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The anionic conjugates of bilirubin and bile acids stimulate ATP hydrolysis by *S*-(dinitrophenyl)glutathione ATPase of human erythrocyte

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These studies demonstrate that bilirubin-ditaurate (an analog of bilirubin-diglucuronide), lithocholic acid 3-*O*-sulfate, and lithocholic acid 3-*O*-glucuronide, which are believed to be transported from liver into bile through an active transport process stimulate ATP hydrolysis by purified dinitrophenylglutathione ATPase of human erythrocytes. The K_m and V_{max} values of the enzyme for these substrates are similar to those for dinitrophenylglutathione indicating the transport mechanisms for bilirubin conjugates, and anionic bile acid-conjugates from hepatocytes to bile and transport of GSH-conjugates from erythrocytes may be mediated by similar mechanisms.

Glutathione-conjugates; Dinitrophenylglutathione ATPase; Bilirubin-conjugates; Bile acid-conjugates; Human liver; Human erythrocytes

1. INTRODUCTION

Glutathione (GSH)-conjugates of electrophilic compounds are transported from human erythrocytes through an ATP-dependent primary active transport process [1–5] which is mediated by an ATPase distinct from the known ion pump ATPases [6–8]. This ATPase stimulates ATP hydrolysis in the presence of dinitrophenyl glutathione (Dnp-SG) and other GSH-conjugates and has been designated as Dnp-SG ATPase [6–8]. We have previously purified Dnp-SG ATPase from human erythrocytes and demonstrated that it is also expressed in some of the other human tissues including liver [7]. In rat liver, the transport of GSH-conjugates, as well as the transport of the conjugates of bilirubin and certain bile acid derivatives, along with other organic anions is also an energy dependent process utilizing ATP [9–15]. The interrelationship(s) among the transport mechanisms for the conjugates of GSH, bilirubin, bile acids, and other organic anions from liver is not completely understood. The results presented in this communication for the first time demonstrate that bilirubin ditaurate, an analog of bilirubin diglucuronide, and dianionic bile acid-conjugates as well as other organic anions, stimulate ATP hydrolysis by human erythrocyte Dnp-SG ATPase. The kinetics of this reaction have been studied using purified erythrocyte Dnp-SG ATPase, and relative substrate specificities of the enzyme towards a number of organic anions have been determined.

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2. MATERIALS AND METHODS

Unless otherwise specified, sources of all the chemicals in the present study were the same as those used in our previous studies [7]. [γ-^{32}P]ATP was procured from DuPont-NEN (Boston, MA). Dnp-SG was synthesized enzymatically according to our previous method [3]. After establishing its purity by TLC and HPLC, Dnp-SG was linked to CNBr-activated Sepharose 4B by the method of Porath et al. [16]. Bilirubin, hematin, β-estradiol 17-(β-D-glucuronide) sodium salt, 17 β-estradiol-3-(β-D-glucuronide) sodium salt and lithocholic acid 3-*o*-sulfate disodium salt were purchased from Sigma Chemical Co., St. Louis, MO. Bilirubin ditaurate was purchased from Porphyrin Products Inc., Logan, Utah. Lithocholic acid 3-*o*-glucuronide, disodium salt, was synthesized as described previously [17].

2.1. *Preparation of vesicles from human erythrocytes*

Human blood was obtained from the blood bank of the University of Texas Medical Branch at Galveston, TX. All studies were performed using blood less than one week old collected from normal healthy subjects. Erythrocyte ghosts were prepared by a modification of the procedure of Dodge et al. [18] as described by us recently [5]. Membrane vesicles from the ghosts were prepared with a slight modification of the method of Kasahara and Hinkle [19] as reported earlier [5]. Since Lubrol PX was present, protein content was determined utilizing the dye binding assay described by Minamide and Bamburg [20]. ATPase activity with all substrates was determined by the method of Knowles and Leng [21] with modifications described by us previously [5]. Enzyme activity unit was expressed as one nmol of ATP hydrolyzed per minute at 37°C.

2.2. *Purification of Dnp-SG ATPase from erythrocyte vesicles*

Dnp-SG ATPase was purified by the method described earlier [7] using Dnp-SG linked CNBr-activated Sepharose 4B affinity chromatography. In brief, erythrocyte membrane vesicles were suspended in 0.5% Lubrol PX in 10 mM Tris-HCl, pH 7.4, containing 2.0 mM EDTA and 2.8 mM β-mercaptoethanol. The mixture was incubated for 20 min at 4°C with occasional shaking and centrifuged at 48000 × *g* for 2 h. The supernatant was subjected to affinity chromatography over a column (1 cm × 10 cm) of Dnp-SG coupled to CNBr-activated Sepharose 4B described previously [7]. The enzyme was eluted from the column with 10 mM Tris-HCl, pH 7.4, containing

2.8 mM β-mercaptoethanol, 0.25% Lubrol, 2 mM $MgCl_2$, 2 mM ATP, and 0.2 mM Dnp-SG and dialyzed extensively against 10 mM, Tris-HCl, pH 7.4, containing 2.8 mM β-mercaptoethanol and 0.1 mM ATP. ATPase activity was monitored during the purification using Dnp-SG as well as bilirubin ditaurate by the method described by us previously [5]. SDS-polyacrylamide gel electrophoresis was performed using the buffer system described by Laemmli [22]. The stacking and resolving gels contained 7.1% and 12.5% acrylamide, respectively.

3. RESULTS AND DISCUSSION

Results of the present studies for the first time demonstrate that bilirubin ditaurate and 2 conjugates of bile acids which are thought to be transported from liver into bile through an ATP-dependent transport system [9-15] stimulate ATP hydrolysis by Dnp-SG ATPase of erythrocytes. Bilirubin ditaurate, lithocholic acid 3-*o*-sulfate, and lithocholic acid 3-*o*-glucuronide stimulated ATP hydrolysis in the presence of ouabain and EGTA, by erythrocyte membrane vesicles prepared by the method of Kasahara and Hinkle [19] in a similar manner as reported by us previously for Dnp-SG and other GSH-conjugates [5,6]. Erythrocyte membrane vesicles prepared from different human subjects caused a 30-50% stimulation of ATP hydrolysis over the non-specific baseline hydrolysis of ATP in the presence of ouabain and EGTA when bilirubin ditaurate, lithocholic acid 3-*o*-sulfate or lithocholic acid 3-*o*-glucuronide were included in the reaction mixture. Thus, the effect of these compounds is similar to that of Dnp-SG, which caused a 20-50% stimulation of ATP hydrolysis over the non-specific ATP hydrolysis by membrane vesicles [5,6]. The stimulation of ATPase hydrolysis by these substrates was linear in relation to protein concentration (Fig. 1) and with time of incubation up to 60 min (data not presented). Heat treated vesicles showed complete loss of stimulation of ATP hydrolysis with all these substrates. When the K_m of the enzyme in the vesicles was determined for bilirubin ditaurate at a fixed concentration of ATP (1.6 mM), the double reciprocal plots (Fig. 2) indicated biphasic kinetics with two K_m and V_{max} values. A single K_m value of 1.33 mM was however found for ATP. Both K_m values for bilirubin ditaurate were in the micromolar (25 μM and 117 μM) range as described by us previously for Dnp-SG [5]. The crude enzyme in the vesicles also showed biphasic kinetics with the two lithocholic acid conjugates used in this study with two K_m values comparable to those of bilirubin ditaurate (data not presented).

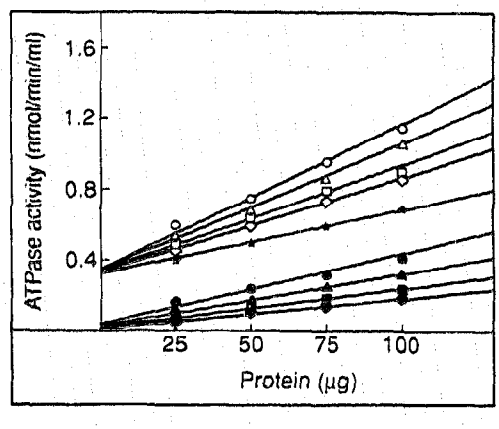

Fig. 1. Dependence of Dnp-SG ATPase activity on protein measured in the presence of different substrates. The enzymatic hydrolysis of [^{32}P]ATP to form $^{32}P_i$ was catalyzed by increasing aliquots of erythrocyte vesicles during a 60 min incubation. (*) without any substrates; (◇) with lithocholic acid 3-*o*-sulfate; (□) Dnp-SG; (△) lithocholic acid 3-*o*-glucuronide; (○) bilirubin ditaurate. The difference between ATP hydrolysis measured in the presence and absence of various substrates is shown by corresponding closed symbols in the same order. Details are given in the text.

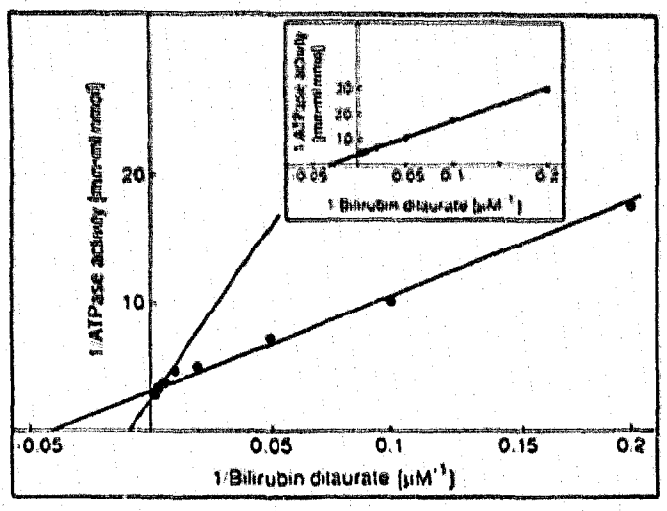

Fig. 2. Double reciprocal plots of the human erythrocyte vesicle ATPase activity obtained with increasing concentrations of bilirubin ditaurate at a fixed concentration of ATP (1.6 mM). *Inset:* double reciprocal plots of purified human erythrocyte Dnp-SG ATPase with bilirubin ditaurate.

Dnp-SG ATPase was purified from erythrocyte membranes by affinity chromatography on Dnp-SG linked to CNBr-activated Sepharose 4B as described by us recently [7]. Consistent with previous findings [7], an apparently homogenous protein showing a single band in denaturing gels corresponding to a subunit M_r value of 38 000 was obtained (data not presented). Specific activities of the enzyme using bilirubin ditaurate, Dnp-SG and other compounds as substrates are presented in Table I. The K_m and V_{max} values of the purified enzyme for different substrates are also presented in Table I. In contrast to the biphasic kinetics of the crude enzyme in vesicles, the purified enzyme showed monophasic kinetics for bilirubin ditaurate (Fig. 2 inset) and with Dnp-SG (graph not presented) with K_m values for bilirubin ditaurate and Dnp-SG to be 32 μM and 59 μM, respectively. As shown in Table I, K_m values of the purified enzyme corresponded to the K_m values of the higher affinity ATPase component present in the vesicles. This may indicate that at least 2 ATPases having varying affinity for bilirubin-conjugates are present in the erythrocytes, and that Dnp-SG affinity chromatography yields

Table I

Specific activities and Kinetic parameters of purified Dnp-SG ATPase from human erythrocytes for bilirubin and bile acid conjugates

Substrates	Sp. Activity (nmol/min/mg)	K_m (μM)	V_{max} (mol/mol/min)*
Bilirubin ditaurate	7.6	32	380
Lithocholic acid 3-α-sulfate	8.3	18	421
Lithocholic acid 3-α-glucuronide	10.4	17	522
17 β-Estradiol 3-(β-D-glucuronide)	10.2	6	408
β-Estradiol 17-(β-D-glucuronide)	9.1	12	364
S-(2,4-dinitrophenyl) glutathione	6.4	59	288

* Based upon subunit M_r value of 38 kDa because molecular weight of holoenzyme is not known

only one of these enzymes. It is likely that the ATPase described by Kondo et al. [23,24] which has two subunits with M_r values of 85 000 and 62 000 may also utilize bilirubin and bile acid-conjugates as substrates. The purified enzyme did not stimulate ATP hydrolysis in the presence of taurocholic acid and taurolithocholic acid. Hematin also did not stimulate ATP hydrolysis by the enzyme. Inability of the enzyme to utilize mono-anionic bile acids as substrates is consistent with separate mechanisms for the transport of mono-anionic bile acids on the one hand, and glucuronides of bilirubin and bile acid 3-*o*-glucuronides and sulfates on the other, and is compatible with observations made in vivo [25,26]. In agreement with our previous finding, GSSG did not stimulate ATP hydrolysis by the purified enzyme.

Results of the present study suggest a direct relationship at the molecular level between the mechanisms for the transport of bilirubin-conjugates and other anions by liver canalicular membranes and the transport of GSH-conjugates from erythrocytes. Utilization of anionic conjugates of bilirubin and bile acids as substrates by human erythrocyte Dnp-SG ATPase strongly suggest that at least the ATP hydrolyzing component of the transport systems for GSH-conjugates and bilirubin-conjugates, and bile acid-conjugates in humans is similar. Since a protein with a similar subunit M_r value and immunological properties as the erythrocyte Dnp-SG ATPase is expressed in liver [7], it is more than likely that Dnp-SG ATPase may be involved in the transport of conjugates of bilirubin and bile acids from the hepatocyte into bile. Purification and characterization of the transporter(s) from human liver is needed to establish the exact relationship(s) among these transporters and erythrocyte Dnp-SG ATPase.

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