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A COMPARATIVE STUDY OF NUCLEAR AND MICROSOMAL UDP-GLYCURONIC ACID PYROPHOSPHATASE OF RAT LIVER

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SUMMARY

1. The uridine 5′-diphosphoglucuronic acid (UDPGlcUA) pyrophosphatase isolated from the nuclei and microsomes of rat liver were subjected to the same purification procedure. This included the solubilization of the respective cellular fractions obtained by differential centrifugation with Triton X-100, followed by fractionation by column chromatography using DEAE-cellulose and Sepharose 4B.

2. The nuclear and microsomal enzymes were found to be similar in many respects. Their elution patterns from both columns were identical. The partially purified enzymes exhibited a pH optimum of 9.4 and were inhibited by various nucleotides and EDTA. Their affinities for UDPGlcUA were of the same order of magnitude, the apparent K_m values being 0.40 and 0.25 mM, respectively. NAD^+, NADH and $NADP^+$ were also hydrolyzed by these enzymes and the ratios of pyrophosphatase activity of the nuclear to microsomal fractions on these nucleotides were the same as that obtained with UDPGlcUA as substrate.

3. It was concluded that the nuclear and microsomal pyrophosphatase are one and the same enzyme, and that it is a plasma membrane enzyme because of its bimodal distribution.

INTRODUCTION

The hydrolysis of uridine 5′-diphosphoglucuronic acid (UDPGlcUA) at the pyrophosphate bond has been observed in rat kidney [1, 2], skin [3] and liver [2]. It was thought that this reaction, catalyzed by UDPGlcUA pyrophosphatase may be important in L-ascorbic acid biosynthesis [2] and that its high activity in rat liver homogenate could be responsible for the low transglucuronidation in vitro observed in this animal [4, 5]. We have demonstrated that there was indeed an inverse relationship between the hepatic activity of the UDPGlcUA pyrophosphatase and glucuronyl transferase measured in liver homogenates of four species of animals [6]. Our preliminary attempts to fractionate this enzyme from liver homogenate showed that pyro-

Abbreviations: UDPGlcUA, uridine 5′-diphosphoglucuronic acid; UDPGlcNAc, uridine 5′-diphospho-*N*-acetylglucosamine; UDPGlc, uridine 5′-diphosphoglucose; UDPGal, uridine 5′-diphosphogalactose; GDPGlc, guanosine 5′-diphosphoglucose; CDPGlc, cytidine 5′-diphosphoglucose.

phosphatase activity was present in both the nuclear and microsomal fractions. Previously, Schliselfeld et al. [7] had suggested that the nuclear enzyme could be a precursor of the microsomal enzyme. Ogawa and his co-workers [8], however, contended that the microsomal enzyme is different from the nuclear enzyme; this was based on their anomalous metal ion requirement and substrate specificity. In this study, both the nuclear and microsomal enzymes were purified and their characteristics compared. From kinetic and inhibition studies performed on the partially purified UDPGlcUA pyrophosphatase obtained from these two cellular fractions, it was concluded that they could be one and the same enzyme, possibly of plasma membrane origin.

MATERIALS

UDPGlcUA (triammonium salt), uridine 5′-diphosphoglucose (UDPGlc) (sodium salt), uridine 5′-diphospho-*N*-acetylglucosamine (UDPGlcNAc) (sodium salt), guanosine 5′-diphosphoglucose (GDPGlc) (disodium salt), cytidine 5′-diphosphoglucose (CDPGlc) (disodium salt), NAD^+, NADH (disodium salt), $NADP^+$ (monosodium salt), NADPH (disodium salt), ATP (disodium salt), ADP (barium salt), UDP (sodium salt), UMP (disodium salt), uridine, glucuronic acid, glucuronic acid-1-*P*, Triton X-100, glucose-6-phosphate dehydrogenase, type XII (Torula Yeast, 180 units/mg protein) and alcohol dehydrogenase (yeast, 306 units/mg protein) were obtained from Sigma Chem. Co., St. Louis, Mo., U.S.A.; harmol·HCl from Fluka, A.G., Buchs, Switzerland; cellulose MN-300 for thin-layer chromatography from Macherey, Nagel and Co., Germany; DEAE-cellulose (Whatman DE 32, microgranular) from W. and R. Balston Ltd, England; Sepharose 4B from Pharmacia, Uppsala, Sweden and Aquacide from Calbiochem., U.S.A.

METHODS

Cell fractionation

Sprague–Dawley rats were used in the preparation of enzyme. A 10% liver homogenate was prepared by homogenizing the tissue in 9 vol. of 0.25 M sucrose solution for 1 min with a Teflon pestle. The fractionation procedure of Hogeboom [9] was followed with one modification: the microsomal fraction was subjected to centrifugation at 105 000 × *g* for 60 min in the Spinco Model L ultracentrifuge.

Purification procedure

The particulate fractions (nuclear and microsomal) were solubilized by the procedure of Miller [10]. The microsomal and nuclear pellets, obtained from 5 g fresh wt liver, were homogenized at 4 °C in 100 ml 0.01 M Tris–HCl buffer (pH 7.0) containing 0.3% (v/v) Triton X-100. After centrifugation at 105 000 × *g* for 60 min, the solubilized extract was applied on to a DEAE-cellulose column (25 cm × 1.5 cm) which had previously been equilibrated with the same Tris–HCl buffer. Elution was carried out with 0.5 M NaCl and 0.5% (v/v) Triton X-100 in Tris–HCl buffer.

The active fractions were concentrated to 2 ml with aquacide and this was next applied on to a column (80 cm × 1.5 cm) containing Sepharose 4B. Pre-equilibration was carried out as before. The enzyme was eluted with Tris–HCl buffer.

Assay conditions for nucleotide pyrophosphatase

With UDPGLcUA as substrate. The fluorimetric procedure of Wong and Lau [6] was employed with slight modifications. The reaction mixture contained 100 μg UDPGlcUA (0.14 mM), 800 μl 0.08 M veronal buffer (pH 9.4) and 100 μl of enzyme solution as prepared above, in a total vol. of 1.0 ml. Incubation was carried out for 10 min at 37 °C. The reaction was stopped by boiling for 2 min in a water-bath, and the denatured protein removed by centrifugation. An aliquot of 250 μl of the supernatant was carried over for the estimation of the unreacted UDPGlcUA. For the subsequent transglucuronidation reaction, the procedure of Wong and Sourkes [11] was followed, but harmol, instead of harmalol was used as the aglycone (for details of the fluorimetric analysis of harmol glucuronide, see ref. 12).

With NAD$^+$ and NADH as substrate. The reaction with NAD$^+$ and NADH as substrates were assayed by the reversible alcohol dehydrogenase reaction according to the methods of Racker [13] and Jacobson and Kaplan [14], respectively.

With NADP$^+$ as substrate. The procedure of Chung and Langdon [15], using glucose-6-phosphate dehydrogenase was adopted.

Protein determination

The protein content was measured by the procedure of Lowry et al. [16].

RESULTS

Cellular distribution

A study of the cellular distribution of UDPGlcUA pyrophosphatase of rat liver showed that the enzyme resides predominantly in the microsomal and nuclear fractions in a proportion of 1.0:1.2.

Elution patterns

Under the same conditions of chro[illegible]AE-cellulose and Sepharose 4B the microsomal and nu[illegible]r en[illegible]ntially similar elu-

TABLE I

PURIFICATION OF UDPGlcUA PYROPHOSPHATASES

Specific activity is expressed as μmole: UDPGlcUA hydrolyzed/h per mg enzyme protein.

Fraction	Specific activity	Fold of purification	Total activity	Recovery (%)
Nuclear				
Particulate	2.65	0.0	129.1	100
Triton X-100 extract	4.36	1.6	144.6	112
DEAE-cellulose fractionate	7.51	2.8	85.1	66
Sepharose 4B fractionate	12.80	4.8	44.4	34
Microsomal				
Particulate	3.50	0.0	106.5	100
Triton X-100 extract	5.10	1.5	89.7	84
DEAE-cellulose fractionate	9.10	2.6	68.1	64
Sepharose 4B fractionate	14.20	4.1	34.7	33

tion patterns. In both cases, the major protein peak coincided with the peak of enzyme activity; a purification of only 4.5-fold was achieved. The activities of the two pyrophosphatases obtained at each step of purification are summarized and compared in Table I.

Kinetic studies

The two partially purified enzyme preparations were subjected to the following kinetic and inhibition studies:

Effect of pH. The pH optima for both the nuclear and microsomal enzymes were 9.4 in veronal or borax buffer of 0.032 M concentration.

Affinity for UDPGlcUA. From the Lineweaver–Burk plot [17] of Fig. 1, the apparent K_m values of the nuclear and microsomal enzymes for UDPGlcUA were 0.40 and 0.25 mM, respectively.

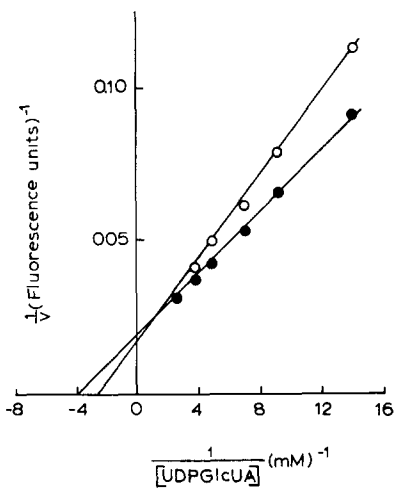

Fig. 1. Lineweaver–Burk plots of the hydrolysis of UDPGlcUA by the partially purified nuclear (○—○) and microsomal (●—●) pyrophosphatase with varying amount of substrate (50–400 μg or 0.07–0.57 mM UDPGlcUA). Standard assay procedure (see text) was employed. The reaction velocity (v) is expressed in arbitrary fluorescence units.

Substrate specificity. Apart from UDPGlcUA, the nucleotides, NAD^+, NADH and $NADP^+$ were found to be hydrolyzed by these enzyme preparations. It is interesting to note that the ratios of the pyrophosphatase activity of the microsomal to nuclear fractions range between 1.1 to 1.3 for all the above nucleotides (Table II).

Inhibition studies. The effects of the metabolites of UDPGlcUA, various nucleotides, Mg^{2+} and EDTA on the pyrophosphatase activity are shown in Table III. Where there is inhibitory action, the same degree of inhibition was exerted on both the nuclear and microsomal enzymes. The sugar catabolites of UDPGlcUA did not affect the enzymic activity; neither did Mg^{2+}, L-ascorbic acid nor nicotinamide at 0.3 mM concentration. On the other hand, uridine, UMP and the other uridine nucleotides, UDP and UDPGlc, were slightly inhibitory. The adenine nucleotides, ADP and ATP, and the reduced forms of the coenzymes NAD^+ and $NADP^+$ were strongly inhibitory. The inhibition by EDTA observed previously [6] was confirmed.

TABLE II

SUBSTRATE SPECIFICITY STUDY ON PARTIALLY PURIFIED UDPGlcUA PYROPHOSPHATASE

Pyrophosphatase activity is expressed as μmoles substrate hydrolyzed/h per mg enzyme protein.

Substrate	Concentration (mM)	Time of incubation (min)	Pyrophosphatase activity		
			Nuclear	Microsomal	Microsomal/Nuclear
NAD^+	0.3	10	7.6	8.6	1.13
		20	6.4	7.4	1.15
NADH	0.3	10	22.4	26.6	1.18
		20	16.4	21.1	1.28
$NADP^+$	0.3	10	10.2	11.6	1.13
		20	7.9	9.4	1.19
UDPGlcUA	0.29	10	19.3	20.0	1.04
					1.28*

* Figure obtained from data in Table I.

TABLE III

EFFECTS OF UDPGlcUA METABOLITES AND NUCLEOTIDES ON THE ACTIVITY OF UDPGlcUA PYROPHOSPHATASE

The concentration of UDPGlcUA in the reaction was 0.14 mM. Values given show the percentage inhibition of enzyme activity in the presence of inhibitor, the activity without inhibitor being taken as reference.

Inhibitor	Concentration (mM)	Inhibition of activity (%)		
		Nuclear*		Microsomal
Glucuronic acid-1-*P*	0.3	2	1	0
Glucuronic acid	0.3	2	1	0
Uridine	0.3	8	12	14
UMP	0.3	26	27	28
UDP	0.3	48	46	75
ADP	0.3	81	83	89
ATP	0.3	84	73	82
GDPGlc	0.3	73	—	58
CDPGlc	0.3	60	—	49
UDPGal	0.3	22	32	24
UDPGlc	0.3	17	20	18
UDPGlcNAc	0.3	13	9	13
NAD^+	0.3	59	59	68
NADH	0.3	83	79	71
$NADP^+$	0.3	39	35	51
NADPH	0.3	80	68	69
Nicotinamide	0.3	1	—	3
Nicotinamide	3.0	32	—	23
L-Ascorbic acid	0.3	0	—	0
EDTA	0.3	85	80	100
$MgCl_2$**	0.3	4	—	5
$MgCl_2$**	3.0	0	—	0

* Where two values are shown they were obtained from duplicate experiments.

** Reaction was stopped by addition of 10 mM EDTA (final concentration) as heating accelerates non-enzymic degradation of UDPGlcUA in the presence of Mg^{2+}.

DISCUSSION

The pyrophosphatases isolated from the nuclei and microsomes of rat liver were found to be identical in all parameters studied. Our kinetic and inhibition data suggested that they are one and the same enzyme. It is possibly of plasma membrane origin because of its bimodal distribution, a feature commonly observed for plasma membrane enzymes [18]. Skidmore and Trams [19] had compared the activity of the nucleotide pyrophosphatase of rat liver that acted upon UDPGlc and NADH with that of 5′-nucleotidase, a plasma membrane marker enzyme. The similar degree of purification of both enzymes indicated that the nucleotide pyrophosphatase is also a membrane enzyme. Their data on inhibition studies with NAD^+, UDPGlc and EDTA are similar to our results with these same nucleotides on UDPGlcUA pyrophosphatase.

In view of the fact that the substrates of nucleotide pyrophosphatase are important intermediary metabolites, this hydrolase had been extensively studied. This includes its action on the following nucleotides: ATP [7, 20], NAD^+ [7, 19, 21, 22], UDPGlc [7, 19, 23, 24], UDPGlcUA [1–3, 8] and UDPGlcNAc [25]. From an analysis of the data obtained on these nucleotides and from our own studies on UDPGlcUA pyrophosphatase, we are of the opinion that the same nucleotide pyrophosphatase is involved in all these reactions. The constant ratio (1.2) of pyrophosphatase activity obtained for the microsomal to nuclear enzymes with respect to four nucleotides, NAD^+, $NADP^+$, NADH and UDPGlcUA strongly supported this consensus of opinion. The functional role of this enzyme is difficult to assess in view of its non-specific nature. Its action on ATP, one of the main metabolite effectors in the liver could contribute to its participation in control mechanisms. Whether its localization on the plasma membrane is of any attribute in this regulatory capacity remains to be seen. The possibility of the degradative products of the pyrophosphoryltic split gaining ready access into the cell and therein being re-synthesized could be an important facet which has not been considered.

With regard to transglucuronidation, the presence of the nucleotide pyrophosphatase on the cell membrane made it unlikely to influence glucuronidation in vivo; the latter process occurs mainly in the endoplasmic reticulum. However, its action on transglucuronidation in vitro must be accounted for as our results showed that about half of the total activity of UDPGlcUA pyrophosphatase is present in the microsomal fraction. UDPGlcNAc at a concentration double that of UDPGlcUA present in the reaction medium inhibited the pyrophosphatase by only 13%. This suggested that its "protective" action on the conjugating agent cannot satisfactorily explain the large activation on the glucuronyl transferase normally observed. On the other hand, an average of 70–80% of the pyrophosphatase activity was inhibited by ATP and NADPH. It is conceivable that this action could be partly responsible for the stimulation of glucuronyl transferase in vitro, although it is believed that the enhancement of *p*-nitrophenol conjugation by NADPH is effected by some other mechanism which is at present unknown [26].

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