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Characterization studies of glucose dehydrogenase[1]

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Summary. Porcine liver β-D-glucose dehydrogenase has been isolated using Triton X-114 to release it from the endoplasmic reticulum. The purified enzyme contains a limited amount (1.7%) of lipid material, including cholesterol, fatty acids, mono and diglycerides, phosphatidylcholine, phosphatidylethanolamine, and cholesterol esters. This enzyme is a tetrameric protein containing an extensive number of hydrophobic residues. This form of glucose dehydrogenase is capable of turning over both β-D-glucose and α-D-glucose-6-phosphate in vivo as indicated from a steady state kinetic analysis at 37 °C.

Glucose dehydrogenase (E.C.1.1.1.47) is a membrane bound protein that catalyzes the oxidation of β-D-glucose and α-D-glucose-6-phosphate to their corresponding 1,5-gluconolactones along with the simultaneous reduction of NAD and NADP, respectively[2]. This enzyme is reported to be located on the luminal side of the endoplasmic reticulum, as is glucose-6-phosphatase[3].

In this work, we report the results of a quantitative lipid and amino acid analysis of the purified enzyme. These results, together with other evidence suggest that this hydrophobic protein may not be limited to the cisternal side of the endoplasmic reticulum. Furthermore, a steady-state kinetic study indicates the ability of this enzyme to function in 2 catabolic pathways simultaneously. This latter result is consistent with glucose dehydrogenase providing NADH and NADPH for the microsomal electron transport systems[4,5].

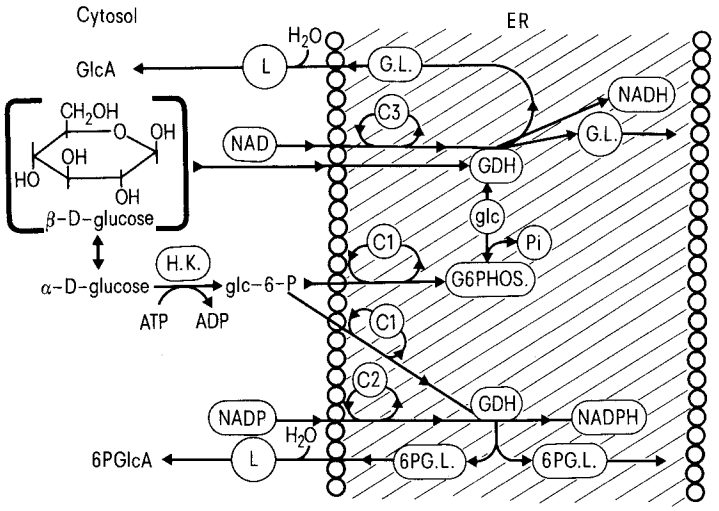
Materials and methods. Glucose dehydrogenase was purified by the method of Campbell et al.[2], with the following modifications. Triton X-114 was substituted for Triton X-100 in view of its ability to solubilize membrane proteins and its convenient separation from these proteins[1]. Finally, a Whatman SE-53 cation exchange column, previously equilibrated with 5 mM pH 6.0 phosphate buffer, was used prior to the DEAE column. The enzyme was eluted from the SE-53 column with 5 mM pH 7.0 phosphate buffer, added to the DEAE column, and eluted with 50 mM pH 7.0 phosphate buffer.

Spectrophotometric assays were made at pH 7.5 in 0.05 M tris/HCl buffer and at pH 10.0 in 0.05 M glycine/NaOH buffer. All buffers were 1 mM in EDTA and 10^{-4} M in dithioerythritol. Absorbance readings at 340 nm were recorded after initiation of the reaction by addition of the sugar solution.

Total lipid extracts were made with 2:1 chloroform-methanol. 20 vols of 2:1 chloroform-methanol were added to the sample, mixed well and allowed to stand for 30 min. The sample was then centrifuged for 20 min at 12,000 × g. The organic layer containing lipid material was removed and evaporated with dry nitrogen. The various lipids were separated by TLC using Adsorbisil plates in a chloroform/methanol/acetic acid/water (75:45:12:6) mixture and silica gel G plates in a hexane/ethyl ether/acetic acid (80:20:1) solvent mixture. The plates were charred and scanned on a Helena Quick Scan Densitometer with cholesterol acetate incorporated as an internal standard.

Hydrolysis for amino acid analysis was carried out in vacuo at 110 °C in 6 N HCl with 0.2% (v/v) 2-mercaptoethanol for 24, 48, 72, and 96 h. Amino acid analyses were determined on a Dionex D-300 amino acid analyzer with a ninhydrin detection system. Threonine, serine and tryptophan values were obtained by extrapolation to zero time. Cysteine was determined separately[7] as was tyrosine[8].

Sodium dodecyl sulfate electrophoresis[9] was carried out on 12% gels using Coomassie brilliant blue R as a stain. Standards used included bovine serum albumin (67,200), catalase (60,000) and mol.wt markers (14,300, 28,600, 42,900, 57,200 and 71,500) furnished by the Gallard-Schlesinger Chemical Corp. SDS electrophoresis of glucose dehydrogenase gives a single subunit band (not shown) of mol.wt 58,000 (±1100). In view of the previously determined mol.wt of 235,000[10], porcine liver glucose dehydrogenase is apparently a tetrameric protein consisting of similar subunits.



Summary of glucose dehydrogenase (GDH) activity in the endoplasmic reticulum (ER). L, gluconolactonase; G.L., 1,5-gluconolactone; 6PG.L., 6-phospho,1,5-gluconolactone; H.K., hexokinase; G6PHOS., glucose-6-phosphatase, C1, C2 and C3, carrier molecules.

The steady-state kinetic data were analyzed using the program POLFIT[2]. A minimum of 50 data points were used in each kinetic study for a given pair of substrates. All data points (duplicate sets) were given equal weight after they were observed to fall within a 5% error limit during a test of linearity[11,12].

$$\frac{1}{v} = \frac{K_{ia}K_b}{V_1AB} + \frac{K_b}{V_1B} + \frac{K_a}{V_1A} + \frac{1}{V_1} \quad (1)$$

Results and discussion. Table 1 contains typical results of the various column chromatographic separations. Similar results have been obtained for more than ten different samples obtained from both male and female animals. The apparent purity of the final enzyme sample was first indicated by the presence of a single protein band observed during acrylamide gel electrophoresis of a 4 µg sample on a 4% gel at pH 8.9. Finally, a single band was observed during SDS electrophoresis at pH 6.6 as described in 'Materials and methods'.

The results of a lipid analysis of glucose dehydrogenase by quantitative TLC is given in table 2. The presence of phosphatidyl ethanolamine is unexpected, as this lipid is generally associated with the cytoplasmic side of the endoplasmic reticulum[13]. This observation conflicts with conclusions drawn from a study with rat liver glucose dehydrogenase[2], in which it was concluded that this enzyme is located on the luminal side of the endoplasmic reticulum. In view of the fact that gluconolactonase is a cytosolic protein[14-16], it appears likely that some glucose dehydrogenase is either located on the cytoplasmic side of the endoplasmic reticulum or vectorially oriented such that 1,5-gluconolactone is ultimately released into the cytosol.

Table 3 contains the results of the amino acid analysis. The mol.wt calculated from this table is 222,400. Adding to this total is the lipid material given in table 2 equal to approximately 4000, giving a mol.wt of approximately 226,400. The value compares favorably with the subunit analysis of 58,000, previously mentioned. As is indicated in table 3, this enzyme contains in excess of 41% hydrophobic residues, a result that is consistent with its location as a membrane bound protein.

The pH vs velocity curves for the purified enzyme gave a maximum at pH 10 at 37 °C in 50 mM glycine buffer, regardless of substrate or coenzyme used. This led to a steady state kinetic analysis at 37 °C, the results of which are given in table 4. The coenzyme dissociation constants (K_{ia}) and the K_m (K_b) values are considerably lower than those obtained previously for the enzyme isolated with an acetone powder method[12]. Finally, both the K_{ia} and K_b values are considerably lower than their respective cellular concentration levels[17].

The kinetic constants obtained from the steady-state experiments are interesting from several perspectives. For the first time, porcine liver glucose dehydrogenase has been isolated in a form whose affinity for all substrates is high enough to indicate that it is catalytically active in vivo. Furthermore, only specific combinations of substrates and cofactors appear to lead to efficient reactions in vivo. From table 4, it is evident that K_b for β-D-glucose is less than the in vivo concentration of β-D-glucose (7.1 mM) only when NAD is the cofactor for the reaction. Glucose-6-phosphate is only oxidized by NADP, and the K_b for this substrate is again less than the physiological concentration (200 µM) of the compound. Thus we have the interesting implied rela-

Table 1. Purification scheme and results

Step	Activity (units/ml)	Protein (mg/ml)	Volume (ml)	Specific activity (units/mg)	Overall purification
1. Homogenization, Triton X-114, acid treatment, and centrifugation	1.12	57.2	213	0.0198	–
2. Ammonium sulfate fractionation	2.17	99.0	65	0.0219	1.1
3. Sephacryl S-200	0.29	5.40	416	0.0531	2.7
4. Sephacryl S-300	0.26	0.82	137	0.318	16.2
5. SE cellulose	0.25	0.59	78	0.417	21.3
6. DEAE cellulose	0.45	0.126	39	3.57	182

1 unit of activity is defined as the amount of enzyme required to produce 1 µmole of NADH/min at pH 10, 37 °C.

Table 2. Lipid analysis by quantitative TLC

Lipid[a]	µg lipid/mg GDH	moles lipid/mole GDH
Cholesterol (387)	1.30	0.79
Fatty acids (262)	2.38	2.13
Diglycerides (580)	1.73	0.70
Monoglycerides (336)	2.72	1.90
Phosphatidyl choline (745)	2.17	0.68
Phosphatidyl ethanolamine (702)	5.58	1.87
Cholesterol esters (631)	1.06	0.39

[a] Figures in parentheses are average molecular weights.

Table 3. Amino acid composition of glucose dehydrogenase

Amino acid	Residues per mole of enzyme
Asx	211 (asp + asn)
Thr	137
Ser	175
Glx	243 (glu + gln)
Pro	46
Gly	247
Ala	348
Val	142
Met	56
Ile	73
Leu	155
Tyr	48
Phe	61
Lys	52
His	21
Arg	21
Trp	66
Cys	52
NH_3	337 (asp + gln)

Table 4. Kinetic constants

Temperature, pH Substrates	K_{ia} (μM)	K_a (μM)	K_b	V_l (μM/min)
37 °C, 7.5				
Glc-6-P, NADP	6.93 ± 0.98	2.09 ± 0.14	3.59 μM ± 0.26	1.03 ± 0.08
Glc, NADP	4.89 ± 0.35	10.62 ± 0.63	12.75 mM ± 0.77	7.46 ± 0.54
Glc, NAD	2.42 ± 0.17	2.43 ± 0.19	5.06 mM ± 0.46	1.61 ± 0.12
37 °C, 10.00				
Glc-6-P, NADP	4.66 ± 0.47	7.17 ± 0.23	61.7 μM ± 2.5	2.51 ± 0.25
Glc, NADP	5.45 ± 0.21	32.0 ± 1.6	28.4 mM ± 1.4	11.00 ± 0.43
Glc, NAD	6.21 ± 0.40	3.17 ± 0.32	4.56 mM ± 0.41	1.26 ± 0.08

E_o is 0.027 μM. NAD, NADP varied 1–50 μM.

tionship that the enzyme catalyzes reactions between mutually phosphorylated or non-phosphorylated substrates and cofactors without significant cross-reaction in vivo.

This relationship appears to hold independent of pH at 37 °C, and suggests that NADP and NAD bind to different subunits. Thus, the oxidation of glucose and glucose-6-phosphate would be catalyzed by distinct subunits, a result consistent with the total lack of cooperativity displayed by this enzyme[2,10,12].

If we temporarily ignore the questions of metabolite penetration of the endoplasmic reticulum and of product inhibition, it is possible to approximate the relative concentration levels of the various enzyme-substrate complexes by the relationship below[2]

$$[\text{Complex}] \propto \frac{[\text{Sugar}]\,[\text{cofactor}]\,[\text{E}]}{K_{ia}\,K_b} \qquad (2)$$

in which one calculates the relative number of enzyme molecules in each of the three possible complexes, [E-NAD-glucose], [E-NADP-glucose], and [E-NADP-glucose-6-phosphate]. Using the rat liver physiological concentrations[17] of NAD = 760 μM, NADP = 76 μM, glucose = 7.1 mM, and glucose-6-phosphate = 200 μM, one arrives at the relative amounts of 441 [E], 8[E], and 539 [E] for the glucose-NAD, glucose-NADP and glucose-6-phosphate-NADP containing complexes, respectively. This calculation suggests that at 37 °C and pH 7.5, glucose dehydrogenase is 45% occupied with glucose and NAD; the remaining enzyme being occupied almost completely by glucose-6-phosphate and NADP. At pH 10.0 and 37 °C, values of 191 [E], 3 [E], and 47 [E] are determined for the same 3 complexes.

In view of these results and the fact that both glucose dehydrogenase and glucose-6-phosphatase are located in the endoplasmic reticulum, it is possible to postulate a model of glucose dehydrogenase's role in sugar metabolism. Unfortunately, there is the question of metabolite penetration into the endoplasmic reticulum, which is presently unresolved. Nilsson et al.[18] determined that glucose and sucrose freely penetrate the microsomes, whereas glucose-6-phosphate and other charged substrates only weakly diffuse into the endoplasmic reticulum; results with NAD and NADP were less definitive. In a study of rat liver glucose dehydrogenase[3], it was concluded that the microsomal membrane is freely permeable to glucose-6-phosphate and only weakly permeable to NADP. Despite these somewhat conflicting reports, and the presence of complicated product inhibition relationships, it is still possible and useful to consider a general model of this enzyme's functional role in the endoplasmic reticulum, as shown in the figure.

We will assume that both glucose and glucose-6-phosphate penetrate the endoplasmic reticulum and that glucose-6-phosphate crosses the membrane via a transport system[19,20].

We will assume that glucose dehydrogenase and glucose-6-phosphatase work together to maintain a certain ratio of glucose to glucose-6-phosphate and that this ratio will be controlled by the levels of both NADH and NADPH or vice-versa. Until the levels of various metabolites in the endoplasmic reticulum are known, determination of this ratio will be approximate, at best.

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