Purification of an α-*N*-Acetylglucosaminyltransferase from the Yeast *Kluyveromyces lactis* and a Study of Mutants Defective in This Enzyme Activity†

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ABSTRACT: An enzyme activity in *Kluyveromyces lactis* that catalyzes the transfer of *N*-acetylglucosamine from uridine diphosphate *N*-acetylglucosamine to αMan(1→3)αMan(1→2)αMan(1→2)Man to yield αMan(1→3)[αGlcNAc(1→2)]-αMan(1→2)αMan(1→2)Man, a mannoprotein side-chain unit, has been solubilized by Triton X-100 and purified 18 000-fold by a combination of ion-exchange chromatography, gel filtration, hydrophobic chromatography, and adsorption to a lectin column. The enzyme activity from a *K. lactis* mutant (*mnn2-2*) that made mannoprotein lacking *N*-acetylglucosamine in its side chains, but that possessed a normal level of transferase activity in cell extracts, was purified and compared with the enzyme from the wild-type strain. Both transferase activities are integral membrane proteins found in particles associated with endoplasmic reticulum. The two purified enzymes had the same apparent size, heat stability, Mn^{2+} requirement, and K_m for donor and acceptor and a similar V_{max}. Wild-type and mutant cells had similar pool sizes of sugar nucleotide donor, and they incorporated labeled *N*-acetylglucosamine into chitin at similar rates. No evidence was obtained for an inactive enzyme precursor in mutant cells that was activated upon breaking the cells, nor did the mutant cells contain a transferase inhibitor or a hexosaminidase that could remove the sugar from the mannoprotein during processing and secretion. The *mnn2-2* locus appears to be allelic with a second mutant, *mnn2-1*, that has the same phenotype but that lacks transferase activity in cell extracts. This suggests that the two mutations affect the structural gene for the transferase, and we conclude that the *mnn2-2* mutant could contain an altered enzyme that fails to function because it is improperly localized or oriented in the membrane.

The polymannose chains of yeast mannoproteins are highly polymorphic (Ballou & Raschke, 1974) and show structures as varied as the bacterial O-antigen (Lüderitz et al., 1966) and mammalian blood group substances (Watkins, 1972). The mannoprotein of *Kluyveromyces lactis* (Raschke & Ballou, 1972) is similar to that of *Saccharomyces cerevisiae* (Ballou, 1976) except that it lacks mannosyl phosphate groups on the side chains and instead possesses some α1→2-linked *N*-acetylglucosamine units. We have reported an enzyme activity in *K. lactis* that catalyzes transfer of *N*-acetylglucosamine from UDP-*N*-acetyl-D-glucosamine[1] to a mannotetraose acceptor and have described the isolation of mutants that fail to add hexosamine to the polymannose side chains of the secreted mannoprotein (Smith et al., 1975). One of the mutants, designated *mnn2-1*, lacks the transferase activity in cell extracts, whereas the other (*mnn2-2*) shows a normal wild-type level of enzyme activity. This unusual observation of an enzyme activity that is present in the cell but that is not expressed in the structure of the secreted macromolecule led to the present study in which we have purified the α1→2-*N*-acetylglucosaminyltransferase from both wild-type and mutant cells and compared them in an effort to rationalize this crypticity. No difference could be demonstrated between highly purified samples of both enzymes, and no difference could be detected in the synthesis or utilization of *N*-acetylglucosamine between the wild-type and *mnn2-2* mutant cells. Our conclusion is that the defect in the mutant probably involves some alteration in membrane orientation of the transferase or compartmentation of the substrates that prevents the normal processing of the mannoprotein.

Experimental Procedures

Materials. UDP-*N*-acetyl-D-[1-^{14}C]glucosamine (53.4 Ci/mol) and D-[6-^{3}H]glucosamine hydrochloride (20.7 Ci/mmol) were from New England Nuclear, whereas UDP-*N*-acetyl-D-[U-^{14}C]glucosamine (300 Ci/mol) came from Amersham. UDP-*N*-acetyl-D-glucosamine, cetyltrimethylammonium bromide (Cetavlon), and dolichol were from Sigma. Lauryldimethylamine oxide was provided by Dr. G. F. L. Ames of this department. Microbeads (class V-A, 500 μm) came from Ferro Corp. Fluorescein coupled to wheat germ agglutinin came from Calbiochem. Concanavalin A, wheat germ agglutinin, and chitin were provided by Dr. C. L. Reading of this laboratory, chitosan and colloidal chitin were from laboratory stocks, and mannotetraose was prepared by partial acetolysis of *S. cerevisiae* X2180 mannoprotein (Lee & Ballou, 1965). Sodium methylsulfinylmethyl carbanion was prepared according to Conrad (1972).

Dowex resins, Cellex D, hydroxylapatite Bio-Gel HT, Bio-Beads SM-2, and Bio-Gels were purchased from Bio-Rad, Amberlite XAD-2 was from Mallinckrodt, and hydroxylapatite (Tiselius et al., 1956) was provided by Dr. T. Nakajima of this laboratory. UDP–Sepharose and concanavalin A–Sepharose were from Sigma. Ethyl-, butyl-, hexyl-, octyl-, and decylamines coupled to Sepharose were obtained from Miles Laboratories, and Sepharose 4B and DEAE-Sephadex A-25 were from Pharmacia, whereas phosphocellulose P11 was from Whatman. Concanavalin A, wheat germ agglutinin, UDP-hexanolamine (Barker et al., 1972), and mannoprotein were coupled to Sepharose 4B according to March et al. (1974). Glusulase (a crude β1→3-glucanase from *Helix pomatia*) was

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[1] Abbreviations: UDP, uridine 5′-diphosphate; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.

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from Endo Laboratories, zymolyase 5000 (a crude β1→3-glucanase from *Arthrobacter luteus*) was purchased from the Kirin Brewing Co., Tokyo, Japan, and endo-α1→6-mannanase was purified from *Bacillus circulans* ATCC 29101 (Nakajima et al., 1976).

General Methods. Total carbohydrate was determined by the phenol–sulfuric acid method (Dubois et al., 1956), hexosamine by a modification of the Elson–Morgan method (Ghosh et al., 1960), and reducing sugar by the Nelson–Somogyi procedure (Spiro, 1966). Protein was measured by the method of Lowry et al. (1951), with bovine serum albumin as the standard. The phenol reagent precipitates when Triton X-100 is present, which was prevented by adding sodium dodecyl sulfate (Dulley & Grieve, 1975; Wang & Smith, 1975).

Electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate was done according to Ames (1974) and native gel electrophoresis was according to Ornstein (1964) and Brewer & Ashworth (1969). Native gels were stained as described (Holbrook & Leaver, 1976). Triton X-100 was removed from samples by binding to Bio-Beads SM-2 (Holloway, 1973) or Amberlite XAD-2 (Cheetham, 1979). Radioactivity was determined in Bray's solution (Bray, 1960) with a Beckman LS 3150 liquid scintillation counter.

Descending paper chromatography was done on Whatman No. 1 filter paper by using the solvents (A) ethyl acetate–pyridine–water (5:3:2), (B) absolute ethanol–1 M ammonium acetate (5:2), or (C) 1-butanol–acetic acid–1 N ammonium hydroxide (2:3:1). Hexoses and hexitols were detected with an alkaline silver nitrate dip reagent (Treveleyan et al., 1950). Radioactivity on paper chromatograms was determined directly, with a Packard radiochromatogram scanner, or by cutting the chromatogram into 1-cm horizontal strips that were moistened with 1 mL of water and then counted in 10 mL of Bray's solution. Oligosaccharide mixtures were separated by gel filtration on Bio-Gel P-2, P-4, or P-6 and by high-pressure liquid chromatography on a Waters Associates Model ALC/GPG 201 instrument with a μBondapak carbohydrate column (0.4 × 30 cm) with CH_3CN–water mixtures. Purity of mannooligosaccharides was substantiated by paper chromatography with solvent A. Partially methylated alditol acetates were separated by gas chromatography at 160 °C on a 3% OV-210 column (3 mm × 120 cm) with a Varian Aerograph 1400 instrument coupled to a Du Pont 21-491 mass spectrometer operating at an ionizing voltage of 70 eV.

Bulk cell mannoproteins were isolated by Fehlings' precipitation (Peat et al., 1961) and by Cetavlon fractionation (Lloyd, 1970). Acetolysis was usually for 8–12 h, but for production of larger mannooligosaccharide fragments the acetolysis reaction was terminated after 1 h (Rosenfeld & Ballou, 1975).

Yeast Cultures and Growth Conditions. Kluyveromyces lactis Y-58a *his4c* and Y-43 *his3* were obtained from Dr. James Haber, Brandeis University (Tingle et al., 1968). Strains possessing the *K. lactis mnn1*, *mnn2-1*, and *mnn2-2* mutations (Smith et al., 1975) were from the laboratory collection. *Saccharomyces cerevisiae* X2180 and the *mnn2* mutant strain were provided by D. L. Ballou of this laboratory. The mannoprotein structures for the carbohydrate outer chains of these strains are illustrated in Figure 1. Yeast strains usually were grown at 30 °C in shaking liquid cultures consisting of 1% yeast extract, 2% bactopeptone, and 2% glucose (YEPD). Cultures were maintained on solid YEPD media containing 2% bacto-agar.

Genetic Analysis. The genetic map distance separating the *K. lactis mnn2-1* and *mnn2-2* mutations was determined by

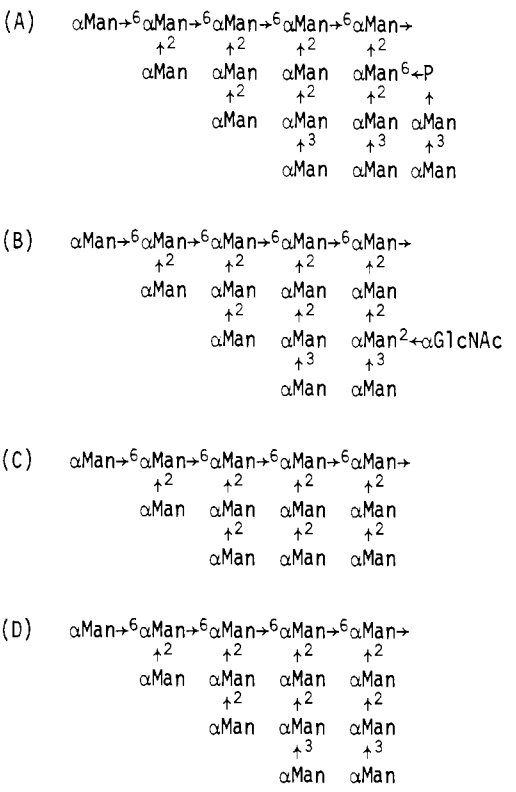

FIGURE 1: Comparison of the outer chain carbohydrate mannoprotein structures: *S. cerevisiae* X2180 (A), *K. lactis* Y-58a (B), and *K. lactis mnn1* (C), and *mnn2* (D) mutants. The *mnn1* mutant lacks terminal *N*-acetylglucosamine because the acceptor side chain is not made, whereas the *mnn2* mutants either lack the transferase activity (*mnn2-1*) or it is not expressed (*mnn2-2*).

random spore analysis. *K. lactis* Y-43α(1-17) *mnn2-1* cells were mass mated with *K. lactis* Y-58a(54) *mnn2-2* cells on malt extract agar plates (2% malt extract, 3% bacto-agar). On this medium, *K. lactis* cells mate and then spontaneously sporulate (Tingle et al., 1968). After 5–7 days, a portion of the sporulated culture was digested with glusulase, and the spores were collected by centrifugation, washed with water, and spread in the middle of a YEPD agar plate. Individual spores were separated from the mixture by micromanipulation, and the colony derived from each spore was tested for its mannoprotein phenotype with specific antisera and f1-WGA binding (Douglas & Ballou, 1980). The validity of the analysis was checked by following segregation of the histidine markers in the parent cells by growth on minimal plates (2.2 g of yeast nitrogen base without amino acids or ammonium sulfate, 5 g of ammonium sulfate, 20 g of glucose, and 20 g of bacto-agar in 1 L) and on minimal plates supplemented with 20 mg/L L-histidine.

α1→2-N-Acetyl-L-glucosaminyltransferase Assay. The transferase activity was assayed according to Smith et al. (1975). Enzyme was incubated for 10 min with 75 nmol of mannotetraose acceptor and 1.25 μmol of $MnCl_2$ in 4 mM imidazole hydrochloride, pH 6.5. The reaction was started by addition of 0.13 nmol of UDP-*N*-acetyl-D-[1-^{14}C]glucosamine (53.4 Ci/mol), which brought the volume to 125 μL. After a fixed reaction time, generally 30 min, the mixture was applied to a Dowex AG 1-X2 column (0.5 × 6 cm). The neutral material, collected into a scintillation vial by washing the column with two 0.5-mL portions of water, was counted in 10 mL of Bray's solution. One unit of enzyme activity catalyzes the formation of 1 pmol of product/min.

Preparation and Lysis of Protoplasts. Protoplasts of *K. lactis* cells were prepared according to Cabib (1971). The

reaction was shaken at 30 °C until greater than 90% of the cells were converted to protoplasts (about 1 h). The protoplasts were washed twice with citrate–phosphate buffer containing 10% mannitol and then were lysed by suspending them in 0.1 M imidazole hydrochloride, pH 6.5. The resulting membrane particles, pelleted by centrifugation at 30000*g* for 30 min, were used for transferase assay and isolation (Smith et al., 1975).

Cell Breakage, Differential Centrifugation, and Enzyme Solubilization. Freshly harvested cells (300 g or more) were washed twice with saline, suspended in 2 volumes of cold 0.01 M imidazole hydrochloride buffer, pH 6.5, containing 5% glycerol (imidazole–glycerol buffer) and 1 mM phenylmethanesulfonyl fluoride, and then passed through a Manton–Gaulin laboratory homogenizer 5–10 times. The effluent was cooled in a dry ice bath after each passage to maintain the temperature between 5 and 20 °C. Cell breakage was monitored microscopically. Smaller quantities of cells were broken in a Braun cell homogenizer, MSK. A 50% suspension (wet weight/v) of washed cells in imidazole–glycerol buffer, containing 1 mM phenylmethanesulfonyl fluoride, and 40 g of glass beads were homogenized in a 75-mL bottle at 4000 rpm in three 1-min bursts, while the sample was cooled continuously.

The resulting homogenates were subjected to differential centrifugation. Whole cells, mitochondria, and large membrane fragments were removed by centrifugation at 20000*g* for 20 min in a Beckman J-21C centrifuge. Membranous material remaining in the supernatant fraction was pelleted by centrifugation for 3 h at 100000*g*. Following ultracentrifugation, the top lipid layer and the clear yellow supernatant fraction were removed, and the pellet was suspended in imidazole–glycerol buffer to a protein concentration of approximately 50 mg/mL. The suspended membrane fraction was diluted with imidazole–glycerol buffer to a protein concentration of 3–8 mg/mL, and Triton X-100 was added to a final concentration of 2% (w/v). The mixture was stirred overnight and then centrifuged at 100000*g* for 3 h. The top lipid layer was removed, and the clear Triton X-100 solubilized extract was isolated. Additional activity could be obtained by reextracting the pellet in the same way.

Chromatography and Isoelectric Focusing. Detergent-solubilized enzyme extract, diluted until the conductivity approximated that of the column buffer, was added to a preequilibrated DEAE-cellulose or DEAE-Sephadex A-25 column (1 mL of packed resin/mg of protein), which was washed with the above buffer until the eluant was free of protein. Stepwise elution was done with imidazole–glycerol buffer containing 1% Triton X-100 and 0.2 M KCl, whereas linear gradients were from 0 to 0.3 M KCl in the same buffer. When DEAE-cellulose chromatography was used to remove Triton X-100 from samples, the column was first equilibrated in imidazole–glycerol buffer. Following application of the sample, the column was washed with the buffer until the absorbance of the effluent at 280 nm was below 0.1. The transferase activity was then eluted with buffer containing 0.2 M KCl.

For chromatography on phosphocellulose P11, the column was equilibrated in imidazole–glycerol buffer. Following application of the sample, the column was washed with the buffer until no more protein was eluted, and bound protein was then eluted with buffer containing 1 M KCl. Hydroxylapatite was equilibrated in imidazole–glycerol buffer, and, following introduction of the sample, the column was washed and eluted with a linear gradient of 0–1.0 M KCl. For batch extraction, 2 mL of enzyme extract was incubated with 3 mL of hydroxylapatite resin for 4 h, the resin was removed by centrifugation, and the solution was dialyzed overnight against buffer before assay.

The transferase activity that was eluted from DEAE-cellulose was pooled, concentrated, and applied to either a Bio-Gel A-0.5m column (2 × 100 cm) or a Bio-Gel A-5m column (2 × 100 cm). An approximate molecular weight for the transferase was determined by chromatography on a Sepharose 4B column (1 × 100 cm) equilibrated in the same buffer.

Binding of the transferase to ethyl-, butyl-, hexyl-, octyl-, and decyl-Sepharose was tested by applying a 0.1-mL sample, purified by chromatography on Bio-Gel A-5m, to a 1-mL column equilibrated with imidazole–glycerol buffer that was then washed with 2 mL of buffer and eluted with 2 mL of buffer containing 0.4 M KCl (Er-el et al., 1972). For enzyme purification on ethyl-Sepharose equilibrated in imidazole–glycerol buffer, the sample was applied and the column (1.4 × 30 cm) was washed with buffer. The transferase was eluted with buffer containing 0.15 M KCl or with a linear salt gradient from 0 to 0.2 M KCl.

Binding of the transferase to UDP–Sepharose, UDP-hexanolamine–Sepharose, wheat germ agglutinin–Sepharose, and concanavalin A–Sepharose was tested in small columns (1 × 5 cm) equilibrated in imidazole–glycerol buffer with or without 1% Triton X-100. Binding to the UDP–Sepharose and UDP-hexanolamine–Sepharose columns was also tested in the presence of 10 mM $MnCl_2$. The transferase was purified by adsorption to a concanavalin A–Sepharose column (2 × 25 cm), followed by elution with buffer containing 0.2 M sucrose or 0.3 M methyl α-D-mannoside.

Isoelectric focusing of the transferase was done on an LKB 8101 apparatus, at 5 °C, in a column stabilized by a sucrose density gradient (0-50%) that contained 2.5% carrier ampholytes, pH 3–6, 2% Triton X-100, and 2 M urea.

Kinetic Measurements. Kinetic data, determined at optimal $MnCl_2$ concentration, were obtained by varying the concentration of one of the substrates with the second substrate at saturating concentration. The K_m and V_{max} were estimated by computer fit of the data to the Michaelis–Menton equation (Hanson et al., 1967). Estimated K_m values for priming the iterative process were determined graphically (Eisenthal & Cornish-Bowden, 1974). Heat inactivation of the transferase was studied in the assay mixture minus both donor and acceptor. Mannotetraose and UDP-*N*-acetylglucosamine were then added, and the assay was performed as usual.

Subcellular Fractionation. Protoplasts were lysed and the components were fractionated on a sucrose step gradient prepared by layering 5 mL each of 50%, 40%, 30%, 20%, 10%, and 5% sucrose solutions (w/v) in 10 mM imidazole hydrochloride, pH 6.5, in a 34-mL centrifuge tube. Two milliliters of sample was layered on top, and the tube was centrifuged at 90000*g* for 1 h with an SW 25.1 rotor. Fractions from the six "shelf" regions and the pellet were collected and assayed.

Braun homogenates were separated on continuous Renografin gradients (Cortat et al., 1973; Lehle et al., 1977). Washed cells, in 10 mM imidazole hydrochloride, pH 6.5, containing 0.5 M sorbitol, were disrupted by two 8-s treatments in the Braun homogenizer (10 g of cells/40 g of beads in 5 mL of buffer). The glass beads were removed on a sintered glass funnel and washed with 4 volumes of homogenization buffer, and unbroken cells were removed by low-speed centrifugation (3000*g* for 10 min). The supernatant fraction was separated by differential centrifugation into pellet P20 (20 min at 20000*g*) and pellet P100 (1 h at 100000*g*). Each pellet was suspended in 5 mL of homogenization buffer, 1 mL was layered on the top of a 10-mL linear Renografin gradient (5–35%)

Table I: Purification of *K. lactis* *N*-Acetylglucosaminyltransferase[a]

fraction	volume (mL)	total protein (mg)	total act.[b] (units)	sp act. (units/mg of protein)	purification (*x*-fold)	yield (%)
crude extract	420	23520	7100	0.3	1	100
Triton X-100 extract[c]	112	11700	24500	2.1	7	345
DEAE-cellulose	1357	429	21200	49.4	165	299
Bio-Gel A-5m	233	266	24900	93.6	310	350
ethyl-Sepharose	163	27	16500	611	2032	232
concanavalin A-Sepharose	18	1	5600	5600	18500	79

[a] From 240 g of stationary phase Y-58a cells broken in a Braum homogenizer. [b] One unit of activity catalyzes the formation of 1 pmol of product/min in the standard assay. [c] The increase in the total activity is a consequence of detergent solubilization, but it is not dependent on the presence of detergent in the assay.

prepared in homogenization buffer, and the tube was centrifuged at 100000*g* for 1 h in a SW-41 rotor.

UDP-N-acetylglucosamine Concentration and Glucosamine Incorporation. The intracellular concentration of UDP-*N*-acetylglucosamine was measured on a hot water extract of whole cells (Kurtz & Binkley, 1961). Lyophilized cells were extracted by 5 mL of boiling water/g of cells for 5 min, and the cells were pelleted by centrifugation. The pellet was washed and the combined supernatant fractions were adjusted to pH 3.0. The precipitated protein was removed by centrifugation at 20000*g* for 20 min, and the supernatant fraction was adjusted to pH 7.0 with NaOH and lyophilized. A portion of the extract was chromatographed on a Bio-Gel P-2 column (1 × 100 cm) along with 0.1 μCi of UDP-*N*-acetyl[^{14}C]-glucosamine as a marker. A sample of the radioactive peak was hydrolyzed and the glucosamine content determined (Ghosh et al., 1960). A second portion was analyzed by paper chromatography in solvent C.

To measure incorporation of glucosamine into the ethanol-soluble pool (UDP-*N*-acetylglucosamine), mannoprotein, and chitin, we suspended the cells from a *K. lactis* culture of 10 mL grown to a density of 5 × 10^8 cells/mL in 6 mL of a medium containing 1% yeast extract, 2% bactopeptone, and 0.5% glucose and incubated them with gentle shaking at 30 °C for 30 min. [^{3}H]Glucosamine (10 μCi) was added to the culture, 1-mL samples were transferred at intervals to tubes that were immediately centrifuged, and the pellets were washed with 1 mL of saline and then frozen in a dry ice–acetone bath.

For analysis, the pellets were thawed and 0.1 mL of toluene was added to each. Following incubation at 35 °C for 30 min, 1 mL of 95% ethanol was added, and the samples were heated for 5 min at 90 °C and centrifuged to give the ethanol-soluble extract. The resulting pellets were washed with 95% ethanol, and 1 mL of 0.2 N NaOH containing 0.5 mg/mL of colloidal chitin was added as a carrier. The tubes were flushed with N_2, capped, and heated at 98 °C for 2 h (Kuo & Lampen, 1976). Centrifugation of the samples yielded an alkali-soluble fraction and a pellet of chitin. To the supernatant fraction was added 3 mg of *K. lactis* wild-type mannoprotein to facilitate precipitation by Fehlings' reagent. The precipitate was collected by centrifugation, suspended in 3 N HCl, and reprecipitated with methanol–acetic acid (8:1 v/v), and the ^{3}H radioactivity in the mannoprotein fraction was determined.

The [^{3}H]chitin-containing pellets were washed twice with water and then hydrolyzed in 1.5 mL of 7 N HCl under N_2 at 110 °C for 5 h. The hydrolyzed samples were evaporated under vacuum over KOH, the residue was suspended in 1 mL of water, insoluble material was removed by centrifugation, and the ^{3}H radioactivity in the extract was determined.

Characterization of the N-Acetylglucosaminyltransferase Products. Neutral labeled product from the enzymic transfer of *N*-acetylglucosamine to mannotetraose was obtained by incubating 7.5 mL of 10 mM imidazole hydrochloride, pH 6.5, containing 10 mM $MnCl_2$, 3 mg of mannotetraose, transferase (80 units), 3 mg of UDP-*N*-acetylglucosamine, and 0.5 μCi of UDP-*N*-acetyl[^{14}C]glucosamine for 24 h at 23 °C. The reaction mixture was applied to a Dowex AG 1-X2 column (1.5 × 20 cm), and the neutral product was eluted with water. The radioactive eluate was fractionated on a Bio-Gel P-2 column (1 × 100 cm), and the single radioactive peak obtained was analyzed by paper chromatography in solvent A and by methylation (Hakomori, 1964; Stewart & Ballou, 1968). The product of the transferase from the *K. lactis mnn2-2* mutant was obtained in a similar way by incubating 0.8 mL of 10 mM imidazole hydrochloride, pH 6.5, containing 10 mM $MnCl_2$, 5% glycerol, 3 mg of mannotetraose, 3 mg of UDP-*N*-acetylglucosamine, *mnn2-2* transferase (21 units), and 0.4 μCi of UDP-*N*-acetyl[^{14}C]glucosamine at 23 °C for 24 h.

Results

(A) N-Acetyl-D-glucosaminyltransferase Purification. Although the transferase assay was performed under nonsaturating conditions to conserve substrates, the rate was linear and less than 20% of the substrates were consumed during the 30-min assay. The results of one purification, summarized in Table I, show the optimum results obtained, but routine purifications of 4000-fold were achieved.

(1) Cell Breakage and Differential Centrifugation. The method of Smith et al. (1975) for producing membrane fractions from *K. lactis* protoplasts is inconvenient for large-scale enzyme purification, so mechanical methods of cell breakage were explored. Because the enzyme activity was stable for several weeks in 5% glycerol at 4 °C, this concentration was employed. To minimize degradation by proteases (Jones, 1977), we included 1 mM phenylmethanesulfonyl fluoride in homogenization buffers, and crude extracts were centrifuged immediately to separate the membrane-bound transferase from soluble proteases.

Although the Manton–Gaulin homogenizer could process large volumes of cells quickly, the membrane preparations had lower activity than those made with the Braun homogenizer. Microscopic examination of both preparations revealed good cell breakage, but the Manton–Gaulin procedure yielded larger fragments and much of the activity sedimented at low speed. Extracts prepared by Braun homogenization were centrifuged at 20000*g* for 30 min to remove cell wall fragments, whole cells, and mitochondria. Sedimentation of the supernatant fluid at 100000*g* for 3 h then gave a pellet with two discrete layers, a lower clear reddish amber one and an upper turbid brown one. These layers were combined for the following step.

(2) Detergent Solubilization. Salt washes or EDTA extraction did not solubilize the *N*-acetylglucosamine transferase, whereas Triton X-100 did (Schwyzer & Hill, 1977a; Sadler et al., 1979). Treatment of the membrane fraction with 2%

Table II: Inhibitors of *K. lactis* *N*-Acetylglucosaminyltransferase

inhibitor	concn for 50% inhibition	inhibitor	concn for 50% inhibition
UDP	0.4 mM	imidazole	70 mM
UDP-hexylamine	0.6 mM	Tris	60 mM
phosphate[a]	<40 mM	Tween 80	1.5%
NaCl	300 mM	copper[a]	<0.8 mM
NaN_3	15 mM		

[a] 100% inhibition at this concentration.

Triton X-100, followed by centrifugation at 100000*g* for 3 h, stimulated the transferase activity 3–4-fold and displaced it into the supernatant fraction. Although other detergents (Nonidet P-40, Tween 80, and lauryldimethylamine oxide) also solubilized the transferase, none was as effective as Triton X-100, whereas the activity was inhibited 50% in 1.5% Tween 80. A porcine liver *N*-acetylglucosaminyltransferase can be solubilized by organic solvent extraction (Hudgin & Schachter, 1971), but the yeast enzyme was inactivated under similar conditions. Inclusion of urea with detergent did not facilitate solubilization.

(*3*) *Ion-Exchange Chromatography.* Significant purification of the transferase was obtained by chromatography on DEAE-cellulose (Table I). Following application of the detergent-solubilized extract, the Triton X-100 was removed by washing the column with buffer, and the enzyme activity was then eluted with KCl. Although this procedure gave 10–20-fold purification, optimal recoveries were obtained only when the column was equilibrated, washed, and eluted in the presence of 1% Triton X-100.

Because the transferase is inhibited by phosphate (Table II), phosphocellulose and hydroxylapatite columns were tested as affinity adsorbents. The transferase activity did not bind to phosphocellulose, but 50–75% of the protein in the preparation was bound, which gave a 2–4-fold purification. Similar results were obtained with hydroxylapatite. Phosphate, eluted from the resin, inhibited the transferase activity, but assay of each fraction after dialysis revealed that the transferase was not bound.

(*4*) *Gel-Filtration Chromatography.* The Triton X-100 solubilized transferase activity was slightly included during chromatography on Bio-Gel A-0.5m, and it was eluted in the middle of the resolving space on Bio-Gel A-5m (Figure 2A), which gave moderate purification of the transferase with good recovery (Table I).

(*5*) *Hydrophobic Chromatography.* Because the *N*-acetylglucosaminyltransferase is membrane bound, hydrophobic chromatography (Er-el et al., 1972) was explored for purification. None of the transferase activity was bound to Sepharose, whereas nearly all of activity and the 280-nm absorbing material were bound to the ethyl- and higher alkyl-Sepharose columns. All of the activity could be eluted from the ethyl-Sepharose column by 0.4 M KCl, but only 25% was recovered after decyl-Sepharose chromatography. A 6.5-fold purification was obtained by a salt gradient elution from the ethyl-Sepharose column (Figure 2B). A disadvantage of this technique was that the Triton X-100 had to be removed from samples prior to the hydrophobic chromatography, which resulted in aggregation and loss of enzyme activity.

(*6*) *Affinity Chromatography.* Neither UDP-hexanolamine–Sepharose (Barker et al., 1972) nor UDP–Sepharose, coupled through the ribose moiety, bound the transferase with or without 1% Triton X-100 or 10 mM $MnCl_2$. The transferase bound to concanavalin A–Sepharose and was eluted with methyl α-D-mannoside (Figure 2C). Although this step resulted in a large purification (Table I), the recovery was poor. The transferase did not bind to wheat germ agglutinin–Sepharose, indicating that if the enzyme is a glycoprotein, it does not possess accessible *N*-acetylglucosamine residues.

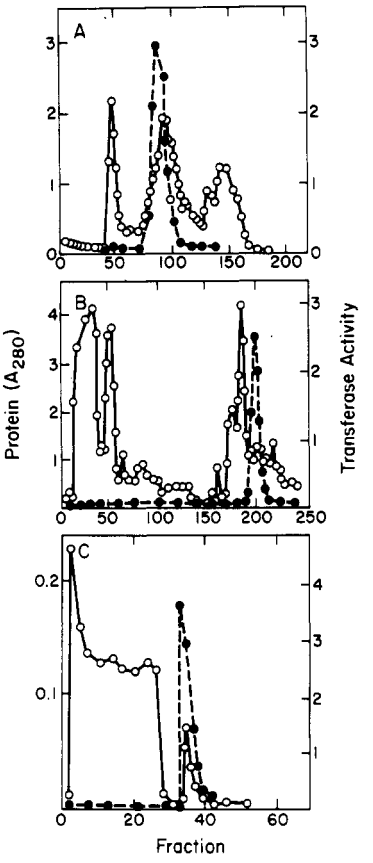

FIGURE 2: Chromatographic purification of the wild-type *N*-acetylglucosaminyltransferase. (Panel A) Enzyme, recovered from the DEAE-cellulose column by elution with 0.5 M KCl, was fractionated on a Bio-Gel A-5m column (6 × 90 cm) by elution with 20 mM imidazole hydrochloride buffer, pH 6.5, containing 5% glycerol. Fractions of 10 mL were collected. (Panel B) The active fractions from (A) were combined (230 mL) and applied to an ethyl-Sepharose column (1.5 × 65 cm), which was then eluted with 10 mM imidazole hydrochloride buffer, pH 6.5, containing 5% glycerol until the A_{280} of the eluent was reduced (150 fractions), at which time a linear gradient of 0–0.75 M KCl was applied in the same buffer. Fractions of 10 mL were collected. (Panel C) The active fractions from (B) were combined (160 mL) and applied to a concanavalin A–Sepharose column (1 × 11 cm), which was eluted with 10 mM imidazole hydrochloride buffer, pH 6.5, containing 5% glycerol and 10 mM $MnCl_2$ until the A_{280} of the effluent was reduced to a low value, at which time elution was carried out with 0.3 M methyl α-D-mannoside in the same buffer. Fractions of 6 mL were collected. In each panel, protein (A_{260}) is the solid line and transferase activity (10^{-3} cpm of *N*-acetyl-D-[1-^{14}C]glucosamine transferred from the sugar nucleotide to mannotetraose acceptor in the standard assay) is the dashed line.

Although the procedure outlined in Table I led to a substantial purification of the transferase, the preparation was not homogeneous. Native gel electrophoresis in the presence of Triton X-100 and urea revealed four major bands, the transferase activity coinciding with a diffuse band at the top of the gel (data not shown). Electrophoresis in the presence of sodium dodecyl sulfate indicated even greater heterogeneity.

(*B*) *Properties of the Wild-Type Transferase.* The Triton X-100 solubilized *N*-acetylglucosaminyltransferase, like that in protoplast particles, had an absolute requirement for Mn^{2+}. The optimal concentration was 10 mM and higher concentrations inhibited the enzyme. This requirement was not replaced by Mg^{2+} or Ca^{2+}. Unlike the *S. cerevisiae* mannosylphosphate transferase, which is activated by Co^{2+} (Karson

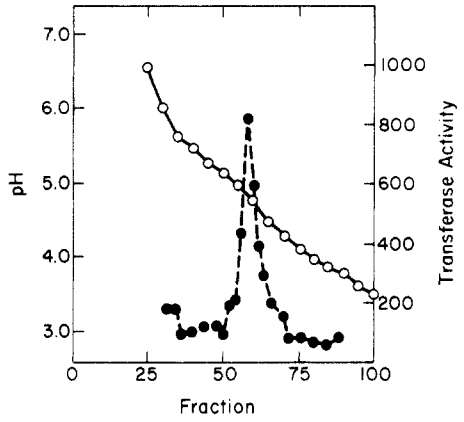

FIGURE 3: Isoelectric focusing of *K. lactis* wild-type transferase. Material recovered from the DEAE-cellulose column was focused for 36 h in a sucrose-stabilized column of pH 3–6 ampholines containing 2% Triton X-100 and 2 M urea. The pH gradient (solid line) and transferase activity (dashed line) are shown.

& Ballou, 1978), the *K. lactis* *N*-acetylglucosamine transferase is completely inhibited by 1 mM Co^{2+} even in the presence of 10 mM Mn^{2+}. The transferase was stable when stored in imidazole–glycerol buffer containing 2% Triton X-100, and it retained 50% activity after 80 days at 4 °C. Activity was lost at 45 °C with a half-time of 4 min, but 100% of the activity remained after 2 h at 35 °C.

The pH optimum of 6.5 found for protoplast particles in imidazole hydrochloride buffer (Dr. W. Smith, unpublished results) was confirmed for the solubilized transferase. An inhibition observed by citrate buffer was probably due to chelation of the Mn^{2+} in the assay. The K_m for UDP-*N*-acetylglucosamine is 44.7 ± 1.4 μM, whereas the K_m for mannotetraose is 11.0 ± 0.7 mM and agrees with that of 13 mM determined on membrane-bound enzyme (Smith et al., 1975). A V_{max} of 1.2 nmol min^{-1} (mg of protein)$^{-1}$ was obtained at saturating levels of both substrates and optimal Mn^{2+}.

The apparent molecular weight of the detergent-solubilized enzyme, determined by chromatography on a Sepharose 4B column (1 × 100 cm) equilibrated in detergent-free buffer, was approximately 300 000. This value is uncertain because membrane-bound glycosyltransferases may bind large amounts of Triton X-100 (Sadler et al., 1979), and the inhomogeneity of the transferase preparation prevented determination of the ratio of enzyme protein to Triton X-100. In the presence of 2% Triton X-100 and 2 M urea, the transferase activity focused into a fairly sharp band at a pH of 4.9 (Figure 3).

(*C*) *Analysis of Product Formed by Purified Wild-Type Enzyme.* The product, from the reaction using wild-type transferase with mannotetraose as acceptor and UDP-*N*-acetyl[^{14}C]glucosamine as donor, was purified by chromatography on Bio-Gel P-2. The labeled product was larger than the acceptor (Figure 4, top), and on paper chromatography (solvent A) a carbohydrate reagent revealed one spot that was coincident with the scanned radioactivity and that had an R_f identical with that for authentic $Man_4GlcNAc$. The sample was reduced, methylated, hydrolyzed, and again reduced, and the resulting permethylated alditols were acetylated and separated by gas chromatography (Conrad, 1972). The products (Table III) were identical with those obtained from a similar analysis of $Man_4GlcNAc$ isolated from the yeast mannoprotein (Raschke & Ballou, 1972).

(*D*) *K. lactis mnn2-2 N-Acetyl-*D*-glucosaminyltransferase Purification.* Cells were grown and harvested as for the wild type. Following cell breakage, differential centrifugation, and solubilization, the mutant enzyme preparation had a specific

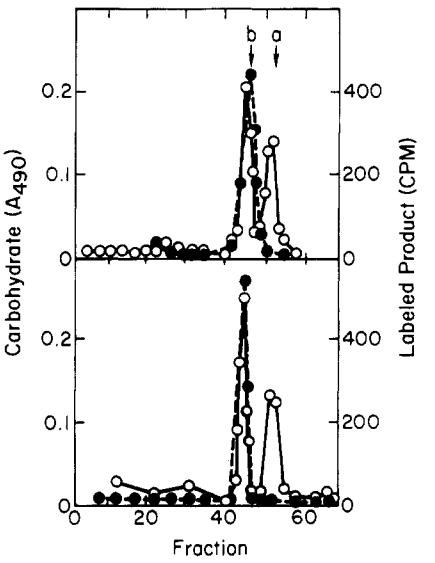

FIGURE 4: Isolation of the *N*-acetylglucosaminyltransferase products formed with exogenous donor and acceptors. The products from the wild-type enzyme (top) and *mnn2-2* mutant enzymes (bottom) were separated on a Bio-Gel P-2 column (1 × 100 cm) by elution with water. Carbohydrate (solid line) and radioactivity (dashed line) are shown. The elution positions of the mannotetraose acceptor (a) and the standard $Man_4GlcNAc$ (b) isolated from *K. lactis* mannoprotein are shown.

Table III: Methylation Analysis of *K. lactis* *N*-Acetylglucosaminyltransferase Oligosaccharide Products[a]

acetylated mannitol derivatives	oligosaccharide source: wild-type manno-protein[b]	wild-type enzyme product	mutant *mnn2-2* enzyme product
1,3,4,5,6-penta-*O*-methyl	0.9	0.8	0.9
2,3,4,6-tetra-*O*-methyl	1.0	1.0	1.0
3,4,6-tri-*O*-methyl	0.8	1.0	1.0
4,6-di-*O*-methyl	0.7	1.0	1.0

[a] Values normalized to 1.0 residue of the 2,3,4,6-tetra-*O*-methyl derivative. [b] Taken from Raschke & Ballou (1972).

activity comparable to that from wild-type cells. The transferase from *mnn2-2* cells is bound by DEAE-cellulose and is eluted by the same salt concentration as the wild-type transferase. The *mnn2-2* transferase was eluted in the center of the resolving space on Bio-Gel A-5m, and its behavior was similar to the wild-type transferase during chromatography on ethyl-Sepharose and concanavalin A–Sepharose columns.

Differences in purification for the wild-type and mutant enzymes were more apparent in the large loss of activity in the mutant extracts on DEAE-cellulose and Bio-Gel A-5m chromatography. The enzyme from the mutant cells, however, is as stable in Triton X-100-solubilized extracts as the wild-type enzyme. Although the purification obtained for the transferase from *mnn2-2* cells was not as large, the enzyme from the wild-type and mutant cells had similar chromatographic properties, and no difference was seen in the proteins resolved by gel electrophoresis.

The mutant transferase reaction product was eluted from Bio-Gel P-2 in the same fraction as authentic $Man_4GlcNAc$ (Figure 4, bottom), and it comigrated with $Man_4GlcNAc$ on paper chromatography using solvent A. Methylation yielded the same permethylated alditol acetates as are found in $Man_4GlcNAc$ from the wild-type strain and from the mannoprotein (Table III).

(*E*) *Properties of the mnn2-2 N-Acetylglucosaminyl-*

Table IV: Acceptor Specificity of *K. lactis* Wild-Type and *mnn2-2* *N*-Acetylglucosaminyltransferases

acceptor[a]	product formed [pmol min^{-1} (mg of protein)$^{-1}$][b] by wild-type enzyme	*mnn2-2* mutant enzyme
intact *mnn2-1* mannoprotein	0.7	0.5
(A) >Man_{25}	10.3	10.3
(B) Man_{15} to Man_{25}	17.7	17.3
(C) Man_8 to Man_{15}	125	134
(D) Man_6 + Man_7	192	206
(E) Man to Man_5	274	314

[a] Fractions A-E were obtained from a partial acetolysate of *mnn2-1* mannoprotein by separation on a Bio-Gel P-4 column. The approximate size range of each fraction in mannose units is indicated by the subscripts. [b] Activity was measured in the standard assay except that unlabeled UDP-*N*-acetylglucosamine was added to a final concentration of 80 μM, and 0.5 mg of each oligosaccharide or 2 mg of mannoprotein was added to each assay.

transferase. The transferase from the mutant has the same Mn^{2+} requirement (10 mM) and pH optimum (pH 6.5 in imidazole hydrochloride) as the wild-type enzyme. The K_m values for mannotetraose (10.7 ± 2 mM) and UDP-*N*-acetylglucosamine (36.4 ± 4 μM) were similar to those of the wild-type transferase. The V_{max} for the *mnn2-2* transferase, 0.74 nmol min^{-1} (mg of protein)$^{-1}$, was lower than that found for the wild-type transferase and reflects the different purities or stabilities of the preparations. NaCl, NaN_3, Tween 80, and phosphate inhibited the *mnn2-2* and wild-type activities to the same extent, and the *mnn2-2* transferase was inactivated by heat at the same rate as the wild-type transferase. During chromatography on Sepharose 4B, the *mnn2-2* transferase was eluted in the same fraction as that from the wild type.

(F) Other Comparisons of K. lactis Wild-Type and mnn2-2 Cells. (1) Subcellular Localization of Transferase Activity. One explanation for the failure of the *N*-acetylglucosaminyltransferase to be expressed in whole cells could be that the enzyme is not properly localized. When membranes from lysed wild-type and mutant protoplasts were separated on sucrose gradients, the majority of the transferase activity from both cell types sedimented to the 50% sucrose shelf. Similarly, membrane fractions from both cell types, briefly homogenized with glass beads and fractionated on linear Renografin gradients, gave indistinguishable patterns. The density to which the enzyme activity sedimented, 1.06–1.10 g/mL, corresponds to endoplasmic reticulum (Cortat et al., 1973; Lehle et al., 1977), a membrane fraction in which the mannosyltransferases also sedimented. Plasma membrane sedimented at a density of 1.20–1.25 g/mL.

(2) Substrate Specificity. To test the possibility that the natural acceptor was an oligosaccharide larger than mannotetraose and that the two transferases had different affinities for it, we produced such potential acceptors by a 1-h acetolysis of mannan from *K. lactis mnn2-1* cells. The use of Cetavlon-isolated mannan, rather than Fehlings' precipitated mannan, was important because traces of Cu^{2+} inhibit the transferases (Table II). The results (Table IV) reveal no difference in acceptor specificity between the two transferases, although it is apparent that the smaller oligosaccharides are better acceptors than the larger ones even if the data are normalized with regard to the amount of terminal α1→3-linked mannose. A similar preference for smaller oligosaccharides has been noted for the *S. cerevisiae* mannosyltransferases (Nakajima & Ballou, 1975) and mannosyl-

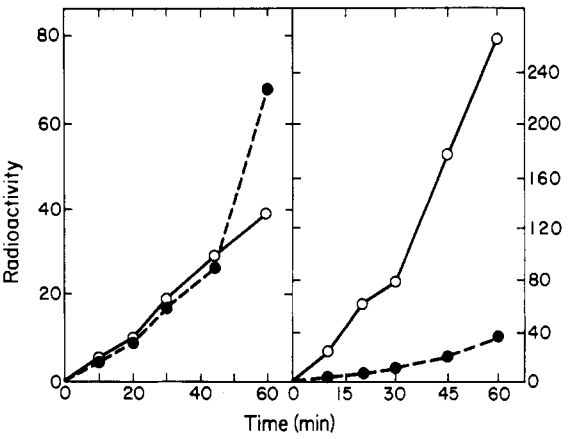

FIGURE 5: Incorporation of [^{3}H]glucosamine by *K. lactis* cells. Radioactivity incorporated into chitin (left) and into mannoprotein (right) by wild-type cells (solid line) and *mnn2-2* mutant cells (dashed line). Radioactivity is given as cpm × 10^{-2} per 10^8 cells.

phosphate transferase (Karson & Ballou, 1978).

(3) Metabolism of [^{3}H]Glucosamine by Whole Cells. To determine whether the intracellular level of UDP-*N*-acetylglucosamine was reduced in the mutant, we compared the concentrations of this sugar nucleotide in the wild-type and mutant cells in hot water extracts of cells. Analyses revealed 20.6 μg/g of glucosamine in wild-type and 22.8 μg/g in *mnn2-2* cell extracts. Paper chromatography (solvent C) confirmed that the material was UDP-*N*-acetylglucosamine, not free glucosamine.

The incorporation of [^{3}H]glucosamine into UDP-*N*-acetylglucosamine, chitin, and mannoprotein was measured (Figure 5). No difference was seen in the rates of incorporation into UDP-*N*-acetyl[^{3}H]glucosamine or chitin (Figure 5A). The wild-type cells, however, incorporated glucosamine into mannoprotein (Figure 5B) at 8 times the rate of the mutant, and the small amount of glucosamine label in the *mnn2-2* mannoprotein is probably in the core units that link the oligosaccharides to asparagine.

To determine whether mutant cells contained a hydrolase that cleaved the *N*-acetylglucosamine from the mannan, we incubated Man_4[^{14}C]GlcNAc with crude cell homogenates at 25 °C for 1 h. The reactions were applied to a Dowex AG 1-X2 column, and the neutral material was chromatographed on Bio-Gel P-2. Free *N*-acetylglucosamine was not detected and all of the starting radioactivity was accounted for in the Man_4GlcNAc peak. Although it is imaginable that the mutant contains a hydrolase that can act only on the intact mannoprotein, this seems unlikely.

(G) Genetic Linkage of K. lactis mnn2-1 and mnn2-2 Loci. Genetic analysis in *K. lactis* is complicated because the diploids spontaneously sporulate (Tingle et al., 1968). Random spores can be isolated, however, and the rate of recombination between two genes determined. Clones from isolated random spores from the cross between *K. lactis mnn2-1* and *mnn2-2* mutants were tested for wild-type recombinants by their ability to bind fluorescent wheat germ agglutinin (Douglas & Ballou, 1980). Three wild-type recombinants were found among 270 spores, which indicates that the *mnn2-1* and *mnn2-2* mutations are separated by 1.1 map units and may be in the same genetic locus (Mortimer & Hawthorne, 1969). The validity of the analysis was confirmed by the recombination frequency (0.25) of the two unlinked histidine markers.

Discussion

Mannoprotein biosynthesis is a complex process that involves the action of many glycosyltransferases. Synthesis could be

regulated indirectly by the differential locations, concentrations, and substrate specificities of the individual enzymes, or the process could be controlled by more complex mechanisms, including feedback inhibition, subunit interactions, and transcriptional or translational controls. The dominance of the *S. cerevisiae mnn4* mutation (Karson & Ballou, 1978; Ballou et al., 1973; Ballou, 1974) suggests that some steps in mannoprotein biosynthesis may be regulated by the latter mechanisms.

The α1→2-*N*-acetylglucosaminyltransferase is a convenient enzyme for study because it can be assayed independently from the mannosyltransferases in a reaction that utilizes exogenous acceptors and labeled UDP-*N*-acetylglucosamine. Like other enzymes involved in yeast mannoprotein biosynthesis, the transferase is an integral membrane protein; but it can be solubilized by 2% Triton X-100, as supported by the facts that such enzyme preparations could not be sedimented at 100000*g* for 3 h, and the activity was included during chromatography on Bio-Gel A-0.5m in presence of detergent.

The *N*-acetylglucosaminyltransferase activity was stimulated severalfold upon solubilization, a fact noted for most membrane-bound glycosyltransferases (Karson & Ballou, 1978; Lehle & Tanner, 1975; Schwyzer & Hill, 1977b). This stimulation may result from membrane disruption that allows enzyme molecules on the inner side of vesicles to gain access to substrate. The increase in total units during differential centrifugation may reflect removal of endogenous cell wall acceptors that compete in the assay with exogenous acceptor.

The properties of the solubilized enzyme are identical with those reported for the transferase in protoplast particles (Smith et al., 1975). The two preparations have the same pH optimum, Mn^{2+} dependence, and K_m for exogenous mannotetraose acceptor. Although it was reported (Smith et al., 1975) that the membrane-bound transferase could not be saturated with UDP-*N*-acetylglucosamine, we find a K_m of about 40 μM for the nucleotide sugar with both the membrane-bound and the solubilized transferase. The kinetic properties of the *K. lactis* transferase are similar to those for other glycosyltransferases, which often show a higher K_m for the oligosaccharide substrate than for the sugar nucleotide and a dependency on Mn^{2+}, an ion that becomes inhibitory at high concentration. Mannoproteins appear to be synthesized in preference to chitin in an *S. cerevisiae* glucosamine auxotroph deprived of this amino sugar (Ballou et al., 1977), a priority that could result because the transferases involved in mannoprotein biosynthesis have a greater affinity for UDP-*N*-acetylglucosamine than does chitin synthetase. The *K. lactis N*-acetylglucosamine transferase, in fact, has a K_m for UDP-*N*-acetylglucosamine that is 10-fold lower than the *S. cerevisiae* chitin synthetase K_m (Keller & Cabib, 1971).

The transferase from *K. lactis* Y-58a is an anionic protein with an apparent molecular weight of 300000, although the large size could result from aggregation during gel filtration or from binding of detergent. Other membrane-bound glycosyltransferases, with polypeptide molecular weights of 46000–100000, initially appeared much larger on gel filtration due to the binding of detergent (Schwyzer & Hill, 1977a; Sadler et al., 1979).

The binding of the transferase to concanavalin A suggests that it is a mannoprotein, although the failure to bind wheat germ agglutinin implies that there is no accessible *N*-acetylglucosamine on the enzyme. The binding specificities of immobilized lectins in the presence of Triton X-100 are the same as the soluble lectins (Lotan et al., 1977). That a glycosyltransferase is itself a glycoprotein is not uncommon, two examples being the membrane-bound ovine galactosyltransferase (Smith & Brew, 1977) and the porcine *N*-acetylglucosaminyltransferase (Schwyzer & Hill, 1977a).

Table V: Possible Defects in *mnn2-2* Mutant Cells

(1) alteration in the K_m for one of the substrates
(2) difference in the availability of one of the substrates
(3) increased activity of a degradative pathway
(4) overproduction of an inhibitor
(5) alteration in the affinity of the transferases for an inhibitor or effector
(6) temperature sensitivity of the enzyme
(7) alteration in the oligosaccharide-acceptor structure
(8) failure to activate a zymogen form of the transferase
(9) change in the ability of the transferase to interact with other glycosyltransferases
(10) alteration in the intracellular localization of the transferase
(11) alteration of the orientation of the transferase within the lipid layer

Attempts to purify the *N*-acetylglucosaminyltransferase to homogeneity encountered several difficulties. Because removal of the detergent was required before hydrophobic chromatography could be performed, the transferase aggregated and activity was lost upon concentration of fractions eluted from the ethyl-Sepharose columns. Recovery of the activity was also low if the protein concentration was too high when the detergent was removed on Bio-Beads SM-2 or Amberlite XAD-2. Another problem was the sensitivity of the transferase to high salt concentrations, and activity was often lost during salt elution of columns. Although protamine sulfate precipitation of nucleic acids followed by ammonium sulfate precipitation of the enzyme was an effective purification step, the yields were very low. The *S. cerevisiae* mannosylphosphate transferase shows a similar sensitivity to salt (Karson & Ballou, 1978). Schwyzer & Hill (1977a) have purified two membrane-bound glycosyltransferases on nucleotide–agarose columns, but they found that the conditions must be precisely determined. Although we made several unsuccessful attempts to employ such columns, further effort may be warranted because the *K. lactis* enzyme is completely inhibited by uridine nucleotides.

The *N*-acetylglucosaminyltransferase apparently does not require a lipid intermediate because our purification of the enzyme should have removed any endogenous lipid oligosaccharide pool (Jung & Tanner, 1973). Moreover, the addition of yeast dolichol had no effect on the transferase activity. This agrees with other findings that lipid oligosaccharides do not play a role in building the outer chain of yeast mannoproteins (Nakajima & Ballou, 1975).

The *K. lactis mnn2-2* mutant is not the only example in which the affected enzyme is active in extracts but inactive in the cell. A chinese hamster ovary cell line (clone 13), selected for resistance to wheat germ agglutinin, fails to add galactose to glycoproteins or glycolipids, although it shows appropriate levels of nucleotide sugar, glycoprotein and glycolipid acceptors, and galactosyltransferase activity in cell extracts (Briles et al., 1977). Some alternatives to rationalize the phenotype observed in the *K. lactis mnn2-2* mutant are listed in Table V, and most of these possibilities have been eliminated. The catalytic and physical properties of the wild-type and mutant transferases are very similar: they have identical substrate specificities and affinities for oligosaccharide acceptors, UDP-*N*-acetylglucosamine, and Mn^{2+}, and both enzymes produced identical products with the mannotetraose acceptor, which in turn were identical with the side-chain unit found in the mannoprotein. No evidence was obtained that the transferase was produced as a zymogen that was activated

in the mutant only upon cell disruption. The transferases had the same apparent molecular size, and both were located in the same subcellular membrane fraction. Both cell types had the same quantity of transferase activity, and the activities behaved similarly upon purification and were similarly affected by inhibitors and heat inactivation. Both transferases appear to be glycoproteins, and since neither enzyme bound to wheat germ agglutinin–Sepharose beads, we conclude that they could not differ in the presence or absence of terminal *N*-acetylglucosamine in the carbohydrate chains. Some insight to the importance of the carbohydrate chains on these enzymes might come from a study of the effects of tunicamycin and polyoxin, but we have not attempted such an investigation.

Mixing experiments ruled out the possibility that the *mnn2-2* cells were overproducing an inhibitor of the transferase. The intracellular concentration of UDP-*N*-acetylglucosamine and the metabolism of [^{3}H]glucosamine were the same in the wild-type and mutant cells except, as expected, [^{3}H]glucosamine was not rapidly incorporated into the mannoprotein of the *mnn2-2* mutant in intact cells. The possibility that the mutant had an increased activity of an enzyme that cleaved *N*-acetylglucosamine from the mannoprotein was also eliminated, and the *mnn2-2* mannoprotein had a normal density of acceptor sites.

Two properties of the *mnn2-2* mutation should be emphasized. The transferase is totally inactive in the cell, although in extracts it is as active as that from the wild type. The second important property is that the *mnn2-2* locus is tightly linked to the *mnn2-1* mutation. Extracts of the *mnn2-1* mutant completely lack transferase activity, and the mutation possibly is in the structural gene for the *N*-acetylglucosaminyltransferase. Although not conclusive, these results indicate that the *mnn2-2* mutation is allelic with *mnn2-1* and that it also affects the transferase directly.

References

Ames, G. F.-L. (1974) *J. Biol. Chem. 249*, 634–644.

Ballou, C. E. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol. 40*, 239–270.

Ballou, C. E. (1976) *Adv. Microb. Physiol. 14*, 93–158.

Ballou, C. E., & Raschke, W. C. (1974) *Science (Washington, D.C.) 184*, 127–134.

Ballou, C. E., Kern, K. A., & Raschke, W. C. (1973) *J. Biol. Chem. 248*, 4667–4673.

Ballou, C. E., Maitra, S. K., Walker, J. W., & Whelan, W. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4351–4355.

Barker, R., Olsen, K. W., Shaper, J. H., & Hill, R. C. (1972) *J. Biol. Chem. 247*, 7135–7147.

Bray, G. A. (1960) *Anal. Biochem. 1*, 279–285.

Brewer, J. M., & Ashworth, R. R. (1969) *J. Chem. Educ. 46*, 41–44.

Briles, E. B., Li, E., & Kornfeld, S. (1977) *J. Biol. Chem. 252*, 1107–1116.

Cabib, E. (1971) *Methods Enzymol. 22*, 120–122.

Cheetham, P. S. J. (1979) *Anal. Biochem. 92*, 447–452.

Conrad, H. E. (1972) *Methods Carbohydr. Chem. 7*, 71–75.

Cortat, M., Matile, P., & Kopp, F. (1973) *Biochem. Biophys. Res. Commun. 53*, 482–489.

Douglas, R. H., & Ballou, C. E. (1980) *J. Biol. Chem. 255*, 5979–5985.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350–356.

Dulley, J. R., & Grieve, P. A. (1975) *Anal. Biochem. 64*, 136–141.

Eisenthal, R., & Cornish-Bowden, A. (1974) *Biochem. J. 139*, 715–720.

Er-el, Z., Zaidenzaig, Y., & Shaltiel, S. (1972) *Biochem. Biophys. Res. Commun. 49*, 383–390.

Ghosh, S., Blumenthal, H. J., Davidson, E., & Roseman, S. (1960) *J. Biol. Chem. 235*, 1265–1273.

Hakomori, S. (1964) *J. Biochem. (Tokyo) 55*, 205–208.

Hanson, R. K., Ling, R., & Havir, E. (1967) *Biochem. Biophys. Res. Commun. 29*, 194–197.

Holbrook, I. B., & Leaver, A. G. (1976) *Anal. Biochem. 75*, 634–636.

Holloway, D. W. (1973) *Anal. Biochem. 53*, 304–308.

Hudgin, R. L., & Schachter, H. (1971) *Can. J. Biochem. 49*, 829–857.

Jones, E. W. (1977) *Genetics 85*, 23–33.

Jung, P., & Tanner, W. (1973) *Eur. J. Biochem. 37*, 1–6.

Karson, E. M., & Ballou, C. E. (1978) *J. Biol. Chem. 253*, 6484–6492.

Keller, F. A., & Cabib, E. (1971) *J. Biol. Chem. 246*, 160–166.

Kuo, S. C., & Lampen, J. O. (1976) *Arch. Biochem. Biophys. 172*, 574–581.

Kurtz, E., & Binkley, S. B. (1961) *Biochim. Biophys. Acta 46*, 595–597.

Lee, Y. C., & Ballou, C. E. (1965) *Biochemistry 4*, 257–264.

Lehle, L., & Tanner, W. (1975) *Biochim. Biophys. Acta 399*, 364–374.

Lehle, L., Bauer, F., & Tanner, W. (1977) *Arch. Microbiol. 114*, 77–81.

Lloyd, K. O. (1970) *Biochemistry 9*, 3446–3453.

Lotan, R., Beattie, G., Hubbell, W., & Nicholson, G. L. (1977) *Biochemistry 16*, 1787–1794.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Lüderitz, O., Staub, A. M., & Westphal, O. (1966) *Bacteriol. Rev. 30*, 192–255.

March, S. C., Parkh, I., & Cuatracasas, P. (1974) *Anal. Biochem. 60*, 149–152.

Mortimer, R. K., & Hawthorne, D. C. (1969) in *The Yeasts* (Rose, A., & Harrison, J. S., Eds.) Vol. 2, pp 386–460, Academic Press, New York.

Nakajima, T., & Ballou, C. E. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3912–3916.

Nakajima, T., Maitra, S. K., & Ballou, C. E. (1976) *J. Biol. Chem. 251*, 174–181.

Ornstein, L. (1964) *Ann. N.Y. Acad. Sci. 121*, 321–349.

Peat, S., Whelan, W. J., & Edwards, T. E. (1961) *J. Chem. Soc.*, 28–35.

Raschke, W. C., & Ballou, C. E. (1972) *Biochemistry 11*, 3807–3816.

Rosenfeld, L., & Ballou, C. E. (1975) *Biochem. Biophys. Res. Commun. 63*, 571–579.

Sadler, J. E., Rearick, J. I., Paulson, J. C., & Hill, R. C. (1979) *J. Biol. Chem. 254*, 4434–4443.

Schwyzer, M., & Hill, R. (1977a) *J. Biol. Chem. 252*, 2338–2345.

Schwyzer, M., & Hill, R. (1977b) *J. Biol. Chem. 252*, 2346–2355.

Smith, C. A., & Brew, K. (1977) *J. Biol. Chem. 252*, 7294–7299.

Smith, W. L., Nakajima, T., & Ballou, C. E. (1975) *J. Biol. Chem. 250*, 3426–3435.

Spiro, R. G. (1966) *Methods Enzymol. 8*, 3–26.

Stewart, T. S., & Ballou, C. E. (1968) *Biochemistry 7*, 1855–1863.

Tingle, M., Herman, A., & Halvorson, H. O. (1968) *Genetics 58*, 361–371.

Tiselius, A., Hjerter, S., & Levin, O. (1956) *Arch. Biochem. Biophys. 65*, 132-155.
Treveleyan, W. E., Proctor, D. P., & Harrison, J. S. (1950) *Nature (London) 166*, 444-445.
Wang, C., & Smith, R. L. (1975) *Anal. Biochem. 63*, 414-417.
Watkins, W. M. (1972) in *Glycoproteins* (Gottschalk, A., Ed.) part B, pp 830-891, Elsevier, New York.

Enthalpy and Heat Capacity Changes for the Reduction of Insulin†

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ABSTRACT: The enthalpy changes for the reduction of three disulfide bonds of insulin by dithiothreitol (DTT) were calorimetrically measured at various temperatures ranging from 289 to 308 K. The reduction was performed in three different buffer solutions of pH 9.6, and the observed heat changes were corrected for the ionization heats of the buffer components to obtain the net heats of reduction of insulin with DTT. By subtracting the enthalpy of DTT oxidation reported in the previous paper [Fukada, H., & Takahashi, K. (1980a) *J. Biochem. (Tokyo) 87*, 1105-1110], we determined the standard enthalpy of reduction of insulin to be ΔH_r = 93.4 ± 7.8 kJ (mol of insulin)$^{-1}$ at 298 K. The heat capacity change was $\Delta C_{p,r}$ = 3.2 ± 0.3 kJ mol^{-1} K^{-1}. Using the heat of oxidation of the cysteine residue, we estimated the enthalpy change for the conformational transition of insulin induced by the cleavage of three disulfide bonds to be ΔH_{conf} = 91 kJ mol^{-1} at 298 K. The heat capacity change was 2.1 kJ mol^{-1} K^{-1}. These results imply that the conformational transition taking place during the reduction of three disulfide bonds is thermodynamically of the same nature as the thermal denaturation observed for other globular proteins.

Many calorimetric measurements have been made to obtain the thermodynamic parameters for protein denaturation processes (Tsong et al., 1970; Privalov, 1974, 1979; Privalov & Khechinashvili, 1974; Pfeil & Privalov, 1976a-c). However, the data hitherto accumulated are limited to those for thermally and chemically induced unfolding and do not cover denaturation induced by the reduction of disulfide bonds. In our previous study (Fukada & Takahashi, 1980b), we have carried out calorimetric measurements on the reduction of insulin with dithiothreitol (DTT)[1] at 298 K and have shown that the enthalpy of the conformational change of an insulin molecule induced by the cleavage of disulfide bonds has a value very similar to those reported for the thermal or chemical denaturation of other globular proteins.

It has been shown that many reactions involving proteins are accompanied by large changes in the apparent heat capacity (Sturtevant, 1977). In this respect, it would be worthwhile to determine the heat capacity change as well as the enthalpy change also with the present system. In this study, further calorimetric measurements were made on the reduction of insulin with DTT to obtain the net heat of reduction of insulin at different temperatures, and the heat capacity change associated with the conformational transition of insulin taking place during the reduction of the three disulfide bonds was evaluated.

Materials and Methods

Bovine zinc insulin (molecular weight 5700) and DTT were purchased from Sigma Chemical Co. and were used without further purification. All other chemicals used were commercial preparations of reagent grade. Solutions were prepared with distilled and deionized water which was previously saturated with nitrogen to remove oxygen before use.

Crystalline insulin (zinc content 0.5%, lot no. 24C-3130) was dissolved in 0.01 N HCl to a concentration of about 10 mg cm^{-3} and dialyzed against the same acid solution at 5 °C to remove zinc according to the method described by Cunningham et al. (1955). The solution was then exhaustively dialyzed against 1 mmol dm^{-3} sodium carbonate, pH 9.6, buffer and was used as a stock solution. The final buffer pH was adjusted at the temperature of the measurements.

The insulin stock solutions were diluted with the appropriate buffer solution of pH 9.6 (0.4 mol dm^{-3} carbonate, glycine, or ammonium buffer, each containing 1 mmol dm^{-3} EDTA) to give an insulin concentration of 0.16 mmol dm^{-3} which was determined spectrophotometrically by using an absorption coefficient of 1.05 cm^2 mg^{-1} at 276 nm and at pH 7 (Frank & Veros, 1968).

The DDT solution was prepared in the same buffer as described in the previous paper (Fukada & Takahashi, 1980a). The DDT concentration in the reactant solution was 15 mmol dm^{-3}, being 94-fold molar excess over insulin.

Calorimetric Measurements. The flow microcalorimeter described previously (Fukada & Takahashi, 1980a) was used with further modification. The calorimeter cell consisted of 2 m of Teflon tubing instead of 1 m as in the original design to attain a longer residence time. The sensor unit, consisting of a set of 12 Sanyo thermocouple plates in the prototype, was replaced by four Melcor thermoelectric modules (C.P. 1.4-71-06L) to improve the time constant of heat exchange between the cell and the aluminum heat sink.

The calorimeter was calibrated by the heat of neutralization, by using ΔH = −56.58 kJ mol^{-1} at 298 K (Ackermann, 1958). For other temperatures, values were obtained by using Ackermann's equation.

The solutions were usually delivered at a flow rate of 0.2 cm^3 min^{-1} which was frequently determined by weighing the

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[1] Abbreviations: DTT, dithiothreitol; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin.

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