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Isolation and the complete amino acid sequence of lumenal endoplasmic reticulum glucose-6-phosphate dehydrogenase

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ABSTRACT **I have isolated glucose-6-phosphate dehydrogenase from rabbit liver microsomes and determined its complete amino acid sequence. Sequence determination was achieved by automated Edman degradation of peptides generated by chemical and enzymatic cleavages. The microsomal enzyme consists of 763 residues and is quite dissimilar from the previously characterized cytosolic enzymes. The N terminus of the microsomal enzyme is blocked by a pyroglutamyl residue. Carbohydrate is attached at Asn-138 and Asn-263, implying that the bulk of the protein is oriented on the lumenal side of the endoplasmic membrane. The amino acid sequence of the microsomal protein shows limited homology to the extensively sequenced cytosolic glucose-6-phosphate dehydrogenases. Clusters of up to six identical residues can be identified in four regions: peptide segments at residues 10–21, 154–163, and 173–261. In addition, another array of identical residues, requiring a 100-residue deletion in the sequence of the microsomal enzyme, spans residues 436–462 and corresponds to residues 348–373 of the cytosolic protein. Two segments with a Gly-Xaa-Gly-Xaa-Xaa-Gly motif, related to a coenzyme binding fold, were identified at Gly-399 and Gly-491. In the cytosolic enzymes, a variation of this sequence motif occurs at Gly-37 and Gly-241. The 300-residue C-terminal segment of the microsomal enzyme is unique and has no counterpart in the cytosolic or the bacterial enzymes. An unexpected finding with regard to the microsomal enzyme is that it lacks an identifiable membrane-spanning region or the lumenal-protein C-terminal consensus sequences Lys-Asp-Glu-Leu or His-Ile/Thr-Glu-Leu. Thus, the mode of transport and retention of this protein in the lumen of endoplasmic reticulum remains to be determined.**

The isolation and the amino acid sequences of a number of microsomal membrane proteins have been reported, including cytochrome b_5 (1) and its reductase (2), cytochrome P-450 (3–5), epoxide hydrase (6), stearyl-CoA desaturase (7), the flavin-containing monooxygenases (8, 9), and two forms of esterases present in the lumen of microsomal membranes (10, 11). During the purification of the flavin-containing monooxygenases, a 90-kDa glycoprotein with a high affinity for 2′,5′-ADP-Sepharose was identified. Furthermore, this protein could generate NADPH or NADH for the flavin-containing monooxygenase-catalyzed reactions in the presence of glucose 6-phosphate (Glc-6-*P*). Initial structural studies indicated that this 90-kDa microsomal protein is related to the cytosolic Glc-6-*P* dehydrogenase (G6PD) and is a lumenal protein.

Cytosolic G6PDs have been extensively characterized from mammalian and bacterial sources (12, 13). They are highly conserved proteins of 56 kDa. The monomer molecular mass of the microsomal enzyme, however, is ≈90 kDa. The obvious function of the microsomal G6PD is to provide NADH/NADPH to reductases oriented on the lumenal side of the microsomal membrane. While Glc-6-*P* can be translocated from the cytosol to the lumen, NADP or NAD cannot cross the microsomal membrane (14). However, reactions that utilize the lumenal reducing equivalents or are involved in the lumenal electron transport system are at present unknown.

Although a 108-kDa hexose-6-phosphate dehydrogenase has been purified from rat liver microsomes (15, 16) and the enzyme has been identified in microsomes from adipose tissue, liver, and steroidogenic cells (17), the primary structure of microsomal enzyme from any source has not been reported. To increase our understanding of this group of dehydrogenases and to establish the structural relationship between the lumenal and the cytosolic enzymes, I report here the covalent structure of the G6PD present in the lumen of endoplasmic membrane.*

MATERIALS AND METHODS

Materials. Detergents, enzyme substrates, cofactors chromatographic media, and chemicals, unless stated otherwise, were obtained from Sigma. Hydroxyapatite-agarose (HA-Ultragel) was a product of IBF. Trypsin and pepsin were obtained from Worthington. Endoproteinases Asp-N and Glu-C and pyroglutamate aminopeptidase were obtained from Boehringer Mannheim. *Achromobacter* protease Lys-C was obtained from Biochemical Diagnostics (Edgewood, NY). Endo-*N*-acetylglucosaminidase H (endo H) was a product of Genzyme. G6PD from Torula yeast was obtained from Sigma. Liver microsomal flavin-containing monooxygenase form 1 was isolated as described (8). Anhydrous trifluoromethanesulfonic acid was obtained from Oxford Glyco-Systems (Rosedale, NY). Solvents for HPLC and gel filtrations were from Burdick and Jackson.

Isolation of G6PD from Rabbit Liver Microsomes. Liver microsomes from male New Zealand rabbits were isolated and solubilized as described (18). The solubilized preparation was passed over a column containing 200 ml of DEAE-cellulose equilibrated with 10 mM potassium phosphate (pH 7.4) containing 20% (vol/vol) glycerol, 1 mM EDTA, 0.1 mM dithiothreitol (buffer A), and 0.5% Nonidet P-40 (NP-40). Unbound material eluting from the column was applied to a column containing 100 ml of CM-Sepharose equilibrated with buffer A, containing 0.2% NP-40. The unbound material was then applied to a column containing 100 ml HA-Agarose equilibrated with buffer A containing 0.1% NP-40. The column was developed with buffer A containing 125 mM potassium phosphate and 0.1% NP-40. Fractions containing G6PD were pooled and dialyzed against 1 liter of buffer A, containing 5 mM potassium phosphate (pH 7.0) and 0.1% NP-40. The dialyzed material was absorbed to 20 ml of CM-

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Abbreviations: Glc-6-*P*, glucose 6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; NP-40, Nonidet P-40; endo H, endo-*N*-acetylglucosaminidase H.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. A44520).

Sepharose equilibrated with the dialysis buffer. G6PD was eluted with buffer A containing 10 mM potassium phosphate (pH 7.4) and 0.1% NP-40. The reductase-containing fractions were dialyzed against 1 liter of 20 mM Tris acetate (pH 8.1) containing 20% glycerol, 1 mM EDTA, 0.1 mM dithiothreitol, and 0.1% NP-40 (buffer B). The dialyzed material was then applied to a column containing 7 ml of 2',5'-ADP-Sepharose equilibrated with buffer B. The column was washed with 50 ml of buffer B, followed by 30 ml of buffer B containing 100 mM KCl. Reductase was eluted with 30 ml of buffer B containing 100 mM KCl and 0.5 mM NADPH. The yield of homogeneous G6PD was about 1 mg per rabbit liver.

Measurement of G6PD Activity. Dehydrogenase activity was measured by monitoring Glc-6-*P*-dependent NADP reduction at 340 nm, at room temperature in a 1-ml volume containing 50 mM Tris acetate (pH 7.4), 0.25 mM NADP, and 10 μl of enzyme. The reaction was started by the addition of Glc-6-*P* (1.25 mM). The specific activity of the final preparation was 5 μM per min per mg, and the enzyme had a K_m of 2 μM for either NADP and NAD. Assay of flavin-containing monooxygenase form 1 with thiourea as the substrate and microsomal or yeast G6PD as the NADPH-generating system was carried out as described (8, 19).

Sequence Analysis. Protein/peptide hydrolyses were performed with 6 M HCl in the gas phase, at 150°C for 1 h, and analyses were performed using a Beckman model 6300 amino acid analyzer (20).

Reduction, carboxymethylation, and succinylation were performed as described (20). Enzymatic and chemical cleavages were carried out as described (8).

Peptide mixtures were first separated using a 1.5 × 100 cm column of LH-60 Sephadex equilibrated with formic acid/ethanol, 3:7 (vol/vol), as the solvent, as described (8). Peptide mixtures from the gel-filtration column were further resolved by reverse-phase HPLC. The latter methodology has been described in detail (9, 21). Reverse-phase columns employed for HPLC separations include Vydac (Hesperia, CA) C_4 (15 × 0.46 cm) or Waters C_{18} μBondapak (30 × 0.39 cm). Peptide mixtures were dissolved in 88% (vol/vol) formic acid prior to injection on columns. Solvent A was 0.1% trifluoroacetic acid and solvent B was 0.1% trifluoroacetic acid in 75% (vol/vol) acetonitrile. A linear gradient from 0% to 100% of solvent B in 70 min was applied at a flow rate of 1.0 ml/min.

Sequence analysis of peptides was carried out on an Applied Biosystems model 470A sequencer equipped with model 120A phenylthiohydantoin analyzer according to manufacturer's instructions. Solid-phase sequencing of peptides was carried out on a 6600 ProSequencer system (MilliGen/Biosearch, Novato, CA) (8). Data were collected and analyzed using the MilliGen/Biosearch PTH-Maxima chromatography package. Chromatograms were optimally aligned, using alignment algorithms, and then subtracted to give a difference trace for successive sequencer cycles.

The pyroglutamate residue from intact protein or peptides was removed by the appropriate peptidase (11). Lyophilized calf liver pyroglutamate aminopeptidase (2 mg; 6×10^{-3} unit at 0.5 unit/80 mg of lyophilized material) was added to 200 μl of 50 mM potassium phosphate (pH 7.4), containing 5% glycerol, 10 mM EDTA, and 0.5 mM dithiothreitol (reagent A). A 100-μl aliquot of reagent A was added to 100 μl (40 μg) of G6PD or 0.5 nmol of dried peptide. The reaction mixture was incubated at 4°C for 16 h under nitrogen, and the protein was precipitated by the addition of 20 vol of ice-cold 0.2% HCl in acetone. The resulting pellet was dissolved in 88% formic acid and subjected to automated Edman degradation. Peptides treated with the aminopeptidase were lyophilized and subjected to HPLC prior to the sequence analyses.

Digestion of HCl/acetone-precipitated protein with endo H was performed as described (11). Peptides R-6 and CB-7 were chemically deglycosylated by treatment with anhydrous trifluoromethanesulfonic acid (22). About 20% of the peptide was deglycosylated by this method. Cleavage of the Asp-Pro bond in peptide CB-7 was not observed during the deglycosylation reaction.

RESULTS

Purification of the G6PD was achieved by solubilizing microsomes with sodium cholate and fractionating with polyethylene glycol. The 6–12% (wt/vol) polyethylene glycol fraction was subjected to ion-exchange chromatography in the presence of nonionic detergent and glycerol. The enzyme was eluted in void volume of the first DEAE-cellulose and CM-Sepharose columns. Purification of the enzyme to homogeneity was realized by further chromatography of the unretained material on hydroxyapatite, CM-Sepharose, and finally, on a 2',5'-ADP-Sepharose column. Upon SDS/PAGE, the purified enzyme gave a single band with a subunit molecular mass of ≈90 kDa (Fig. 1*A*). The enzyme obtained after the affinity-chromatography step can be stored at 4°C for several months without significant loss of activity. *In vitro*, the microsomal G6PD was equally effective as the yeast enzyme in generating NADPH for the flavin-containing monooxygenase-catalyzed reactions.

Automated Edman degradation of microsomal G6PD indicated a blocked N terminus. Attempts to fragment the intact protein into domains by limited proteolysis were unsuccessful. Proteolysis conditions yielding partial fragmentation of the intact protein were not found with trypsin, thrombin, or endoprotease Lys-C or Glu-C.

The general strategy of sequence analysis of this dehydrogenase involved the reduction and carboxymethylation of the protein, followed by chemical and enzymatic cleavages. Resolution of the digest was completed by fractionation on an LH-60 column followed by HPLC of individual fractions. The resolution of the CNBr digest by LH-60 column chromatography is seen in Fig. 2*A*. Except for peptide CB-10, all the expected peptides were isolated from this digest by HPLC. Peptide CB-11 (residues 446–701) was eluted in the void volume of the gel-filtration column in an essentially pure form. The N terminus of peptide CB-1 was blocked, but digestion with pyroglutamyl aminopeptidase removed the blocking group, and 20 cycles of Edman degradation established the N-terminal sequence of peptide CB-1. Digestion of the intact protein with pyroglutamyl aminopeptidase, followed by eight cycles of sequencer analysis of the HCl/acetone-precipitated protein, confirmed that peptide CB-1 represents the N terminus of the native protein.

The order of all peptides obtained from CNBr cleavage was determined from isolation and sequence analysis of peptides obtained by cleavage of the reduced and alkylated protein with endopeptidase Lys-C and the succinylated protein with trypsin (Fig. 2 *B* and *C*). Some 20% of lysyl and arginyl bonds

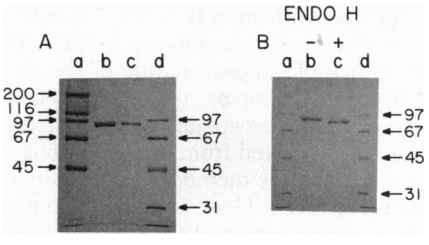

FIG. 1. (*A*) SDS/PAGE analysis of purified microsomal G6PD. Lanes: a and d, molecular mass markers (myosin, 200-kDa; β-galactosidase, 116-kDa; serum albumin, 67-kDa; ovalbumin, 45-kDa; carbonic anhydrase, 31-kDa); b and c, rabbit liver microsomal G6PD. (*B*) Lanes: a and d, molecular mass markers; b and c, SDS/PAGE analysis of G6PD before (−) and after (+) digestion with endo H.

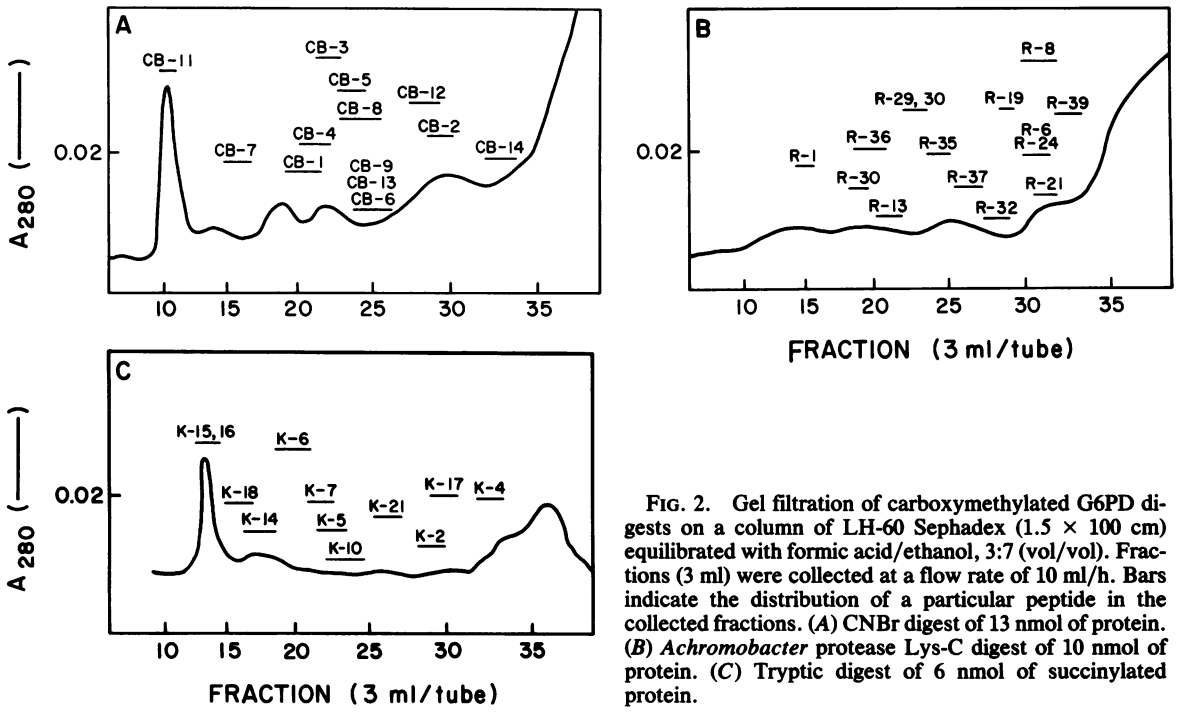

FIG. 2. Gel filtration of carboxymethylated G6PD digests on a column of LH-60 Sephadex (1.5 × 100 cm) equilibrated with formic acid/ethanol, 3:7 (vol/vol). Fractions (3 ml) were collected at a flow rate of 10 ml/h. Bars indicate the distribution of a particular peptide in the collected fractions. (*A*) CNBr digest of 13 nmol of protein. (*B*) *Achromobacter* protease Lys-C digest of 10 nmol of protein. (*C*) Tryptic digest of 6 nmol of succinylated protein.

were resistant or only partially cleaved by endopeptidase Lys-C or trypsin. Some of these partially cleaved peptides, however, provided important overlaps. The carboxymethylated protein was resistant toward endoproteinase Glu-C. However, a partial cleavage of the alkylated protein with Glu-C protease, at an enzyme/substrate ratio of 1:10 was observed at residues 112, 179, and 384. Peptide E-4 was obtained from this digest, and subcleavage of E-4 with trypsin provided peptide E-4; T-26. It provided the essential overlap for peptides CB-8 and CB-9. Endoproteinases Glu-C and Asp-N were effective in cleaving the large peptides obtained by CNBr, endoproteinase Lys-C, or tryptic cleavages. Solid-phase and gas-phase Edman degradation of the purified peptides established the complete amino acid sequence of this protein. The presence of two residues were observed at positions 69 (Arg/Lys), 75 (Leu/Arg), and 437 (Met/Lys).

DISCUSSION

The amino acid sequence of the 763-residue microsomal G6PD is summarized in Fig. 3. A phenylthiohydantoin derivative was not identified at residues 138 and 163. The presence of seryl residues two positions downstream of these sites suggested a glycosylation site of the Asn-Xaa-Ser/Thr type at these residues. Chemical deglycosylation of peptides R-7 and CB-7 with anhydrous trifluoromethanesulfonic acid, followed by HPLC and sequencer analysis, confirmed the predicted glycosylation at Asn-138 and Asn-163. Treatment of the native protein with endo H resulted in a decrease of its apparent molecular mass, as analyzed by SDS/PAGE, indicating the presence of oligosaccharide of the high-mannose type rather than of the complex type (Fig. 1*B*) (23). The endo H sensitivity of the microsomal G6PD indicates that the protein was not transported from the site of oligosaccharide addition in the microsome membrane to the site of oligosaccharide transferases in the Golgi (23). The high mannose type of carbohydrate attachment in the microsomal G6PD implies orientation toward the lumenal side of the endoplasmic membrane. This further suggested that the N-terminal blocking group of this protein is unlikely to be an acyl residue, since the enzyme responsible for the acetylation of N termini of a number of proteins is located on the ribosome at the cytoplasmic surface of rough endoplasmic reticulum (24). Therefore, the possibility of a pyroglutamyl residue at the N terminus was explored, and the residue blocking the N terminus of native protein was removed with the pyroglutamyl aminopeptidase.

Takahashi and Hori (15) and Hino and Minakami (16) have reported the purification of G6PD from rat liver microsomes. Because this enzyme can also utilize galactose 6-phosphate, it was designated as a hexose-6-phosphate dehydrogenase (16, 17). These preparations have enzymatic properties similar to our enzyme, with respect to the NAD/NADP utilization and the lack of steroid inhibition. They differ, however, in the molecular mass and the chromatographic properties. The minimum molecular mass of their enzyme, as determined by SDS/PAGE, is ≈108 kDa rather than 90 kDa. Since structural analysis of these hexose-6-phosphate dehydrogenases has yet to be obtained, the relationship between these and the lumenal G6PD reported here is not known. The possibility exists that the enzyme I have isolated represents a postranslationally modified form that has been processed to remove "trafficking" sequence. The amino acid sequence of G6PD reported here will provide a starting point for the molecular cloning of the gene for this unique enzyme and clarify whether additional residues are present in this group of enzymes.

Amino acid sequences of the cytosolic G6PDs are known for enzymes from humans (12), rats (25), *Drosophila* (26), *Saccharomyces cerevisiae* (27), *Escherichia coli* (28), *Leuconostoc mesenteroides* (29), and *Zymomonas mobilis* (13). These enzymes have a polypeptide chain of some 503–523 residues. The human and the yeast cytosolic enzymes have an acetylated N terminus (27). The rabbit microsomal G6PD consists of 763 residues and has a pyroglutamate at the N terminus. The amino acid sequences of rat and human cytosolic G6PDs are essentially identical. Comparison of the cytosolic enzyme sequences from human, rat, fruit fly, and yeast proteins shows that 200 of the 511 residues are highly conserved. Fig. 4 shows the sequence identity among the rat cytosolic and the rabbit microsomal G6PDs. The identical residues are found in 2- to 6-residue arrays in peptide segments at residues 10–21, 154–163, and 173–261. The latter set of identical residues includes Lys-190, which in the cytosolic enzymes has been implicated in Glc-6-*P* binding.

FIG. 3. Complete amino acid sequence of microsomal G6PD. Peptides obtained by CNBr, *Achromobacter* protease Lys-C, or tryptic cleavage of succinylated protein are designated by CB, K, and R, respectively. Subcleavage of peptides by endoproteinases Asp-N, Glu-C, pepsin, or trypsin is designated by D, E, P, or T, respectively. Prefix DP marks peptides obtained by cleavage with 88% formic acid, and W marks peptides cleaved at tryptophan bonds by excess CNBr. The glutaminyl residue at the N terminus of the protein is pyroglutamate, and PGA denotes pyroglutamate aminopeptidase. Residues identified by automated Edman degradation are identified by a solid line. The carbohydrate binding sites are at Asn-138 and Asn-263. The presence of two residues was observed at positions 69 (Arg/Lys), 75 (Leu/Arg), and 437 (Met/Lys).

Alkylation of human erythrocyte G6PD with pyridoxal phosphate results in 80% loss of activity and modification of Lys-205 (31). Inclusion of the substrate Glc-6-*P* during the alkylation prevents the loss of activity, suggesting that Lys-205 is close to the substrate binding site (31). Another segment of identical residues, although requiring a 100-residue deletion in the microsomal enzyme sequence, is observed at residues 436–461. The function of the identical residues in this segment is not known. Segments of residues identical to the cytosolic enzymes are not evident in the C-terminal 300 residues. The mammalian cytosolic enzymes are uncompetitively inhibited by specific 17- and 20-keto steroids with respect to both NADP and Glc-6-*P* (32). In contrast, the microsomal enzyme is not inhibited by steroids. The microsomal enzyme can utilize both NAD and NADP, as can the enzymes from *L. mesenteriodes* and *Z. mobilis*, whereas the human cytosolic and *E. coli* enzymes are active only with NADP (32). In the microsomal enzyme, two

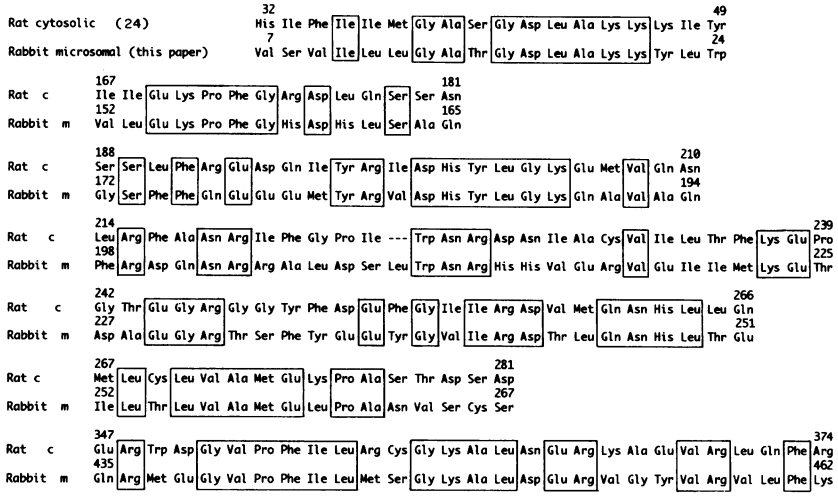

FIG. 4. Comparison of amino acid sequences of cytosolic and microsomal G6PDs. The sequence of rat cytosolic enzyme is from ref. 25.

segments with a Gly-Xaa-Gly-Xaa-Xaa-Gly motif, related to the coenzyme binding fold, can be identified beginning at Gly-399 and Gly-491. In the rat cytosolic enzyme, a motif Gly-Xaa-Gly-Xaa-Xaa-Ala/Gly occurs beginning at residues 37 and 241.

On the whole, the cytosolic and the microsomal enzymes display distinct amino acid sequences, but segments of identical residues present in the cytosolic and microsomal G6PDs are clearly evident. Probing of these residues by site-directed mutagenesis and group-specific reagents may identify and distinguish the structural and regulatory features that enable a dehydrogenase to use dual nicotinamide coenzymes and the residues involved in the steroid inhibition.

An unusual feature of the microsomal G6PD is an absence of a distinct membrane-spanning segment (Fig. 5). The only segment of appreciable hydrophobicity is around residue 260. This segment, however, is unlikely to span the membrane, since a carbohydrate moiety is present at residue 263. The native protein also lacks the C-terminal sequence of Lys-Asp-Glu-Leu present in a number of proteins bound to the lumen (34) or the His-Ile/Thr-Glu-Leu C-terminal sequences present in the esterases of the lumen of endoplasmic membrane (10, 11). The absence of sequences that are similar to the cytosolic enzymes in the 300-residue C-terminal segment of the microsomal G6PD raises questions about its function. Is this domain responsible for the retention of the protein in the lumen?

Stanton *et al.* (35) reported that epidermal growth factor or platelet-derived growth factor releases bound G6PD activity from a variety of cell types including rat renal cortical cells. These results are consistent with previous reports that a significant fraction of G6PD activity is bound to a structural intracellular element (36, 37). Further work is needed to determine whether the bound form may be the cytosolic or the lumenal enzyme.

Great progress has been made in the understanding of the microsomal electron transport enzymes involved in drug metabolism, chemical carcinogens, steroid metabolism, and fatty acid desaturation. The known NADPH/NADH-utilizing reductases and the flavin-containing monooxygenases have their catalytic domains oriented to the cytosolic side of the membrane (8, 30, 38). The NADPH for these reactions is likely provided by the cytosolic G6PD. The existence of an active NADPH/NADH-generating system in the lumen of the microsomal membrane implies that it may be involved in reductive enzymatic reactions occurring on the lumenal side of the microsomal membrane. Further studies will be necessary to identify the reductases that are present on the lumenal side of the microsomal membrane. The lumenal G6PD might also play a role in supplying NADPH/NADH to the cytosol, particularly to the reductases involved in the steroid synthesis and metabolism, the substrates and products that are inhibitory to the cytosolic G6PD. The mechanism for tunneling reducing equivalents from the lumen to the cytosol at the present is unknown. The structural features responsible for directing this enzyme to the lumen as well as the signals responsible for its retention in the lumen of the endoplasmic membrane are yet to be elucidated.

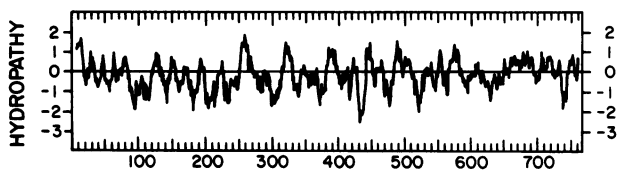

FIG. 5. Hydrophobicity plot of the liver microsomal G6PD. The hydrophobicity plot was determined by the Kyte–Doolittle method (33).

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