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N-Acetylglucosamine-1-phosphate Transferase from Hen Oviduct: Solubilization, Characterization, and Inhibition by Tunicamycin†

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ABSTRACT: UDP-*N*-acetylglucosamine (UDPGlcNAc): dolichyl phosphate, *N*-acetylglucosamine-1-phosphate transferase, the first enzyme of the dolichol cycle, has been solubilized from crude hen oviduct membranes by using 0.5% sodium deoxycholate. Accompanying solubilization was an 8- to 50-fold increase in the dependence of enzyme activity on the addition of dolichyl phosphate. The enzyme activity was stimulated by $MgCl_2$ (10 mM optimum) and KCl (0.4 M optimum) and exhibited a pH optimum around 8.0. Bisubstrate kinetic analysis indicated that the enzyme follows a sequential mechanism. The K_m values for UDPGlcNAc and dolichyl phosphate were determined to be 4 and 9 μM, respectively. The inhibition of the enzyme by tunicamycin (TM) occurred at low concentrations of inhibitor (10^{-8}–10^{-9} M) and was time dependent. These findings precluded conventional kinetic analysis to determine the mechanism of inhibition. By employing pseudo-first-order conditions, we determined a second-order rate constant of 7×10^4 M^{-1} s^{-1} for the association of the enzyme with TM at 23 °C. Plots of enzyme activity vs. inhibitor concentration were consistent with TM acting as a reversible tight-binding inhibitor. The I_{50} for tunicamycin inhibition was found to vary with protein concentration. At a protein concentration of 11 mg/mL solubilized enzyme, the I_{50} was 7×10^{-9} M. The inhibition by tunicamycin is apparently competitive since high concentrations of UDPGlcNAc relative to TM protected the enzyme from inhibition. Based on the known structure of TM and its inhibitory properties, it is proposed that the antibiotic acts as a multisubstrate analogue in which the branched hydrocarbon side chain mimics dolichyl phosphate and the uracil–carbohydrate–GlcNAc backbone mimics UDPGlcNAc. This proposal explains why other GlcNAc transferases are not inhibited by TM. Since these transferases do not utilize dolichyl phosphate as an acceptor, it is unlikely that the bulky hydrocarbon side chain of TM would fit into the active site of these enzymes.

Work in the last decade has shown that glycosylation of asparagine to form N-linked glycoproteins proceeds via an en bloc mechanism in which a portion of the oligosaccharide chain is preassembled on a long-chain polyisoprenoid carrier, dolichyl phosphate, prior to transfer of the entire chain to a nascent polypeptide [for a review, see Waechter & Lennarz (1976)]. Since dolichyl phosphate is presumably regenerated subsequent to oligosaccharide transfer, the process of preassembly and transfer is often collectively referred to as the dolichol cycle.

Our laboratory has recently embarked on an investigation into the regulation of the dolichol cycle in hen oviduct. The oviduct is an excellent tissue for studying glycoprotein biosynthesis since it secretes copious quantities of glycoproteins and since synthesis can be induced in immature chicks by administration of estrogenic hormones (Kohler et al., 1969). We have begun our investigations by examining the first enzyme of the dolichol cycle, UDPGlcNAc:dolichyl phosphate (Dol-P), *N*-acetylglucosamine-1-phosphate transferase (GlcNAc-1-P transferase) (eq 1). This enzyme has previously

$$\text{UDPGlcNAc} + \text{Dol-P} \rightleftharpoons \text{Dol-PP-GlcNAc} + \text{UMP} \quad (1)$$

been studied by Heifetz & Elbein (1977), who solubilized it from porcine aorta membranes and demonstrated that the endogenous substrate is dolichyl phosphate. In the present study we report on the solubilization and partial characterization of the enzyme from oviduct membranes. We find that the enzyme carries out a sequential reaction; i.e., both substrates must be bound to the enzyme before product release.

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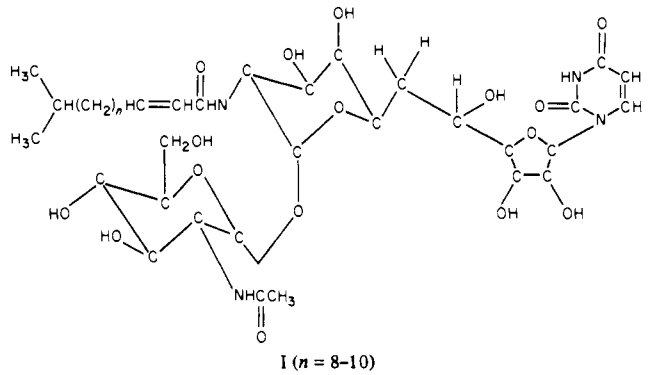
We also show that tunicamycin (I; Takatsuki et al., 1977),



I (n = 8–10)

a specific inhibitor of this enzyme, acts as a tight-binding inhibitor, exhibiting time-dependent inhibition. We further show that the inhibition by tunicamycin is apparently competitive and offer an explanation as to why other *N*-acetylglucosaminyl transferases are not inhibited by tunicamycin.

Materials and Methods

Chemicals. UDP[6-^{3}H]GlcNAc (6.6 Ci/mmol) was obtained from New England Nuclear. Dolichyl phosphate was prepared from hen oviduct dolichol by the procedure of Wedgwood et al. (1974). The concentration of dolichyl phosphate was determined by phosphate assay (Ames, 1966). Tunicamycin was a gift from Robert Hamill, Eli Lilly Research Laboratories. Its purity was verified by UV spectral analysis and thin-layer and high-pressure liquid chromatography. Concentrations of tunicamycin were determined by using the ϵ_{max} for uridine. Pinaprenol phosphate was a gift from Dr. Lee Adair of this department. Pinaprenol phosphate is a polyisoprenoid phosphate similar to dolichyl phosphate but unsaturated in the α-isoprene residue. It was prepared by the chemical phosphorylation of pinaprenol (isolated from *Pinus caribaea*). All other chemicals were reagent grade from various sources.

Tissue Fractionation. All operations were carried out at 4 °C. Oviducts, fresh or frozen, from actively laying White Leghorn chickens were homogenized in 5 volumes of ice-cold homogenizing buffer (0.25 M sucrose, 0.01 M Tris-HCl, pH 8.0, and 0.001 M DTT) per gram of original tissue using a Polytron homogenizer (Brinkmann) at a setting of "7" for 30 s. The resulting suspension was further homogenized in a glass–teflon homogenizer (Glenco) using 5 up-and-down strokes. The final homogenate was centrifuged at 1000g for 10 min. The supernatant fluid was removed, and the pellet was resuspended in 5 volumes of homogenizing buffer and centrifuged as above. The resulting pellet (P_1) was suspended in 1.5 volumes of homogenizing buffer. The supernatant fluids from the first and second centrifugation were pooled and centrifuged at 140000g for 45 min. The resulting pellet (P_2) was resuspended in 1.5 volumes of homogenizing buffer.

Solubilization of GlcNAc-1-P Transferase. To the resuspended P_2 pellet was added 1/19 volume of 10% sodium deoxycholate so that the final deoxycholate concentration was 0.5%. The suspension was stirred for 20 min at 4 °C and then centrifuged at 140000g for 45 min to yield the solubilized enzyme.

Removal of Deoxycholate. Deoxycholate was removed from the enzyme by using cholestyramine according to Agnew & Popjak (1978). Solubilized enzyme was stirred in the presence of 10 mg/mL cholestyramine (Questran, Mead Johnson) for 20 min at 4 °C. Cholestyramine resin was then removed by centrifugation for 10 min at 2000 rpm in a refrigerated centrifuge.

Enzyme Assay. Assay reactions contained the following components in a final volume of 0.1 mL: UDP[^{3}H]GlcNAc (0.5 μCi, 0.076 nmol); $MgCl_2$, 10 mM; Tris-HCl, 0.1 M, pH 8.0; AMP, 5 mM; KCl, 0.4 M; and an appropriate amount of membranes or solubilized enzyme. Dolichyl phosphate (5.0 μg, equal to ~36 μM), when added, was dissolved by using the method of Verman et al. (1977). After incubation for 20 min at 37 °C, the reactions were terminated by the addition of 1 mL of chloroform–methanol (2:1) and were extracted according to Verman et al. Incorporation of radioactivity in reactions carried out in the absence of dolichyl phosphate reflects the amount of endogenous dolichyl phosphate in the enzyme preparation.

Protection Experiments. Various compounds were preincubated with the enzyme for 20 min on ice before addition of tunicamycin (50 nM) and were further incubated for 30 min on ice. The samples were then gel-filtered over small (0.5 × 5 cm) G-50 columns to remove free tunicamycin and protecting compounds. Void volume fractions, containing identical concentrations of protein, were assayed under standard conditions in the presence of dolichyl phosphate. Control samples were preincubated with buffer alone or tunicamycin alone.

Chemical Procedures. To determine the glycosyl residues transferred to lipids, samples were subjected to mild acid hydrolysis in 0.05 N HCl in 50% 2-propanol at 100 °C for 20 min. The reaction mixture was extracted with chloroform as described by Bligh & Dyer (1959) with the aqueous phase being removed for chromatography (see below).

Chromatographic Methods. Thin-layer chromatography of ^{3}H-labeled lipids was carried out on silica gel 60 plates (Merck) in chloroform–methanol–water (65:25:4). Labeled lipids were detected by using a Packard radiochromatogram scanner. Unlabeled lipids were detected by using an anisaldehyde spray reagent (McSweeney, 1965). Carbohydrates released from glycolipids by mild acid treatment were analyzed by using descending chromatography on Whatman No. 1 paper in 1-butanol–pyridine–water (60:40:30) for 24 h. Standard sugars were detected as fluorescent spots after spraying with 0.5 N NaOH in ethanol (Roseman, 1958).

Analytical Methods. Protein was determined by using the biuret procedure (Lee & Lardy, 1965) with bovine serum albumin as a standard. Radioactivity measurements were carried out in a scintillation counter (Packard Tri-Carb Model 3390) using ACS (Amersham) scintillation fluid.

Results

Enrichment of GlcNAc-1-P Transferase Activity by Differential Centrifugation. Preliminary experiments indicated that oviduct homogenates catalyzed the transfer of [^{3}H]-GlcNAc from UDP[^{3}H]GlcNAc into chloroform–methanol-soluble material. Investigations carried out in other laboratories (Heifetz & Elbein, 1977; Waechter & Harford, 1977; Molnar et al., 1971) suggested that the product was *N*-acetylglucosaminylpyrophosphoryldolichol (Dol-PP-GlcNAc). As a first step in the partial purification of this activity, we carried out differential centrifugation of the homogenate. The fractionation procedure described under Materials and Methods resulted in greater than 70% recovery of activity in the P_2 fraction concomitant with a substantial purification (Table I). Since the P_2 fraction is quite heterogeneous with respect to organelle composition, it is difficult to draw conclusions concerning the subcellular localization of the enzyme. It should be noted, however, that Czichi &

Table I: GlcNAc-1-P Transferase in Oviduct Subcellular Fractions[a]

fraction	vol (mL)	pmol of GlcNAc incorporated per mL	protein (mg/mL)	sp act. (pmol/mg)
P_1	7.5	190	30	6.3
P_2	13.8	270	23	11.7
supernate	60	1	24	0.04

[a] Tissue fractionation and assay of enzymatic activity were performed as described under Materials and Methods.

Table II: Solubilization of GlcNAc-1-P Transferase[a]

treatment	% act. in 100000*g* supernate: −dolichyl phosphate	% act. in 100000*g* supernate: +dolichyl phosphate[b]
starting material (P_2) before centrifugation	78	100[c]
no addition	1	4.5
TX 100 (0.5%)	1.3	32
(1.0%)	1.3	4
Tergitol (0.1%)	2.0	2.0
(0.5%)	1.5	1.5
deoxycholate (0.5%)	8.2	65
(1.0%)	3	35
KCl (2 M)	ND[d]	22
phospholipase (10 μg/mL, 15 min, 37 °C)	ND	0
BuOH (4%)	ND	0
(20%)	ND	0

[a] P_2 membranes were resuspended in homogenizing buffer (1.5 mL/g) and stirred for 30 min at 4 °C with the various reagents before centrifugation. [b] Assays were carried out as described under Materials and Methods. Dolichyl phosphate concentration was 36 μM. [c] Starting material had an activity of 23 000 cpm (4 pmol) incorporated/[min (0.05 mL of enzyme)]. [d] Not determined.

Lennarz (1977) have shown that GDP-mannosyl transferase, another enzyme of the dolichol cycle, is located in the rough endoplasmic reticulum of oviduct.

Characterization of the Reaction Product. The product formed upon incubation of UDP[^{3}H]GlcNAc with the P_2 fraction was extracted as described under Materials and Methods and chromatographed on silica gel thin layers in chloroform–methanol–water (65:25:4). A single radioactive band was observed at R_f 0.25, consistent with that reported in the literature for Dol-PP-GlcNAc (Lehle & Tanner, 1975). Mild acid hydrolysis of the labeled product released in excess of 90% of the radioactivity into the aqueous phase. This radioactivity migrated with standard GlcNAc after paper chromatography, well resolved from chitobiose (data not shown). These results indicated that only the mono-*N*-acetylglucosaminylpyrophosphoryldolichol was being generated and thus demonstrated that only the first enzyme of the dolichol cycle was being assayed. It should be noted, however, that at higher concentrations of UDPGlcNAc (>10 μM) some chitobiosyl derivative was detected.

Solubilization of GlcNAc-1-P Transferase. Several solubilization procedures were examined for their ability to liberate the transferase from the P_2 fraction. The criterion employed for solubilization was recovery of activity in the supernatant fluid after centrifugation at $(6 \times 10^6)g$·min. As can be seen in Table II, 0.5% sodium deoxycholate extraction alone resulted in significant release of enzyme activity. Higher concentrations of deoxycholate proved inhibitory. An unexpected finding, which can be seen in Table II, was the large increase in dolichyl phosphate dependence of the solubilized enzyme. Some preparations were stimulated as much as 50-fold by addition of dolichyl phosphate. This effect was not dependent on removal of the solubilized enzyme from the deoxycholate-treated membranes. Thus, if membranes were simply treated with 0.5% deoxycholate and not centrifuged, large increases (>8-fold) in dolichyl phosphate dependence were observed (data not shown). Hence, deoxycholate either lowered the affinity of the enzyme for dolichyl phosphate or physically separated the enzyme from dolichyl phosphate. The first possibility seems unlikely since removal of deoxycholate from the solubilized enzyme by cholestyramine treatment (see Materials and Methods) did not alter the dependency of activity on dolichyl phosphate (results not shown).

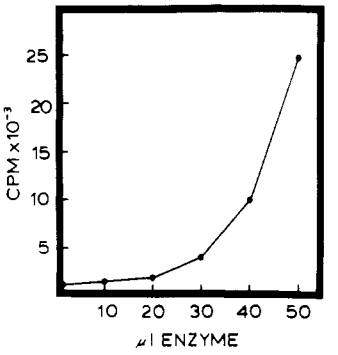


FIGURE 1: Plot of velocity vs. enzyme concentration. Aliquots of enzyme in the amounts indicated were added to standard reaction mixtures and assayed in the presence of dolichyl phosphate as described under Materials and Methods. In this experiment the concentration of deoxycholate was kept constant at 0.25%.

P_2 membranes assayed with saturating dolichyl phosphate in the presence of deoxycholate were always observed to exhibit greater activity than membranes assayed in the absence of deoxycholate (data not shown). In some instances, this increase approached twice the initial activity. However, quantitative release of activity (assayed in the presence of deoxycholate and saturating dolichyl phosphate) into the $(6 \times 10^6)g$·min supernatant fluid was never observed.

Stability. The solubilized enzyme retained full activity when stored for 2 months at −20 °C.

Characteristics of the Solubilized Enzyme. The incorporation of ^{3}H from UDP[^{3}H]GlcNAc into the lipid-soluble Dol-PP-GlcNAc product was linear with time through 20 min. Approximately 10% of the substrate was converted into product under these conditions. We found that the addition of 5 mM AMP to the reaction, as recommended by Waechter & Harford (1977), increased the length of time in which the reaction proceeded in the linear range. The result was an overall increase in radioactivity incorporated of ~40% in a 20-min reaction. Presumably, this effect was due to the protection of UDPGlcNAc from phosphatases by AMP (Waechter & Harford, 1977).

Plots of reaction velocity vs. concentration of solubilized enzyme were concave upward (Figure 1). These results suggested either that the enzyme underwent a loss of activity upon dilution or that there was an irreversible inhibitor associated with the assay reagents. The following procedures were attempted to obtain a linear velocity vs. enzyme concentration curve: (1) addition of 10 mg/mL bovine serum albumin or ovalbumin to assay reactions; (2) varying the concentration of deoxycholate so that the deoxycholate/protein ratio remained the same or the absolute amount of deoxycholate remained constant; (3) treatment of the enzyme with cholestyramine (see Materials and Methods) to remove deoxycholate (recoveries after cholestyramine treatment were on the order of 75%); (4) varying the concentrations of all

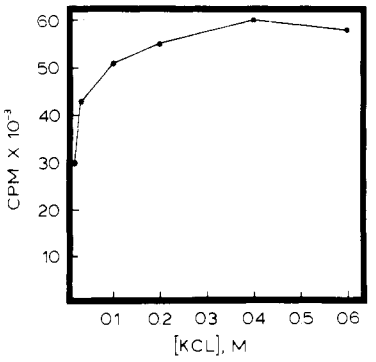


FIGURE 2: Effect of KCl on enzyme activity. Standard assays were performed in the presence of saturating dolichyl phosphate and varying concentrations of KCl according to the procedure described under Materials and Methods.

reagents of the reaction to explore the possibility of a nonspecific inhibition in the reagents. Thus far, none of these procedures have significantly altered the pattern obtained in Figure 1.

To examine the possibility that the loss of activity upon dilution is due to dissociation, we diluted a solubilized enzyme sample 10-fold in homogenizing buffer containing 0.5% deoxycholate and concentrated it to its original volume by ultrafiltration using a P-10 membrane (Amicon Corp.). The total activity of the diluted enzyme was 36% of the original sample before dilution, whereas the total activity of the concentrated enzyme was 57% of the original. Hence, it appears that at least a portion of the activity lost upon dilution is due to some type of dissociation.

The reaction was stimulated by the addition of divalent cation. Magnesium proved superior to manganese, with the optimum concentration of magnesium determined to be around 10 mM. The optimum pH for the enzyme was between 8 and 8.5. Activity was dependent on salt, maximum activity being achieved at a KCl concentration of 0.4 M (Figure 2).

Specificity of Lipid Acceptor. Mankowski et al. (1975) have reported that liver microsomal UDP-glucose transferase can partially utilize (~25%) fully unsaturated polyisoprenoids in place of dolichyl phosphate, which is saturated in the α-isoprene residue. We found a similar effect with GlcNAc-1-P transferase. At saturating concentrations of pinaprenol phosphate (equivalent to α-dehydrodolichyl phosphate), ~20% of the transferase activity was observed relative to the activity observed in the presence of saturating levels of dolichyl phosphate (data not shown). Hence, the enzyme does not have absolute specificity for a saturated α-isoprene residue.

Kinetics. Since solubilization of the enzyme resulted in large increases in the dependence of the transferase activity on dolichyl phosphate, we were able to carry out bisubstrate kinetic analysis of the enzyme. By first determining activity as a function of exogeneous dolichyl phosphate, it was possible to extrapolate and estimate the amount of dolichyl phosphate present in the enzyme sample. Generally, this amount was negligible compared to that which was added. As shown in Figure 3a, double-reciprocal plots of $1/v$ vs. $1/$[UDPGlcNAc] at varying concentrations of dolichyl phosphate yielded a series of intersecting lines, indicative of a sequential reaction mechanism. The lines appear to intersect on the $1/v$ axis, suggesting that the reaction may be rapid equilibrium ordered. However, for reasons to be considered under Discussion, it is felt that further evidence is necessary to unequivocally establish an ordered mechanism for the enzyme.

Figure 3b shows the double-reciprocal plot of $1/v$ vs. $1/$[Dol-P]. When the slopes of Figure 3a were plotted against

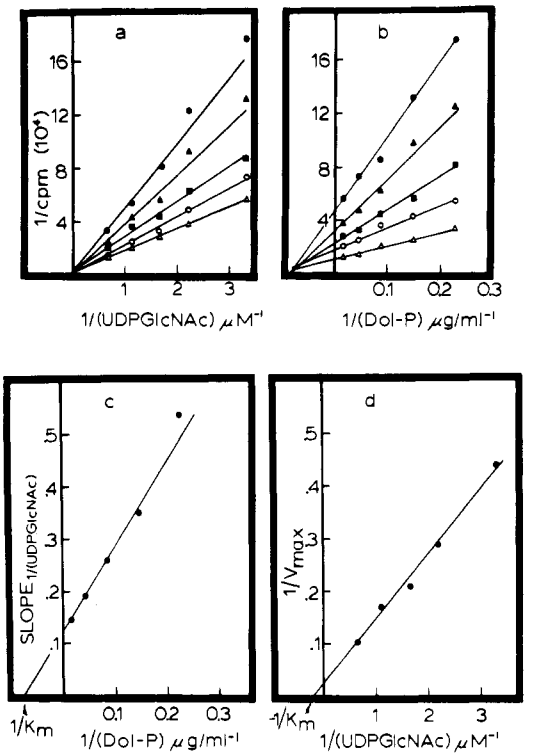


FIGURE 3: (a) Double-reciprocal plots of $1/v$ vs. $1/$[UDPGlcNAc] at various concentrations of dolichyl phosphate. Standard assays were carried out and the reactions terminated after 10 min. (b) The data in part a were used to plot $1/v$ vs. $1/$[Dol-P] at varying concentrations of UDPGlcNAc. (c) The slopes from part a were plotted against the reciprocal of the dolichyl phosphate concentration to determine the K_m for dolichyl phosphate. (d) The intercepts of part b were plotted against the reciprocal of the UDPGlcNAc concentration to determine the K_m for UDPGlcNAc.

$1/$[Dol-P] and the intercepts of Figure 3b were plotted against $1/$[UDPGlcNAc], replots were obtained (parts c and d of Figure 3) which yielded K_m values of 4 μM for UDPGlcNAc and 13 μg/mL (equivalent to 9 μM assuming M_r = 1400) for dolichyl phosphate. However, the K_m for dolichyl phosphate should be viewed with caution since, in vivo, this substrate is probably associated with membrane lipids during catalysis.

Since the enzyme activity was found to vary nonlinearly with dilution (Figure 1), it was possible that the kinetic parameters might also vary. However, experiments carried out using two different concentrations of enzyme (equivalent to 20 and 50 μL of Figure 1) indicated no significant difference in the K_m values of UDPGlcNAc and dolichyl phosphate (data not shown). Thus, the loss of activity observed with dilution appears to be an effect on the V_{max} of the enzyme.

Inhibition by Tunicamycin. The inhibition of GlcNAc-1-P transferase by tunicamycin was first reported by Tkacz & Lampen (1975) and confirmed by several investigators (Takatsuki et al., 1975; Struck & Lennarz, 1977). Since the tunicamycin molecule has moieties resembling both uridine and *N*-acetylglucosamine, it was speculated that tunicamycin might act as a substrate analogue and hence behave as a tight-binding inhibitor [as described by Cha (1975)]. This was found to be the case. When low concentrations of tunicamycin were added to enzyme preparations, a time-dependent loss in enzyme activity occurred. Due to this effect, classical kinetic analysis to determine K_I could not be applied since steady-state conditions would not prevail and since the amount of inhibitor bound to the enzyme could not be neglected with respect to the total inhibitor concentration. Cha (1975) has recommended a method for accurately determining

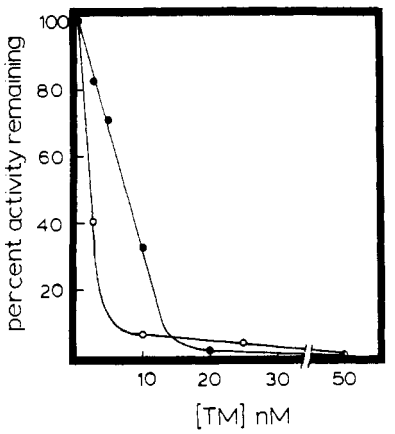


FIGURE 4: Effect of tunicamycin on enzyme activity. Varying amounts of tunicamycin were preincubated with enzyme in the standard reaction mixture at 37 °C for 20 min in the absence of UDPGlcNAc. Assays were started with UDP[^{3}H]GlcNAc and carried out for 15 min: (○) 20 μL of enzyme, (●) 50 μL of enzyme.

the K_I of a tight-binding inhibitor. The procedure involves measuring the I_{50} of the inhibitor at various concentrations of enzyme. Unfortunately, such analysis requires linear velocity vs. enzyme concentration plots, which are not observed with the solubilized enzyme preparation (Figure 1). Hence, it was not possible to obtain K_I values for tunicamycin using Cha's procedure.

An alternative method to determine the K_I for tunicamycin would be to measure the association and dissociation rate constants. These parameters could then be used to calculate the K_I since $K_I = k_1/k_{-1}$. To determine the k_1 for tunicamycin binding, we incubated high concentrations (relative to the I_{50}) of tunicamycin with transferase preparations for up to 20 min. Aliquots were removed at various times and diluted 10-fold into an assay reaction. Assays were run for short time periods (1.5 min) to minimize further binding of tunicamycin. The high concentrations of tunicamycin employed were necessary in order that the binding of the inhibitor to the enzyme followed pseudo-first-order kinetics (Cha, 1975). Plots of log (percent activity remaining) vs. time were linear, indicating that pseudo-first-order conditions were achieved. From these data (not shown) a k_1 for tunicamycin binding was calculated to be 7×10^4 M^{-1} s^{-1} at 23 °C. At 41 °C, the k_1 for binding was found to be 32×10^4 M^{-1} s^{-1}.

Attempts to measure the k_{-1} for tunicamycin dissociation, including dialysis, gel filtration, or exposure to charcoal, have thus far failed to result in recovery of enzyme activity. Hence, the off-rate for tunicamycin appears to be relatively slow. In fact, if the K_I for tunicamycin is in the range of K_I values for typical tight-binding inhibitors (i.e., $\sim 10^{-10}$ M), the dissociation rate constant (k_{-1}) can be computed to be 10^{-6} s^{-1}. This would yield a $t_{1/2}$ for dissociation of 8 days. Hence, the inability to measure the off-rate of tunicamycin is not surprising.

Although the structure of tunicamycin suggests that the antibiotic is a tight-binding inhibitor, the possibility existed that tunicamycin acts as a true irreversible inhibitor by covalently binding to the enzyme. To examine this possibility, we preincubated the enzyme with increasing concentrations of tunicamycin and determined the residual enzyme activity. A plot of the percent activity remaining vs. tunicamycin concentration (Figure 4) exhibited a sharp curvature close to the abscissa, consistent with a reversible tight-binding inhibitor (Morrison, 1969). The formation of a covalent irreversible complex would have been expected to give a straight line plot intersecting the abscissa at the concentration of inhibitor equal to the enzyme concentration.

The I_{50} for tunicamycin inhibition was found to vary with protein concentration (Figure 4). This effect has been observed with GlcNAc-1-P transferases from other sources (Ericson et al., 1977) and is consistent with the antibiotic acting as a tight-binding inhibitor (Cha, 1975). Under the standard assay conditions of 11 mg/mL of solubilized oviduct enzyme, the I_{50} was 7×10^{-9} M.

Competitive Nature of Tunicamycin Binding. The time-dependent binding exhibited by tunicamycin precluded employment of classical kinetic analysis to determine whether the inhibition was competitive or noncompetitive. Cha (1976) has described a method in which the nature of a tight-binding inhibitor can be determined by measuring pseudo-first-order rate constants at various concentrations of substrate and inhibitor. Such measurements are straightforward if the course of the enzymic reaction can be constantly monitored (e.g., by spectrophotometry). In the case of GlcNAc-1-P transferase, a constant recording assay is unavailable and the reaction can only be monitored by assaying at given time points. Clearly, the transferase assay, due to its expense and inherent inaccuracy, is not amenable at the present time to Cha's type of analysis. As an alternative approach to determine if tunicamycin binding is competitive, protection experiments were carried out (see Materials and Methods) in which various compounds were preincubated with the enzyme in order to assess their ability to subsequently protect the enzyme from inhibition by tunicamycin. Under conditions in which enzyme activity was reduced to 5% of control by incubation with tunicamycin (50 nM), preincubation of the enzyme with either UDPGal, GDPMan, or UMP (all at 400 μM) prior to incubation with tunicamycin resulted in no greater than 15% of control activity. However, preincubation with 400 μM UDPGlcNAc yielded a preparation having 93% of control activity. These data strongly suggest, but do not prove (see Discussion), that the inhibition by tunicamycin is competitive.

Dolichyl phosphate was also examined for its ability to protect the enzyme from inhibition. Preincubation with dolichyl phosphate in concentrations up to 360 μM offered essentially no protection. To examine the possibility of breakdown, we added [^{3}H]dolichyl phosphate as a tracer during preincubation. Subsequent extraction and thin-layer chromatography indicated that greater than 90% of the radioactivity was unmodified by the incubation procedure.

Discussion

As part of an investigation into the regulation of glycoprotein biosynthesis in the oviduct, we have carried out preliminary studies on the first enzyme of the dolichol cycle, GlcNAc-1-P transferase. Deoxycholate treatment was found to be the most effective method of solubilizing the enzyme. This is in contrast to the work of Heifetz & Elbein (1977), who found that treatment of aorta membranes with Tergitol resulted in maximum solubilization. Since the procedures employed for solubilization were similar in the two studies, we attribute this discrepancy to the different tissues employed. A second difference between the oviduct and aorta enzymes is that the oviduct enzyme is stable to storage at −20 °C, whereas Heifetz & Elbein reported that the aorta enzyme is unstable after solubilization, losing all activity in 6 days.

The use of 0.5% sodium deoxycholate to solubilize the enzyme has hampered purification since the presence of detergent prevents adsorption of the enzyme to ion-exchange or hydroxylapatite columns. We have therefore employed cholestyramine treatment as described by Angew & Popjak (1978) for removal of deoxycholate from solubilized preparations of squalene synthetase. Although cholestyramine

treatment results in preparations which will adsorb to ion-exchange columns, enzyme samples so treated appear to undergo aggregation and subsequent precipitation. A further problem concerning purification is the loss of activity when the enzyme undergoes dilution. At least some of this loss appears to be due to a dissociation phenomenon since a portion of the activity which is lost upon dilution can be recovered by concentration. We are currently trying to determine if the loss of activity represents dissociation of protein subunits or loss of an essential lipid component.

Bisubstrate kinetic analysis of GlcNAc-1-P transferase (Figure 3) indicated that substrate binding follows a sequential mechanism. That is, both substrates must be bound to the enzyme before any products are released. The data obtained in Figure 3a suggest that, at extremely high concentrations of UDPGlcNAc, the concentration of dolichyl phosphate does not influence the reaction velocity. This is consistent with a rapid equilibrium ordered mechanism (Segal, 1975) in which dolichyl phosphate is the leading substrate. However, there are two factors which indicate that such conclusions be considered preliminary. First, the enzyme preparation with which we are working is very crude. Although the enzyme is considered "soluble" by standard criteria, it is probably enveloped in a micelle of deoxycholate. Second, one of the substrates, dolichyl phosphate, is insoluble in aqueous solutions and must be presented to the enzyme in a micellular form. In view of these limitations, we are reluctant to draw any conclusions as to whether the reaction is ordered or random. Future studies with a more purified enzyme preparation are required to unequivocally establish the order of substrate addition to the enzyme.

In view of the potent and time-dependent inhibition of GlcNAc-1-P transferase by tunicamycin, we suggest that this antibiotic acts as a tight-binding inhibitor, as recently studied by Cha and co-workers (Cha, 1975, 1976; Cha et al., 1975). The curved activity vs. inhibitor concentration plot obtained is consistent with this proposal (Morrison, 1969). As in the case of other tight-binding substrate analogues, we expected the inhibition by tunicamycin to be competitive.

$$EI \underset{k_4}{\overset{I,\,k_3}{\rightleftharpoons}} E \underset{k_2}{\overset{S,\,k_1}{\rightleftharpoons}} ES \qquad (2)$$

The fact that the enzyme is protected from inhibition by UDPGlcNAc is consistent with a competitive mechanism. An alternative possibility is that tunicamycin acts in a non-competitive manner, binding to both free enzyme and the enzyme–substrate complex.

$$EI \underset{k_4}{\overset{I,\,k_3}{\rightleftharpoons}} E \underset{k_2}{\overset{S,\,k_1}{\rightleftharpoons}} ES \underset{k_6}{\overset{I,\,k_5}{\rightleftharpoons}} ESI \qquad (3)$$

Apparent protection by substrate could occur if the binding of tunicamycin to the free enzyme were much tighter than to the ES complex (i.e., $k_3 \gg k_5$). However, in view of the structural similarities between tunicamycin and UDPGlcNAc and the near total protection observed in the presence of high concentrations of UDPGlcNAc, we favor the simplest possibility, i.e., that tunicamycin and UDPGlcNAc compete for the same site on the enzyme.

The tight binding of tunicamycin suggests that it may be more than simply a UDPGlcNAc analogue. Closer inspection of the antibiotic reveals that when the branched hydrocarbon side chain is drawn in an extended form (Figure 5b), it resembles the polar end of dolichyl phosphate (Figure 5a). This raises the possibility that tunicamycin may act as a bisubstrate analogue (Wolfenden, 1972) by mimicking the orientation of both substrates during catalysis (Figure 5, compare parts a and b). Although the lack of protection from inhibition by dolichyl phosphate might appear inconsistent with this proposal, the fact that pinaprenol phosphate [and presumably other prenyl phosphates (Mankowski et al., 1975)] acts as an acceptor suggests that the hydrophobic domain which binds dolichyl phoshate does not have a strict structural requirement and that the primary specificity of the enzyme resides in the binding of the sugar nucleotide. Hence, it is not surpising that tunicamycin, with its uracil–carbohydrate–GlcNAc backbone, can completely displace dolichyl phosphate during inhibition. If tunicamycin acts as a bisubstrate analogue, then this offers an explanation as to why other GlcNAc transferases are not inhibited by tunicamycin. Since these enzymes do not utilize dolichyl phosphate as an acceptor, it is unlikely that the bulky hydrocarbon side chain of tunicamycin would fit into the active site of these transferases.

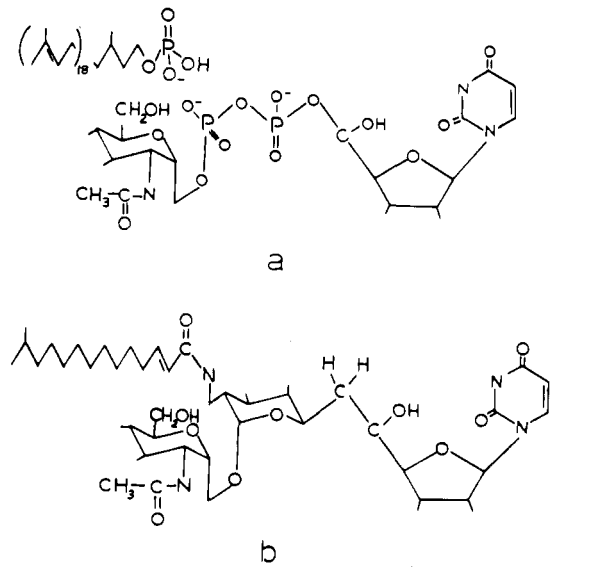


FIGURE 5: Comparison of structures of (a) UDPGlcNAc and dolichyl phosphate (upper left) with (b) tunicamycin [A form (Takatsuki et al., 1977)].

An important finding in this study was the large increase in dolichyl phosphate dependence of the solubilized enzyme. Hence, the enzyme may be employed to determine the amount of dolichyl phosphate in an unknown sample (Behrens & Tabora, 1978). An assay for dolichyl phosphate should prove useful in investigating the possible fluctuations of tissue dolichyl phosphate levels which may accompany changes in the rate of glycoprotein synthesis (Spiro et al., 1976; Lucas & Levin, 1977). Such investigations are now underway in this laboratory.

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Isolation and Characterization of the Histone Variants in Chicken Erythrocytes†

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ABSTRACT: Chicken erythrocyte histones 2A, 2B, and 3 can be resolved into nonallelic primary structure variants by polyacrylamide gel electrophoresis in the presence of Triton X-100. These variants were isolated and characterized by analysis of their tryptic and thermolytic peptides. The major variants of chicken H2A and H2B differ from the analogous component of calf thymus by a small number of conservative amino acid substitutions in the basic terminal regions, which interact with DNA. This moderate rate of allelic evolution of the slightly lysine-rich histones contrasts with the complete conservatism found in the arginine-rich histones. Chicken H4 and both chicken H3 variants are identical with their corresponding components in mammals. The amino acid substitutions distinguishing histone variants are located within the highly conserved hydrophobic regions, which are involved in histone–histone interactions.

Eucaryotic chromatin is arranged into similar repeating units, nucleosomes, each consisting of ca. 200 base pairs of DNA associated with two molecules each of histones 2A, 2B, 3, and 4 and variable amounts of H1 and/or H5 (Kornberg, 1974; Olins & Olins, 1974; Oudet et al., 1975; Van Holde et al., 1974). While the nucleosomal "spacer region", which is associated with H1–H5, is variable between cells of the same species and different species, the nucleosomal "core region", which contains the histone octamer (H2A, H2B, H3, H4)$_2$ and 140 base pairs of DNA, is extremely uniform (Felsenfeld, 1978). It is therefore not surprising that H1-like histones have been found to be quite variable, while the nucleosomal core histones have been highly conserved in evolution (De Lange, 1978; De Lange & Smith, 1975; Elgin & Weintraub, 1975). This conservatism is particularly stringent within the hydrophobic regions of the histones, which may be involved in important intermolecular interactions (Kootstra & Bailey, 1978).

We have previously shown that mammalian histones 2A, 2B, and 3 can be resolved into nonallelic primary structure variants by polyacrylamide gel electrophoresis in the presence of the nonionic detergent Triton X-100 (Franklin & Zweidler, 1977; Zweidler, 1976, 1978; A. Zweidler and S. Franklin, unpublished experiments). Since the H2A and H3 variants appear to have been preserved unchanged throughout the evolution of mammals, it was important to determine if the same variants already existed in nonmammalian species. We report here on the isolation and characterization of two variants each of chicken erythrocyte histones 2A, 2B, and 3.

Materials and Methods

Preparation of Cells and Isolation of Nuclei. All procedures were conducted at ~2 °C unless specified. Blood from a White Leghorn rooster was collected from a severed jugular

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