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ENZYMATIC DEPHOSPHORYLATION OF DOLICHYL PYROPHOSPHATE ——— THE BACITRACIN-SENSITIVE, RATE-LIMITING STEP FOR DOLICHYL MANNOSYL PHOSPHATE SYNTHESIS IN RAT LIVER MICROSOMES

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SUMMARY: The incorporation of [^{14}C]mannose from GDP-[^{14}C]mannose into dolichyl mannosyl phosphate in rat liver microsomes showed a biphasic time-course; an initial rapid incorporation of mannose which ceased within 2 min and a much slower incorporation which continued for 30 min. In the presence of 0.18 mM (250 μg/ml) bacitracin, the rapid incorporation proceeded normally whereas the slow incorporation was inhibited by about 70 %. Upon addition of dolichyl pyrophosphate, the microsomes catalyzed the dephosphorylation of the added compound which was also inhibited by bacitracin. The results, coupled with several other observations, suggest that the rapid reaction represents the transfer of mannose to endogenous dolichyl phosphate whereas the bacitracin-sensitive, slow reaction represents a more complex process in which the enzymatic dephosphorylation of dolichyl pyrophosphate is involved as a rate-limiting step.

While many details are known about the participation of Dol-P as a glycosyl-group carrier in the assembly of asparagine-linked oligosaccharides of glycoprotein in animal cells (see Refs. 1-3 for reviews), it is not yet clear whether the carrier lipid is regenerated from Dol-P-P after each oligosaccharide transfer so as to participate catalytically in a second cycle of glycosylation. In bacterial systems, undecaprenyl phosphate has been shown to be regenerated from undecaprenyl pyrophosphate and reutilized in the reaction cycle for cell wall synthesis (4). The antibiotic bacitracin forms complexes with undecaprenyl pyrophosphate and thereby prevents cleavage of the pyrophosphate and reutilization of the lipid-phosphate for a second cycle (5). In eukaryotic system, some reports indicate that bacitracin can interfere with the formation of some dolichol-linked sugar derivatives. However, the evidence so far obtained is conflicting; in different membrane systems, inhibition of formation of Dol-P-Man, Dol-P-P-GlcNAc, or Dol-P-P-$(GlcNAc)_2$ has been described (6-9). In these cases, 50 % inhibition was observed at bacitracin concentrations between 0.5 mM and 7.0 mM, much higher than concentrations causing 50 % inhibition of bacterial cell wall synthesis. Therefore, the inhibition of synthesis of dolichyl glycosyl phosphates might well be secondary to an effect on the membranes and caused by relatively

Abbreviations: Dol-P, dolichyl phosphate; Dol-P-P, dolichyl pyrophosphate; Dol-P-Man, dolichyl β-D-mannosyl phosphate.

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nonspecific membrane perturbation, rather than primary and caused by a specific reaction with enzymes for Dol-P-sugar or Dol-P-P-sugar formation.

In this paper, we show that at concentrations lower than 0.18 mM (250 µg/ml) the antibiotic inhibited the synthesis of Dol-P-Man in rat liver microsomes by blocking the formation of Dol-P from endogenous precursors. A bacitracin-sensitive phosphatase activity toward Dol-P-P could be detected in the microsomes, consistent with the view that the control of Dol-P concentrations is linked with changes in the rate of Dol-P-P dephosphorylation. Wedgwood and Strominger (10) have also recently described the presence of Dol-P-P phosphatase activity in particulate preparations from human lymphocytes.

MATERIALS AND METHODS

Rat liver microsomes were prepared as described previously (11). The final pellet of microsomes was suspended in 0.25 M sucrose/5 mM Tris-maleate, pH 6.3/5 mM $MgCl_2$ at a protein concentration of about 8 mg/ml and used for enzyme assay.

Dol-P-P was synthesized by the procedure of Warren and Jeanloz (12). Since thin layer chromatography of the material obtained showed the existence of some lipid impurities, further purification was made as follows. The material was dissolved in hexane and applied as a thin band to precoated aluminum sheets of Silica Gel 60 F_{254}. Chromatography was carried out in $CHCl_3/CH_3OH/15$ M NH_4OH/H_2O (65/35/4/4, by volume). Lipids were visualized by staining guide strips with the anisaldehyde reagent (13). Dol-P-P (R_F = 0.14) was then eluted from the remainder of the chromatogram with $CHCl_3/CH_3OH/H_2O$ (10/10/3). The purification by thin layer chromatography was repeated once more to ensure complete elimination of impurities. The final preparation was dissolved in $CHCl_3/CH_3OH$ (2/1) and stored at -20°C.

GDP-[U-^{14}C]mannose (210 mCi/mmol) was purchased from New England Nuclear, bacitracin and Dol-P were from Sigma, and precoated glass plates and aluminum sheets of Silica Gel 60 F_{254} were from Merck.

Measurement of Dol-P-Man synthesis: For the measurement of mannosyltransferase activity toward endogenous lipid, the incubation mixture contained 6×10^4 cpm of GDP-[^{14}C]mannose, 1 µmol of $MnCl_2$, 1 µmol of $MgCl_2$, 0.3 µmol of 5'-AMP (Na salt), 0.3 µmol of NaF, 2.5 µmol of Tris-maleate (pH 6.8), and 0.2 mg (as protein) of microsomes, in a total volume of 100 µl. When necessary, bacitracin was added as indicated in individual experiments. Controls contained heat-killed microsomes. The mixtures were incubated at 37°C, and the reaction was stopped by the addition of 1 ml of ice-cold 5 % trichloroacetic acid. After removal of the supernatant by centrifugation at 2°C, the precipitate was washed twice with 1 ml each of ice-cold 5 % trichloroacetic acid. The washed precipitate was dissolved in 100 µl of 0.1 M NaOH, spotted on a paper disc, and counted.

For the measurement of activity toward exogenously added Dol-P, 6 nmol of Dol-P in CCl_4 was mixed with 4 µl of 5 % Triton X-100 and dried under N_2. The standard incubation mixture (see above) was then added to each reaction tube containing the dried material (final detergent concentration = 0.2 %), and incubation was carried out at 37°C. The amount of Dol-P-[^{14}C]Man formed was determined as above.

Measurement of phosphatase activity toward Dol-P-P: Dol-P-P, 2 nmol, dissolved in $CHCl_3/CH_3OH$ (2/1) was mixed with 4 µl of 5 % Triton X-100 and dried under N_2. The mixture containing microsomes was then added and incubation was carried out at 37°C for 30 min. The microsome-containing

mixture was the same as above except for the absence of GDP-[^{14}C]mannose. Controls contained heat-killed microsomes. The reaction was stopped with 500 µl of $CHCl_3/CH_3OH$ (2/1). After thorough mixing and centrifugation, the organic phase was washed with $CHCl_3/CH_3OH/H_2O$ (3/48/47) and dried under N_2. The sample contained some lipid impurities (probably acyl glycerides and phosphatidates derived from the microsomes); therefore, 100 µl of $CHCl_3$, 150 µl of CH_3OH, and 250 µl of 0.2 M methanolic NaOH were successively added with vigorous shaking and the mixture was allowed to stand for 15 min at room temperature. Under these conditions acyl glycerides and phosphatidates are deacylated (14). The alkali-treated sample was mixed with 100 µl of $CHCl_3$, 400 µl of CH_3OH, and 450 µl of H_2O and, after thorough mixing, the aqueous phase containing deacylated materials was removed by centrifugation. The organic phase was washed twice with 250 µl each of CH_3OH/H_2O (10/9) and dried under N_2. The dried sample was dissolved in two drops of hexane and subjected to thin layer chromatography to estimate Dol-P-P and Dol-P (see the legend to Fig. 3 for details).

For the measurement of inorganic phosphate released by phosphatase action, 100 µl of the incubation mixture containing 0.3 µmol of NaF, 2.5 µmol of Tris-maleate (pH 6.8), 0.3 µmol of EDTA, and 0.2 mg (as protein) of microsomes was added to each reaction tube containing 5.0 nmol of dried Dol-P-P in Triton X-100 (see above). After incubation at 37°C for 30 min, the reaction was stopped with 500 µl of $CHCl_3/CH_3OH$ (2/1) and the aqueous phase was analyzed for inorganic phosphate by the method of Lowry et al. (15). The blank value obtained in the absence of Dol-P-P was subtracted.

RESULTS AND DISCUSSION

The microsomes from rat liver catalyze the incorporation of mannose from GDP-[^{14}C]mannose into Dol-P-Man (see Ref. 11 for the characterization of reaction products). Using this microsome preparation, a time course experiment was done as shown in Fig. 1. It can be seen that the incorporation of [^{14}C]mannose follows a very rapid course between 0- and 2-min

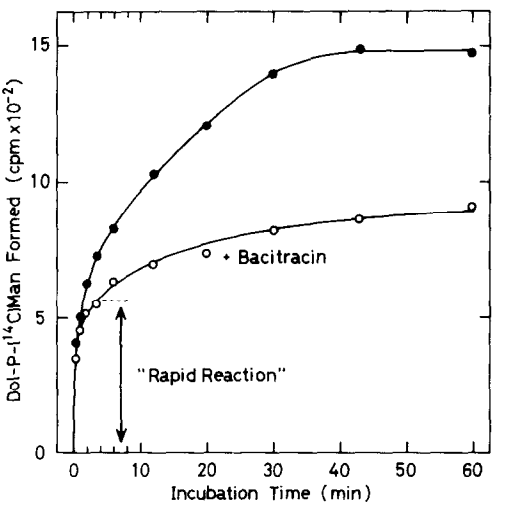
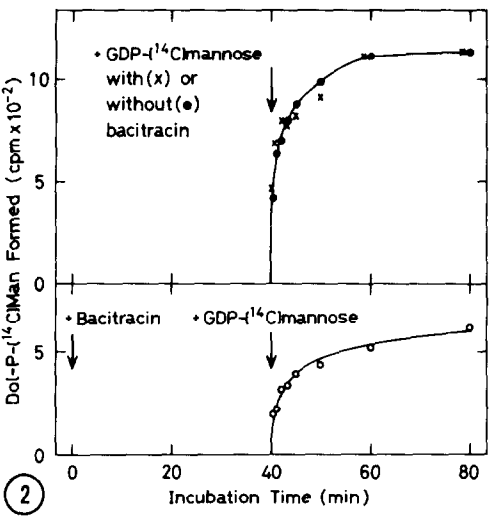


Fig. 1. Time course of Dol-P-[^{14}C]Man synthesis from GDP-[^{14}C]mannose and endogenous lipid of rat liver microsomes. Incubations with (○) or without (●) bacitracin (250 µg/ml) were analyzed at the indicated times as described under "Materials and Methods".

incubation time ("rapid reaction") and slows down markedly thereafter ("slow reaction"). A distinction between the rapid and slow reactions was established by experiments using the antibiotic bacitracin. Thus, in the presence of bacitracin (250 µg/ml) the formation of Dol-P-Man was not affected during the initial rapid reaction period but thereafter it was inhibited by about 70 %. Experiments with different bacitracin concentrations showed that even at a level of 1,500 µg/ml the antibiotic caused no inhibition of the rapid reaction. In contrast, the concentration of bacitracin which was necessary to inhibit the slow reaction by 50 % was only 50 µg/ml.

When the microsomes were preincubated for 40 min at 37°C and subsequently assayed for Dol-P-Man synthesis from GDP-[^{14}C]mannose, the amount of [^{14}C]mannose incorporated within 2 min was increased about 2-fold, with a corresponding reduction in the share of "slow reaction" (Fig. 2, panel a). Bacitracin had no effect on the mannose incorporation throughout a 40-min time course. If, however, the preincubation of the microsomes was carried out in the presence of bacitracin, no such enhancement of the rapid reaction was observed (Fig. 2, panel b); this suggests that bacitracin has an indirect effect due to depletion of lipid substrate, such as that observed in bacterial cell-wall synthesis (5), rather than a direct effect on the mannosyltransferase itself. Consistent with this hypothesis, bacitracin (250 µg/ml) had little effect on the transfer of mannose from GDP-[^{14}C]mannose to exogenous Dol-P by microsomal mannosyltransferase. Thus, addition of Dol-P (plus Triton X-100, see "Materials and Methods" for the conditions) to the standard incubation mixture resulted in a 40-fold increase in the amount of ^{14}C incorporated into Dol-P-Man within 10 min, regardless of whether or not bacitracin was included in the incubation mixture. After this "rapid" reaction, ^{14}C was not incorporated into the lipid in detectable quantity even in the absence of bacitracin. The preponderance of the rapid reaction appears to mask the bacitracin-sensitive, "slow" synthesis from endogenous precursors that might otherwise be revealed.

These results suggest that the mode of action of bacitracin in the microsomes may to some extent be similar to its mode of action in inhibiting bacterial cell wall synthesis, i.e. the concentration of Dol-P in the microsomes is probably rate-limiting for the glycosylation process and the antibiotic may block the formation of Dol-P from endogenous precursors, thereby inhibiting the "slow" synthesis of Dol-P-Man. The following experiments show that a reaction which may be particularly relevant to the formation of Dol-P is the dephosphorylation of Dol-P-P.



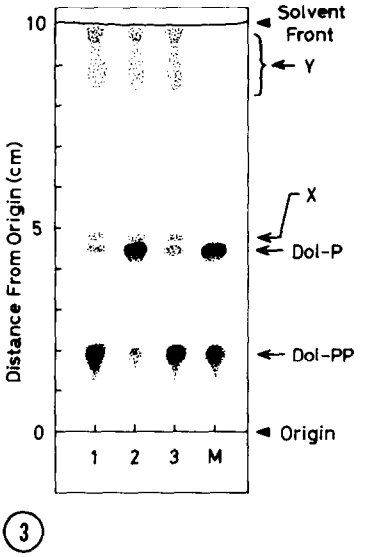


Fig. 2. Effect of the preincubation of microsomes on Dol-P-[^{14}C]Man synthesis from GDP-[^{14}C]mannose and endogenous lipid. Incubation mixtures without GDP-[^{14}C]mannose were prepared and preincubated for 40 min at 37°C in the absence (panel a) or presence (panel b) of bacitracin (250 µg/ml). The incubations were placed in ice and GDP-[^{14}C]mannose (panel a, •——•), GDP-[^{14}C]mannose plus bacitracin (panel a, x——x), or GDP-[^{14}C]mannose (panel b) were added. The mixtures were incubated at 37°C and analyzed at the indicated times as described under "Materials and Methods".

Fig. 3. Separation of the products of Dol-P-P dephosphorylation catalyzed by rat liver microsomes. Anisaldehyde stain of a Silica Gel 60 thin layer chromatogram (solvent = $CHCl_3/CH_3OH/15$ M NH_4OH/H_2O, 65/35/4/4) is shown. Dol-P-P was incubated for 30 min with 1, heat-killed microsomes; 2, fresh microsomes; and 3, fresh microsomes plus bacitracin (250 µg/ml). M, mixture of standard Dol-P-P and Dol-P. X and Y, unidentified lipids derived from the microsomes.

In contrast to the results with exogenous Dol-P, bacitracin (250 µg/ml) had a significant effect on the formation of Dol-P-[^{14}C]Man in the presence of exogenous Dol-P-P (about 0.6 nmol); the amount of Dol-P-[^{14}C]Man formed in 50 min was 1.25×10^3 cpm in the presence of bacitracin, compared to 2.30×10^3 cpm in the absence of bacitracin.

Exogenous Dol-P-P was incubated with rat liver microsomes in the presence of 3 mM NaF (an inhibitor for most acid phosphatases) and 3 mM 5'-AMP, and the reaction mixture was examined by thin layer chromatography as shown in Fig. 3. It is clear that after 30-min incubation the amount of a compound behaving as Dol-P was greatly increased with a concomitant reduction in the amount of Dol-P-P. In addition to these two components, several minor components, presumably derived from endogenous lipids in the microsomes, were detected, but their positions and amounts were not significantly altered upon incubation. Bacitracin (250 µg/ml) caused almost complete

inhibition of both the formation of Dol-P and the disappearance of Dol-P-P. The fact that neither NaF nor 5'-AMP interfered with Dol-P-P hydrolysis suggests that the reaction was brought about by an enzyme different from most acid phosphatases.

In a separate experiment, 3 mM EDTA was shown to stimulate the dephosphorylation of Dol-P-P. Under these optimized conditions (see "Materials and Methods") the release of inorganic phosphate from exogenous Dol-P-P could be demonstrated; upon 30-min incubation, 4.5 nmol of inorganic phosphate was released from 5.0 nmol of added Dol-P-P. Effect of bacitracin on the release of phosphate was not investigated, since EDTA is known to abolish the inhibition by bacitracin of the enzymatic dephosphorylation of undecaprenyl pyrophosphate (5). Attempts to detect inorganic phosphate released in the absence of EDTA have so far been unsuccessful. Wedgwood and Strominger (10) have reported a [^{32}P]Dol-P-P phosphatase activity in membranes from human lymphocytes which is stimulated by EDTA. These authors indicated that the reaction was inhibited by bacitracin when the inhibitor was preincubated with [^{32}P]Dol-P-P for 5 min before addition of membranes and EDTA; this inhibition was not observed when bacitracin was preincubated with EDTA.

The precise mechanism of bacitracin action on the rat liver microsomes is not yet clear. Any interference which may lead to decreased amounts of Dol-P in the microsomes could conceivably be a factor responsible for inhibition of Dol-P-Man synthesis, but our observations, together with those reported by Wedgwood and Strominger (10), suggest that bacitracin forms a complex with Dol-P-P and thereby prevents cleavage of the pyrophosphate and reutilization of the Dol-P for Dol-P-Man synthesis. This proposal has been supported by our recent observations that Dol-P-P does indeed form a complex with bacitracin in 0.025 M Tris-maleate, pH 6.8; the complex was readily distinguished from free Dol-P-P by thin layer chromatography ($R_{Dol\text{-}P\text{-}P}$ of the complex = 2.50) or by solubility test (the complex did not dissolve in hexane and required alkali, pH 13, for dissociation).

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REFERENCES

1. Waechter, C. J., and Lennarz, W. J. (1976) Annu. Rev. Biochem. 45, 95-112.
2. Parodi, A. J., and Leloir, L. F. (1979) Biochim. Biophys. Acta 559, 1-37.
3. Hemming, F. W. (1977) Biochem. Soc. Trans. 5, 1223-1231.

4. Siewert, G., and Strominger, J. L. (1967) Proc. Natl. Acad. Sci. U.S.A. 57, 767-773.
5. Stone, K. J., and Strominger, J. L. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 3223-3227.
6. Herscovics, A., Bugge, B., and Jeanloz, R. W. (1977) FEBS Lett. 82, 215-218.
7. Spencer, J. P., Kang, M. S., and Elbein, A. D. (1978) Arch. Biochem. Biophys. 190, 829-837.
8. Reuvres. F., Boer, P., and Steyn-Parvé, E. P. (1978) Biochem. Biophys. Res. Commun. 82, 800-804.
9. Elbein, A. D. (1979) Annu. Rev. Plant Physiol. 30, 239-272.
10. Wedgwood, J. F., and Strominger, J. L. (1980) J. Biol. Chem. 255, 1120-1123.
11. Kato, S., Tsuji, M., Nakanishi, Y., and Suzuki, S. (1980) J. Biochem. 87, 929-939.
12. Warren, C. D., and Jeanloz, R. W. (1975) Biochemistry 14, 412-419.
13. Dunphy, F. J., Kerr, J. D., Pennock, J. F., White, K. J., and Feeney, J. (1967) Biochim. Biophys. Acta 136, 136-147.
14. Kates, M. (1972) in Laboratory Techniques in Biochemistry and Molecular Biology (Work, T. S. and Work, E., eds.), vol. 3, pp. 267-610, North-Holland Publishing Co., Amsterdam.
15. Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M. L., and Farr, A. L. (1954) J. Biol. Chem. 207, 1-17.