Tridecaptin stimulates the formation of lipid-linked saccharides in aorta

Alan D. ELBEIN

Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX 78284, U.S.A.

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The peptide antibiotic tridecaptin caused a 2–4-fold stimulation in the incorporation of mannose from GDP-[^{14}C]mannose and glucose from UDP-[^3H]glucose into lipid-linked monosaccharides by both the particulate and the soluble enzyme fractions from pig aorta. In both cases, the major products and the ones stimulated by antibiotic were dolichyl phosphate mannose and dolichyl phosphate glucose. The stimulation in activity was unaffected by increasing concentrations of dolichyl phosphate, GDP-mannose, UDP-glucose, Mn^{2+} or the detergent Nonidet P40. Tridecaptin stimulation was apparently not due to protection of sugar nucleotide substrate, since addition of various concentrations of sugar nucleotides did not alter the stimulation. Nor did the addition of tridecaptin result in any increase in the amount of radioactive sugar nucleotide recovered from incubation mixtures. Tridecaptin bound to the particulate enzyme and could not be removed by centrifugation of the particles.

The tridecaptins are peptide antibiotics produced by strains of *Bacillus polymyxus* that are active against Gram-positive and Gram-negative bacteria (Kato *et al.*, 1979). This group of antibiotics are acyl-tridecapeptides and represent a mixture of compounds A, B and C that differ in both amino acid and fatty acid composition (Kato *et al.*, 1978; Shoji, 1978). The mechanism of action of these antibiotics on bacterial cells has not been reported. In the present paper, we describe the effect of tridecaptin on the synthesis of lipid-linked saccharides by using enzymes isolated from aorta tissue. In this system, tridecaptin stimulated the formation of both dolichyl phosphate mannose and dolichyl phosphate glucose in both particulate and soluble enzyme systems. The antibiotic also stimulated the incorporation of sugars into the lipid-linked oligosaccharides.

Materials and methods

Materials

GDP-[^{14}C]mannose (sp. radioactivity 278 Ci/mol), UDP-[^3H]glucose (sp. radioactivity 6.6 Ci/mmol) and UDP-[^3H]N-acetylglucosamine (sp. radioactivity 1.5 Ci/mmol) were obtained from New England Nuclear. Dolichyl phosphate and unlabelled sugar nucleotides were purchased from Sigma Chemical Co., and silica gel F254 plates were from Brinkman Instrument Co. Bio-Gel P-4 and P-6 were from Bio-Rad Laboratories. All other chemicals were from commercial sources and were

of the best grade available. Tridecaptin was a generous gift from Dr. J. Shoji, Shionogi Chemical Co., Tokyo, Japan.

Preparation and assay of aorta enzyme

The intimal layer of fresh pig aorta was removed and homogenized in 50 mM-Tris buffer, pH 7.5, as described previously (Chambers & Elbein, 1975; Chambers *et al.*, 1977). The homogenate was filtered through cheesecloth and the filtrate was centrifuged at 5000 g for 10 min to remove large particles. The supernatant from this centrifugation was centrifuged at 100 000 g for 90 min to obtain a high-speed pellet. This pellet was suspended in approximately one-tenth of the original volume of Tris buffer and gently homogenized. This preparation represented the particulate enzyme fraction.

Solubilized enzyme was prepared by mixing the above particulate enzyme with 0.5% Nonidet P40 for 2–3 min followed by centrifugation at 100 000 g for 45 min. The supernatant was removed and used as the solubilized enzyme (Heifetz & Elbein, 1977).

For assay of the particulate enzyme, incubation mixtures contained the following components in a final volume of 0.4 ml: 10 μmol of Tris buffer, pH 7.5; 2.5 μmol of MnCl_2 ; 100 000 c.p.m. of sugar nucleotide (GDP-[^{14}C]mannose, UDP-[^3H]glucose or UDP-[^3H]N-acetylglucosamine); various amounts of the antibiotic tridecaptin as indicated; 100 μl of particulate enzyme (usually 1 or 2 mg of protein). Assay tubes were incubated at 37°C for the indicated times and reactions were stopped by the

addition of 2 ml of chloroform/methanol and 0.6 ml of water and thorough mixing. The layers were separated by centrifugation and the various classes of lipid-linked saccharide intermediates were isolated as described in previous papers (Chambers & Elbein, 1975; Heifetz & Elbein, 1977).

For the assay of the solubilized enzyme, the reaction mixtures were similar to those above except that dolichyl phosphate had to be included as a glucosyl acceptor. In these cases, 5 μ g of dolichyl phosphate (usually in 25 μ l of chloroform) was added to each tube and the solvent was removed under a stream of air. Nonidet P40 (100 μ l) was added to each tube to suspend the lipid, and the tubes were mixed vigorously. The remaining reaction components were added as follows: 10 μ mol of Tris buffer, 150 μ l of water, 2.5 μ mol of MnCl_2 , various amounts of tridecaptin as indicated and 100000 c.p.m. of sugar nucleotide. The tubes were mixed vigorously between each addition. The reaction was initiated by the addition of 100 μ l of soluble enzyme (0.5–1 mg of protein) and the tubes were incubated at 37°C for the indicated times. Reactions were stopped by the addition of 2 ml of chloroform/methanol (1:1, v/v) and 0.6 ml of water, and the lipid-linked saccharides were isolated as indicated above.

Analytical and chemical methods

Radioactivity was measured in a Beckman liquid-scintillation counter with either a Scintiline or Scintiverse counting solution (Fisher Scientific Co.). Paper chromatograms were scanned with a Packard radiochromatogram scanner. Protein was measured by the Lowry method (Lowry *et al.*, 1951), and hexose by the anthrone procedure (Loewus, 1952). Mild acid hydrolysis of lipid-linked saccharides was done in 50% propanol in 0.05 M-HCl at 95°C for 15 min. After hydrolysis, the reaction mixtures were partitioned by the addition of chloroform to give a chloroform/propanol/water (1:1:1, by vol.) mixture. The aqueous phase, containing the oligosaccharides and monosaccharides, was removed, taken to dryness and chromatographed.

Chromatographic procedures

Descending paper chromatography of radioactive oligosaccharides was done on Whatman 3MM paper in solvent A (n-butanol/pyridine/water, 4:3:4, by vol.). Standard sugars were detected with the silver nitrate dip (Trevelyan *et al.*, 1950). T.l.c. of lipids was done on silica gel plates (2 mm thick) in the following solvents: solvent B, chloroform/methanol/water (65:25:4, by vol.); solvent C, chloroform/methanol/ NH_3 (75:25:4, by vol.); solvent D, chloroform/methanol/acetic acid/water (25:15:4:2, by vol.). Lipids were detected with I_2 vapour and radioactive areas were detected by

cutting the plate into 1 cm areas and counting each area for radioactivity in the scintillation counter.

In some cases, the dolichyl-linked sugars were purified on DEAE-cellulose as previously described (Chambers & Elbein, 1975; Chambers *et al.*, 1977). Lipid-linked monosaccharides were run on columns of DEAE-cellulose that had been washed with chloroform/methanol (1:1, v/v), whereas lipid-linked oligosaccharides were run on DEAE-cellulose prepared in chloroform/methanol/water (10:10:3, by vol.). After washing the columns well with these solvents, the lipids were eluted with gradients of ammonium formate in 99% methanol. Radioactive peaks were pooled, extracted with chloroform and used in further studies.

Results

The effect of tridecaptin on the particulate enzyme

The effect of tridecaptin on the incorporation of sugars from sugar nucleotides into lipid-linked saccharides was studied with the aorta particulate enzyme. Fig. 1 shows that this antibiotic greatly stimulated the incorporation of mannose from GDP-[^{14}C]mannose into dolichyl phosphate mannose and also the incorporation of glucose from UDP-[^3H]glucose into dolichyl phosphate glucose. Maximum stimulatory activity was observed at 100–200 μ g of tridecaptin per incubation mixture. However, only slight stimulation of *N*-acetylglucosamine incorporation from UDP-[^3H]*N*-acetylglucosamine was observed. In the particulate enzyme, tridecaptin also stimulated the incorporation

Fig. 1. The effect of tridecaptin concentration on the incorporation of mannose, glucose and *N*-acetylglucosamine into lipid by the particulate enzyme

Reaction mixtures were as described in the text and contained either GDP-[^{14}C]mannose (●), UDP-[^3H]glucose (○) or UDP-[^3H]*N*-acetylglucosamine (Δ) and various amounts of tridecaptin. After an incubation of 15 min lipid-linked saccharides were isolated as described in the text.

of mannose and glucose into the lipid-linked oligosaccharides. Thus, whereas 50 μ g of tridecaptin doubled the incorporation of mannose into dolichyl phosphate mannose (from 7400 c.p.m. to 14400 c.p.m.), it increased the incorporation into lipid-linked oligosaccharides about 30–40% (from 800 c.p.m. to 1200 c.p.m.). Likewise, glucose incorporation into dolichyl phosphate glucose was tripled by 50 μ g of tridecaptin (from 2500 c.p.m. to 7700 c.p.m.), whereas incorporation into lipid-linked oligosaccharides increased about 30% (from 690 c.p.m. to 980 c.p.m.). The increase in incorporation into lipid-linked oligosaccharides may be due to the greater availability of the substrates dolichyl phosphate mannose and dolichyl phosphate glucose.

The lipids extracted into the chloroform layer with chloroform/methanol/water (1:1:1, by vol.) were examined by t.l.c. to see whether tridecaptin had any effect on the nature of the products. The results of one of these chromatograms are shown in Fig. 2. Lipids formed in the presence and absence of antibiotic were run in solvent B and the plates were

cut into 1 cm sections for the determination of radioactivity. In the case of the products formed from GDP-[14 C]mannose, in either the presence or absence of tridecaptin, only one radioactive band was detected and this band had the same migration as authentic dolichyl phosphate mannose. However, there was much more radioactivity in this band when the antibiotic was present in the reaction mixtures. Similar results were obtained when these lipids were chromatographed in solvents C or D. When the products obtained from UDP-[3 H]glucose were examined by t.l.c., two radioactive areas were detected. The major radioactive area migrated with dolichyl phosphate mannose, and a small peak (10–15% of the total radioactivity) of radioactivity migrated more rapidly (Fig. 2). Based on previous studies, this faster migrating peak is probably a glycosylceramide (Kang *et al.*, 1979). In the presence of tridecaptin, there was much more radioactivity in the dolichyl phosphate glucose area, but the radioactivity in the faster area remained approximately the same.

The lipid-linked oligosaccharides isolated by extraction with chloroform/methanol/water (10:10:3, by vol.) were subjected to mild acid hydrolysis and the oligosaccharides were chromatographed on Whatman 3MM paper in solvent A. No major differences in the oligosaccharide profiles were seen between those incubations having tridecaptin and those lacking it (results not shown). Thus for mannose incorporation, the radioactivity was mostly in large oligosaccharides that migrated slower than the stachyose standard and probably contain 9–12 sugar residues (Chambers *et al.*, 1977). For glucose incorporation, three radioactive peaks were seen in either case, one of which remained at the origin and two others that moved a short distance from the origin. These peaks have not been further characterized yet.

Fig. 2. Thin-layer chromatographic identification of lipid-linked monosaccharides formed from either GDP-[14 C]mannose (a and b) or UDP-[3 H]glucose (c and d). Reactions were run in the presence (a and c) and absence (b and d) of tridecaptin and products are compared. Plates were run in solvent B. The bar above scan (a) indicates the position of dolichyl phosphate mannose (for all the scans).

The effect of tridecaptin on dolichyl phosphate mannose and dolichyl phosphate glucose formation by the solubilized enzyme

Tridecaptin was used to determine its effects on mannose and glucose incorporation using the aorta solubilized enzyme. Fig. 3 shows a time course of incorporation of mannose from GDP-[14 C]mannose into dolichyl phosphate mannose in the presence and absence of antibiotic. In the absence of tridecaptin, the incorporation of mannose was linear for about 5 min and then levelled off. However, in the presence of 100 or 250 μ g of antibiotic, mannose incorporation was stimulated 2–3-fold. The product formed in these incubations was shown to be dolichyl phosphate mannose by t.l.c. in three different solvent systems. A time course of glucose incorporation from UDP-[3 H]glucose into dolichyl

Fig. 3. Time course of dolichyl phosphate mannose formation by the soluble enzyme in the absence (Δ) or in the presence of 100 ($\circ$) or 250 μ g ($\bullet$) of tridecaptin. Incubation mixtures were as described in the text and contained dolichyl phosphate and 100 μ l of soluble enzyme (0.5–1 mg of protein). Dolichyl phosphate mannose was isolated as described in the text.

phosphate glucose showed essentially the same results (results not shown). Thus in the absence of antibiotic, incorporation of glucose was linear for about 5–10 min and then levelled off. However, in the presence of antibiotic, the incorporation was stimulated 3–4-fold and the curves were similar to those shown for mannose incorporation.

Tridecaptin was also studied to determine its effect on mannose and glucose incorporation in the presence and absence of dolichyl phosphate. Fig. 4 shows the results obtained with respect to mannose incorporation. With the soluble enzyme, only small amounts of dolichyl phosphate mannose were formed in the absence of dolichyl phosphate but this incorporation was stimulated to some extent by antibiotic. However, when dolichyl phosphate was included in the incubation mixtures, there was a pronounced stimulation in the formation of dolichyl phosphate mannose with increasing amounts of tridecaptin. The maximum stimulation, which resulted in a 3–4-fold increase in product, occurred at about 200 μ g of tridecaptin. The effect of dolichyl phosphate concentration on the synthesis of dolichyl phosphate mannose in the presence and absence of tridecaptin is shown in Fig. 5. In the

Fig. 4. The effect of dolichyl phosphate on the tridecaptin stimulation of dolichyl phosphate mannose formation

Various amounts of tridecaptin were added to incubation mixtures either lacking dolichyl phosphate ($\bullet$) or containing 5 μ g of this lipid ($\circ$). Other reaction components were the same as indicated in the text and reactions were initiated by the addition of 100 μ l of soluble enzyme. Incubations were for 15 min.

Fig. 5. The effect of dolichyl phosphate concentration on the tridecaptin stimulation of dolichyl phosphate mannose

Reaction mixtures with soluble enzyme were as described in the text, except that the amount of dolichyl phosphate was varied as indicated in the Figure; 10 μ l of dolichyl phosphate represents 5 μ g. Incubations were for 15 min.

Fig. 6. The effect of GDP-mannose concentration on the formation of dolichyl phosphate mannose in the presence and absence of tridecaptin

Incubation mixtures with soluble enzyme were as described in the text, except that the amount of GDP-mannose was varied. Incubations were for 15 min.

absence of antibiotic, product formation increased with increasing amounts of dolichyl phosphate up to about 5–10 μg of this substrate per incubation (K_m approx. 1 μM). In the presence of tridecaptin, the formation of dolichyl phosphate mannose was greatly increased but the shape of the curves were the same as those of the control. That is, the reactions levelled off at about 20 μg of dolichyl phosphate and this substrate, even at high concentrations, had no effect on the tridecaptin stimulation. The same results were observed with regard to the formation of dolichyl phosphate glucose by the solubilized enzyme (results not shown). That is, tridecaptin greatly stimulated the formation of dolichyl phosphate glucose and dolichyl phosphate, even at high concentrations, had no effect on this stimulation.

The effect of adding increasing amounts of GDP-[^{14}C]mannose on the tridecaptin stimulation was also examined with the soluble enzyme as shown in Fig. 6. In the control, dolichyl phosphate mannose formation was proportional to GDP-mannose concentration to about 1 nmol and then levelled off. The K_m for GDP-mannose was estimated to be about 0.1 μM . In the presence of antibiotic, the formation of dolichyl phosphate mannose was stimulated at all concentrations of GDP-mannose. However, at 100 μg of tridecaptin the shape of the curves and the estimated K_m were essentially the same as in the controls. At higher concentrations of antibiotic, dolichyl phosphate mannose formation continued to increase as the GDP-mannose was increased. In these experiments, as in those already discussed with the particulate enzyme, the only radioactive product was shown to be dolichyl phosphate mannose (or dolichyl phosphate glucose) by t.l.c. in several different solvent systems.

Table 1. Effect of substrate concentration on tridecaptin stimulation

In this experiment, the radioactive sugar nucleotide (UDP-glucose in *a*, GDP-mannose in *b*) was mixed with the indicated amount of unlabelled sugar nucleotide (UDP-glucose in *a*, GDP-mannose in *b*) and this mixture was incubated with the soluble enzyme in the assay mixtures described in the text. Incorporation of radioactivity into dolichyl phosphate glucose (*a*) and dolichyl phosphate mannose (*b*) was measured in the presence and absence of antibiotic.

Amount of unlabelled sugar nucleotide (nmol)	Radioactivity incorporated (c.p.m.)	
	– tridecaptin	+ tridecaptin
(a) 0	665	2337
2	574	2402
10	349	1371
20	284	1336
100	197	557
250	111	467
(b) 0	5051	11137
2	1877	3368
10	1255	1341
20	498	814
100	266	412
250	108	205

The effect of Nonidet P40 and Mn^{2+} concentrations were studied with respect to tridecaptin stimulation of dolichyl phosphate glucose formation. As the Nonidet P40 concentration was increased, the amount of dolichyl phosphate glucose decreased, but again tridecaptin stimulated to the same extent at all detergent concentrations (results not shown). Finally, increasing or decreasing the Mn^{2+} concentration had no effect on the stimulation by this antibiotic.

Effect of tridecaptin on sugar nucleotides

Since it seemed possible that tridecaptin could be stimulating these reactions by protecting the substrates from degradation, we examined the effect of adding various unlabelled nucleotides on the synthesis of dolichyl phosphate mannose and dolichyl phosphate glucose. In these experiments, the solubilized enzyme fraction was used and the radioactive sugar nucleotides (either GDP-[^{14}C]mannose or UDP-[^3H]glucose) were diluted by adding increasing amounts of the same unlabelled sugar nucleotide (i.e. GDP-mannose was added to GDP-[^{14}C]mannose and UDP-glucose to UDP-[^3H]glucose). The amount of radioactive sugar nucleotide added in each of these incubations was approx. 0.4 nmol, so the unlabelled material represented a 5-fold or greater excess. Table 1 presents the results of this experiment. In either case, as the radioactive

sugar nucleotide was diluted with the same unlabelled sugar nucleotide, the radioactivity incorporated into dolichyl phosphate mannose or dolichyl phosphate glucose decreased as expected. However, in every case tridecaptin still stimulated the incorporation to the same extent, suggesting that its stimulation was not a result of its increasing substrate availability.

In another type of experiment, soluble enzyme was incubated with either GDP-[14 C]mannose or UDP-[3 H]glucose and Mn^{2+} in the absence or presence of 50, 100 or 200 μ g of tridecaptin. These incubations did not contain dolichyl phosphate. After incubation for 15 min, protein was precipitated by the addition of trichloroacetic acid to a final concentration of 10%. Precipitate was removed by centrifugation and the supernatant liquid was extracted five or six times with diethyl ether to remove the trichloroacetic acid. The supernatant liquid was concentrated to a small volume and chromatographed on Whatman 3MM paper in ethanol/1M-ammonium acetate (7:3, v/v). In both the control incubations and those with antibiotic, the major radioactive band corresponded to UDP-glucose or GDP-mannose and there was no significant difference between any of these incubations. Thus, tridecaptin does not appear to stimulate as a result of protecting the sugar nucleotide substrates from degradation.

The effect of tridecaptin on dolichyl phosphate metabolism was also tested. In this case, [3 H]-dolichyl phosphate was incubated with soluble enzyme in the presence of Tris buffer and Mn^{2+} , and with and without antibiotic. The incubations were examined by t.l.c. of the chloroform-extracted lipids. Very little dolichyl phosphate was degraded in the controls and tridecaptin had no effect on the stability of this substrate.

Binding of tridecaptin to the particulate enzyme

To determine whether tridecaptin bound to the particulate enzyme, various amounts of antibiotic were mixed with the particulate enzyme and, after centrifugation, the pellet was resuspended and assayed for activity with either GDP-[14 C]mannose or UDP-[3 H]glucose (see the legend to Fig. 7). As a control, enzyme was assayed in the presence of the same concentrations of tridecaptin but without centrifugation. The results of this experiment are shown in Fig. 7. For GDP-mannose transfer to dolichyl phosphate mannose, tridecaptin stimulated the reaction at least 2-fold and this stimulation was equally evident in those samples that had been centrifuged. Similar results were observed with respect to glucose incorporation in that equal stimulation of dolichyl phosphate glucose formation was observed regardless of whether the antibiotic/enzyme mixture was centrifuged or not. These data

Fig. 7. *Binding of tridecaptin to the particulate enzyme* Particulate enzyme was mixed with various amounts of tridecaptin and, after standing for 10 min on ice, one set of tubes was diluted 10-fold with buffer and centrifuged at 20000 g for 45 min to isolate the particles. The particles were resuspended and assayed for their ability to catalyse the incorporation of mannose (●) and glucose (×) into lipid-linked saccharides. The control particles (incubated with antibiotic, but not centrifuged) were also assayed with GDP-mannose (○) and UDP-glucose (▲).

indicate that the antibiotic binds to the particulate enzyme and is not removed by washing and centrifuging.

Discussion

Generally one expects an antibiotic to inhibit a given reaction, so it is somewhat surprising to find one that causes an increase in product formation. In this case, the peptide antibiotic tridecaptin causes a 2–3-fold stimulation in the incorporation of mannose from GDP-[14 C]mannose into dolichyl phosphate mannose and of glucose from UDP-[3 H]-glucose into dolichyl phosphate glucose. This stimulation occurred in the particulate enzyme, which utilizes endogenous lipid acceptors (i.e. dolichyl phosphate), and also in the solubilized enzyme fraction, which requires exogenous dolichyl phosphate as an acceptor.

The mechanism of tridecaptin stimulation is not

clear at this time but the possibility that the antibiotic stimulates by protecting the substrates from degradation appears to be eliminated on the basis of several experiments described in the present paper. Also the antibiotic does not appear to interact directly with the substrates since alterations in the concentrations of any of the reaction components does not affect the tridecaptin stimulation. In a previous study with the antibiotic showdomycin, we found that this compound stimulated the incorporation of glucose into lipid by yeast membrane preparations (Kang *et al.*, 1979). However, this stimulation did appear to be a result of this antibiotic protecting the substrate UDP-glucose from degradation. The glucolipid formed in those experiments was a neutral lipid and was probably a glucosylceramide. The tridecaptin stimulation described in the present paper apparently involves a different mechanism.

Tridecaptin binds to the particulate enzyme. Thus, after mixing tridecaptin and enzyme, the tridecaptin stimulation cannot be removed by centrifuging and resuspending the particles. It is possible that tridecaptin binds and removes some inhibitory substance rather than acting on the enzyme itself, but this has not been determined. It is noteworthy that several other peptide antibiotics, such as amphomycin (Kang *et al.*, 1979) and tsushimysin (Elbein, 1981), also bind to the particulate enzyme, as evidenced by their continued activity even in cen-

trifuged particles. However, these two antibiotics inhibit rather than stimulate the formation of lipid-linked saccharides.

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References

- Chambers, J. & Elbein, A. D. (1975) *J. Biol. Chem.* **250**, 6904–6915
- Chambers, J., Forsee, W. J. & Elbein, A. D. (1977) *J. Biol. Chem.* **252**, 2498–2506
- Elbein, A. D. (1981) *Biochem. J.* **193**, 477–484
- Heifetz, A. & Elbein, A. D. (1977) *J. Biol. Chem.* **252**, 3057–3063
- Kang, M. S., Spencer, J. P. & Elbein, A. D. (1978) *J. Biol. Chem.* **253**, 8860–8866
- Kang, M. S., Spencer, J. P. & Elbein, A. D. (1979) *J. Biol. Chem.* **254**, 10037–10043
- Kato, T., Hindo, H. & Shoji, J. (1978) *J. Antibiot.* **31**, 652–661
- Kato, T., Sakazaki, R., Hindo, H. & Shoji, J. (1979) *J. Antibiot.* **32**, 305–312
- Loewus, F. (1952) *Anal. Chem.* **24**, 219
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Shoji, J. (1978) *Adv. Appl. Microbiol.* **24**, 187–214
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950) *Nature (London)* **166**, 444