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A Stolz, … , J Kuhlenkamp, N Kaplowitz

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Research Article

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3α-Hydroxysteroid Dehydrogenase Activity of the Y' Bile Acid Binders in Rat Liver Cytosol

Identification, Kinetics, and Physiologic Significance

Andrew Stolz, Hajime Takikawa, Yuichi Sugiyama, John Kuhlenkamp, and Neil Kaplowitz
Liver Research Laboratory, Medical and Research Services, Wadsworth Veterans Administration Hospital Center and the UCLA School of Medicine, Los Angeles, California 90073

Abstract

Rat Y' bile acid binders (33 kD) have been previously recognized as cytosolic bile acid binding proteins (Sugiyama, Y., T. Yamada, and N. Kaplowitz, 1983, *J. Biol. Chem.*, 258:3602–3607). We have now determined that these Y' binders are 3α-hydroxysteroid dehydrogenases (3α-HSD), bile acid–metabolizing enzymes. 3α-HSD activity copurified with lithocholic acid–binding activity after sequential gel filtration, chromatofocusing, and affinity chromatography. Three peaks of 3α-HSD activity (I, II, III) were observed in chromatofocusing and all were identified on Western blot by a specific Y' binder antiserum. 3α-HSD-I, the predominant form, was purified and functioned best as a reductase at pH 7.0 with a marked preference for NADPH. Michaelis constant values for mono- and dihydroxy bile acids were 1–2 μM, and cholic acid competitively inhibited the reduction of 3-oxo-cholic acid. Under normal redox conditions, partially purified 3α-HSD-I and freshly isolated hepatocytes catalyzed the rapid reduction of 3-oxo-cholic to cholic acid without formation of isocholic acid, whereas the reverse reaction was negligible. The Y' bile acid binders are therefore 3α-HSD, which preferentially and stereospecifically catalyze the reduction of 3-oxo-bile acids to 3α-hydroxy bile acids.

Introduction

The translocation of bile acids by the liver from sinusoidal blood to bile is of critical importance in the generation of bile flow. Recent work has begun to elucidate the mechanisms for membrane transport of bile acids at the sinusoidal and canalicular poles of the hepatocytes (1–3). However, the mechanism for intracellular transfer of bile acids from one surface to the other is poorly understood (4, 5). To better understand the intracellular handling of bile acids, we have identified, purified, and characterized the binding affinities of cytoplasmic proteins in rat liver that interact with bile acids (6, 7). Two unrelated families of proteins seem to bind bile acids with high affinity, namely ligandins (45–50-kD dimers) (8) and Y' proteins (33-kD monomers) (6). Ligandins are glutathione S-transferases that bind bile acids at a nonsubstrate, high-affinity site. In this report we have sought evidence for enzymatic activity of the Y' binders. We particularly focused on the possibility that the Y' binders were forms of 3α-hydroxysteroid dehydrogenase because we were struck by the similarities in molecular weight, isoelectric point, and amino acid composition between our previous results for Y' binders and others' results for 3α-hydroxysteroid dehydrogenase (9, 10). Our approach was to examine the copurification of bile acid–binding activity and enzymatic activity. Three forms of 3α-hydroxysteroid dehydrogenase were identified as the Y' binders and their kinetic properties and physiologic action on bile acids were further analyzed.

Address reprint requests to Andrew Stolz, M.D., W151N Liver Research Lab, Wadsworth Veterans Administration Hospital, Los Angeles, CA 90073.

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Methods

The following materials were obtained from the stated sources: Sephadex G-75 superfine, Polybuffer exchanger-94, Polybuffer-74 and 3'-phosphoadenosine 5'-phosphate-agarose, type 3 (Pharmacia Fine Chemicals, Piscataway, NJ); Sep Pak C_{18} cartridges (Waters Associates, Milford, MA); bovine serum albumin, EGTA, 1-anilino-8-naphthalene sulfonate (ANS),[1] bis–Tris, glutathione, NAD^+, $NADP^+$, NADH, NADPH, molecular weight standards (SDS-7), cholylglycine hydrolase, 3α-hydroxysteroid dehydrogenase (bacterial), and collagenase (Sigma Chemical Co., St. Louis, MO); Biorad electroblot apparatus, Tris, nitrocellulose membrane, Tween-20, goat antirabbit affinity-purified horse radish peroxidase (HRP) linked antibody, HRP color development reagent (containing 4-chloro-1-naphthol), TEMED, N'N'-bis methylene acrylamide, gelatin, and acrylamide (Bio-Rad Laboratories, Richmond, CA); Spectropor #2 membrane (10–14-kD exclusion size) (Spectrum Medical Industries, Inc., Los Angeles, CA), [^{14}C,24-C]lithocholic acid (50 mCi/mmol) and chenodeoxycholic acid (45 mCi/mmol) (Amersham Corp., Arlington Heights, IL); [^{3}H]cholic acid (23 Ci/mmol) (New England Nuclear, Boston, MA). [^{3}H]3-oxo-7α,12α-dihydroxy-5β-cholanic acid (3-oxo-cholic acid) was prepared from radiolabeled cholic acid by incubation with bacterial 3α-hydroxysteroid dehydrogenase and NAD^+, followed by extraction and purification on thin-layer chromatography (TLC) (System A). All labeled bile acids were > 98% pure on TLC. Unlabeled bile acids were obtained from Calbiochem-Behring Corp., La Jolla, CA, except for 3,7-dioxo-5β-cholanic acid and 3α-hydroxy-7-oxo-5β-cholanic acid (Steraloids, Inc., Wilton, NH), 3-oxo-7α-hydroxy-5β-cholanic acid (a gift from Dr. K. Uchida, Shionogi Research Laboratory, Osaka, Japan) and 3β,7α,12α-trihydroxy-5β-cholanic acid(isocholic) (a gift from Dr. A. F. Hofmann, University of California, San Diego). These were recrystallized and > 99% pure on TLC.

Protein purification

Chromatofocusing of Y' fraction. Y' fraction in 0.01 M sodium phosphate, pH 7.4 (buffer 1) was prepared from Sephadex G75 superfine gel filtration of cytosol (33% wt/vol) harvested from male Sprague–Dawley rats (200–250 g), as previously described (6, 11). 100 ml of pooled Y' was applied by gravity onto a 1 × 60-cm column of Polybuffer Exchange 94 equilibrated with 0.025 M bis-Tris-HCl, pH 6.4. The column was eluted with 600 ml of a 1:10 dilution of Polybuffer 74–HCl, pH 4.5, at a flow rate

1. *Abbreviations used in this paper:* ANS, 1-anilino-8-naphthalene sulfonate; HRP, horse radish peroxidase; 3-oxo-cholic acid, 3-oxo-7α,12α-dihydroxy-5β-cholanic acid; PAP, 3'-phosphoadenosine 5'-phosphate; TLC, thin-layer chromatography.

of 30 ml/h. 2.8-ml fractions were collected and assayed for protein absorbance, enzymatic activity, lithocholic acid binding and pH.

3'-Phosphoadenosine 5'-phosphate (PAP)–agarose affinity chromatography. Pooled fractions of enzyme activity and bile acid binding after chromatofocusing of the Y' were applied to a 1 × 10–cm column of PAP–agarose equilibrated with buffer 1. The column was initially eluted with buffer 1 followed by a linear salt gradient consisting of 150 ml of buffer 1 and 150 ml of buffer 1 containing 0.2 M sodium chloride. The column was eluted at 12 ml/h. 3-ml fractions were collected and assayed for protein absorbance, bile acid binding, and enzymatic activity.

Enzymatic activities

Glutathione S-transferase. Glutathione S-transferase activity was measured by monitoring the conjugation of 20 μM 1-chloro-2,4-dinitrobenzene and 0.67 mM GSH, as in prior publication (6, 11).

3α-Hydroxysteroid dehydrogenase. 3α-hydroxysteroid dehydrogenase activity was determined by monitoring the consumption or production of the cofactor NADPH or NADH as the respective rate of decrease or increase in A_{340} at 37°C. For measurement of reductase activity, assays were performed in 0.05 M sodium phosphate, pH 7.0, and dehydrogenation was determined in 0.05 M glycine-sodium hydroxide, pH 9, according to the method of Oesch (9). Bile acids were dissolved in absolute ethanol; cofactors were dissolved in water. Enzymatic reaction was initiated by the addition of sample to 1.9 ml of buffer after adding 50 μl of bile acid substrate and 50 μl of nicotinamide cofactor.

Binding studies

Lithocholic acid binding by equilibrium dialysis. Individual column fractions or pooled protein peaks (0.5 ml) were dialyzed in Spectropor #2 membrane placed in 1 l of buffer 1 with 60 nmol of [^{14}C]lithocholic acid (3 μCi) for 40 h at 4°C. Bound over free ratio was determined as previously described (11).

ANS displacement method for bile acid binding. Competitive displacement of the fluorescent probe, ANS was performed as previously described (6, 7) with varying concentrations of lithocholic acid and chenodeoxycholic acid added to purified form I (0.1 μM) and ANS (15 μM). Inhibition constant (K_i) values were calculated as previously described (6).

Electrophoresis

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis. SDS–PAGE was performed according to the method of Laemmli using 12.5% gel (12) and then silver stained according to the method of Wayne (13).

Electroblotting and immuno-overlay. SDS gels were electroblotted on nitrocellulose membrane as previously described (11) according to the method of Towbin (14).

Enzyme kinetics

Kinetics of purified 3α-hydroxysteroid dehydrogenase for bile acids and cofactors. Kinetic parameters for cofactors and bile acids were determined by monitoring absorbance change (A_{340}) of cofactor at 25°. Kinetic parameters were determined in 1.0 ml of 0.05 M sodium phosphate, pH 7.0. When the reactions were carried out at pH 9.0, maximum velocity (V_{max}) was altered but Michaelis constant (K_m) was unchanged. To determine the K_m for $NADP^+$ and NAD^+, 312 μM cholic acid was utilized as cosubstrate, and 125 μM 3-oxo-cholic acid was used for NADH and NADPH. The following range of cofactor and protein concentrations were utilized to determine K_m for cofactors: $NADP^+$ (0.1–40 μM) with 0.8 μM protein, NAD^+ (0.3–5 mM) with 1.5 μM protein, NADPH (0.05–10 μM) with 0.5 μM protein and NADH (5–80 μM) with 1.5 μM protein. To determine the K_m for bile acids, 1.0 ml of 0.05 M glycine sodium hydroxide, pH 9.0, containing 100 μM of $NADP^+$ and 0.05 μM protein, and the following concentration range of bile acids were used: lithocholic acid (0.1–5 μM), cholic acid (25–250 μM), chenodeoxycholic acid (0.5–10 μM), taurolithocholic acid (0.5–8 μM) and taurochenodeoxycholic acid (0.8–25 μM). To determine the K_m for 3-oxo bile acids, we used total volume of 1.0 ml of 0.05 M sodium phosphate, pH 7.0, containing 3 μM NADPH and 0.3 μM protein with the following concentration range of bile acids: 3-oxo-cholic acid (5–75 μM) and 3-oxo-5β-cholanic acid (0.25–8 μM). All reactions were performed in duplicate. Data were analyzed by nonlinear least squares fit of the data to the Michaelis–Menton equation.

Kinetics of inhibition of reduction of 3-oxo-cholic acid by cholic acid. Kinetics of reduction of 3-oxo-cholic acid (3–80 μM) were determined in 0.05 M sodium phosphate, pH 7.0, at 25°C with 0.7 μM purified 3α-hydroxysteroid dehydrogenase-I and 3 μM NADPH in the presence and absence of 100 μM cholic acid. The data in the presence and absence of the inhibitor were fit by nonlinear least squares to the Michaelis–Menton equation. K_i was determined by the equation: $K_i = [i] \times (K_{m\ app} - K_m / K_m)$, where [i] is the inhibitor concentration and $K_{m\ app}$ is K_m in the presence of an inhibitor.

Identification of reaction products of the 3α-hydroxysteroid dehydrogenase in incubations with partially purified form and in hepatocytes

5 μM [^{3}H]cholic acid or its 3-oxo,7α,12α-dihydroxy-5β-cholanic acid derivative were incubated with partially purified bile acid binder I in 2 ml of 0.05 M sodium phosphate, pH 7, with physiological concentrations of NAD^+ (800 μM), NADH (200 μM), $NADP^+$ (100 μM or 20 μM) and NADPH (400 μM). Timed samples were collected and reaction was terminated by boiling the sample for 30 s. Bile acids were extracted by initially adjusting the pH to 1 with 2 N hydrochloric acid followed by sequential extraction with ether. The ether fraction was then washed with water, evaporated, and redissolved in methanol and applied onto a silica TLC plate, and developed in solvent system A consisting of isooctane/ethyl acetate/acetic acid (50:50:0.7) (15). The retardation factor (R_f) of reaction products was compared with standards. Standards were identified by 10% phosphomolybdic acid in ethanol and radioactivity was determined in 0.5-cm scraping of each entire lane. In addition, 5 μM of [^{14}C] C_{24}-chenodeoxycholic acid was incubated with purified enzyme with 230 μM $NADP^+$ in 0.05 M sodium phosphate buffer, pH 7, for 1 min at 37°C. Bile acids were extracted as above and applied to a silica gel TLC developed in solvent system A. R_fs were compared with 3,7-dioxo-5β-cholanic acid, 3α-hydroxy-7-oxo-5β-cholanic, 3-oxo-7α-hydroxy-5β-cholanic acid and chenodeoxycholic acid. Relative R_f values of these compounds were 0.19, 0.44, 0.72, 1, respectively.

Bile acids were extracted from isolated hepatocytes by initially separating cells from the medium with 2,000-*g* centrifugation. Cell pellets were then suspended in 1 N potassium hydroxide for 24 h at room temperature. After incubation and centrifugation, the supernatant was neutralized to pH 7 with dilute hydrochloric acid and applied onto a Sep Pak C_{18} cartridge. The cartridge was initially washed with water and then bile acids were eluted with methanol. Half of the methanol extract was analyzed on a TLC using the solvent system B consisting of chloroform/methanol/acetic acid/water (65:24:15:9) to assess conjugation (16). The other half of the methanol was evaporated and the residue was deconjugated with cholyglycine hydrolase in sodium acetate buffer, pH 5.6, for 1 h (16). After deconjugation the mixture was acidified to pH 1 with 2 N hydrochloric acid, and bile acids were extracted with ether. Aliquots were applied to TLC plates and developed in solvent system A. Bile acids in the medium were analyzed in the same way but without the treatment with potassium hydroxide.

Preparation of isolated hepatocytes. Isolated hepatocytes were prepared according to the method of Moldeus (17) as previously described (18). During the course of 30 min incubation, > 95% viability was maintained as assessed by the exclusion of the supravital dye, trypan blue.

Results

Copurification of bile acid binders and 3α-hydroxysteroid dehydrogenase. Initially, rat hepatic cytosol was fractionated by molecular weight gel filtration and the eluate was screened for 3α-hydroxysteroid dehydrogenase activity, glutathione S-transferase activity and lithocholic acid binding (Fig. 1). 3α-hydroxysteroid dehydrogenase was monitored using taurochenodeoxy-

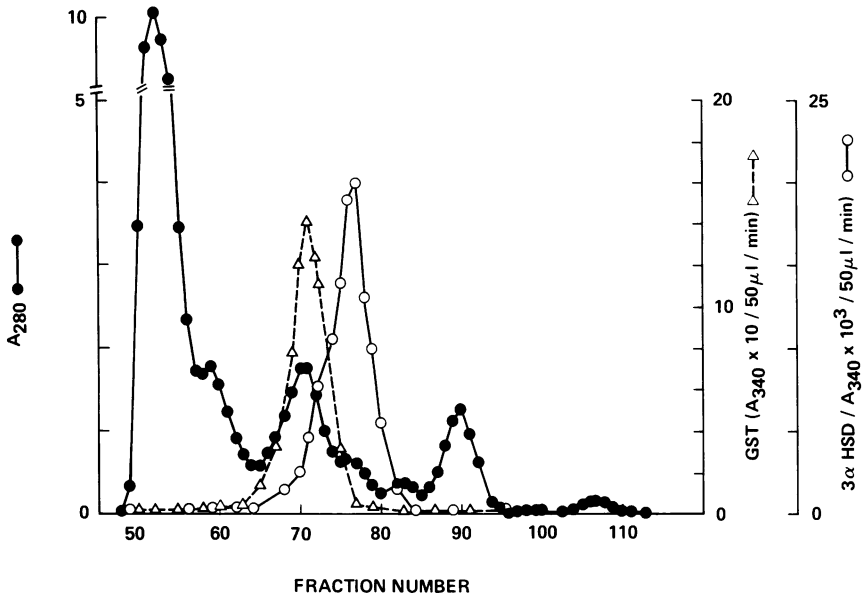

Figure 1. Elution profile of 3α-hydroxysteroid dehydrogenase and GSH S-transferase activity in gel filtration of rat hepatic cytosol. 8 ml of 33% wt/vol cytosol was applied to a 2.5 × 120-cm column of Sephadex G-75 superfine and eluted with buffer 1 (12 ml/h) (see Methods for details). 2.8-ml fractions were collected and analyzed for protein absorbance (A_{280}) (*solid circle*), glutathione S-transferase activity (*open triangle*), and 3α-hydroxysteroid dehydrogenase (*open circle*) as $\Delta A_{340}/50$ μl/min using 75 μM taurochenodeoxycholic acid and 230 μM $NADP^+$ as substrates.

cholic acid as substrate (Fig. 1). The same elution pattern was observed for dehydrogenase activity with conjugated and unconjugated mono-, di-, and trihydroxy bile acids and for reductase activity with 3-oxo-derivatives of these bile acids. The elution of these activities corresponded exclusively to fractions previously identified as the Y′ fraction (6); the peak eluted after the GSH S-transferases (Y fraction). As we have previously reported (6), lithocholic acid–binding activity was eluted as two peaks corresponding to the Y and Y′ fractions (not shown). Y′ fraction prepared from preparative chromatography of cytosol on Sephadex G75 superfine (5 × 100 cm) demonstrated a similar elution profile and was used for further purification of the Y′ bile acid binder.

Pooled Y′ fraction was examined for substrate specificity for 3α-hydroxysteroid dehydrogenase. Since this enzyme catalyzes a bidirectional reaction, substrates for dehydrogenation (Table I) or reduction were examined separately (Table II). Activity is listed as nanomoles of NADPH consumed or produced per minute. Substrate concentrations were arbitrarily chosen based on binding constants from previously reported studies (6). Dehydrogenase activity was specific for 3α-hydroxy sterols since there was no activity with either estradiol or 7α,12α-dihydroxy-5β-cholanic acid. Conversely, reductase activity was dependent on presence of a keto group at the 3 position except for testosterone, which differs from the other sterol by its 4-ene structure. These results with the pooled Y′ fraction suggested that dehydrogenase activity was specific for the hydroxy group in the 3 alpha position and that 3-oxo group was required for reductase activity in the absence of a 4-ene structure. The presence of reductase activity with either 5α or 5β androsterone suggests that the 4-ene structure prevents the reduction of the 3-oxo group by the 3α-HSD.

In accordance with the purification protocol used in the initial identification of the Y′ bile acid binder (6), pooled Y′ fraction was further fractionated by chromatofocusing. A pH gradient from 6.5 to 4.5 was utilized so as to improve the resolution of

Table I. Dehydrogenase Activity of the Crude Y′ Fraction

Substrate	Activity
Lithocholic acid (.005)	2.41
Chenodeoxycholic acid (0.125)	1.90
Taurochenodeoxycholic acid (0.075)	2.92
Taurocholic acid (1.5)	6.48
Cholesterol (.005)	ND
3α,7α-Dihydroxy-12-oxo-5β-cholanic acid (0.5)	6.70
Androsterone (0.35)	5.04
Tauro-7α,12α-dihydroxy-3-oxo-5β-cholanic acid (0.5)	ND
7α,12α-dihydroxy-5β-cholanic acid (0.5)	ND
Estradiol (0.35)	ND
Estrone (0.35)	ND

Substrate concentrations are listed in parentheses in millimolar. Reaction was performed in 0.05 M glycine-sodium hydroxide, pH 9.0 with 230 μM $NADP^+$ at 37°C with 50 μl of crude Y′ fraction (31 μg of protein). The substrate concentrations used in this screening procedure were arbitrarily selected on the basis of dissociation constant value for bile acid binding from previous results (6). The results are expressed as nmoles of NADPH produced per minute. Nondetectable (ND) is defined as < .05 nmol of NADPH produced per minute.

Table II. Reductase Activity of the Crude Y′ Fraction

Substrate	Activity
Tauro-7α,12α-dihydroxy-3-oxo-5β-cholanic acid (0.5)	1.42
7α,12α-Dihydroxy-3-oxo-5β-cholanic acid (0.5)	0.67
5α-Androstan-3,17-dione (0.5)	0.34
5β-Androstan-3,17-dione (0.5)	0.99
Testosterone (0.035)	ND
3α,7α-Dihydroxy-12-oxo-5β-cholanic acid (0.5)	ND

Concentration of substrates are listed in parentheses in millimolar concentration. Reaction was performed in 0.1 M sodium phosphate, pH 7.0 with 20 μM NADPH at 37°C with 50 μl of crude Y′ fraction (31 μg of protein). Results are expressed as nanomoles of NADPH consumed per minute per milligrams protein. Nondetectable (ND) is defined as < 0.10 nmol of NADPH consumed per minute.

this method whereas the initial purification protocol utilized a pH gradient from 7 to 4 (6). Fig. 2 illustrates the elution profile of the pooled Y′ fraction. Three distinct peaks corresponding to lithocholic acid binding and 3α-hydroxysteroid dehydrogenase activity were observed eluting at pH 5.40, 5.35, and 5.20, using either the substrate 3-oxo-cholic acid or cholic acid (3α,7α,12α-trihydroxy-5β-cholanic acid). The elution pattern of enzymatic activity was identical for unconjugated and conjugated mono-, di-, and trihydroxy bile acids (not shown). The pH at which bile acid binders I and II eluted in our previous report (pH 5.6 and 5.5, respectively) are comparable with these results. However, improved resolution now reveals three peaks as opposed to the two previously identified. These three peaks are now designated 3α-hydroxysteroid dehydrogenase-I, II, and III. 3α-Hydroxysteroid dehydrogenase-I (bile acid binder I) is the predominant form, constituting 60–70% of the total 3α-hydroxysteroid dehydrogenase activity in the Y′ fraction. Coelution of both bile acid binding and 3α-hydroxysteroid dehydrogenase activity strongly suggested that the Y′ bile acid binders are in fact 3α-hydroxysteroid dehydrogenase.

Further purification of lithocholic acid binding and 3α-hydroxysteroid dehydrogenase activity corresponding to the major form (I) was performed with two different approaches. As in our previous report (6), hydroxyapatite chromatography of pooled 3α-hydroxysteroid dehydrogenase/bile acid binder I from chromatofocusing was performed. Both binding and enzymatic activity coeluted along with the bulk of the protein and this fraction exhibited a single band on SDS–PAGE with M_r 33 kD (not shown).

Recovery of enzyme activity from gel filtration and chromatofocusing was > 80%. However, recovery from hydroxyapatite was only 20%. Thus, to insure not missing other forms of the enzyme, the pooled form I from chromatofocusing was alternatively purified with affinity chromatography on PAP–agarose. This approach has been successively employed by others for purification of dehydrogenases. Fig. 3 depicts the elution of lithocholic acid binding and enzymatic activity as a single peak corresponding to the bulk of protein. Recovery of enzymatic activity was 40%. The protein from the pooled fractions corresponding to this peak was homogeneous on SDS–PAGE (Fig. 4, *lane C′*) with M_r 33 kD. Thus, only one peak of enzymatic and bile acid–binding activity was identified with either hydroxyapatite or PAP–agarose for purification of 3α-hydroxysteroid dehydrogenase-I from chromatofocusing. The purification (~300-fold) of 3α-hydroxysteroid dehydrogenase-I to homogeneity is summarized in Table III.

Immunochemical identification. Confirmation that the purified 3α-hydroxysteroid dehydrogenase-I is identical to the previously purified Y′ bile acid binder was determined by immuno-overlay experiments. The SDS gel of the purified 3α-hydroxysteroid dehydrogenase-I after PAP–agarose affinity chromatography and pooled fractions of 3α-hydroxysteroid dehydrogenase-I, II, and III from chromatofocusing were electroblotted onto nitrocellulose and probed with a previously characterized polyclonal rabbit antiserum specific for Y′ bile acid binders (11) (Fig. 4). No binding was identified with preimmune serum (not shown). The purified protein and 3α-hydroxysteroid dehydrogenase-I, II, and III from chromatofocusing were recognized by the antiserum.

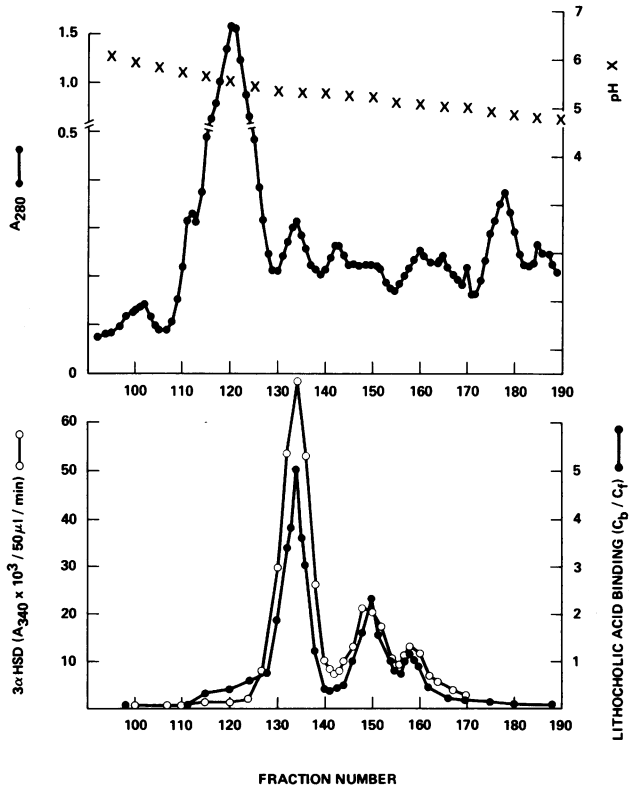

Figure 2. Chromatofocusing of the Y′ fraction. *Upper panel* represents the elution profile of protein absorbance A_{280} (*solid circle*) and pH gradient (*x*). Lower panel illustrates the corresponding activities: lithocholic acid binding as C_b/C_f (*solid circle*) and 3α-hydroxysteroid dehydrogenase activity (*open circle*) as in Fig. 1.

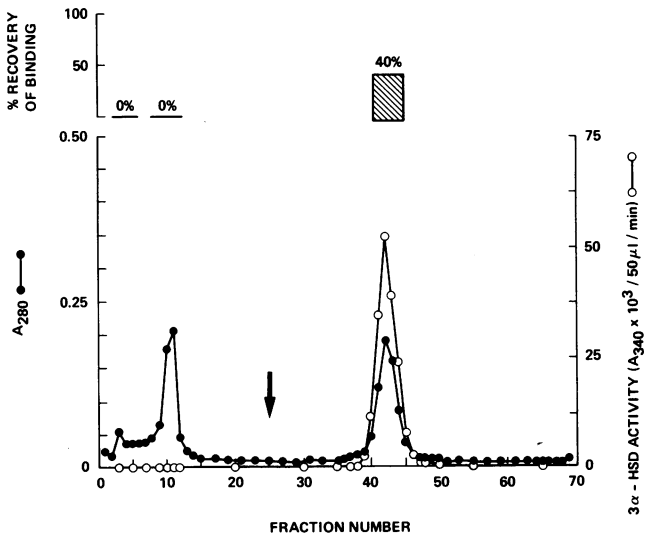

Figure 3. Affinity chromatography of 3α-hydroxysteroid dehydrogenase-I on PAP–agarose. 35 ml of pooled 3α-hydroxysteroid dehydrogenase-I from chromatofocusing was applied to a 1 × 10–cm column of PAP–agarose and eluted with a linear salt gradient (see Methods for detail). Arrow identifies the initiation of the salt gradient. Fractions (2.9 ml) were assayed for protein absorbance (*solid circle*) and 3α-hydroxysteroid dehydrogenase activity (*open circle*) as in Fig. 1. Pooled protein peaks were assayed for lithocholic acid binding and results are expressed as percent of total binding activity applied to the column (*upper panel*).

Table III. Purification of 3α-Hydroxysteroid Dehydrogenase

Purification step	Vol	Total protein	Total activity*	Specific activity	Purification factor
	ml	*mg*		*U/mg protein*	
Cytosol	120	3000	955	0.32	1
Sephadex G75 sf	100	184	563‡	3.06	9.62
Chromatofocusing 3α-hydroxysteroid dehydrogenase					
I	45	10.80	249	23.06	72.5
II	30	6.90	150	21.74	68.4
III	24	5.28	107	20.19	63.5
PAP-agarose I	15	1.33	124	93.23	293.2

* Unit of enzyme activity is defined as 1 μmol of NADP consumed per minute using 75 μM taurochenodeoxycholic acid and 230 μM $NADP^+$ in 0.05 M glycine-sodium hydroxide, pH 9.0.

‡ Only ~ 70% of 3α-hydroxysteroid dehydrogenase activity was used for further purification.

pH optima and kinetics of 3α-hydroxysteroid dehydrogenase-I. Fig. 5 illustrates the effects of pH on both reductase and dehydrogenase activity of form I. A broad pH optimum from 5 to 8 for reductase activity with 3-oxo-cholic acid was observed, indicating optimum activity at physiologic pH. The peak dehydrogenase activity with cholic acid was limited to a narrow pH range between 8.5 and 9.5, as has been previously reported with other substrates (9).

Table IV summarizes the K_m values for various cofactors. $NADP^+$ and NADPH were the preferred cofactors over NAD^+ and NADH for the dehydrogenase and reductase activities, respectively. K_m for various bile acids are also listed in Table IV. Mono- and dihydroxy bile acids in the unconjugated or conjugated form all had similar K_m (1–2 μM) and these values agreed with binding affinities (K_i) as determined by competitive displacement of ANS. Cholic acid was a much poorer substrate (K_m 105 μM) compared with its 3-oxo derivative (K_m 8 μM). Dehydrogenase activities at pH 9.0 were greater than pH 7.0 due to an increase in V_{max}. pH had no effect on the K_m values for bile acids (not shown).

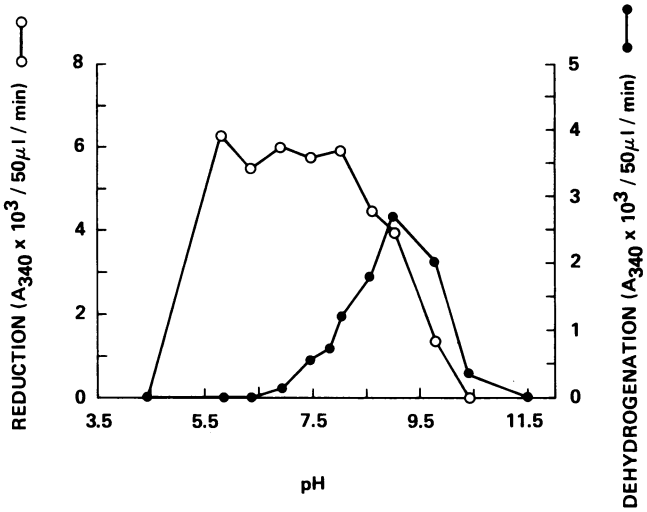

Figure 5. Optimum pH for reductase and dehydrogenase activity of the purified 3α-hydroxysteroid dehydrogenase-I. Reductase (*open circle*) or dehydrogenase (*solid circle*) activity of the purified 3α-hydroxysteroid dehydrogenase (0.5 μM protein) was determined in either 0.05 M phosphate buffer (pH 4.42–8.3) or 0.05 M glycine-sodium hydroxide buffer (pH 8.6–11.5) by monitoring A_{340} at 25°. Reduction of 3-oxo-cholic (10 μM) with NADPH (10 μM) demonstrated a broad pH range of activity whereas the dehydrogenation of cholic acid (310 μM) with $NADP^+$ (100 μM) demonstrated a narrow, optimal pH around pH 9.0.

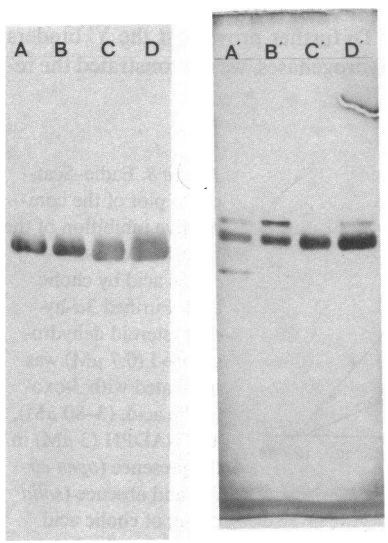

Figure 4. Western blot and silver stain of 3α-hydroxysteroid dehydrogenases I, II, and III with specific polyclonal rabbit antiserum for bile acid binder I. SDS–PAGE of 1 μg each of pooled peaks of 3α-hydroxysteroid dehydrogenase-III, II, and I activity from chromatofocusing, *lanes A, B, D* respectively, and purified 3α-hydroxysteroid dehydrogenase-I from PAP-agarose (*lane C*) were electroblotted onto nitrocellulose and probed with a previously characterized (11) specific rabbit antiserum for bile acid binder I (see Methods for detail). *Lanes A'–D'* depict the silver stain of the corresponding gel after electroblotting. No binding was observed with preimmune serum (not shown). Only a 33 kD protein from all three forms of partially purified 3α-hydroxysteroid dehydrogenase and homogeneous form I were recognized by the antiserum.

Identification of products. The enzymatic activity of bile acid binder as 3α-hydroxysteroid dehydrogenase was also confirmed by examining the bile acid products of the reaction. Purified 3α-hydroxysteroid dehydrogenase-I was incubated at pH 7.4 with

Table IV. Summary of the Kinetic Properties of 3α-Hydroxysteroid Dehydrogenase-I

Cofactor	K_m	Bile acids	K_m	K_i
	μM		*μM*	*μM*
NADPH	0.1	Lithocholic acid	2.6	2.3
$NADP^+$	0.9	Taurolithocholic acid	1.3	—
NAD^+	43	Chenodeoxycholic acid	1.2	3.5
NADH	470	Taurochenodeoxycholic acid	2.5	—
		Cholic acid	105	—
		3-Oxo-cholic acid	8.3	—
		3-Oxo-lithocholic acid	2.0	—

Activities were monitored by ΔA_{340} of cofactors. Activities with 3-oxo-bile acids were determined in 0.05 M sodium phosphate buffer, pH 7, whereas activities with 3α-hydroxy-bile acids were determined in 0.05 M glycine-sodium hydroxide buffer, pH 9.0. The K_i values were determined by competitive displacement of ANS in 0.05 M sodium phosphate buffer, pH 7. See Methods for experimental details.

radiolabelled [^{14}C]chenodeoxycholic acid (5 μM) and NADP$^+$ (230 μM) for 1 min at 37°C. Bile acids were then extracted from the incubation medium and analyzed by TLC compared with reference bile acids (see Methods). 85% conversion of chenodeoxycholic acid to 3-oxo-7α-hydroxy-5β-cholanic acid was observed in 1 min. No 3α-hydroxy,7-oxo-5β-cholanic acid was generated under these conditions. No conversions occurred in the absence of protein or with boiled enzyme. Thus, 3α-hydroxysteroid dehydrogenase is specific for the 3α-hydroxy sterol position.

In addition to incubation of the purified enzyme with bile acid and a single cofactor, a mixture of the four nicotinamide cofactors mimicking the normal redox state in the liver was employed (19, 20). Radiolabelled cholic and 3-oxo-cholic acids (10 μM) were separately incubated with this mixture of cofactors and radiolabelled products identified by TLC. Fig. 6 depicts the complete and rapid reduction of 3-oxo-cholic to cholic acid under these conditions, whereas minimal conversion of cholic to 3-oxo-cholic acid was observed.

3α-Hydroxysteroid dehydrogenase activity in isolated hepatocytes. Isolated hepatocytes (2×10^6/ml) were incubated with radiolabelled cholic (100 μM) or 3-oxo-cholic acid (10 μM). Fig. 7 illustrates the rapid and complete conversion of 3-oxo-cholic to cholic acid whereas minimal conversion of cholic to 3-oxo-cholic acid was observed. No 3-oxo-cholic acid was detected in the extracts from hepatocytes indicating rapid reduction after uptake by the cells. Uptake of cholic acid by the cells was confirmed by the finding that nearly all the cholic acid was in a conjugated form by 30 min. No isocholic acid was detected in either the medium or within the hepatocyte. Thus, in the intact hepatocyte, 3α-hydroxysteroid dehydrogenases preferentially function as 3-oxo-bile acid reductases.

Competitive inhibition of reduction of 3-oxo-cholic acid by cholic acid. The effect of presence or absence of cholic acid (100 μM) on the reduction of 3-oxo-cholic acid (range 3–80 μM) by purified 3α-hydroxysteroid dehydrogenase-I (0.7 μM) with NADPH (3 μM) was examined. The data are depicted in Fig. 8 in an Eadie–Scatchard plot demonstrating competitive inhibition by cholic acid. The calculated K_i value for cholic acid (137 μM) is comparable with the K_m for cholic acid (105 μM), suggesting that cholic acid is capable of binding with the same affinity to 3α-hydroxysteroid dehydrogenase whether it is a substrate or an inhibitor. Thus, bile acids may still bind and inhibit 3α-hydroxysteroid dehydrogenase even though the enzyme preferentially functions as 3-oxo-bile acid reductase under normal physiological conditions.

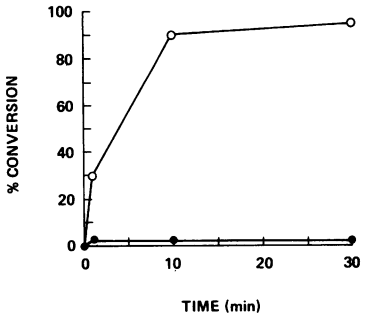

Figure 6. Activity of partially purified 3α-hydroxysteroid dehydrogenase under normal redox conditions. Partially purified 3α-hydroxysteroid dehydrogenase (form I from chromatofocusing) was incubated with a mixture of NADP$^+$ (100 μM), NADPH (400 μM), NAD$^+$ (800 μM), and NADH (200 μM) and either [^{3}H] 3-oxo-cholic (10 μM) (*open circle*) or cholic acid (10 μM) (*solid circle*). Bile acids were extracted and identified on TLC. Results are the mean of duplicate experiments. The results were nearly identical with 20 μM NADP$^+$ (not shown).

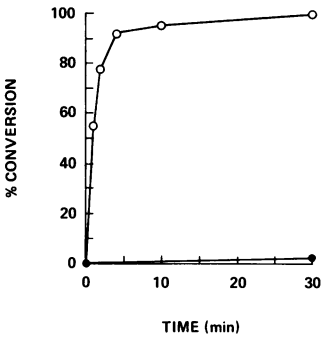

Figure 7. Activity of 3α-hydroxysteroid dehydrogenase in isolated hepatocytes. Isolated hepatocytes were incubated with either 10 μM [^{3}H] (*open circle*) 3-oxo-cholic or 100 μM [^{3}H] cholic acid (*solid circle*). Bile acids in hepatocytes and medium were extracted, deconjugated, and identified on TLC. Data for medium and cells are combined in this depiction. Results are the mean of two experiments. When incubations were carried out with only tracer concentrations of [^{3}H] cholic or [^{3}H] 3-oxo-cholic acid, nearly identical results were obtained (not shown).

Discussion

Our laboratory previously identifed the Y′ bile acid binders in hepatic cytosol. Recently we were struck with the similarities between the proteins we had identified (6) and published characteristics of 3α-hydroxysteroid dehydrogenase (9). Since the latter can catalyze oxidoreduction of bile acids we were even more suspicious that the binding activity represented the interaction of bile acids with an active site on an enzyme. Therefore, we identified the elution of 3α-hydroxysteroid dehydrogenase activity in gel filtration. Enzyme activity coeluted with the Y′ lithocholic acid binders. We then proceeded to purify both enzyme and binding activity exactly as we had previously described for the binders and in a modification of the procedure. In both cases, copurification was demonstrated. The improved resolution of our current techniques permitted identification of one major and two minor forms of binding activity. Previously we had observed only two forms. To further prove that the Y′ binders are 3α-hydroxysteroid dehydrogenases, we demonstrated the re-

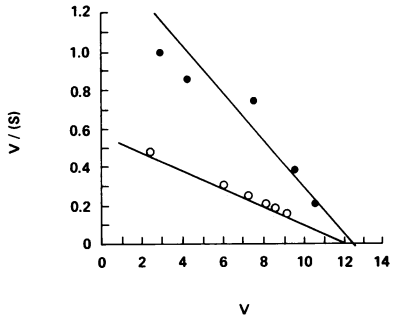

Figure 8. Eadie–Scatchard plot of the competitive inhibition of the reduction of 3-oxo-cholic acid by cholic acid. Purified 3α-hydroxysteroid dehydrogenase-I (0.7 μM) was incubated with 3-oxo-cholic acid, (3–80 μM), and NADPH (3 μM) in the presence (*open circle*) and absence (*solid circle*) of cholic acid (100 μM). Reactions were carried out at 25° in 0.05 M sodium phosphate buffer, pH 7.0 (see Methods for detail). V represents (A_{340} $\times 10^4$/min) and (S) is the 3-oxo-cholic concentration (μM). V_{max} with cholic and without cholic acid was nearly identical whereas K_m for 3-oxo-cholic acid in the presence of cholic was more than twice the value in the absence of cholic acid. K_i for cholic acid (137 μM).

action of binder-I antisera with an identical molecular weight form of 3α-hydroxysteroid dehydrogenase in peaks I, II, and III from chromatofocusing and the homogeneous 3α-hydroxysteroid dehydrogenase-I. Other investigators also have noted multiple molecular forms of 3α-hydroxysteroid dehydrogenase (21).

Having proved the identity of the Y′ bile acid binders and 3α-hydroxysteroid dehydrogenase, we performed a series of experiments to characterize the types of reaction catalyzed by the pure enzyme and the nature of the enzymatic activity in intact hepatocytes. The purified enzyme could catalyze a bidirectional oxidoreduction. Oxidation (dehydrogenase activity) had a very high, unphysiologic pH optimum whereas reductase activity was most active in the physiologic pH range. Furthermore, the enzyme exhibited a marked preference for pyridine nucleotides, especially NADPH. This preference for NADPH has been noted by other investigators (21, 22). Since NADPH is the predominant form under normal redox conditions and the reductase activity is optimal at normal intracellular pH, we predicted that the enzyme would function mainly as a reductase in cells. This hypothesis was confirmed in two types of experiments, one using purified enzyme and a mixture of cofactors to mimick physiologic redox conditions and the other using freshly isolated hepatocytes. In both cases, there was a rapid reduction of 3-oxo-cholic acid to cholic acid but the reverse reaction was negligible. Thus, our data suggests that 3α-hydroxysteroid dehydrogenase is a reductase and not a dehydrogenase under physiologic conditions.

We found that the enzyme had a K_m of 1–2 μM for lithocholic and chenodeoxycholic acids and their taurine conjugates but a much higher K_m (~100 μM) for cholic acid. In examining the conversion of 3-oxo-cholic acid to cholic acid in the presence of excess NADPH, we found that cholic acid could competitively inhibit the reductase activity. The K_i value for inhibition by cholic acid was comparable with the K_m value for cholic acid as a substrate for the dehydrogenase activity. This indicates that 3α-hydroxy bile acids can bind to the enzyme's active site without undergoing biotransformation. Therefore, it remains possible that these enzymes serve a dual role: reductases for 3-oxo-bile acids and cytoplasmic bile acid binders. There is another possible important feature of bile acid binding and inhibition of 3-oxo-bile acid reductase activity. In bile acid synthesis, a common 7α-hydroxy or 7α,12α-dihydroxy-cholestan-4-ene-3-one precursor is believed to undergo reduction by 3α-hydroxysteroid dehydrogenase (23). Theoretically, this step may be a point of regulation in which high concentrations of bile acids periodically present in hepatocytes inhibit this key step in bile acid synthesis. More work will be required to examine this hypothesis.

The reductase activity of 3α-hydroxysteroid dehydrogenase, aside from being important in bile acid synthesis, may be important in reducing 3-oxo-bile acids returning to the liver after colonic bacterial metabolism (24) or produced by an NAD^+-dependent hepatic microsomal 3β-hydroxy bile acid dehydrogenase (25–27) which acts on 3β-hydroxy bile acids produced by bacteria (28, 29). Although 3β-hydroxy bile acids have been identified in portal blood, they are absent in bile (28). Therefore, a conserted action of two hepatic enzymes may account for this efficient conversion: microsomal NAD^+-3β-hydroxysteroid dehydrogenase and cytosolic 3α-hydroxysteroid dehydrogenase (NADPH 3-oxo reductase). The cofactor preference of these two different enzymes is probably responsible for the epimerization at the 3 position. NAD^+ predominates over NADH and is the known preferred cofactor for the microsomal enzyme (25). NADPH predominates over $NADP^+$ and is the preferred cofactor for the 3α-hydroxysteroid dehydrogenase. The microsomal enzyme does not oxidize 3α-hydroxy-bile acids (26 and Stolz et al., unpublished observations).

An extensive amount of literature exists on 3α-hydroxysteroid dehydrogenase. Tomkins was the first to characterize the mammalian enzyme with the reversible conversion of dihydro and tetra-hydrocortisone (30). Most of the older work used impure preparations. More recently, several groups have purified a form of 3α-hydroxysteroid dehydrogenase from rat liver (9, 21, 22). The enzyme has been implicated in the biosynthetic oxidoreduction of the 3-position of a wide variety of steroid hormone substrates. In addition, 3α-hydroxysteroid dehydrogenase has been shown to catalyze dihydrodiol dehydrogenase activity that may be important in the detoxification of dihydrodiol carcinogens (31, 32).

The enzymatic characteristics of the cytosolic 3α-hydroxysteroid dehydrogenase with regard to bile acid metabolism have not been as intensively studied as with steroid hormones or dihydrodiol substrates. Penning and Talalay (22) and Vogel and Oesch (9), although working with a purified form of the enzyme, have not examined bile acids as substrates. Studies examining either the reduction or dehydrogenation of bile acids by the cytosolic 3α-hydroxysteroid dehydrogenase have been limited because of the use of crude cytosolic extracts or impure protein preparations. Usui and coworkers partially purified 3α-hydroxysteroid dehydrogenase by ammonium sulfate precipitation of cytosol followed by gel filtration. They identified complete stereospecific reduction of tri-oxo cholic acid (3,7,12-tri-oxo-5β-cholanic acid) to 3α-hydroxy-7,12-di-oxo-cholic acid with preference for the cofactor, NADPH (26). Berseus partially purified 3α-hydroxysteroid dehydrogenase and characterized its substrate specificity and kinetics (23). The K_m for cofactor preference and pH optimum were similar to our results. More recently, Ikeda et al. identified multiple forms of 3α-hydroxysteroid dehydrogenase in rat liver and purified two forms (21, 33). Their purification scheme consisted of sequential ammonium sulfate precipitation, anionic and cationic ion exchange chromatography, chromatofocusing, and red-sepharose affinity chromatography. Since their procedure differs from ours, it is difficult to directly compare the forms of 3α-hydroxysteroid dehydrogenase that we and they have identified. There is close agreement between the results of the two laboratories with respect to molecular weight and pH optima. However, our enzyme kinetic data differ markedly from theirs in showing an order of magnitude higher affinity (lower K_m) for mono- and dihydroxy bile acids. In addition our K_m values for NADPH and $NADP^+$ are two to three orders of magnitude lower (higher affinity) than theirs. The reason for this discrepancy may be due to differences in stability of their preparation with loss of affinity or strain differences. They studied Wistar and we used Sprague–Dawley rats.

In summary, we have proved that the previously identified Y′ bile acid binders are 3α-hydroxysteroid dehydrogenases. On the basis of pH optima and cofactor preferences, we have shown that this enzyme, both in purified form and in cells, functions as a 3-oxo-bile acid reductase. Since the enzyme can bind bile acids at the active site without biotransformation, 3α-hydroxysteroid dehydrogenase activity might be regulated by bile acid flux through the liver, and these enzymes may still serve as cytosolic binders under normal redox conditions.

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