DEPHOSPHORYLATION OF C_{55}-ISOPRENYL-MONOPHOSPHATE BY NON-SPECIFIC PHOSPHATASES

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1. Introduction

The biosynthesis of peptidoglycan and several other components of bacterial cell-walls proceeds via sugar precursors bound by a pyrophospho- or a monophosphobridge to a C_{55}-isoprenyl carrier lipid [1].

More than 90% of the endogenous C_{55}-isoprenyl lipid of *Staphylococcus aureus* consists of the non-functional free C_{55}-isoprenoid-alcohol (30 nmol/g wet weight bacteria, [2]). Similar amounts of free isoprenoid-alcohol and the derived non-functional fatty acyl ester were found in *Streptococcus faecalis* [3]. *Myxococcus fulvus* (Kleinig, H., personal communication), *Lactobacillus plantarum* [4,5] and in *Listeria monocytogenes* [6]. A preponderance of the pools of non-functional free isoprenoid-alcohol and its fatty acyl ester has also been found in yeast, plant and mammalian species [7].

Bacterial C_{55}-isoprenyl lipids are biosynthesized as the pyrophosphate derivatives, which are dephosphorylated to C_{55}-isoprenyl-monophosphate by specific and non-specific phosphatases [8–11]. In spite of the predominance of the free C_{55}-isoprenoid-alcohol in various bacteria, attempts to cleave long-chain isoprenyl-monophosphates by bacterial enzyme preparations have been unsuccessful [8,12, 13], with the exception of a membrane-bound C_{55}-isoprenyl-monophosphate phosphatase activity in *S. aureus* [13].

We now report the dephosphorylation of C_{55}-isoprenyl-monophosphate using non-specific phosphatases from *Escherichia coli, S. aureus,* potato and calf intestine.

2. Experimental

2.1. *Materials*

Commercially purified alkaline phosphatases (grade I) from *E. coli* and calf intestine and acid phosphatase (grade I) from potato were purchased from Boehringer, Mannheim. Loosely membrane-bound acid phosphatase of *S. aureus* was prepared by KCl-elution of intact cells [14]. C_{55}-isoprenoid-alcohol kinase apoprotein (step 6) and ficaprenol were prepared as previously described [15]. Span-20 was obtained from Atlas Chemical Co., Delaware.

2.2. *Preparation of [^{32}P]C_{55}-isoprenyl-monophosphate*

Ficaprenol (15 nmol) and Span-20 detergent (50 μg), dissolved in organic solvents, were first added to the test tube, followed by removal of the solvents in vacuo. C_{55}-isoprenoid-alcohol kinase apoprotein in organic solvent [15] was next added, followed by removal of solvent as above. Buffer (25 μl) and [γ-^{32}P]ATP (1 μl containing 18 nmol and 0.4 μCi) was then added, as previously described [15], and the mixture incubated, under N_2, for 12 h at room temperature. Lipids were extracted by the Bligh-Dyer procedure [16]. The organic phase (100 μl) was washed with five 100 μl portions of 0.9% (w/v) NaCl. A lipid extract pooled from 20 separate incubations was applied to a column (1 × 8 cm) of silica gel (Merck No. 7754) packed in chloroform. Most of the residual Span-20 detergent was eluted with chloroform as examined by colorimetric ester determination [17]. [^{32}P]ficaprenyl-monophosphate was then eluted from the column using methanol; its purity was examined by paper chromatography (see Fig.1).

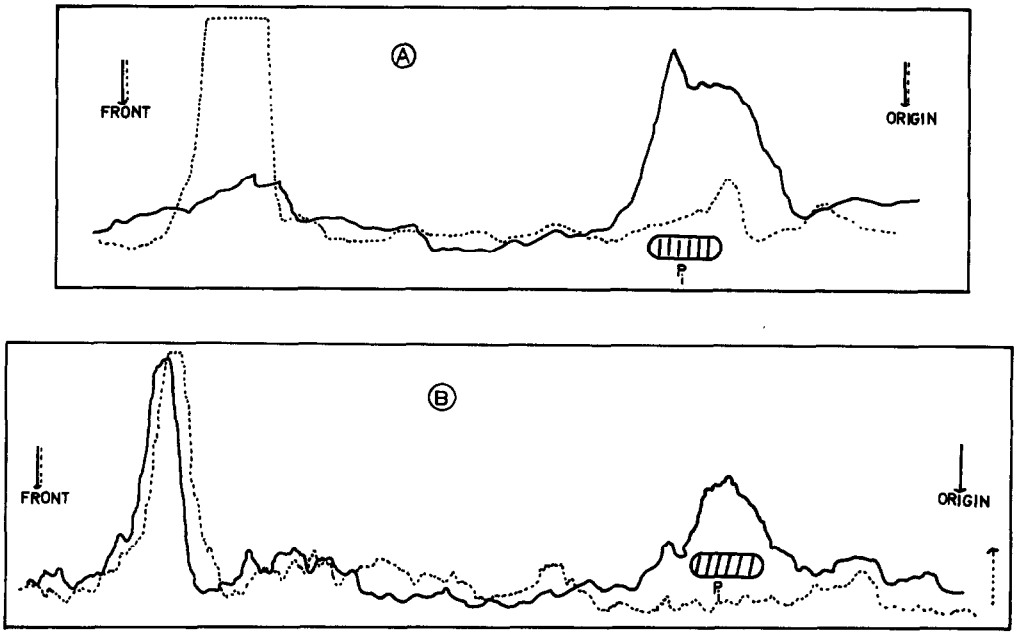

Fig.1. Cleavage of [^{32}P]ficaprenyl-monophosphate by alkaline phosphatase from *E. coli* (A) and by acid phosphatase from potato (B). Incubations were performed as described in Experimental, with (————) or without (– – – – –) addition of enzyme protein. Analysis was by paper chromatography in isobutyric acid/1 M ammonia, 5 : 3 (v/v), and scanning, using a Berthold LB 280 chromatogram scanner. The position of the P_i marker was located using the Hanes–Isherwood spray reagent [30].

The yield of [^{32}P]ficaprenyl-monophosphate was 2–3%. The amount of [^{32}P]C_{55}-isoprenylmonophosphate present in subsequent phosphatase incubations was calculated from the initial specific radioactivity of [γ-^{32}P]ATP. Since dolichol was also phosphorylated by the *S. aureus* kinase (*V* about 20% of *V* for ficaprenol), [^{32}P]dolichyl-monophosphate was prepared in the same way as the ficaprenyl-derivative.

2.3. *Cleavage of [^{32}P]isoprenyl-monophosphate*

An aliquot of a methanolic solution of [^{32}P] ficaprenyl-monophosphate was added to the test tube. Solvent was next removed in vacuo, 50 μl aliquots of the following buffers added (see below), followed by addition of phosphatase dissolved in 10 μl assay buffer and incubation as indicated below:

for *E. coli* phosphatase: 1.5 M Tris-HCl, 5 mM $(NH_4)_2SO_4$, 0.2% (w/v) Triton X-100, pH 8.0 (2 min, 25°C). For intestinal phosphatase: 0.1 M glycine–NaOH, 5 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.2% (w/v) Triton X-100, pH 10.5 (2 min, 25°C). For *S. aureus* phosphatase: 0.1 M sodium citrate 0.1 M KCl, 0.2% (w/v) Triton X-100, pH 5.6 (60 min, 25°C). For potato phosphatase: 0.1 M sodium citrate, 5 mM $(NH_4)_2SO_4$, 0.2% (w/v) Triton X-100, pH 5.6 (1 min, 25°C). Enzyme was omitted in control experiments. Reactions were terminated at the times indicated by addition of 50 μl chloroform/methanol, 1 : 1 (v/v). The entire reaction mixtures were then applied to chromatography paper and developed in isobutyric acid/1 M ammonia, 5 : 3 (v/v). The amount of P_i liberated was determined by scintillation counting [15] of the appropriate area on the chromatogram (see fig.1).

2.4. *Cleavage of p-nitrophenyl-monophosphate*

The conditions for the colorimetric assay of the phosphatases [18–20] are described in the following sequence: buffer used, total volume, temperature, continuous measurement of absorbance at 405 nm or addition of 2 ml 0.5 N NaOH after the stated incubation time, followed by reading at 405 nm. *E. coli* phosphatase: 1.0 M Tris-HCl, 0.1 mM $(NH_4)_2SO_4$,

0.13% (w/v) Triton X-100, pH 8.0, 3 ml 25°C, continuous reading. Intestinal phosphatase: 0.1 M glycine–NaOH, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.1 mM $(NH_4)_2SO_4$, 0.2% (w/v) Triton X-100, pH 10.5, 3 ml, 25°C, continuous reading.

S. aureus phosphatase: 0.1 M sodium citrate, 19 mM KCl, 0.2% (w/v) Triton X-100, pH 5.6, 1 ml, 25°C, terminated after 5 min by addition of NaOH. Potato phosphatase: 0.1 M sodium citrate, 0.3 mM $(NH_4)_2SO_4$, 0.2% (w/v) Triton X-100, pH 5.6, 1 ml, 25°C, terminated after 2 min by addition of NaOH.

The concentration of the substrate, *p*-nitrophenyl-monophosphate, was varied in the above incubation mixtures in the range of 0.03 to 10 mM. The amount of product, *p*-nitrophenolate, was calculated using the molar extinction coefficient of 18 500 M^{-1} cm^{-1} [18]. Values of K_m and V (table 1) were determined by the Lineweaver-Burk procedure.

3. Results

Dephosphorylation of [^{32}P]ficaprenyl-monophosphate by *E. coli* periplasmic [21] alkaline phosphatase and potato acid phosphatase is shown in fig.1.

Apparent K_M and V values were determined by the Lineweaver-Burk procedure for the alkaline phosphatases of *E. coli* and calf intestine, and the acid phosphatases of potato and *S. aureus* (table 1). Corresponding values were determined under similar conditions using the standard substrate, *p*-nitrophenyl-monophosphate (table 1). The apparent K_M values for ficaprenyl-monophosphate ranged from 10^{-5} to 10^{-4} M, and V values were between 1% and 6% of those for *p*-nitrophenyl-monophosphate. V values for both substrates were found to decrease as the concentration of Triton X-100, present in the assay mixture, was further increased.

In preliminary experiments, [^{32}P]dolichyl-monophosphate was cleaved by *E. coli* alkaline phosphatase and by a microsomal preparation from rat liver.

4. Discussion

In the only previous report on bacterial C_{55}-isoprenyl-monophosphate phosphatase activity [13], V was below 0.2 nmol P_i liberated/mg protein min. The non-specific phosphatases now examined appeared to be much more effective (see table 1), although the kinetic constants obtained should only be regarded as order-of-magnitude values. Interestingly, the previously studied activity from *S. aureus* [13] was affected by the pH and metal ions in the same way as the non-specific acid phosphatase from *S. aureus* [14,22–24].

Non-specific phosphatases are of ubiquitous occurrence [25] and might be responsible for the relatively large pool size of dephosphorylated isoprenoid-alcohols. Counteracting isoprenoid-alcohol kinase activities could serve a regulatory function in

Table 1

Values of K_M and V for the cleavage of [^{32}P]ficaprenyl-monophosphate and *p*-nitrophenyl-monophosphate by phosphatases. The values for [^{32}P]ficaprenylmonophosphate were determined from Lineweaver-Burk plots of incubations where substrate concentrations varied between 1 and 40 μM. The values for *p*-nitrophenyl-monophosphate were determined in the same way from colorimetric assays (see Experimental). The amounts of enzyme protein were either calculated from the data sheet supplied by the manufacturer or, in the case of the *S. aureus* phosphatase, determined by a modified Lowry procedure [15]

Enzyme tested	[^{32}P]ficaprenyl-monophosphate		*p*-Nitrophenyl-monophosphate	
	K_M (μM)	V (μmol P_i liberated/ mg protein × min)	K_M (μM)	V (μmol P_i liberated/ mg protein × min)
E. coli phosphatase	10	1.0	100	50
Intestinal phosphatase	22	3.3	2600	400
Potato phosphatase	44	8.3	280	170
S. aureus phosphatase	77	0.003	280	0.045

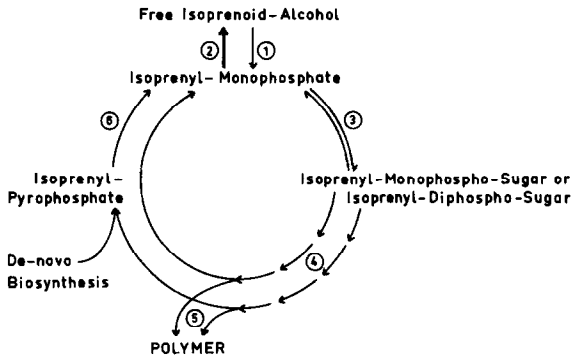

Fig.2. Possible role of isoprenyl-lipids in cell-wall and glycoprotein biosynthesis. The following enzyme reactions are shown: (1) Isoprenoid-alcohol kinase. (2) Isoprenyl-monophosphate phosphatase. (3) Translocase. (4) Modification reactions (optional). (5) Transferase. (6) Isoprenyl-pyrophosphate phosphatase.

cell-wall and glycoprotein biosynthesis [1] by providing isoprenyl-monophosphate, which is required as a carrier lipid (fig.2). So far, C_{55}-isoprenoid-alcohol kinase activity has been demonstrated in *S. aureus* (1) *Klebsiella aerogenes* [26] and *E. coli* (Sandermann, H., unpublished results). An apparent ATP requirement for in vitro peptidoglycan biosynthesis has been reported for *S. aureus* [27] and *E. coli* [28] although this requirement may depend upon how well the membrane preparation was washed since many bacterial phosphatases are only loosely bound to the membrane [21].

Bacterial cell wall biosynthesis is usually depicted as a biosynthetic cycle where the same molecule of C_{55}-isoprenyl-monophosphate is constantly turning over [27,29]. This scheme ignores the preponderance of non-functional C_{55}-isoprenyl lipid pools and the existence of the C_{55}-isoprenoid-alcohol kinase and the counteracting phosphatase activities. Pioneering studies with a membrane preparation capable of peptidoglycan biosynthesis have indicated a turnover number of the carrier lipid of approximately five [10]. An updated scheme for the possible participation of isoprenyl lipids in cell-wall and glycoprotein biosynthesis is shown in fig.2. However, detailed in vivo studies and the enzymatic characterization of cell-wall mutants are required to clarify the regulatory role of the enzyme reactions shown, as e.g., the ratio of the velocities of reaction 1 and 2.

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References

[1] Strominger, J. L., Higashi, Y., Sandermann, H., Stone, K. J. and Willoughby, E. (1972) in: Biochemistry of the Glycosidic Linkage (Piras, R. and Pontis, H. G., eds.), pp. 135–154, Academic Press, New York.
[2] Higashi, Y., Strominger, J. L. and Sweeley, C. C. (1970) J. Biol. Chem. 245, 3697–3702.
[3] Umbreit, J. N., Stone, K. J. and Strominger, J. L. (1972) J. Bacteriol. 112, 1302–1305.
[4] Thorne, K. J. I. and Kodicek, E. (1966) Biochem. J. 99, 123–127.
[5] Gough, D. P., Kirby, A. L. Richards, J. B. and Hemming, F. W. (1970) Biochem. J. 118, 167–170.
[6] Vilim, A., Woods, M. C. and Carroll, K. K. (1973) Can. J. Biochem. 51, 939–941.
[7] Hemming, F. W. (1974) in: MTP International Review of Science, Biochemistry, Series 1, Vol. 4, Biochemistry of Lipids (Goodwin, T. W., ed.), pp. 39–97, Butterworths, London.
[8] Kurokawa, T., Ogura, K. and Seto, S. (1971) Biochem. Biophys. Res. commun. 45, 251–257.
[9] Keenan, M. V. and Allen, C. M. (1974) Arch. Biochem. Biophys. 161, 375–378.
[10] Siewert, G. and Strominger, J. L. (1967) Proc. Natl. Acad. Sci. USA 57, 767–773.
[11] Goldman, R. and Strominger, J. L. (1972) J. Biol. Chem. 247, 5116–5122.
[12] Jankowski, W., Mankowski, T. and Chojnacki, T. (1974) Biochim. Biophys. Acta 337, 153–162.
[13] Willoughby, E., Higashi, Y. and Strominger, J. L. (1972) J. Biol. Chem. 247, 5113–5115.
[14] Malveaux, F. J. and San Clemente, C. L. (1969) J. Bacteriol. 97, 1209–1214.
[15] Sandermann, H. (1974) Eur. J. Biochem. 43, 415–422.
[16] Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
[17] Kates, M. (1972) in: Laboratory Techniques in Biochemistry and Molecular Biology (Work, T. S. and Work, E., eds.), pp. 358–359, North Holland, Amsterdam.
[18] Biochemica Information (1973), (Boehringer Mannheim GmbH, ed.), vol. 1, p. 148.
[19] Biochemica Information (1975) (Boehringer Mannheim GmbH, ed.), vol. II, pp. 115–116.
[20] Bergmeyer, H. U. (1974) in: Methoden der enzymatischen Analyse, vol. 1, p. 529, Verlag Chemie, Weinheim.
[21] Heppel, L. A. (1971) in: Structure and Function of Biological Membranes (Rothfield, L., ed.), pp. 223–247, Academic Press, New York.

[22] Mitchell, P. (1956) Discuss. Far. Soc. 21, 282–283.
[23] Malveaux, F. J. and San Clemente, C. L. (1969) J. Bacteriol. 97, 1215–1219.
[24] Malveaux, F. J. and San Clemente, C. L. (1967) Appl. Microbiol. 15, 738–747.
[25] Morton, R. K. (1965) in: Comprehensive Biochemistry (Florkin, M. and Stotz, E. H., eds.), vol. 16, pp. 55–84, Elsevier, Amsterdam.
[26] Poxton, I. R., Lomax, J. A. and Sutherland, I. W. (1974) J. Gen. Microbiol. 84, 231–233.
[27] Anderson, J. S., Matsuhashi, M., Haskin, M. A. and Strominger, J. L. (1965) Proc. Natl. Acad. Sci. USA 53, 881–889.
[28] Araki, Y., Shirai, R., Shimada, A., Ishimoto, N. and Ito, E. (1966) Biochem. Biophys. Res. Commun. 23, 466–472.
[29] Dankert, M., Wright, A., Kelley, W. S. and Robbins, P. W. (1966) Arch. Biochem. Biophys. 116, 425–435.
[30] Hanes, C. S. and Isherwood, F. A. (1949) Nature 164, 1107.