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URIDINE DIPHOSPHATE GLUCOSE PYROPHOSPHORYLASE: DIFFERENTIAL HEAT INACTIVATION AND FURTHER CHARACTERIZATION OF HUMAN LIVER ENZYME

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SUMMARY

UDPglucose pyrophosphorylase (EC 2.7.7.9) crystallized from human liver will catalyze the biosynthesis of nucleoside diphosphate sugars, including UDP-glucose, UDPgalactose, and UDPxylose.

$$NTP + Sugar\text{-}1\text{-}P \rightleftharpoons NDP\text{-}sugar + PP_i$$

When the crystalline enzyme is heated in solution, activity for the three substrates is differentially decreased with time. Physical and chemical measurements provide evidence for the homogeneity of the enzyme and suggest an octomeric structure with a subunit molecular weight of 56 000. Separately both UDPglucose and UDPgalactose bind to the enzyme in a molar ratio of 8:1. UDPgalactose bound to the enzyme is displaced stoichiopmetrically by UDPglucose. These findings are discussed in relation to a pyrophosphorylase pathway of metabolism in the galactosemic.

INTRODUCTION

Patients with galactosemia have the capacity to metabolize galactose-1-*P* despite the absence of galactose-1-*P* uridylyl transferase. UDPgalactose pyrophosphorylase (EC 2.7.7.10) activity provides an alternative pathway of galactose metabolism in human tissue.

$$Gal\text{-}1\text{-}P + UTP \xrightleftharpoons{\text{pyrophosphorylase}} UDPGal + PP_i \quad (1)$$

$$UDPGal \xrightleftharpoons{\text{epimerase}} UDPGlc \quad (2)$$

$$UDPGlc + PP_i \xrightleftharpoons{\text{pyrophosphorylase}} Glc\text{-}1\text{-}P + UTP \quad (3)$$

$$Gal\text{-}1\text{-}P \rightleftharpoons Glc\text{-}1\text{-}P \quad \text{Sum}$$

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Reaction 1 has been reported in microbes [1, 2] rats, pigeons and humans [3, 4].

UDPglucose pyrophosphorylase (EC 2.7.7.9) is ubiquitous in nature and as purified and crystallized from human liver is relatively non-specific with regard to substrates. With the crystalline enzyme, the pyrophosphorolysis of UDPgalactose is catalyzed at more than 10% of the rate of UDPglucose [5]. During purification of this enzyme, the ratio of activities towards the glucose and galactose containing substrates remains constant, suggesting a high degree of similarity of the enzymes or that both reactions are catalyzed by the same protein [5, 6]. In humans the presence of a distinct UDPgalactose pyrophosphorylase as has been suggested [3] thus becomes questionable.

The existence of UDPgalactose pyrophosphorylase activity in cultured human skin fibroblasts has been reported [7]. While chemical and physical evidence indicated only one protein for both UDPglucose and UDPgalactose activities, thermal stability experiments suggested two different enzymes. When an extract of the tissue was heated at 50 and 56 °C, UDPgalactose activity decreased more rapidly than UDPglucose activity. On this basis it was concluded that two pyrophosphorylases were present in the extract, one specific for glucose and another for galactose containing substrates. As mounting evidence continued to indicate a single non-specific pyrophosphorylase for both substrates, it was of interest to measure thermal inactivation of crystalline enzyme purified from human liver. Further characterization of the human pyrophosphorylase and implications for the metabolism of galactose in the galactosemic is the subject of this report.

MATERIALS AND METHODS

Reagents and purification

UDPglucose pyrophosphorylase was purified and crystallized from human liver according to the procedure of Knop and Hansen [5]. All other chemicals and enzymes were obtained from commercial sources.

Heat inactivation

Solutions of enzyme (0.01–0.1 mg protein/ml in 0.1 M Tris buffer, pH 7.8) were incubated in a water bath at 50 or 56 °C. Aliquots were removed at 0, 5, 10 and 15 min and placed in an ice bath. The aliquots were assayed for UDPglucose, UDPgalactose, and UDPxylose pyrophosphorolysis using the procedure of Verachtert et al. [8] substituting mercaptoethanol for hydrazine. The presence of mercaptoethanol in the incubation mixture had no effect on the results. Ten replications of the experiment were conducted at each temperature and the standard error calculated.

Column chromatography

After incubation, the enzyme–UDPglucose mixture was layered on a Sephadex G-25-80 column (1 cm × 28 cm) and eluted with Tricine buffer. 1 ml samples of eluate were collected and assayed for enzyme activity. 50-μl aliquots were placed in 15 ml of Aquasol in polyethylene vials and counted to 1% error on a Beckman CPM 100 liquid scintillation counter.

Equilibrium dialysis

Dialysis cells using silicone rubber sheeting were made using the procedure of

Reitz and Riley [9]. Half ml cells were routinely used. The equilibrium was monitored by removing 5 μl aliquots and measuring the radioactivity. For estimating substrate concentration in the absence of binding, the enzyme was heat or solvent inactivated and the UDPgalactose equilibrium was measured.

Gel electrophoresis

Gel electrophoresis in the presence of sodium dodecylsulfate was performed according to the procedure of Shapiro et al. [10]. Trypsin, ovalbumin, bovine serum albumin, catalase and glucose oxidase were used as protein standards.

Amino acid analysis

Amino acid analysis on lyophilized enzyme was performed according to the procedure of Zumwalt et al. [11] by the Analytical Biochemistry Laboratory at Columbia, Mo.

Peptide mapping

Lyophilized enzyme was digested with TPCK–trypsin and fingerprinted using the procedures of Ingram [12] and Katz et al. [13]. Peptides and amino acids were visualized with a multiple dipping technique using ninhydrin, Ehrlich's stain, and Sakaguchi reagent [14].

Electron microscopy

Enzyme (0.05 mg/ml in 0.01 M Tricine buffer) was placed on carbon-coated 400 mesh copper grids. The grid was blotted dry after 5 min and negatively stained with 2% potassium phosphotungstate for 1–5 min. The grids were examined with a JEM 100 B3 electron microscope.

Inhibition constants

Inhibition of UDPglucose pyrophosphorolysis by UDPgalactose was determined using assay I of Albrecht et al. [15]. The inhibition constant was calculated according to Dixon and Webb [16].

RESULTS AND DISCUSSION

It is evident from Fig. 1 that heating the solutions of the crystalline enzyme at both 50 and 56 °C, the activity for UDPgalactose pyrophosphorolysis disappeared more rapidly than for UDPglucose pyrophosphorolysis. The decline in activity relative to the two substrates is significantly different as shown by the standard deviations. With only slight variation the results are qualitatively similar to those reported for skin fibroblast homogenates [7]. When UDPxylose is substrate for the enzyme, the heat inactivation rate was intermediate between that of UDPglucose and UDPgalactose.

Thus, the question of one, two or even three UDP-sugar pyrophosphorylases in the crystalline enzyme hinges on its purity. Evidence of homogeneity based on polyacrylamide gel electrophoresis, sucrose density gradient centrifugation and ultracentrifugation [5] has consequently been reviewed and extended.

When the purified enzyme from human liver was subjected to electrophoresis in the presence of sodium dodecylsulfate, only a single protein band could be observed,

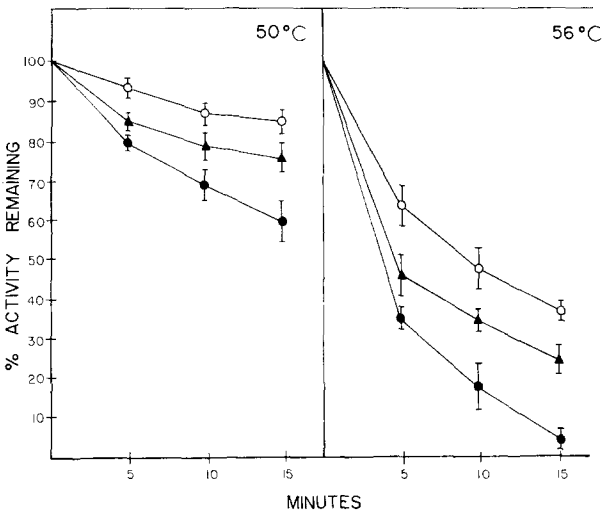

Fig. 1. Heat inactivation of solutions of UDPglucose pyrophosphorylase at 50 and 56 °C showing loss of UDPglucose (○), UDPxylose (▲), and UDPgalactose (●) pyrophosphorolysis activity. The vertical lines indicate standard deviations.

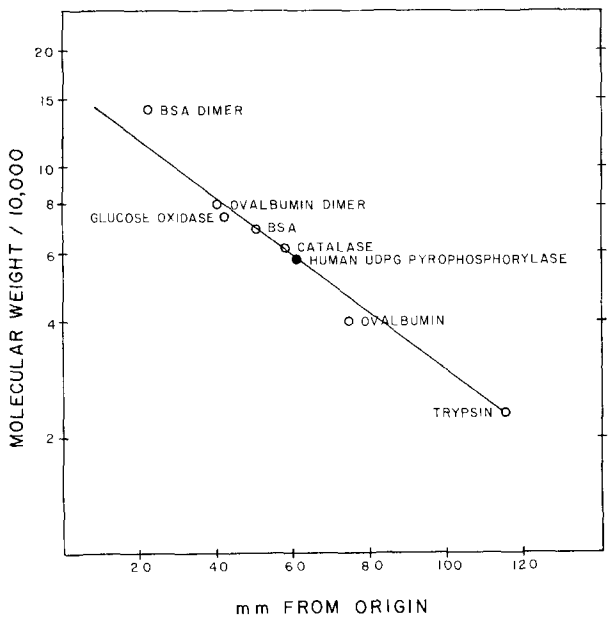

Fig. 2. Migration of UDPglucose pyrophosphorylase on polyacrylamide-sodium dodecylsulfate gel. Electrophoresis progressed with a gel concentration of 5% through a path of 12 cm at 8 V/cm for 135 min. Protein was stained with Coomassie blue. BSA, bovine serum albumin. UDPG, UDP-glucose.

even when the gel was heavily loaded. From comparative analysis (Fig. 2), the molecular weight was estimated at about 56 000, which agrees with the molecular weight of human liver enzyme determined by sedimentation [5] and that isolated from blood [17], assuming the molecule to be an octomer. However, the electron micrographs (Fig. 3) show a tetrametric structure; like the calf liver enzyme [18], the molecule

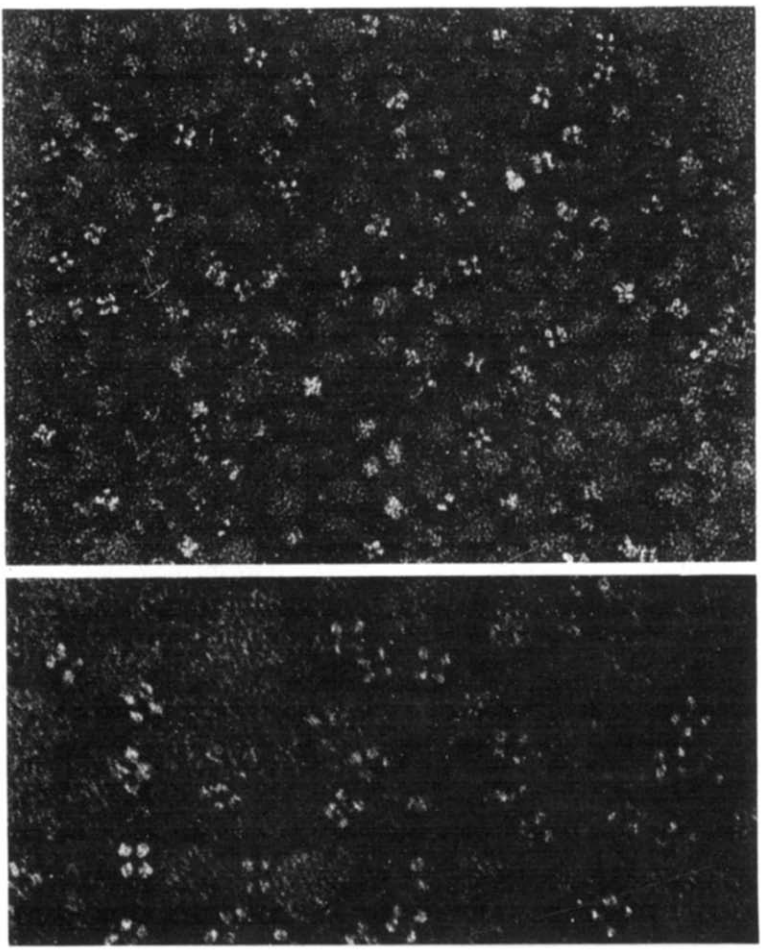

Fig. 3. Electron micrograph of negatively stained UDPglucose pyrophosphorylase (top × 270 000 and bottom × 440 000).

probably consists of two superimposed tetramers, only one of which is visible in the micrographs.

An octameric molecule is also consistent with the results of quantitative substrate binding experiments. The human enzyme binds 8 moles of UDP-glucose for each mole of protein or one mole of substrate per subunit (Fig. 4). UDPgalactose is also bound to human enzyme in the same ratio of 8:1 (Fig. 5), and can be totally displaced by the addition of UDPglucose, indicating that the enzyme, while binding both

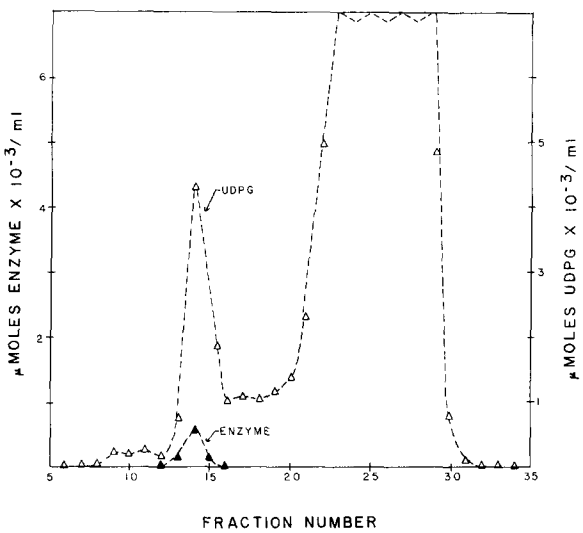

Fig. 4. Elution pattern of UDPglucose and UDPglucose pyrophosphorylase from a Sephadex G-25-80 column. Approx. 0.5 mg of enzyme was incubated for 15 min at 32 °C in 0.6 ml of Tricine buffer (0.01 M, pH 8) containing 0.1 μmole UDPglucose, 0.015 μmole glucose-1-[^{3}H]UDP-glucose (4850 μCi/μmole) and 0.2 μmole Mg^{2+}. Elution was with the same Tricine buffer. The molar ratio of substrate to enzyme was 8:1. UDPG, UDPglucose.

substrates in equal amounts, has a greater affinity for UDPglucose. This displacement is consistent with the K_m of human pyrophosphorylase for UDPglucose which is an order of magnitude less than that for the galactose-containing substrate [5]. Thus if a galactose pyrophosphorylase were present as a contaminant, of necessity, it would have a higher K_m for UDPglucose than for UDPgalactose.

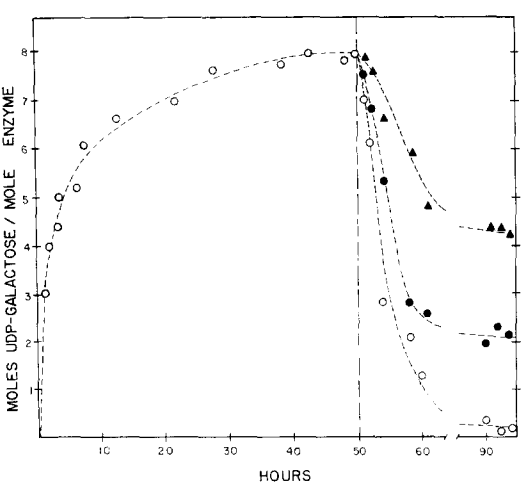

Fig. 5. Equilibrium dialysis of UDPgalactose with enzyme. Both cells were made to volume with $1 \cdot 10^{-4}$ M UDPgalactose, 2 mM Mg^{2+} and 2 nM [^{14}C]UDPgalactose in Tricine buffer. Approx. 2 mg of human UDPglucose pyrophosphorylase were added to one of the cells. At 50 h, UDPglucose [$1 \cdot 10^{-5}$ M (▲), $1 \cdot 10^{-4}$ M (●) and $2 \cdot 10^{-4}$ M (○)] were added to the equilibrium mixture.

Amino acid analysis with peptide mapping gives corroborative evidence for the purity of the crystalline enzyme. Based on a molecular weight of 450 000 (8 × 56 000), 280 lysine and 137 arginine residues were found per molecule. Assuming 8 identical subunits and no repeating peptides, the 54 peptides found after tryptic digestion (Fig. 6) is in agreement with the 53 predicted from the amino acid analysis.

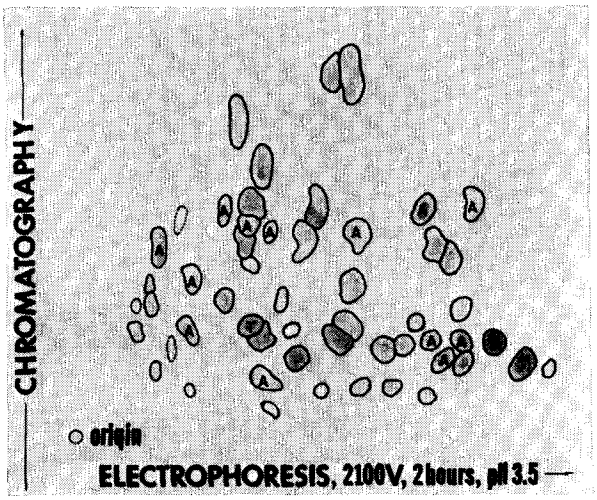

Fig. 6. Fingerprint of enzyme digested with trypsin. A single tryptophan peptide is indicated by the letter T and arginine peptides by the letter A. Dashed lines indicate spots lightly staining with ninhydrin.

14–16 arginine spots were observed, which is in close agreement with the 17 predicted. In both cases, if a contaminating protein were present, one would expect greater disagreement between the predicted number of peptides and those observed.

The purified human liver UDPglucose pyrophosphorylase, thus, cannot be shown to contain significant quantities of contaminating protein. If a UDPgalactose pyrophosphorylase were present, it not only fractionates and crystallizes identically with UDPglucose pyrophosphorylase, but probably has the same molecular weight, identical substructure and electrophoretic behavior. Furthermore, in consideration of the heat inactivation rate with UDPxylose as a substrate, can it be assumed that there are three enzymes with indistinguishable properties? It seems more probable that differential heat inactivation rates toward similar but different substrates may not be adequate demonstration of the presence of multiple enzymes.

That one enzyme may show differential heat stability toward different substrate is of some interest. Since UDPgalactose inhibits UDPglucose pyrophosphorolysis competitively (Fig. 7), both substrates probably compete for the same active site on the enzyme. It remains to be explained how heat inactivation of an enzyme differentially affects the catalytic rate towards various substrates presumably metabolized at the same site on a single protein. This phenomenon is not without a precedent as fructose diphosphatase from rabbit liver behaves similarly in response to urea or dinitrofluorobenzene inactivation with respect to two competing substrates, fructose-1,6-di-*P* and sedoheptulose-1,7-di-*P* [19].

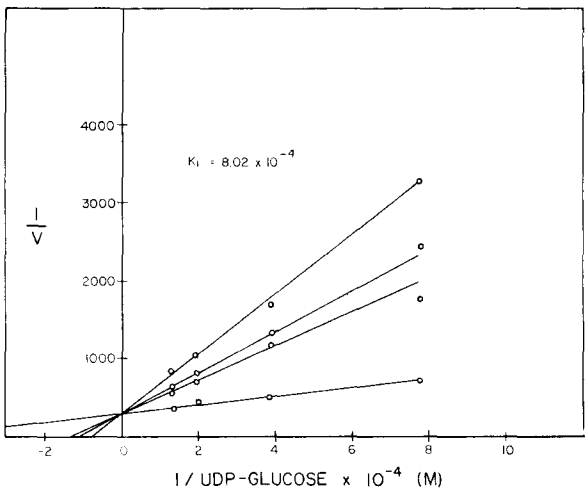

Fig. 7. Lineweaver–Burk plot of UDPgalactose inhibition of UDPglucose pyrophosphorolysis. UDPgalactose concentrations were 0, $2 \cdot 10^{-3}$, $3.2 \cdot 10^{-3}$ and $4 \cdot 10^{-4}$ M. UDPglucose concentrations were $1.3 \cdot 10^{-5}$, $2.6 \cdot 10^{-5}$, $5.2 \cdot 10^{-5}$ and $7.8 \cdot 10^{-5}$ M.

The availability of crystalline pyrophosphorylase from human liver clarifies the understanding of the pathway of galactose metabolism in galactosemics. Antibodies, for purified UDPglucose pyrophosphoryleas, simultaneously precipitate both UDP-glucose and UDPgalactose activities from human red blood cells, consistent with a single antigen protein being responsible for both activities. Antibodies specific for either human or calf liver UDPglucose pyrophosphorylases will prevent galactose-1-*P* metabolism in red blood cells from the galactosemic [20]. Considering the new criteria of the purity of the antibody-inducing enzyme and the binding ratios for the substrates, UDPglucose pyrophosphorylase is most probably responsible for UDPgalactose metabolism in galactosemics.

ACKNOWLEDGMENT

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