Mechanism of Action of Tunicamycin on the UDP-GlcNAc:Dolichyl-Phosphate GlcNAc-1-Phosphate Transferase†

Aaron Heifetz, Roy W. Keenan, and Alan D. Elbein*

ABSTRACT: The enzyme that transfers *N*-acetylglucosamine 1-phosphate from UDP-*N*-acetylglucosamine to dolichyl phosphate forming *N*-acetylglucosaminylpyrophosphoryldolichol was solubilized from a microsomal fraction of pig aorta and was partially purified on DEAE-cellulose [Heifetz, A., & Elbein, A. D. (1977) *J. Biol. Chem. 252*, 3057–3065]. This enzyme was used to study the mode of action of tunicamycin, an *N*-acetylglucosamine containing antibiotic produced by *Streptomyces lysosuperificus*. At low concentrations of enzyme, the reaction was inhibited 50% or greater by antibiotic concentrations of 0.05–0.1 μg/mL, whereas higher enzyme concentrations required progressively more tunicamycin for inhibition. The inhibition was noncompetitive with respect to the concentration of UDP-*N*-acetylglucosamine or of dolichyl phosphate. Tunicamycin also inhibited the reverse reaction; that is, it prevented the formation of UDP-*N*-acetylglucosamine from *N*-acetylglucosaminylpyrophosphoryldolichol. The inhibition was increased by preincubating the enzyme with antibiotic for up to 5 min before the addition of substrates. The addition of phosphatidylcholine, at concentrations up to 20 mM, did not affect the inhibition regardless of whether it was added during the preincubation or at the same time as the substrates. Tunicamycin did, however, bind to heat-denatured microsomal particles of aorta as shown by the fact that preincubation of antibiotic with these particles prevented the inhibition of the *N*-acetylglucosamine-1-phosphate transferase.

Many glycoproteins contain oligosaccharide chains having a common core region of mannose and GlcNAc that is attached to the protein moiety via an *N*-glycosidic linkage to an asparagine residue (Kornfeld & Kornfeld, 1976). The assembly of the oligosaccharide core region, of the general structure

$$(Man)_n\alpha\rightarrow Man\beta\rightarrow(GlcNAc)_2-$$

occurs on a dolichyl carrier lipid before en bloc transfer of this saccharide core to the protein. This lipid-linked pathway has been reviewed (Waechter & Lennarz, 1976).

Recently, the structure of the antibiotic, tunicamycin, was reported by Takatsuki et al. (1977). Tunicamycin specifically inhibits the first step in the lipid-linked oligsaccharide pathway which produces GlcNAc-pyrophosphoryldolichol (Figure 1). Studies from various laboratories have shown that only the transfer of GlcNAc 1-phosphate is inhibited and not other reactions involving the transfer of GlcNAc (Tkacz & Lampen, 1975; Kuo & Lampen, 1975; Struck & Lennarz, 1977; Heifetz & Elbein, 1977a,b; Waechter & Harford, 1977; Lehle & Tanner, 1977). Numerous investigations have now utilized tunicamycin as a probe to assess the biological role of the oligosaccharide moiety that is synthesized by the lipid-linked pathway as well as an aid in elucidating reactions in the pathway itself (Duskin & Bornstein, 1977; Hickman et al., 1977; Ericson et al., 1977; Hasilik & Tanner, 1976).

Due to the increased use of tunicamycin as a valuable experimental tool, the investigation of the biochemical nature of its action is of widespread interest. The present study is the first to utilize a solubilized and partially purified UDP-GlcNAc:dolichyl-phosphate GlcNAc-1-phosphate transferase to study in detail the enzymatic mechanism of tunicamycin action. We report here that the inhibition with tunicamycin is noncompetitive with respect to both UDP-GlcNAc and dolichyl phosphate over a wide range of different concentrations and is unaffected by the addition of exogenous phospholipid. The antibiotic also inhibited the reaction in the reverse direction. This suggested that tunicamycin acts as an irreversible inhibitor of the GlcNAc-1-phosphate transferase by being a substrate–product transition-state analogue. The binding of tunicamycin to biological membranes from porcine aorta was also investigated.

Experimental Procedures

Materials. UDP-[^{3}H]GlcNAc (6.6Ci/mmol) and GDP-[^{14}C]mannose (270 Ci/mol) were purchased from New England Nuclear. Dolichyl phosphate was prepared as described (Rupar & Carroll, 1976) or was from Calbiochem. UDP-GlcNAc, GDP-mannose, and DEAE-cellulose were from Sigma Chemical Co. Tunicamycin was a generous gift of Dr. Robert Hamill, Eli Lilly Co. It was dissolved at 5 mg/mL in 0.01 N NaOH and then was diluted 10- and 100-fold in water. Tunicamycin solutions were kept at −20 °C. The initial samples of tunicamycin used in this study were not of high purity and required as much as 5–10 μg/mL for 50% inhibition. However, recent samples are highly purified and require as little as 0.05 μg/mL for 50% inhibition. Phosphatidylcholine was prepared by alumina chromatography of egg yolk phospholipids. This fraction also contained small amounts of sphingomyelin and lysophosphatidylcholine. The fatty acid composition of similar preparations was determined by gas–liquid chromatography to be 38% 16:0, 2.4% 16:1, 15% 18:0, 31% 18:1, and 13% 18:2. All other chemicals were from commercial sources and were of the best grade available.

Preparation of the Enzyme. Porcine aortas were obtained from a local slaughterhouse and were maintained at 4 °C during all subsequent procedures. The intima-media layer was removed and homogenized in a Waring blender in 5 volumes of 50 mM Tris buffer, pH 7.5. After centrifugation at 20000*g* for 15 min, the resulting supernatant fluid was centrifuged at 88000*g* for 60 min. The pellet from this centrifugation was resuspended in 50 mM Tris buffer at about 6 mg of protein/mL.

† From the Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78284. *Received July 17, 1978; revised manuscript received February 28, 1979.* This study was supported by grants from the National Heart and Lung Institute (HL-17783) and the Robert A. Welch Foundation to Alan D. Elbein and a grant from the National Institutes of Health (AM-17897) to Roy W. Keenan.

0006-2960/79/0418-2186$01.00/0

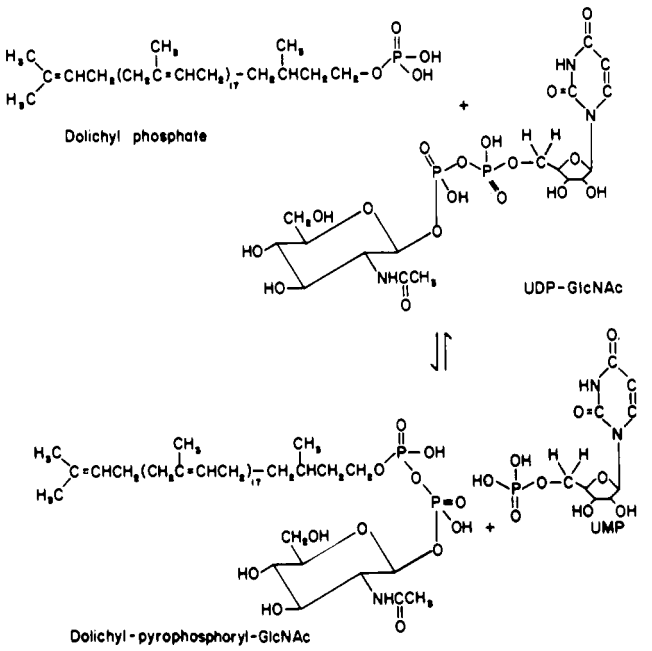

FIGURE 1: Reaction for the synthesis of dolichylpyrophosphoryl-GlcNAc.

To the above particulate enzyme, Nonidet P-40 (NP-40) was added to a final concentration of 0.5%. After vortexing for several min, the mixture was centrifuged for 60 min at 100000*g*. The supernatant liquid from this centrifugation contained the GlcNAc-1-phosphate transferase for forming dolichylpyrophosphoryl-GlcNAc. The enzyme was partially purified on columns of DEAE-cellulose as previously described (Heifetz & Elbein, 1977a,b).

Enzyme Assays. To determine the effect of tunicamycin, various amounts of this antibiotic were incubated with different amounts of enzyme at room temperature for 5 min in a final volume of 200 μL. Following this preincubation, a mixture containing UDP-[^{3}H]GlcNAc (50000 cpm), 0.5% NP-40, $MnCl_2$ (1 μmol), dolichyl phosphate (3 μg), and Tris buffer (10 μmol) in a final volume of 300 μL was added. Incubations were performed at 37 °C for varying times but usually 10 min was used. The reaction was stopped by the addition of 2 mL of $CHCl_3$:CH_3OH (1:1) and 0.6 mL of H_2O. The GlcNAc–lipid was extracted as previously described and its formation was determined by counting an aliquot of the organic phase (Heifetz & Elbein, 1977a,b). In some experiments, NaF, ATP, or UDP-glucose was added at various concentrations to try to inhibit UDP-GlcNAc degradation. However, these compounds did not stimulate the incorporation of GlcNAc into the GlcNAc-pyrophosphoryldolichol. In some cases, UDP-GlcNAc was reisolated at various times during the incubations (see Results). After a 10-min incubation, 50% of the UDP-GlcNAc could still be recovered.

Analytical Methods. Protein was determined by the method of Lowry et al. (1951) and phosphate by the method of Bartlett (1959). Radioactivity was determined by counting samples in a liquid scintillation spectrometer in a Triton–toluene scintillator field. Radioactivity on paper was located with a radiochromatogram scanner.

Chromatography and Electrophoresis. UDP-GlcNAc and GlcNAc 1-phosphate were separated from each other by high voltage paper electrophoresis on Whatman 3MM in 0.05 M ammonium formate, pH 3.5. Radioactivity in these areas was determined by cutting the papers into strips and counting in a liquid scintillation counter. GlcNAc-pyrophosphoryldolichol and *N,N'*-diacetylchitobiosylpyrophosphoryldolichol were separated from each other by thin-layer chromatography on silica gel plates in $CHCl_3$:CH_3OH:H_2O (65:25:4). Mono- and di-GlcNAc-lipids could also be determined by separation of GlcNAc and *N,N'*-diacetylchitobiose on Whatman 3MM paper in ethyl acetate:HOAc:formic acid:water (18:3:1:4). These sugars were released from the lipids by mild acid hydrolysis and quantitated by counting in a liquid scintillation counter.

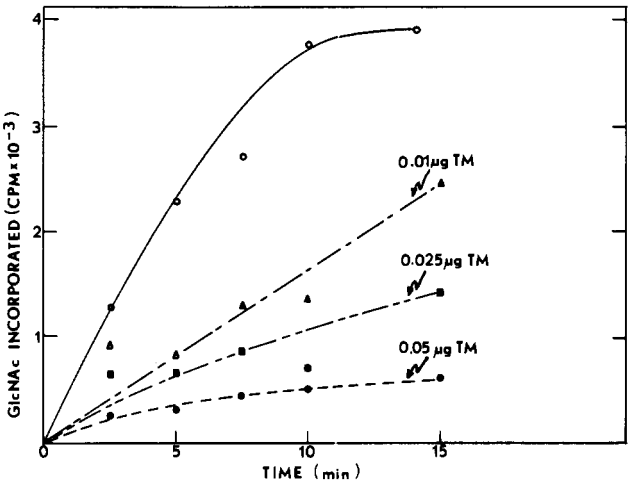

FIGURE 2: The effect of tunicamycin on the time course of formation of dolichylpyrophosphoryl-GlcNAc by the solubilized enzyme from aorta. Preincubation mixtures contained 100 μL of solubilized enzyme (100 μg of protein) and 100 μL of 50 mM Tris buffer, pH 7.5. Tunicamycin was added in the amounts shown in the figure. After 5 min, 200 μL of a mixture containing 3 μg of dolichyl phosphate, 0.5% NP-40, 1 μmol of $MnCl_2$, 50000 cpm of UDP-[^{3}H]GlcNAc, and 5 μmol of Tris buffer was added to each tube. A tube was removed at each of the times shown in the figure and dolichylpyrophosphoryl-GlcNAc was extracted with $CHCl_3$:CH_3OH as described.

Results

Effect of Tunicamycin on the Particulate Enzyme. The effect of tunicamycin was first examined by using the particulate enzyme fraction from pig aorta. The incorporation of GlcNAc from UDP-[^{3}H]GlcNAc and of mannose from GDP-[^{14}C]mannose into lipid-linked saccharides as a function of time of incubation in the presence or absence of tunicamycin was examined (data not shown). As has been described in other systems (Tkacz & Lampen, 1975; Struck & Lennarz, 1977; Takatsuki et al., 1971; Waechter & Harford, 1977; Ericson et al., 1977), this antibiotic effectively inhibited the formation of dolichylpyrophosphoryl-GlcNAc, but had no effect on the synthesis of dolichylphosphorylmannose. A concentration curve showed that about 0.25–0.5 μg/mL of tunicamycin was required to cause a 50% inhibition when 10 μL of particulate enzyme (120 g of protein) was used, whereas 0.5–1 μg of antibiotic was necessary when 25 μL of enzyme was used.

Effect of Tunicamycin on the Solubilized Enzyme. The UDP-GlcNAc:dolichyl-phosphate GlcNAc-1-phosphate transferase was solubilized from aorta membranes and partially purified on DEAE-cellulose as previously described (Heifetz & Elbein, 1977a). This enzyme preparation required the addition of exogenous dolichyl phosphate for activity and represented a tenfold purification over the particulate enzyme. The partially purified enzyme was much more sensitive to tunicamycin than was the particulate fraction. This enzyme preparation was used in all subsequent experiments. Figure 2 shows a time course of incorporation of GlcNAc from UDP-GlcNAc into dolichylpyrophosphoryl-GlcNAc in the absence of antibiotic or in the presence of three different

Table I: Distribution of Radioactivity from UDP-[^{14}C]GlcNAc during Incubation

time of incubation (min)	radioact (cpm) recovered in[a]		
	GlcNAc-lipid	UDP-[^{14}C]GlcNAc	GlcNAc-1-P
5	47 000	198 269	90 757
10	68 600	169 945	129 137
15	91 000	139 052	191 273
20	98 000	67 246	225 364
10 + chase[b]	67 000	108 480	220 463

[a] Incubations were as described in the text except that they were scaled up and contained 0.5 mL of enzyme and UDP-[^{14}C]-GlcNAc (500 000 cpm). GlcNAc-pyrophosphoryldolichol (GlcNAc-lipid) was isolated by extraction with $CHCl_3$:CH_3OH after which the aqueous phase was concentrated to dryness and subjected to high voltage paper electrophoresis to separate UDP-GlcNAc from GlcNAc 1-phosphate. [b] In this experiment, enzyme was incubated for 10 min with UDP-[^{14}C]GlcNAc, after which time 0.1 μmol of unlabeled UDP-GlcNAc was added and incubation was continued for another 10 min.

concentrations of tunicamycin. In these experiments, enzyme and antibiotic were mixed and allowed to stand for 5 min at room temperature. The reaction was initiated by the addition of a mixture of substrates and the incubations were done for various times at 37 °C. It can be seen that, in the absence of antibiotic, the formation of dolichylpyrophosphoryl-GlcNAc was linear for about 10 min and then leveled off. In the presence of 0.01 μg/mL of antibiotic, the incorporation of GlcNAc into lipid was still linear for at least 10 or 15 min but inhibition was 50% or greater at all time periods. Probably the reason that the reaction was linear for a somewhat longer period of time in the presence of antibiotic is that more substrate was available. It may also be that a second GlcNAc is being added during longer incubations (see below) and this may alter the rate. Larger amounts of antibiotic completely inhibited the reaction. Since the reaction was linear for 10 min, this time period was chosen for most of the experiments described here. The effect of time of preincubation of enzyme with antibiotic was examined to determine the optimum time. In one experiment with 0.01 μg/mL of tunicamycin, there was a 25% inhibition without preincubation and this inhibition increased to 50% by preincubation for 5 min. Longer times did not increase the inhibition beyond the 5-min point and, therefore, 5 min was routinely used for preincubation. The time course experiment seen in Figure 2 was also used to determine the stability of UDP-GlcNAc. In this case, UDP-[^{14}C]GlcNAc was used as substrate since it is more readily detected on paper chromatograms, and tunicamycin was not included so that the maximum substrate utilization would occur. Following the extraction of the GlcNAc-lipids, the aqueous phases were isolated, concentrated to dryness, and subjected to paper electrophoresis to separate UDP-GlcNAc from GlcNAc 1-phosphate. Table I shows the amount of radioactivity in each of these components as a function of time. It can be seen that, even after incubation for 10 min (in the absence of antibiotic), there was still considerable UDP-GlcNAc left in the aqueous phase indicating that a 10-min incubation was realistic in terms of product formation and substrate (i.e., UDP-GlcNAc) availability.

By using several different amounts of antibiotic, the effect of enzyme concentration was examined as shown in Figure 3. In this case, varying amounts of enzyme were preincubated with different concentrations of antibiotic and then substrates were added and the mixtures were allowed to incubate for 10 min at 37 °C. It can be seen that, at low concentrations of enzyme, tunicamycin effectively inhibited the formation of

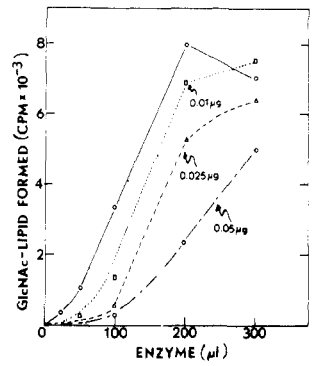

FIGURE 3: The effect of increasing enzyme concentrations on the inhibition by tunicamycin. Preincubations and incubations were done as described in Figure 2, except that the amount of enzyme was varied. In this case, preincubations were done in a final volume of 0.3 mL. Curves are: (O—O) no tunicamycin; (□-----□) 0.01 μg of tunicamycin; (Δ---Δ) 0.025 μg of tunicamycin; (O—·—O) 0.05 μg of tunicamycin.

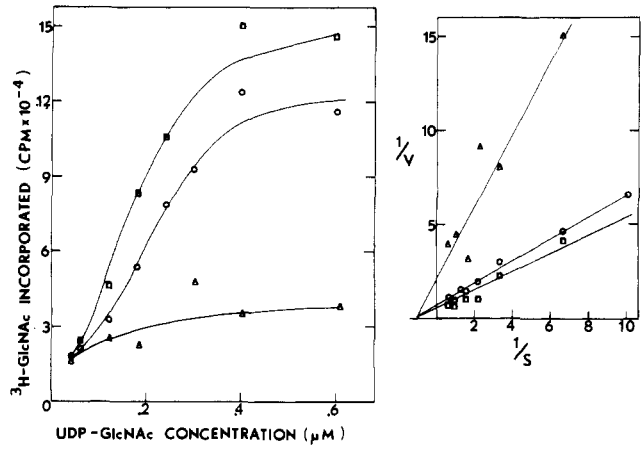

FIGURE 4: The effect of UDP-GlcNAc concentration on the tunicamycin inhibition. Preincubations and incubations were as described in other figures, except that UDP-GlcNAc concentration was varied. Curves are: (□—□) no tunicamycin; (O—O) 0.05 μg of tunicamycin; (Δ—Δ) 0.25 μg of tunicamycin.

dolichylpyrophosphoryl-GlcNAc, but this inhibition was overcome by adding greater and greater amounts of enzyme, suggesting that the antibiotic was binding to and inactivating the GlcNAc-1-phosphate transferase. Similar results were obtained at varying tunicamycin concentrations.

Effect of Substrate Concentration on Tunicamycin Inhibition. In order to determine whether the antibiotic was acting as a competitive inhibitor of the enzyme, the effect of concentration of the substrates, UDP-GlcNAc and dolichyl phosphate was studied. Figure 4 shows the effect of increasing concentrations of UDP-GlcNAc on the formation of dolichylpyrophosphoryl-GlcNAc in the absence of antibiotic as well as in the presence of 0.05 or 0.25 μg/mL of tunicamycin. The reaction was proportional to UDP-GlcNAc concentration to about 0.3 μM, and the K_m was estimated to be about 2×10^{-7} M. It can be seen by the Lineweaver–Burk plot that the K_m was not altered by antibiotic and the inhibition was of a noncompetitive nature with respect to UDP-GlcNAc. This experiment was repeated several times with two- to fivefold higher concentrations of UDP-GlcNAc and the same results were obtained. A similar experiment was done with respect to concentration of dolichyl phosphate as shown in Figure 5. The curves on the top (Figure 5A) show the effect of dolichyl phosphate concentrations from 0 to 3 μg/incubation. The K_m for dolichyl phosphate was estimated to be about 1 μg/mL or about 7×10^{-7} M. It can be seen that the reactions were markedly inhibited by tunicamycin and that this inhibition

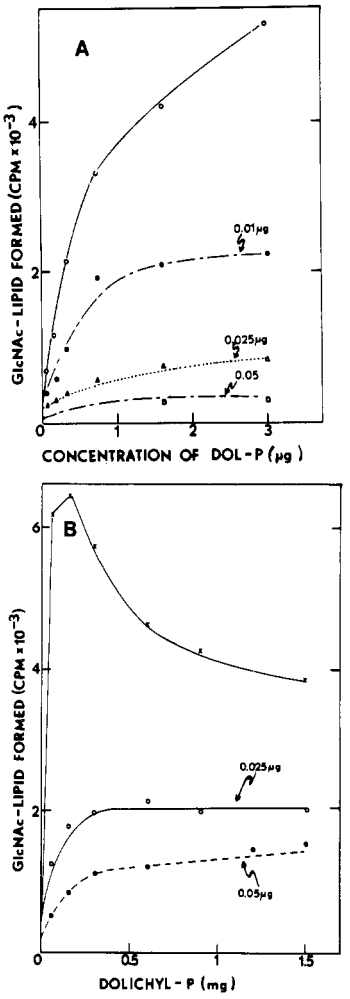

FIGURE 5: The effect of dolichyl phosphate concentration on the inhibition by tunicamycin. Preincubations and incubations were as described except that dolichyl phosphate concentration was varied. The curves on the top (A) show the effect of dolichyl phosphate concentrations from 1 to 3 μg, while those on the bottom (B) are from 0.5 to 1.5 mg. Tunicamycin was used at 0.01, 0.025, and 0.05 μg/mL.

could not be overcome by increasing concentrations of the substrate, dolichyl phosphate. In fact, as shown by the curves on the bottom, even at very high dolichyl phosphate concentrations (i.e., 1–1.5 mg), there was no change in the inhibition indicating again that the inhibition was noncompetitive in nature.

Characterization of Product Formed from UDP-GlcNAc. In order to characterize the lipid product formed from UDP-GlcNAc, UDP-[^{14}C]GlcNAc was incubated with enzyme and dolichyl phosphate for varying time periods (see Table I), and the lipids were extracted as described. In order to determine whether the lipids were GlcNAc- or *N,N'*-diacetylchitobiosylpyrophosphoryldolichol or both, two methods were used. In the first, the lipids were subjected to thin-layer chromatography to separate mono-GlcNAc from di-GlcNAc-lipids, and the amount of radioactivity in each was determined. In the second method, the lipids were subjected to mild acid hydrolysis, and the GlcNAc and *N,N'*-diacetylchitobiose were isolated by paper chromatography. Table II shows that at early times of incubation (up to 10 min) mostly only GlcNAc-pyrophosphoryldolichol was seen, but, when incubations were allowed to proceed to 20 min or were chased with an excess of unlabeled UDP-GlcNAc, *N,N'*-diacetylchitobiosylpyrophosphoryldolichol was observed. But under conditions used in the experiments described here, the product was mostly the mono-GlcNAc-lipid. The other

Table II: Identification of GlcNAc-Lipids Formed during Incubations

time of incubation (min)	radioact (cpm) recovered in[a] GlcNAc-P-P-Dol	$(GlcNAc)_2$-P-P-Dol
5′	2323	321
10′	3198	749
15′	3579	1200
20′	5085	2108
10′ + chase	428	3702

[a] Incubations were as described in Table I, and lipids were isolated as described. In this case, lipids were streaked on silica gel plates and run in $CHCl_3$:CH_3OH:H_2O (65:25:4). Plates were scraped in 1-cm sections and counted in the scintillation counter to identify mono- and di-GlcNAc-lipids. P-P-Dol, pyrophosphoryldolichol.

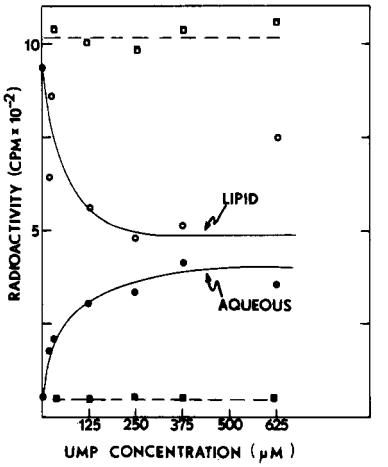

FIGURE 6: Effect of UMP on the formation of UDP-[^{3}H]GlcNAc from [^{3}H]GlcNAc-lipid. [^{3}H]GlcNAc-lipid (1000 cpm) was suspended in 10 μL of 10% NP-40, and 1 μmol of $MnCl_2$, 0.5 mL of 50 mM Tris buffer, partially purified GlcNAc transferase from DEAE-cellulose (150 μg of protein), and varying amounts of UMP were added. After incubation at room temperature for 30 min, mixtures were extracted with $CHCl_3$:CH_3OH as described. Radioactivity in the lipid phase (i.e., GlcNAc-lipid) (○—○) and in the aqueous phase (i.e., UDP-GlcNAc) (●—●) was measured. Controls were run by using pooled fractions from the DEAE-cellulose column that were devoid of GlcNAc-1-P transferase activity (□——□ and ■——■).

method of analysis, i.e., determining radioactivity in the liberated sugars, gave essentially the same results as seen in Table II (data not shown).

Inhibition of the Reverse Reaction by Tunicamycin. Since the GlcNAc-1-phosphate transferase catalyzes a reversible reaction, it was of interest to determine what effect tunicamycin would have on the formation of UDP-[^{3}H]GlcNAc from dolichylpyrophosphoryl-[^{3}H]GlcNAc and UMP. The [^{3}H]GlcNAc-lipid was synthesized by using the partially purified enzyme, and this lipid was isolated and purified as previously described (Heifetz & Elbein, 1977a). Figure 6 shows that, when the [^{3}H]GlcNAc-lipid was incubated with enzyme in the presence of UMP, there was a progressive loss of radioactivity from the lipid soluble material as the UMP concentration was increased. The radioactivity lost from the lipid phase was recovered in the aqueous phase, and this radioactivity was identified as UDP-[^{3}H]GlcNAc by paper chromatography in ethanol:1 M ammonium acetate, pH 7.5 (7:3). The dashed line in Figure 6 shows that no radioactivity was lost from the lipid material or appeared in the aqueous phase in the absence of active GlcNAc-1-phosphate transferase. The reverse reaction was inhibited by tunicamycin as

Table III: Effect of Tunicamycin on the Formation of UDP-[^{3}H]GlcNAc from [^{14}C]GlcNAc-pyrophosphoryldolichol

additions[b]	omissions[b]	amt of UDP-[^{3}H]GlcNAc formed (% of control)
none	none[a]	100
0.25 μg of tunicamycin		85
0.5 μg of tunicamycin		56
1.0 μg of tunicamycin		22
	UMP	5

[a] Incubations were essentially as described in Figure 6, except that 1 mM UMP was used as indicated. [b] To normal incubation.

Table IV: Attempts to Remove Tunicamycin from Enzyme by Dialysis

reaction mixture[a]	not dialyzed	dialysis 6 h	dialysis 16 h
no tunicamycin	11534	2530	1991
0.005 μg of tunicamycin	7901	1439	842
0.025 μg of tunicamycin	1978	644	594
0.1 μg of tunicamycin	384	744	336
1.0 μg of tunicamycin	371	465	457

[a] Enzyme (2 mL) was mixed with the amounts of tunicamycin shown under reaction mixture. A 150-μL aliquot of this mixture was assayed for GlcNAc-1-phosphate transferase activity, and the remainder was placed in a dialysis bag and dialyzed for 6 h against 4 L of 50 mM Tris, pH 7.5 containing 0.4% NP-40. At 6 h, 150 μL was again removed and assayed for activity, and the remainder was dialyzed for another 10 h.

shown in Table III. Thus in the presence of 0.25 μg of antibiotic, there was a 15% decrease in the formation of UDP-GlcNAc and this increased to 78% at 1 μg/mL. In these experiments, larger amounts of antibiotic were used then in previous studies because 0.5 mL of soluble enzyme was used in order to be able to observe a rapid rate of reversal in a short time period. This was necessary since the enzyme preparation did contain degradative enzymes and since UDP-GlcNAc did disappear in long incubations. Thus, tunicamycin did inhibit the reverse reaction as well but this reaction may be somewhat less sensitive to antibiotic. Because of the difficulty of this assay, we did not attempt to determine rates of reaction.

Binding of Tunicamycin to Soluble and Microsomal Enzyme Fractions. Since the mechanism of tunicamycin inhibition was of a noncompetitive nature and could be overcome by increasing the amount of enzyme, it was of interest to examine the binding of this antibiotic to various enzyme preparations. Kuo & Lampen (1974) had previously reported that [^{3}H]tunicamycin bound to yeast protoplasts. In order to determine whether tunicamycin bound irreversibly to the GlcNAc-1-phosphate transferase, we mixed enzyme with various amounts of tunicamycin and then tried to remove antibiotic by dialysis. The results of such an experiment are shown in Table IV. One major difficulty which we encountered with this experiment was the loss of activity by the GlcNAc-1-phosphate transferase upon dialysis. For example in Table IV it can be seen that the enzyme lost more than 75% of its activity upon dialysis for 6 h. Interestingly enough, this loss of activity is much slower when the enzyme is kept at 5 °C without dialysis, indicating that there may be some dialyzable factors necessary for activity. We are attempting to identify this factor(s) at the present time. Nevertheless, it can be seen from Table IV that 0.025 μg of tunicamycin inhibited at least 90% and this inhibition was not altered by dialysis for 6–16 h. Higher concentrations of antibiotic were almost

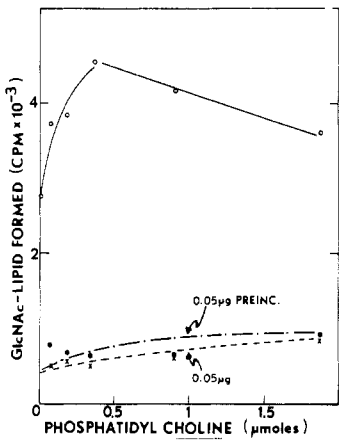

FIGURE 7: Effect of phosphatidylcholine on the inhibition by tunicamycin. To the preincubation mixtures containing 100 μL of soluble enzyme and 0.05 μg of tunicamycin, varying amounts of phosphatidylcholine were added (●—·—●). To another series of tubes, enzyme and phospholipid were preincubated without tunicamycin (○—○), while a third series had enzyme and tunicamycin (X—X). To this latter set, phosphatidylcholine was added after preincubation. Following the preincubation, the mixture of substrates was added as described in Figure 3, and the reaction was allowed to incubate at 37 °C for 10 min. GlcNAc-lipid was isolated as described.

completely inhibitory and in no case could the inhibition be reversed by dialysis. We also attempted to remove tunicamycin from the enzyme on Bio Gel P-10 columns without success (data not shown). Again we had difficulty in maintaining enzyme activity perhaps due to loss of some necessary factor.

We also examined the binding of tunicamycin to the aorta microsomal fraction. Since [^{3}H]tunicamycin was not available, the binding of this antibiotic to aorta particles was determined by measuring the loss of tunicamycin from the supernatant liquid after incubating heat-denatured aorta microsomes with tunicamycin. After the incubation, the particles were removed by centrifugation and the supernatant fluid was tested for its ability to inhibit the incorporation of GlcNAc into dolichylpyrophosphoryl-GlcNAc. In the absence of particles, GlcNAc incorporation was inhibited about 75% and this inhibition was reversed by the addition of heat-denatured microsomes. This indicates that the microsomes were binding tunicamycin and removing it from solution. Interestingly enough the binding curve was biphasic, suggesting that there may be two binding sites for tunicamycin, one which has a high affinity and the other a lower affinity, or the tunicamycin may be composed of two different molecular species, one of which binds more avidly to the microsomes.

Kuo & Lampen (1976) found that the addition of phosphatidylcholine prevented the binding of tunicamycin to protoplasts of yeast. Thus, we decided to examine the effect of phosphatidylcholine on the solubilized enzyme. Phosphatidylcholine vesicles were prepared, and varying amounts of these vesicles were either added to the preincubation mixture along with tunicamycin and enzyme, or the phospholipid was added at the end of the preincubation. The substrate mixture was then added and incubations were done for 10 min at 37 °C. The results of this experiment are presented in Figure 7. In the absence of tunicamycin, phosphatidylcholine caused a 30–40% stimulation in the synthesis of dolichylpyrophosphoryl-GlcNAc when added at a concentration of 1 μmol/mL.

Higher concentrations of phospholipid appeared to be somewhat inhibitory. However, it can be seen that phosphatidylcholine had no effect on the tunicamycin inhibition whether it was added to the enzyme at the same time as the

antibiotic (i.e., preincubation) or at a later time. In fact, in some experiments phospholipid and tunicamycin were mixed first and then enzyme was added, but the same degree of inhibition was observed. These results suggest that tunicamycin is not binding to the phospholipid. The addition of serum albumin from 0.5 to 5 mg/incubation mixture also did not protect the solubilized enzyme from inhibition regardless of whether albumin was added to the preincubation mixture or at any other time. Thus the antibiotic apparently does not bind nonselectively to protein.

Discussion

Tunicamycin is a powerful tool for biochemical studies on the mechanism of assembly, secretion, and function of glycoproteins in both procaryotic and eucaryotic cells. It is now well established that many secretory and membrane glycoproteins in which the oligosaccharide is N-glycosidically attached to protein in a glucosaminylasparagine linkage have a common core region composed of mannose and GlcNAc (Kornfeld & Kornfeld, 1976). This core oligosaccharide is synthesized via dolichyl intermediates (Waechter & Lennarz, 1976). Thus in this assembly process, GlcNAc 1-phosphate is transferred from UDP-GlcNAc to dolichyl phosphate to form dolichylpyrophosphoryl-GlcNAc and then a second GlcNAc is added to form dolichylpyrophosphoryl-*N*,*N*′-diacetylchitobiose. The $(GlcNAc)_2$-lipid then serves as an acceptor for mannose residues, some of which come from GDP-mannose and some from dolichylphosphorylmannose (Chambers et al., 1977; Heifetz & Elbein, 1977b; Waechter et al., 1973; Behrens et al., 1971; Hsu et al., 1974) to form a lipid-linked oligosaccharide which ultimately donates its oligosaccharide moiety to protein.

In vitro studies from a number of laboratories have shown that tunicamycin specifically inhibits the first step in this assembly process (Takatsuki et al., 1975; Tkacz & Lampen, 1976; Struck & Lennarz, 1977; Waechter & Harford, 1977; Ericson et al., 1977). If this step is inhibited in vivo, then the lipid-linked oligosaccharide cannot be formed and glycosylation of the protein is prevented (Hickman et al., 1977; Krag et al., 1977; Leavitt et al., 1977). Tunicamycin has been used for a number of interesting in vivo studies on glycoprotein synthesis and secretion. Oviduct tissue slices incubated with tunicamycin synthesized an ovalbumin polypeptide chain at almost normal rates but this protein was not glycosylated (Struck & Lennarz, 1977). Tunicamycin also has been shown to inhibit the conversion of procollagen to collagen in chick fibroblasts as well as the incorporation of mannose into procollagen (Duskin & Bornstein, 1977). Recently, Hart & Lennarz (1978) demonstrated that tunicamycin abolished keratan sulfate biosynthesis in chick cornea while having little effect on the production of other glycosaminoglycans. Presumably, these other glycosaminoglycans are not biosynthesized by means of lipid-linked saccharide intermediates. In yeast protoplasts, tunicamycin inhibited the incorporation of [^{3}H]glucosamine into mannose peptides without affecting the synthesis of chitin (Kuo & Lampen, 1974).

Using a solubilized and partially purified UDP-GlcNAc:dolichyl-phosphate GlcNAc-1-phosphate transferase, we have explored the mechanism of action of tunicamycin. This is the first report to show that inhibition of this transferase by tunicamycin could not be overcome by high concentrations of either UDP-GlcNAc or dolichyl phosphate, indicating that the inhibition was noncompetitive. This antibiotic also inhibited the reverse reaction, that is the formation of UDP-GlcNAc and dolichylphosphate from dolichylpyrophosphoryl-GlcNAc and UMP. Since the inhibition could be reduced by increasing the amount of enzyme in the preincubation mixture, it appeared likely that the antibiotic was binding to the enzyme and inactivating it. This inhibition also could not be overcome by the addition of phosphatidylcholine or bovine serum albumin, suggesting that tunicamycin specifically binds to the GlcNAc-1-phosphate transferase.

Kuo & Lampen (1974) reported that a 0.2% phospholipid suspension added along with the tunicamycin prevented most of the antibiotic from binding to yeast protoplasts. This interaction was reported to require an unsaturated fatty acid moiety on the intact phospholipid. Also they found that antibiotic binding could not be prevented by phospholipid once the tunicamycin was exposed to sites on the membrane. Takatsuki & Tamura (1971) reported incorporation of tunicamycin into chick fibroblast membranes with much of the incorporation appearing to be nonspecific interactions between the antibiotic and the membrane phospholipid. We report here that tunicamycin was removed from solution by heat-denatured microsomal membranes from porcine aorta, presumably by binding to the GlcNAc-1-phosphate transferase. The binding curve suggested the possibility of two different sites, one of high affinity and one of much lower affinity. Thus it may be that the antibiotic binds preferentially to the transferase but may also interact with lipophilic membrane components as well. This result may help explain the way that tunicamycin acts in vivo and further studies along these lines are in process.

Tkacz & Wong (1978) have recently investigated a family of antibiotics, the mycospocidins, and have found them chemically and functionally identical with tunicamycin. None of the components of tunicamycin generated by acid hydrolysis or acetolysis were effective in inhibiting the membrane bound GlcNAc-1-phosphate transferase from liver. Some correlation between aliphatic side chain of the intact antibiotic and its potency were reported. Kuo & Lampen (1974) found no metabolism of tunicamycin in yeast, again suggesting that the intact molecule is the functional inhibitor.

Thus the use of the soluble, partially purified GlcNAc-1-phosphate transferase has allowed the study of the mechanism of action of tunicamycin in a system free of membrane components. These studies suggest that tunicamycin may act as a substrate–product transition state analogue, binding irreversibly to the UDP-GlcNAc:dolichyl-phosphate GlcNAc-1-phosphate transferase and inactivating the enzyme.

References

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Behrens, N. N., Parodi, A., & Leloir, L. F. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 7857–7860.

Chambers, J., Forsee, W. T., & Elbein, A. D., (1977) *J. Biol. Chem. 252*, 2498–2506.

Duskin, D., & Bornstein, P. (1977) *J. Biol. Chem. 252*, 955–962.

Ericson, M. C., Gafford, J. T., & Elbein, A. D. (1977) *J. Biol. Chem. 252*, 7431–7433.

Hart, G. W., & Lennarz, W. J. (1978) *J. Biol. Chem. 253*, 5795.

Hasilik, A., & Tanner, W. (1976) *Antimicrob. Agents Chemother. 10*, 402–410.

Heifetz, A., & Elbein, A. D. (1977a) *J. Biol. Chem. 252*, 3057–3063.

Heifetz, A., & Elbein, A. D., (1977b) *Biochem. Biphys. Res. Commun. 75*, 20–27.

Hickman, S., Kulczyki, A., Jr., Lynch, R. G., & Kornfeld, S. (1977) *J. Biol. Chem. 252*, 4402–4408.

Hsu, A. F., Baynes, J. W., & Heath, E. C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2391–2395.

Kornfeld, R., & Kornfeld S., (1976) *Annu. Rev. Biochem. 45*, 217–235.
Krag, S., Cifone, M., Robbins, P. W., & Baker, R. M. (1977) *J. Biol. Chem. 252*, 3561–3564.
Kuo, S. C., & Lampen, J. O. (1974) *Biochem. Biophys. Res. Commun. 58*, 277–285.
Kuo, S. C., & Lampen, J. O. (1976) *Arch. Biochem. Biophys. 172*, 574–581.
Leavitt, A., Schlessinger, S., & Kornfeld, S. (1977) *J. Virol. 21*, 375–385.
Lehle, L., & Tanner, W. (1976) *FEBS Lett. 71*, 167–170.
Lowry, O. H., Rosebrough, M. S., Farr, A. L., & Randall, R. S. (1951) *J. Biol. Chem. 193*, 265–275.
Rupar, C. A., & Carroll, K. K. (1976) *Chem. Phys. Lipids 17*, 193–206.
Schwartz, R. J., Rohrschneider, J. M., & Schmidt, M. F. G. (1977) *J. Biol. Chem. 252*, 3561–3564.
Struck, D. L., & Lennarz, W. J. (1977) *J. Biol. Chem. 252*, 1007–1013.
Takatsuki, A., & Tamura, G. (1971) *J. Antibiot., Ser. A 24*, 785–794.
Takatsuki, A., Arima, K., & Tamura, G. (1971) *J. Antibiot., Ser. A 24*, 215–273.
Takatsuki, A., Kohno, K., & Tamura, G. (1975) *Agric. Biol. Chem. 39*, 2089–2091.
Takatsuki, A., Kawamura, K., Okina, M., Kodama, Y., Ito, T., & Tamura, G. (1977) *Agric. Biol. Chem. 41*, 2307–2309.
Tkacz, J. S., & Lampen, J. O. (1975) *Biochem. Biophys. Res. Commun. 65*, 248–257.
Tkacz, J. S., & Wong, A. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 2723.
Waechter, C. J., & Harford, J. B. (1977) *Arch. Biochem. Biophys. 181*, 185–198.
Waechter, C. J., & Lennarz, W. J. (1976) *Annu. Rev. Biochem. 45*, 95–112.
Waechter, C. J., Lucas, J. J., & Lennarz, W. J. (1973) *J. Biol. Chem. 248*, 7570–7579.

Counterion-Induced Condensation of Deoxyribonucleic Acid. A Light-Scattering Study†

Robert Wilfred Wilson and Victor A. Bloomfield*

ABSTRACT: The addition of the trivalent or tetravalent cations spermidine or spermine to a solution of T7 DNA in aqueous solution causes an alteration of the DNA from its extended coil form to a condensed form. If performed at low DNA concentration and at low ionic strengths, this transformation results in a monomolecular collapse to form a particle with a hydrodynamic radius of about 500 Å. We have monitored this change using quasielastic and total intensity light scattering. In a solution of 50% methanol in water, the divalent cations Mg^{2+} and putrescine also can cause the condensation of DNA. Using Manning's (1978) counterion condensation theory, we calculate a striking unity among these disparate ions: the collapse occurs in each case when from 89 to 90% of the DNA phosphate charges are neutralized by condensed counterions.

The majority of DNA in all living organisms is present in a compact form. In higher organisms the histones are responsible for maintaining this packaging, and the interaction between DNA and the histones is the subject of a great deal of current research. In viruses, several different molecules have been implicated in the maintenance of a condensed form of DNA. These include internal proteins (Laemmli, 1975) and the polyamines such as putrescine, spermidine, and spermine (Ames & Dubin, 1960). Several studies have demonstrated the efficacy of these compounds in collapsing DNA in vitro. Collapse has been observed by using electron microscopy (Chattoraj et al., 1978; Laemmli, 1975) and inferred from turbidity, circular dichroism, and hydrodynamic measurements (Gosule & Schellman, 1976, 1978). Collapse has also been induced by using neutral polymers such as poly(ethylene oxide) in the presence of high NaCl concentrations (Lerman, 1973).

We are interested in the packaging of DNA into viruses and therefore wished to investigate the condition under which cations can cause the self-association of DNA and to characterize the particles which result from its monomolecular collapse. We have attempted to study the collapse of DNA under conditions which favor intrachain interactions over intermolecular association. A very sensitive photon counting laser light-scattering instrument enables the rapid determination of the conditions required for DNA condensation even at the very low concentrations used to avoid intermolecular association. Since this is a quasielastic light-scattering apparatus, we have the additional ability to obtain diffusion coefficients for the collapsed particles.

In order to study the effects of cation concentration and valence on the DNA condensation, we have added various combinations of Na^+, Mg^{2+}, spermidine^{3+}, and spermine^{4+} to solutions of the DNA from T7 bacteriophage. The results are discussed in terms of the counterion condensation theory developed by Manning (1978).

Materials and Methods

(*a*) *DNA.* The T7 bacteriophage DNA was obtained by gentle phenol extraction of a phage suspension which had been prepared by using a version of the method of Studier (1969). The DNA fraction was exhaustively dialyzed against a phosphate buffer containing 0.094 M Na^+ and 5×10^{-4} M

† From the Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108. *Received January 5, 1979.* This research was supported in part by National Institutes of Health Grant GM 17855.

0006-2960/79/0418-2192$01.00/0