°C (after 24 h another portion of Pronase (1 mg) was added). Then the suspension was centrifuged and the amount of radioactivity in the supernatant and the residue was measured.

RESULTS AND DISCUSSION

Electron micrographs of thin sections of the vesicle preparation after purification on the sucrose density gradient show that the preparation is fairly homogeneous. As judged also by biochemical criteria, it mainly consists of plasma membranes (G.W. Welten-Verstegen et al., manuscript in preparation).

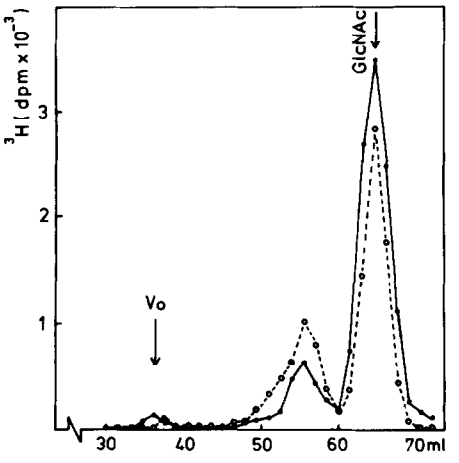

Figure 1. Estimation of the molecular mass of the sugar moieties in the glycolipids by column chromatography. After acid hydrolysis of alkali-stable lipids, the water-soluble radioactivity was applied to a Bio-Gel P2 column. Details are given in the text. (o- -o), no bacitracin; (●——●), with 0.33 mM bacitracin. Fractions of 1 ml were collected and analysed for radioactivity. V_o, void volume measured with Dextran Blue.

When this membrane preparation is incubated with UDP-[^{3}H]GlcNAc under standard conditions 3-5x10^3 dpm [^{3}H]GlcNAc/mg protein are incorporated into the protein fraction. The protein character of this fraction is indicated by its susceptibility to Pronase: more than 90% of the label is solubilized by treatment with this protease. The amount of radioactivity found in glycolipids extractable with $CHCl_3/CH_3OH$ (2:1) is about 10-25x10^3 dpm and varies between membrane preparations. The alkali-stable glycolipids (i.e. polyprenoid glycolipids, resistant to 0.2 M NaOH, 60°C, 20 min) comprise 70 to 90% of the total labelled glycolipids. This percentage is much higher than we have found previously in experiments with a crude membrane preparation: the fraction of a yeast homogenate sedimenting between 1 000 and 40 000xg (13). The alkali-stable lipid fraction consists of two labelled glycolipids: GlcNAc-P-P-Dol and $(GlcNAc)_2$-P-P-Dol. They were identified by (a) TLC chromatography of the lipid fraction and comparison in solvent $CHCl_3/CH_3OH/H_2O$ (60:35:6, v/v) with authentic standards prepared earlier (13); (b) chromatography on Bio-Gel P2 of the sugars released by mild acid hydrolysis (Fig. 1). The ratio between GlcNAc-P-P-Dol and $(GlcNAc)_2$-P-P-Dol is 3:2.

Table I. The effect of bacitracin on the transfer of GlcNAc from UDP-[^{3}H]GlcNAc to alkali-stable lipids (A.S.L.) and protein fraction. The amount of label in $(GlcNAc)_2$-P-P-Dol is given as a percentage of the total incorporated label in alkali-stable lipids.

Bacitracin (mM)	A.S.L. (dpm)	$(GlcNAc)_2$-P-P-Dol (%)	Protein (dpm)
none	11 710	30	1 190
0.16	12 600	24	900
0.33	13 030	19	790
0.67	11 680	13	750
1.67	11 140	7	660

Addition of increasing concentrations of bacitracin does not change the total incorporation of label into alkali-stable lipids but TLC of this fraction shows that the radioactivity incorporated in $(GlcNAc)_2$-P-P-Dol decreases, while label accumulates in GlcNAc-P-P-Dol (Table I). So, in S. cerevisiae bacitracin would seem to inhibit the attachment of a second GlcNAc residue to GlcNAc-P-P-Dol but not the transfer of GlcNAc-1-phosphate from UDP-GlcNAc to polyprenylphosphate. This result is in striking contrast with those obtained with hen oviduct preparations (2) or with pancreas microsomes (3). In yeast, $(GlcNAc)_2$-P-P-Dol is the intermediate from which $(GlcNAc)_2$ is directly transferred to protein *in vitro* (13). Accordingly, we may expect that an inhibition of the formation of this glycolipid will bring about a drop in the label incorporated in the protein fraction. As Table I shows, addition of bacitracin indeed has this result.

The effect of bacitracin on the transfer of GlcNAc residues from UDP-GlcNAc to polyprenoid lipids and protein can also be demonstrated in another manner: yeast (C_{65-85})-polyprenylphosphate (30 µM), added to the reaction mixture in a small volume of CH_3OH, enhances the incorporation of [^{3}H]GlcNAc into GlcNAc-P-P-Dol in the presence of bacitracin more than 13-fold, but not that into $(GlcNAc)_2$-P-P-Dol and the protein fraction.

GlcNAc-containing lipids formed in the presence of bacitracin move faster in TLC. For instance, the R_F value for GlcNAc-P-P-Dol shifts from 0.25 to 0.30.

But the sugar residues attached to the polyprenoid lipids are unchanged, as can be seen in Fig. 1. Upon acid hydrolysis (0.1 M HCl in 50%(v/v) 1-propanol, 100°C 10 min) of the alkali-stable lipids, obtained after incubation with bacitracin (0.33 mM), and gel filtration of the sugars on Bio-Gel P2 (100x1 cm) only GlcNAc and $(GlcNAc)_2$ are found; they run in the same position as when bacitracin is absent. In Fig. 1 we again observe the shift in GlcNAc incorporation from $(GlcNAc)_2$-P-P-Dol to GlcNAc-P-P-Dol: in the absence of bacitracin 34% of the label is present in $(GlcNAc)_2$, but this percentage drops to 17% in 0.33 mM bacitracin. These data confirm those of Table I. In seeking an explanation for the divergent effects of bacitracin on the formation of polyprenoid glycolipids all we can say at present is that the mechanism of action of this antibiotic in eukaryotic systems seems to be different from one type of cell to another. To explain the increase of the R_F values in TLC one could think of complex formation of the glycolipids with the antibiotic.

ACKNOWLEDGMENTS

The authors wish to thank Dr. A. Reinking and Mr. L. Zeegers for performing the isolation and phosphorylation of yeast polyprenols and Ms. C. v.d. Tillaart for taking care of the continuous yeast culture. We are grateful to Ms. C. Habets-Willems and Ms. G. Welten-Verstegen for valuable discussions.

REFERENCES

1. Stone, K.J. and Strominger, J.L.(1971) Proc. Natl. Acad. Sci. U.S.A. 68, 3223-3227
2. Chen, W.W. and Lennarz, W.J.(1976) J. Biol. Chem. 251, 7802-7809
3. Herscovics, A., Bugge, B., and Jeanloz, R.W.(1977) FEBS lett. 82, 215-218
4. Tkacz, J.S. and Lampen J.O.(1975) Biochem. Biophys. Res. Commun. 55, 248-257
5. Lehle, L. and Tanner, W.(1976) FEBS lett. 71, 167-170
6. Burgos, J., Hemming, F.W., Pennock, J.F., and Morton, R.A.(1963) Biochem. J. 88, 470-482
7. Reuvers, F., Boer, P., and Hemming, F.W.(1978) Biochem. J. 169, 504-508
8. Popják, G., Cornforth, J.W., Cornforth, R.H., Ryhage, R., and Goldman, D.S. (1962) J. Biol. Chem. 237, 56-61
9. Van Rijn, H.J.M., Boer, P., and Steyn-Parvé, E.P.(1972) Biochim. Biophys. Acta, 268, 431-441
10. Fuhrmann, G.F., Boehm, C., and Wehrli, E.(1974) Biochim. Biophys. Acta 363, 293-310
11. Fuhrmann, G.F., Boehm, C., and Theuvenet, A.P.R.(1976) Biochim. Biophys. Acta 433, 583-596
12. Lowry, O.H., Rosebrough, N.H., Farr, A.L., and Randall, R.J.(1951) J. Biol. Chem. 193, 265-275
13. Reuvers, F., Habets-Willems, C., Reinking, A., and Boer, P.(1977) Biochim. Biophys. Acta 486, 541-552