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3-Hydroxy-3-methylglutaryl-CoA reductase: A transmembrane glycoprotein of the endoplasmic reticulum with N-linked "high-mannose" oligosaccharides

(UT-1 cells/compactin/cholesterol synthesis/crystalloid membranes/concanavalin A)

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ABSTRACT **3-Hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34) is an abundant protein of the crystalloid endoplasmic reticulum of UT-1 cells, a line of cultured hamster cells that overproduces the reductase as a result of gene amplification. In the current studies, we show that reductase in UT-1 cells is a glycoprotein. The solubilized enzyme (M_r = 97,000) from UT-1 cells, Chinese hamster ovary cells, and rat liver was adsorbed quantitatively and specifically to concanavalin A-Sepharose. UT-1 cells incorporated [1,6-^{3}H]glucosamine into the reductase; after release with endo-*N*-acetylglucosaminidase H most of the radioactivity was found in N-linked "high-mannose" chains, including $Man_6(GlcNAc)_2$, $Man_7(GlcNAc)_2$, and $Man_8(GlcNAc)_2$. The carbohydrate of the reductase was localized to a 30- to 35-kilodalton fragment that was separable proteolytically from a cytoplasmic 53-kilodalton fragment that contained the active site of the enzyme. We conclude that 3-hydroxy-3-methylglutaryl-CoA reductase is a transmembrane glycoprotein with an active site facing the cytoplasm and a carbohydrate-bearing site oriented toward the lumen of the endoplasmic reticulum.**

3-Hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34), the rate-controlling enzyme in cholesterol synthesis, is normally a trace protein of the endoplasmic reticulum (ER) in liver and other tissues (1, 2). Regulation of the enzyme is currently under study in cultured hamster cells selected for growth in compactin, a competitive inhibitor of reductase (3–6). These cells, designated UT-1 cells, have undergone a 15-fold amplification in the gene for reductase (4), and they express ≈500 times the normal amount of the enzyme (3, 4, 6). In UT-1 cells, reductase is located in hexagonally packed, smooth tubular membranes designated crystalloid ER (3, 5).

The reductase of UT-1 cells is an integral membrane protein that can be solubilized only with detergents (3, 6). The M_r of the protein is 97,100 according to the amino acid sequence as deduced from the sequence of a full-length cDNA (unpublished data). A Ca^{2+}-dependent protease in cell extracts cleaves the 97-kilodalton (kDa) reductase to an active 62-kDa form that remains membrane bound (3, 6). Further cleavage by a leupeptin-sensitive protease releases a soluble 50- to 55-kDa fragment that retains full enzymatic activity (3). The soluble 50- to 55-kDa fragment from rat liver is the only form of reductase that has been purified (7, 8). Little is known about the orientation of the reductase in the ER membrane.

One approach to the orientation of reductase is to determine whether it contains asparagine-linked (N-linked) oligosaccharides. Certain transmembrane proteins of the rough ER, such as the ribophorins, contain carbohydrate chains on their luminal side as determined by concanavalin A (Con A) binding (9). Other ER proteins, such as cytochrome *b*5, which do not span the membrane, do not contain carbohydrate (10). In the current studies, we show that reductase from UT-1 cells contains N-linked oligosaccharides of the "high-mannose" type, suggesting that the enzyme is a transmembrane protein.

METHODS

Materials. We obtained Con A-rhodamine and wheat germ agglutinin conjugated with fluorescein isothiocyanate (FITC) from Vector Laboratories (Burlingame, CA); Con A-Sepharose and Sepharose CL-4B from Pharmacia; and isotopes, culture media, and other materials from previously described sources (3, 11–13). Purified Ca^{2+}-dependent protease (Ca^{2+}-protease) from rat liver or beef heart (peak II, M_r = 108,000) was prepared as described (13).

Cells and Microsomes. Cells were grown in monolayer in the absence [Chinese hamster ovary (CHO) cells] or presence (UT-1 cells) of 40 μM compactin in Ham's F-12 medium with 10% (vol/vol) lipoprotein-deficient serum (3, 11). Cells were seeded in Petri dishes (60 × 15 mm) or roller bottles (3, 11) and harvested on day 6. Cells were washed at 4°C with Dulbecco's phosphate-buffered saline, scraped, collected by centrifugation (1,000 × *g*, 10 min, 4°C), and disrupted in one of three ways: (*i*) addition of buffer A (0.15 M NaCl/50 mM Tris·HCl/0.5 mM $MnCl_2$/0.5 mM $MgCl_2$/5 mM EGTA/10 mM dithiothreitol/0.1 mM leupeptin/0.2 mM phenylmethylsulfonyl fluoride at pH 7.5) plus 2% (wt/vol) Zwittergent 3-14; (*ii*) Dounce homogenization (20 times at 4°C) in buffer A; or (*iii*) nitrogen cavitation with a Parr cell disruption bomb [1,000 psi (6.9 MPa), 15 min, 4°C] in buffer A. When cells were disrupted by methods *ii* or *iii*, a 1,000 × *g* supernate was spun at 100,000 × *g* for 60 min at 4°C to obtain a microsomal pellet.

Immunoprecipitation. Cells in Petri dishes were radiolabeled with [^{35}S]methionine or [1,6-^{3}H]glucosamine in 2 ml of medium B [Dulbecco's modified Eagle's medium with reduced concentrations of methionine (50 μM) and D-glucose (100 μM), 10% lipoprotein-deficient serum, and 40 μM compactin]. Each monolayer was washed twice at 4°C with phosphate-buffered saline containing 1 mM methionine and 1 mM *N*-acetylglucosamine (GlcNAc). Then 100 μl of 1% (wt/vol) $NaDodSO_4$/5 mM EGTA/1 mM methionine/1 mM GlcNAc/10 mM dithiothrei-

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Abbreviations: CHO, Chinese hamster ovary; Con A, concanavalin A; endo H, endo-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; kDa, kilodalton(s).

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tol/0.1 mM leupeptin/0.2 mM phenylmethylsulfonyl fluoride was added to the dish, and cells were scraped at 24°C. Immunoprecipitations were carried out with a polyclonal anti-reductase IgG (6, 11).

Animals. Rats were fed 2% (wt/wt) cholestyramine plus 0.1% (wt/wt) mevinolin for 5 days (14). Aliquots of liver (1 g) were homogenized in 20 ml of ice-cold buffer A, and microsomes were prepared (14).

Proteolytic Cleavage of Reductase. Microsomes (100–300 μg of protein) were incubated in buffer C (50 mM Tris·HCl/5 mM dithiothreitol/5 mM $CaCl_2$ at pH 7.5) with 6–18 μg of Ca^{2+}-protease at 24°C for 45 min. Control microsomes were incubated in buffer D (50 mM Tris·HCl/5 mM dithiothreitol/5 mM EGTA/0.1 mM leupeptin/0.2 mM phenylmethylsulfonyl fluoride at pH 7.5) at 4°C for 45 min without protease.

Radiolabeled Glycopeptides. UT-1 cells were radiolabeled with [1,6-^{3}H]glucosamine at 250 μCi/ml (1 Ci = 3.7×10^{10} Bq) in medium B for 12 hr at 37°C, and [^{3}H]reductase was isolated by immunoprecipitation and $NaDodSO_4$ gel electrophoresis. The excised gel slices containing the 97-kDa reductase were treated with Pronase (12). Labeled glycoprotein (12, 15) and oligosaccharides (16, 17) were analyzed as described.

Assays. Samples for electrophoresis were prepared in buffer E (15% $NaDodSO_4$/8 M urea/10% sucrose/62.5 mM Tris·HCl/100 mM dithiothreitol at pH 6.8). $NaDodSO_4$/polyacrylamide gel electrophoresis (6, 14), fluorescence microscopy (3), immunoblotting on nitrocellulose (14), and measurement of enzymatic activity of reductase (6, 11) were performed as indicated. One unit of reductase activity represents the formation of 1 nmol of mevalonate per min at 37°C. Protein was measured by a modified Lowry procedure (18).

RESULTS

Reductase Activity Is Adsorbed to Con A. UT-1 cells were solubilized with detergent and incubated with increasing amounts of Con A-Sepharose. After centrifugation, reductase activity remaining in the supernate was measured. All reductase activity was quantitatively adsorbed from solution with Con A-Sepharose; α-methyl mannoside prevented this binding (Fig. 1*A*). Detergent-solubilized reductase from CHO cells (Fig. 1*A*) and rat liver microsomes (Fig. 1*B*) gave comparable results. Binding of reductase to Con A-Sepharose was abolished by low concentrations of α-methyl mannoside and by somewhat higher concentrations of α-methyl glucoside, but not by L-fucose (Fig. 1*C*). Of the bound reductase, 58% could be eluted from the Con A-Sepharose by subsequent treatment with 100 mM α-methyl mannoside (data not shown).

If the carbohydrate of UT-1 reductase is oriented toward the luminal side of the crystalloid ER, then intact microsomes should not be adsorbed to Con A. When microsomal vesicles were prepared by nitrogen cavitation with or without sonication and then incubated with Con A-Sepharose, reductase activity failed to be adsorbed (Table 1). In contrast, when microsomes were disrupted with Triton/deoxycholate, reductase could be adsorbed and the adsorption was blocked by α-methyl mannoside.

Soluble 53-kDa Fragment of Reductase Lacks Carbohydrate. Intact reductase from UT-1 cells has a molecular weight of 97,100 as determined from the amino acid sequence (unpublished data). A fragment of reductase can be released by endogenous proteases from microsomes as a 50- to 55-kDa protein that retains full enzymatic and antigenic activity (3). To release the reductase proteolytically, we supplemented the endogenous proteases with an exogenous Ca^{2+}-protease from rat liver or beef heart (13), which gave a more reproducible cleavage than did the endogenous proteases alone.

When total microsomes were treated with detergents so as to release intact reductase, 97% of activity was adsorbed to Con A-Sepharose (Table 2, No proteolysis). When microsomes were incubated in the presence of inhibitors of proteolysis and then subjected to centrifugation, essentially all of the reductase activity was recovered in the pellet. After detergent solubilization of this pellet, 56% of the reductase could be bound to Con A-Sepharose. In contrast, when microsomes were incubated under conditions that permitted proteolysis and then solubilized, only 21% of the total activity could be adsorbed to Con A. When the protease-treated microsomes were subjected to centrifugation, most reductase activity was in the supernate, and none of this released activity could be adsorbed to Con A. In contrast, 44% of the small amount of activity recovered in the pellet could be adsorbed to Con A after solubilization. This experiment suggests that the proteolytically released fragment has lost the ability to bind to Con A, because the carbohydrate remains in the membrane-bound portion of the enzyme.

To study the molecular weight of the proteolytically released reductase, aliquots of the fractions from an experiment similar to that in Table 2 were subjected to $NaDodSO_4$ electrophoresis,

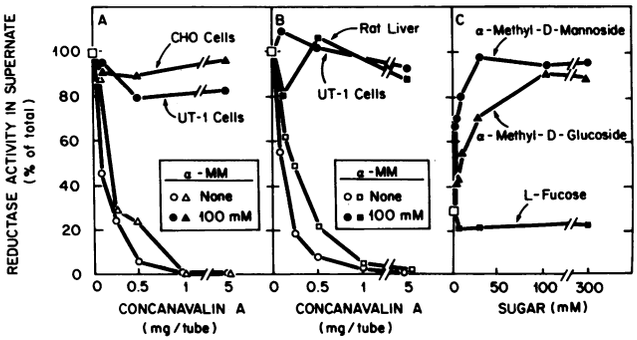

FIG. 1. Binding of reductase to Con A-Sepharose. UT-1 cells, CHO cells, or rat liver microsomes were solubilized in buffer A plus 2% Zwittergent 3-14. Aliquots were incubated for 60 min (*A* and *B*) or 30 min (*C*) at 4°C in 1 ml of buffer A containing 0.5% Zwittergent 3-14, bovine serum albumin at 1 mg/ml, and the indicated amount of Con A-Sepharose. (*A*) UT-1 cells (0.3 unit of reductase activity; 76 units/mg of protein; ○, ●) and CHO cells (0.04 unit; 0.068 units/mg; △, ▲) were incubated in the absence (○, △) or presence (●, ▲) of 100 mM α-methyl D-mannoside (α-MM). (*B*) UT-1 cells (0.4 unit; 41 units/mg; ○, ●) and rat liver microsomes (2.1 units; 25 units/mg; □, ■) were incubated in the absence (○, □) or presence (●, ■) of 100 mM α-methyl D-mannoside. (*C*) UT-1 cells (7.3 units; 145 units/mg) were incubated with 1 mg of Con A-Sepharose in the presence of the indicated concentration of the indicated sugar. After incubation, all mixtures were centrifuged at 35 × *g* for 2 min at 4°C, and aliquots of the supernate were assayed for reductase activity. The amount of Con A-Sepharose is expressed in terms of the Con A content.

Table 1. Differential binding to Con A of reductase from intact and solubilized microsomes

Treatment of microsomes	Reductase activity, units/tube		
		Bound to Con A	
	Total	Without α-MeMan	With α-MeMan
None	4.4	0.2	0
Sonication (three times 10 sec)	4.1	0.9	0.7
1% Triton X-100/0.5% sodium deoxycholate	5.5	5.1	0.8

UT-1 cells were disrupted by nitrogen cavitation at 4°C, and microsomes were prepared in buffer A and treated as indicated. Aliquots (20 μg of protein) were then mixed for 60 min at 4°C with 5 mg of Con A-Sepharose or an equal volume of Sepharose CL-4B in the absence or presence of 0.3 M α-methyl D-mannoside (α-MeMan). The mixtures were centrifuged, and aliquots of the supernate were assayed for reductase activity. Total activity represents reductase in the supernate after incubation with Sepharose CL-4B.

Table 2. Differential binding of soluble and membrane-bound proteolytic fragments of reductase to Con A

Fraction	Reductase activity			
	No proteolysis		With proteolysis	
	Total activity, units/tube	% bound by Con A	Total activity, units/tube	% bound by Con A
Total microsomes	16.5	97	14.4	21
Supernate	0.1	0	11.2	0
Pellet	13.2	56	1.4	44

UT-1 cells were disrupted by nitrogen cavitation at 4°C and microsomes were prepared in buffer A minus $MnCl_2$ and $MgCl_2$. Aliquots (100 μg of protein) were incubated for 45 min either in buffer D at 4°C (No proteolysis) or in buffer C without proteolysis inhibitors and supplemented with 6 μg of Ca^{2+}-protease at 24°C (With proteolysis). After incubation, Na_2CO_3 was added to 0.1 M to remove loosely adherent proteins, and EGTA and leupeptin were adjusted to 5 mM and 0.1 mM, respectively, in all tubes. An aliquot from each tube (80 μg) was centrifuged at 100,000 × *g* for 20 min at 24°C to obtain a supernate and a pellet. Total microsomes, supernates, and pellets were solubilized with 1% Zwittergent 3-14 and incubated for 60 min at 24°C with either 5 mg of Con A-Sepharose or an equal volume of Sepharose CL-4B. The mixtures were centrifuged and aliquots of the supernate were assayed for reductase activity. Total activity represents reductase in the supernate after incubation with Sepharose CL-4B.

and the immunologically active portion of the reductase was visualized by immunoblotting with a monoclonal antibody (Fig. 2). In control microsomes in the absence of proteolysis, reductase was visualized as a 97-kDa protein (lane 1). The radioactive bands above 97 kDa are thought to be aggregates of the 97-kDa reductase (6). When detergent-solubilized reductase was treated with Con A, all of the 97-kDa enzyme was adsorbed by the lectin (lane 2). When intact microsomes were subjected to centrifugation, the supernate did not contain immunodetectable reductase (lanes 3 and 4). Immunodetectable reductase was found in the pellet as a 97-kDa protein with some breakdown to a 62-kDa form that remained membrane bound (lane 5). After detergent solubilization, the majority of the 97-kDa immunoreactive band was removed by treatment with Con A (lane 6).

When microsomes were subjected to proteolysis prior to fractionation, the results shown in the right-hand gel of Fig. 2 were obtained. Most of the reductase in the total microsomes was reduced to a 53-kDa fragment (lane 7). None of this 53-kDa material was removed by incubation with Con A-Sepharose (lane 8). When microsomes were centrifuged prior to electrophoresis, the supernate contained most of the immunodetectable reductase, which appeared as a 53-kDa protein (lane 9) that was not adsorbed by Con A (lane 10). The small amount of reductase recovered in the pellet was also largely in the 53-kDa form and was not adsorbed by Con A (lanes 11 and 12). These results suggest that the proteolytic conversion of reductase from the 97-kDa form to the soluble 53-kDa form is accompanied by loss of the ability to bind to Con A.

Carbohydrate of Reductase Is Located on a 30- to 35-kDa Proteolytic Fragment. UT-1 cells were labeled with either [^{35}S]methionine or [^{3}H]glucosamine (Fig. 3). After solubilization with detergents, reductase was first isolated by immunoprecipitation and then digested *in vitro* with the Ca^{2+}-protease. The digest was subjected to $NaDodSO_4$ electrophoresis. Prior to proteolysis, the immunoprecipitated reductase appeared as a 97-kDa protein that was labeled with [^{35}S]methionine (lane 2) or [^{3}H]glucosamine (lane 5). When the immunoprecipitates were digested with the Ca^{2+}-protease, the [^{35}S]reductase was split into a 53-kDa band and a family of fragments with M_r of 30,000–

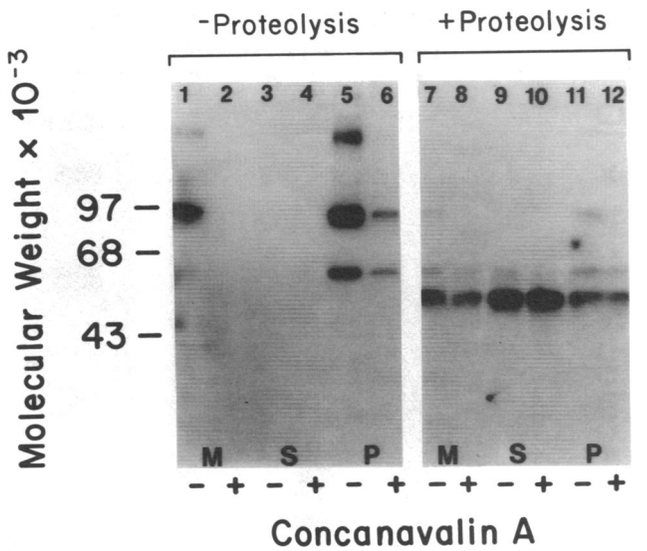

FIG. 2. $NaDodSO_4$ gel electrophoresis and immunoblotting analysis of soluble and membrane-bound proteolytic fragments of reductase after adsorption to Con A. UT-1 cells were disrupted by nitrogen cavitation. Aliquots of microsomes (300 μg of protein; 74 units of reductase activity) were incubated in buffer D with proteolytic inhibitors at 4°C for 45 min (lanes 1–6) or buffer C without proteolytic inhibitors supplemented with 18 μg of Ca^{2+}-protease at 24°C for 45 min (lanes 7–12). After incubation, Na_2CO_3 was added to 0.1 M to remove loosely adherent proteins, and EGTA and leupeptin were adjusted to 5 mM and 0.1 mM, respectively, in all tubes. An aliquot from each tube (240 μg) was centrifuged at 100,000 × *g* for 20 min at 24°C to obtain a supernate and a pellet. Total microsomes (M), supernates (S), and pellets (P) were solubilized with 1% Zwittergent 3-14 and incubated for 60 min at 24°C with either 10 mg of Con A-Sepharose (+Con A) or an equal volume of Sepharose CL-4B (−Con A). The mixtures were centrifuged and aliquots of the supernate were subjected to electrophoresis, immunoblotting with monoclonal mouse anti-reductase IgG-A9 at 10 μg/ml followed by ^{125}I-labeled rabbit anti-mouse IgG (10^6 cpm/ml), and autoradiography for 3 days at −70°C.

35,000 (lane 3). The 53-kDa band did not contain radioactivity from the [^{3}H]glucosamine (lane 6). All of the radioactivity from the [^{3}H]glucosamine was contained in the bands at 30–35 kDa (lane 6). An aliquot of unlabeled enzyme was subjected to the same proteolytic treatment followed by electrophoresis and immunoblotting with the monoclonal anti-reductase antibody. Prior to proteolysis the antibody bound to the 97-kDa protein (and also to the higher molecular weight aggregates of the 97-kDa protein) (lane 8). After proteolysis, the immunoreactive site of reductase was present in the 53-kDa band (lane 9). This experiment indicates that proteolysis of reductase generates two fragments: a 53-kDa fragment that contains the enzymatic and immunologic activities but lacks carbohydrate, and a 30- to 35-kDa fragment that contains the carbohydrate but lacks the immunologically reactive site.

Endo-*N*-acetylglucosaminidase H (endo H) Treatment of Reductase. When the immunoprecipitated [^{35}S]reductase (97-kDa form) was treated with endo H, a slight but reproducible decrease in molecular weight was observed on $NaDodSO_4$ electrophoresis (Fig. 4). The increase in mobility corresponded to an apparent decrease in molecular weight of ≈1,500.

Analysis of N-Linked Oligosaccharides of Reductase. UT-1 cells were grown in the presence of [1,6-^{3}H]glucosamine, and the 97-kDa reductase was purified by immunoprecipitation and $NaDodSO_4$ electrophoresis. The [^{3}H]reductase was digested with Pronase, and the glycopeptides were applied to a column of Con A-Sepharose (Fig. 5 *Inset*). Previous studies have shown that high-mannose oligosaccharides are bound with high affinity by Con A-Sepharose and are eluted efficiently with 100 mM

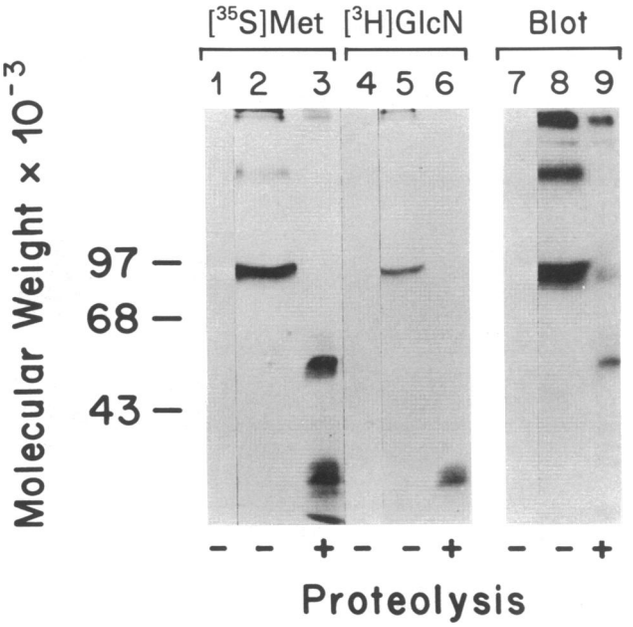

FIG. 3. $NaDodSO_4$ gel electrophoresis of reductase from UT-1 cells after treatment with Ca^{2+}-protease. Twelve hours before harvest, UT-1 monolayers were switched to 2 ml of medium B supplemented with [^{35}S]methionine (lanes 1–3) or [^{3}H]glucosamine (lanes 4–6) at 250 μCi/ml. Cells in lanes 7–9 received no isotope. Cell extracts were solubilized in $NaDodSO_4$ and immunoprecipitated with either nonimmune rabbit IgG (lanes 1 and 4) or polyclonal rabbit anti-reductase IgG (lanes 2, 3, and 5–9). Immunoprecipitates were either eluted immediately from Pansorbin with buffer E or washed once with buffer C and then incubated with 0.36 μg of Ca^{2+}-protease for 15 min at 24°C, after which an equal volume of 2× concentrated buffer E was added. After electrophoresis, the gels in lanes 7–9 were subjected to immunoblotting with either control mouse monoclonal IgG-2001 (lane 7) or anti-reductase IgG-A9 (lanes 8 and 9) at 10 μg/ml followed by ^{125}I-labeled rabbit anti-mouse IgG (10^6 cpm/ml). Gels (lanes 1–6) or nitrocellulose strips (lanes 7–9) were exposed to film at −70°C for 1 day (lanes 1–3), 7 days (lanes 4–6), or 5 days (lanes 7–9).

α-methyl mannoside but not with 10 mM α-methyl glucoside (15, 19). Twenty-two percent of the [^{3}H]reductase peptides did not bind to the lectin; 6% were bound and eluted by α-methyl glucoside; and 72% were bound and eluted by α-methyl mannoside. The glycopeptides eluted with α-methyl mannoside were treated with endo H and reapplied to a column of Con A-Sepharose. Forty-five percent of the radioactivity did not bind to the column, and 55% was bound and eluted by α-methyl mannoside (data not shown). These data are consistent with the known specificity of this glycosidase, which hydrolyzes N-linked high-mannose oligosaccharides between the two internal GlcNAc residues, leaving a single GlcNAc residue attached to asparagine and releasing a high-mannose carbohydrate chain with a single GlcNAc residue (20). The labeled oligosaccharides released by endo H and still bound by Con A-Sepharose were analyzed by HPLC (16). As shown in Fig. 5, the major species corresponded to $Man_6GlcNAc$ (69%), $Man_7GlcNAc$ (12%), and $Man_8GlcNAc$ (20%). The material not bound by Con A-Sepharose (Fig. 5 *Inset*) was hydrolyzed and the labeled monosaccharides were reacetylated and analyzed by descending paper chromatography in a system that separates GlcNAc and GalNAc (17). Greater than 95% of the recovered radioactivity migrated with authentic GlcNAc (data not shown). These data indicate that reductase lacks O-linked oligosaccharides, since GalNAc, a constituent of such oligosaccharides, was not detected.

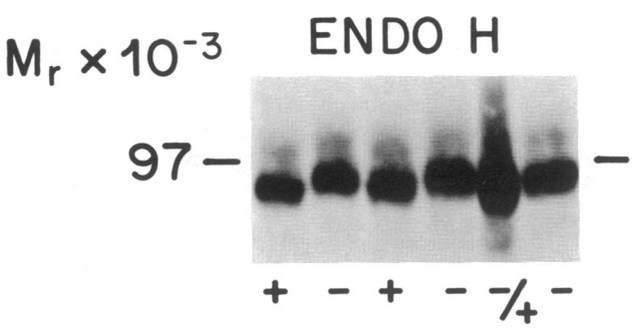

FIG. 4. endo H sensitivity of ^{35}S-labeled reductase. Twelve hours before harvest, UT-1 monolayers were switched to 2 ml of Ham's F-12 medium with decreased methionine (3 μM) and [^{35}S]methionine at 100 μCi/ml. Cells were solubilized and immunoprecipitated. The immunoprecipitates were incubated overnight at 24°C in the absence (−) or presence (+) of 0.05 unit of endo H in 200 μl of 30 mM sodium citrate/0.2% $NaDodSO_4$/1 mM dithiothreitol/0.2 mM phenylmethylsulfonyl fluoride at pH 5.5 and subjected to $NaDodSO_4$ electrophoresis in alternate lanes. Fluorography was performed at −70°C for 18 hr. One lane (−/+) contains an equal mixture of treated and untreated immunoprecipitates.

Crystalloid ER Contains Carbohydrate. UT-1 cells were fixed, made permeable, and then incubated with rhodamine-tagged Con A. Intense fluorescence was observed in the crystalloid ER (Fig. 6*A*), which was abolished by α-methyl mannoside (Fig. 6*C*). The structure that stained with Con A was also stained with an anti-reductase IgG-FITC, as determined by indirect immunofluorescence in the same cells (Fig. 6*B*). The crystalloid ER did not stain with wheat germ agglutinin-FITC, a lectin that binds to terminal sialic acid and GlcNAc residues of processed ("complex") glycoproteins (Fig. 6*D*). The wheat germ agglutinin did, however, stain a focal structure that appeared to be the Golgi complex. These data suggest that the crystalloid ER is rich in high-mannose oligosaccharides but not in processed oligosaccharides.

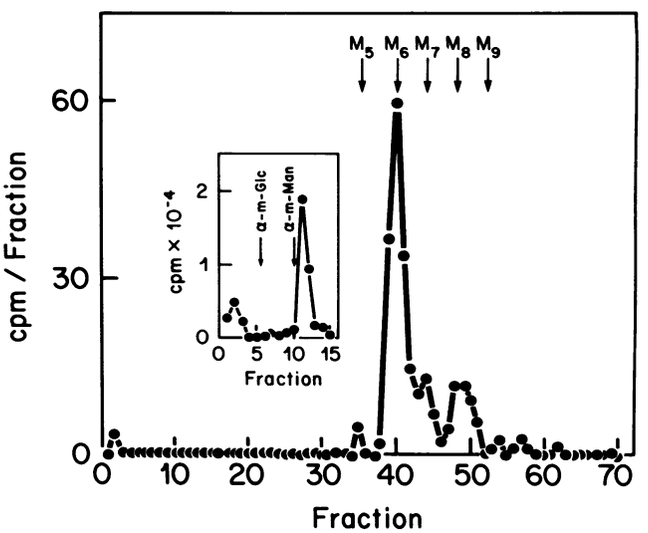

FIG. 5. Separation by HPLC of [1,6-^{3}H]glucosamine-labeled oligosaccharides released by endo H treatment of glycopeptides from reductase in UT-1 cells. UT-1 cells were grown in [^{3}H]glucosamine and the 97-kDa reductase was isolated by digestion with Pronase. The released glycopeptides were applied to a column of Con A-Sepharose (14). (*Inset*) Radioactivity eluted from the column by buffer alone, 10 mM α-methyl glucoside (α-m-Glc), or 100 mM α-methyl mannoside (α-m-Man). The glycopeptides eluted with 100 mM α-methyl mannoside were desalted on Sephadex G-25 and an aliquot was treated with endo H (15), reapplied to a column of Con A-Sepharose, and eluted with 100 mM α-methyl mannoside. An aliquot of this material was analyzed by HPLC (16). The elution positions of standard high-mannose oligosaccharides (M_5–M_9) are shown at the top of the figure.

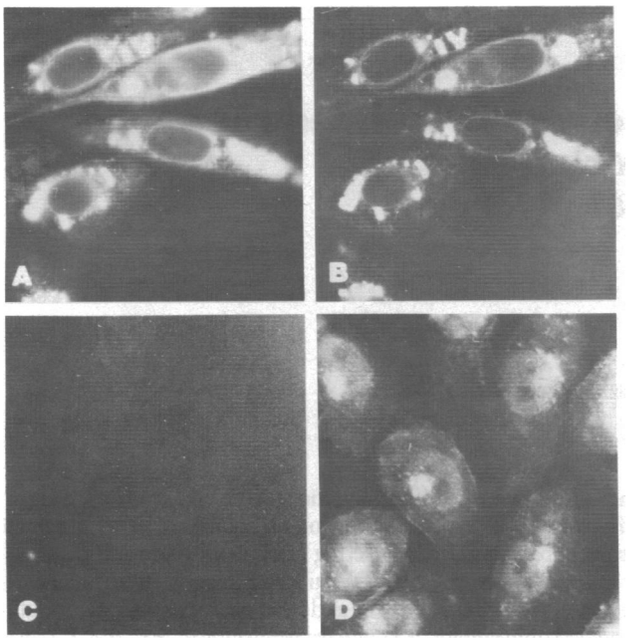

FIG. 6. Visual demonstration of carbohydrate in crystalloid ER in UT-1 cells. Cells were grown on coverslips, fixed, washed, rendered permeable with Triton X-100, then incubated for 1 hr at 37°C as follows: (*A* and *B*) Con A-rhodamine at 3 μg/ml plus polyclonal rabbit anti-reductase IgG at 0.5 mg/ml followed by 1 hr at 37°C with goat anti-rabbit IgG-FITC at 0.5 mg/ml; (*C*) Con A-rhodamine at 3 μg/ml plus 0.2 M α-methyl D-mannoside; or (*D*) wheat germ agglutinin-FITC at 50 μg/ml. The cells used in *A* and *B* were examined with filter packages that detect rhodamine (*A*) and fluorescein (*B*). (×500.)

DISCUSSION

Four lines of evidence indicate that reductase from UT-1 cells is a glycoprotein: (*i*) The intact 97-kDa form of reductase is quantitatively adsorbed from solution by Con A-Sepharose, and this adsorption is blocked by α-methyl mannoside. (*ii*) The apparent molecular weight of reductase is decreased upon treatment with endo H. (*iii*) Reductase can be labeled with [^{3}H]glucosamine, and the ^{3}H-labeled oligosaccharides can be removed with endo H and characterized as N-linked high-mannose chains. Similar results were obtained with [2-^{3}H]mannose (data not shown). (*iv*) Reductase is contained in a structure, the crystalloid ER, that stains specifically with Con A-rhodamine.

The location of the carbohydrate on the reductase was determined by taking advantage of the observation that reductase can be cleaved by proteases into a 53-kDa fragment (3, 6) and several fragments in the 30- to 35-kDa range. The 53-kDa fragment contains the immunologic site that reacts with available monoclonal and polyclonal antibodies; it also retains full enzymatic activity. After proteolysis, the 53-kDa fragment is released in a form that no longer binds to Con A (Table 2 and Fig. 2), suggesting that this fragment is located on the cytoplasmic surface of the ER and does not contain carbohydrate. This result implies that the carbohydrate is located on the 30- to 35-kDa fragment. Indeed, when reductase is labeled with [^{3}H]glucosamine, all of the [^{3}H]glucosamine-derived radioactivity was found in the 30- to 35-kDa fragment (Fig. 3).

Since high-mannose oligosaccharide chains are known to be formed only on the luminal surface of the ER (9, 20), the current results strongly suggest that the reductase is a transmembrane 97-kDa protein. The active site of the enzyme is contained within a 53-kDa fragment that faces the cytoplasmic side of the crystalloid ER, from which it can be released by proteases. The carbohydrate chains of the enzyme are located on a 30- to 35-kDa fragment that must project to some extent into the lumen of the crystalloid ER. Consistent with this formulation was the finding that microsomal reductase failed to be adsorbed to Con A-Sepharose, presumably because the carbohydrate was not accessible on the outer surface of the microsomal vesicles (Table 1).

The conclusions regarding UT-1 reductase likely apply to reductase from CHO cells and rat liver because enzyme activity from all of these sources could be quantitatively and specifically adsorbed to Con A (Fig. 1). The completeness of the adsorption indicates that all active reductase molecules contain at least one carbohydrate chain. This finding may explain the recent observation that reductase activity in C-6 glial cells is diminished when the cells are incubated with tunicamycin, an inhibitor of N-linked glycosylation (21).

In a broad sense the finding of high-mannose carbohydrate on a smooth ER protein raises the question as to how this glycoprotein is segregated in the ER. It seems likely that the high-mannose chains are transferred to the reductase when it is synthesized in the rough ER. What, then, prevents the reductase from moving to the Golgi complex and having its carbohydrates processed in a fashion analogous to that of other high-mannose glycoproteins that eventually move to the plasma membrane? Further studies of the reductase should allow this sorting problem to be solved.

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