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COMPARISON OF DIHYDROPYRIMIDINE DEHYDROGENASE FROM HUMAN, RAT, PIG AND COW LIVER

BIOCHEMICAL AND IMMUNOLOGICAL PROPERTIES

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Abstract—Dihydropyrimidine dehydrogenase (DPD, EC 1.3.1.2) is the initial and rate-limiting enzyme in the catabolic pathway of pyrimidines and has an important role in cancer chemotherapy with fluoropyrimidine drugs. Recently, we purified and characterized this enzyme from human liver and raised a rabbit polyclonal antibody against the purified human enzyme (Lu *et al.*, *J Biol Chem* **267**: 17102–17109, 1992). In the present study, using this purification procedure, DPD was purified to homogeneity from three other mammalian species, i.e. pig, rat, and cow. Comparison of the biochemical properties of these purified enzymes was conducted. Molecular masses of DPD from human, pig, rat, and cow liver were: 210, 204, 210, and 216 kDa, respectively. DPD from all four species appeared to be composed of two subunits. The apparent pI values were 4.6, 4.8, 4.85, and 5.25, respectively. Kinetic studies with uracil, thymine, 5-fluorouracil, and NADPH were carried out with the purified DPD preparation, suggesting species differences in kinetic parameters. Amino acid composition of these purified enzymes also demonstrated slight species differences. In the present study, a rabbit polyclonal antibody against rat liver DPD was raised. Using polyclonal antibodies against human and rat liver DPD, immunoblotting demonstrated cross-reactivity among the four species. In summary, purification and comparison of DPD from different mammalian species will provide a basis for further biochemical and molecular studies of this enzyme.

Dihydropyrimidine dehydrogenase (EC 1.3.1.2, DPD†) is the initial and rate-limiting enzyme in pyrimidine catabolism. The importance of this enzyme has been demonstrated in several previous studies [1–3], particularly its critical role in cancer chemotherapy with fluoropyrimidine drugs. Studies from our laboratory and others have demonstrated that more than 85% of administered 5-fluorouracil (FUra), one of the most frequently used anticancer drugs, is catabolized by this enzyme [1, 2]. It has also been demonstrated that the anticancer efficacy of FUra is related to DPD activity [4]. There is a correlation between DPD activity and plasma FUra concentration in patients treated with FUra [5, 6]. Additional studies with competitive DPD inhibitors [7–9] and in cancer patients with DPD deficiency [10–12] have also shown the importance of this enzyme in cancer chemotherapy.

Since the major site of pyrimidine catabolism is in the liver [13], most studies with DPD have utilized liver tissue. In the last three decades, DPD has been purified to varying degrees from liver of several species, including cow [14], rat [15, 16], and pig [17, 18]. In addition, studies have suggested that DPD may be species specific [14–17]. We have reported recently a new purification procedure and successfully purified human liver DPD to homogeneity, and generated a specific polyclonal antibody against this enzyme [19].

Since most of our understanding of this enzyme has derived from studies with experimental animals [2, 14–18] and potential species differences in this enzyme may exist, it is important to conduct a comprehensive comparative study to investigate biochemical and kinetic properties of DPD from different species. Knowledge acquired from such a study should be helpful in determining the usefulness of data from animal studies for further biochemical and molecular studies of this enzyme and application to clinical cancer chemotherapy.

MATERIALS AND METHODS

Chemicals. Uracil, thymine, FUra, and NADPH were purchased from Sigma (St. Louis, MO). L-Histidine was obtained from Aldrich (Milwaukee, WI). Polybuffer exchanger gel (PBE-94), polybuffer 74, molecular weight markers, and 2′, 5′-ADP-Sepharose 4B were obtained from Pharmacia-LKB (Piscataway, NJ). Coomassie brilliant blue R-250, acrylamide, and pre-stained molecular weight markers were purchased from Bio-Rad (Richmond, CA). Alkaline phosphatase-labeled goat anti-rabbit antibody, nitroblue tetrazolium, and 5-bromo-4-

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† Abbreviations: DPD, dihydropyrimidine dehydrogenase; FUra, 5-fluorouracil; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; and BSA, bovine serum albumin.

chloro-3-indolyl phosphate *n*-toluidine salt were obtained from Southern Biotechnology (Birmingham, AL). [^{3}H]FUra (25 Ci/mmol) was obtained from the New England Nuclear Corp. (Boston, MA). [6-^{14}C]Uracil (55 mCi/mmol) and [12-^{14}C]-thymine (52 mCi/mmol) were obtained from Moravek Biochemicals (Brea, CA). Radiochemicals, uracil, thymine, and FUra were purified by HPLC [19], and their purity was determined by HPLC to be > 99%. All other solvents and reagents were purchased in the highest grade available.

Buffers. The major buffer (buffer A) used in the preparation of this enzyme, DPD assay, and in the kinetic studies of purified enzyme contained 35 mM potassium phosphate, pH 7.4, 2.5 mM magnesium chloride, and 10 mM 2-mercaptoethanol. The equilibration buffer for the chromatofocusing column (histidine buffer) contained 25 mM L-histidine-HCl, pH 6.0, in the presence of 10 mM 2-mercaptoethanol. Elution buffers for the affinity column and the HPLC gel filtration column were prepared from buffer A.

DPD assay. The enzyme activity during purification was determined by measuring the catabolites of FUra using reversed-phase HPLC [19, 20]. The reaction mixture contained 200 μM NADPH, 20 μM [^{3}H]FUra, buffer A, and enzyme solution in a final volume of 2 mL. The mixture was incubated at 37° and 350 μL of the reaction sample was taken out at various times and added into the same volume of ice-cold ethanol to stop the reaction. The sample was then kept in a freezer (−20°) for 30 min and filtered through a 0.2 μm Acro filter (Gelman Sciences, Ann Arbor, MI) prior to HPLC analysis.

Reversed-phase HPLC analysis. Separation of pyrimidines and their catabolites was performed by reversed-phase HPLC using a Hewlett–Packard 1050 HPLC system equipped with a filter spectrometric detector and chromatographic terminal (HP 3396 series II Integrator). Two Hypersil 5 μm columns (Jones Chromatography, Littleton, CO) were used in tandem as the stationary phase. The columns were eluted at a flow rate of 0.5 or 1.0 mL/min with the mobile phase containing 1.5 mM potassium phosphate, pH 8.0 or pH 8.4, with 5 mM tetrabutylammonium hydrogen sulfate [19].

Enzyme purification. All procedures were performed at 4°. The methods used in purification of DPD from rat, cow and pig were a modification of the methods we reported previously [19]. In brief, freshly prepared rat liver (250 g) and frozen pig or cow liver (300 g for each) were used in this study. These liver tissues were minced and homogenized in 4 vol. of buffer A, in the presence of 0.25 M sucrose, 1 mM benzamidine, 1 mM aminoethylisothiouronium bromide, and 5 mM EDTA. The homogenate was centrifuged at 100,000 *g* for 60 min, and the supernatant was removed in order to obtain a cytosolic fraction. Acetic acid (25%, v/v) was added to the resulting supernatant to adjust the pH to 4.85 with constant stirring for 15 min at this pH. After centrifugation at 30,000 *g* for 30 min, the supernatant was removed and adjusted with 0.5 N KOH to pH 7.4. Solid ammonium sulfate was added slowly to the supernatant until a 33% saturation was obtained. The mixture was stirred for 30 min. Following centrifugation at 30,000 *g* for 30 min, the supernatant was removed and additional ammonium sulfate was added to the supernatant until a 55% saturation was obtained with constant stirring for 30 min. After centrifugation at 30,000 *g* for 30 min, the precipitate was dissolved in 125 mL of 25 mM histidine-HCl buffer, pH 6.0, in the presence of 10 mM 2-mercaptoethanol, and dialyzed overnight against 10 L of the same buffer. The dialyzed sample was then centrifuged at 30,000 *g* for 30 min, and the supernatant was loaded onto a chromatofocusing column (1.6 × 100 cm) packed with PBE-94 previously equilibrated with 25 mM histidine-HCl buffer, pH 6.0. The column was then eluted with polybuffer 74 diluted 1:8 with distilled deionized water (final pH adjusted to 4.0 with HCl), in the presence of 10 mM 2-mercaptoethanol. Fractions with DPD activity from the chromatofocusing column were concentrated by an Amicon centriprep 10 concentrator and loaded onto a 2′, 5′-ADP-Sepharose 4B affinity column (1 × 40 cm) previously equilibrated with buffer A. The column was washed with 20 column volumes of buffer A, 10 column volumes of 50 mM KCl–buffer A, 10 column volumes of 100 mM KCl–buffer A, and 2 column volumes of 200 mM KCl–buffer A. Enzyme activity was eluted with 0.1 mM NADPH in buffer A. Fractions containing DPD activity from the affinity column were concentrated in an Amicon centricon 10 concentrator and then injected onto a Bio-Rad TSK-250 gel filtration column (2.15 × 60 cm), previously equilibrated with buffer A. Enzyme activity was eluted by buffer A at a flow rate of 2.5 mL/min. Fractions containing DPD activity were pooled and concentrated in an Amicon centricon 10 concentrator. The purified enzymes were used in subsequent determinations and kinetic studies.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). SDS–PAGE was carried out in a 1.0 mm thick, 7% (w/v) polyacrylamide gel containing 0.375 M Tris–HCl (pH 8.8) and 0.1% SDS. Samples were mixed with an equal volume of sample buffer (0.0625 M Tris–HCl, pH 6.8; 10% glycerol; 0.2% SDS (w/v); 80 mM 2-mercaptoethanol) and then boiled for 5 min. Electrophoresis was performed at a constant current of 30 mA for 45 min at 25°. The gel was fixed in a 5% methanol/7% acetic acid solution for 30 min and then stained overnight using 0.01% (w/v) Coomassie brilliant blue R-250 in a 5% trichloroacetic acid/2.5% methanol/3.5% acetic acid solution.

Molecular weight determination. The molecular weight of native DPD was determined by HPLC gel filtration. A 2.15 × 60 cm TSK-250 gel filtration HPLC column (Bio-Rad) was equilibrated with buffer A, pH 7.4, at a flow rate of 2.5 mL/min. The column was calibrated using known molecular weight standards (Pharmacia) and the retention times of these standards were determined by their peaks of absorbance at 280 nm. The retention times of purified enzymes were then compared with those of the molecular weight standards and the molecular weight of each purified DPD was calculated from the standard curve. The molecular weight of denatured DPD was determined by 7% SDS–PAGE by a

Table 1. Purification of liver dihydropyrimidine dehydrogenase from human [19], pig, rat, and cow

Species	Step	Total protein (mg)	Total activity* (nmol/min)	Specific activity* (nmol/min/mg)	Recovery* (%)	Recovery* (-fold)
Human	Crude supernatant	24,229	4,911	0.203		
	Gel filtration	0.63	999	1,586	20.3	7,824
Pig	Crude supernatant	20,460	12,212	0.597		
	Gel filtration	1.32	2,038	1,544	16.7	2,588
Rat	Crude supernatant	22,500	11,193	0.498		
	Gel filtration	2.6	4,785	1,840	42.7	3,700
Cow	Crude supernatant	29,952	10,205	0.341		
	Gel filtration	2.5	3,125	1,250	30.6	3,669

* All values were calculated using FUra as a substrate.

comparison with molecular weight markers (Bio-Rad).

Kinetic studies. Initial reaction rates were determined at various concentrations (0.5, 1, 2, 3, 4, 5, 7.5, 10, 20, 40, 80, 100, 200, 500 μM) of each substrate, i.e. uracil, thymine, or FUra, in the presence of 200 μM NADPH. Kinetic studies for NADPH were carried out at various concentrations of NADPH (0.5, 1, 2, 3, 5, 7.5, 10, 20, 40, 60, 80, 100, 200, 500 μM) in the presence of 20 μM uracil, thymine or FUra. Reactions were run in buffer A at 37°. The incubation time and protein concentration were adjusted so that no more than 10% of the substrate was consumed. Estimation of the apparent K_m and V_{max} values for each substrate was performed by fitting these data for several concentrations of different substrates to the Michaelis–Menten equation by non-linear regression analysis [21]. Enzyme activity is expressed as micromoles of product formed per minute per milligram of protein.

Protein determination. The amount of protein in the sample was determined by the method of Bradford [22].

Amino acid analysis. Protein samples were carboxymethylated as described by Allen [23]. The amino acid composition was determined by first hydrolyzing the sample at 110° for 20 hr in the presence of 6 N HCl and then analyzed by reversed-phase HPLC using the PICO TAG system (Waters Associates, Milford, MA).

Preparation of polyclonal antibody against rat liver DPD. Male New Zealand rabbits were immunized with subcutaneous injections of purified rat liver DPD. For the primary injection, 50 μg of purified antigen (in 500 μL sterile water) was mixed with an equal volume of Freund's complete adjuvant. Two weeks later, these rabbits were injected with the antigen (50 μg) mixed with an equal volume of Freund's incomplete adjuvant; 3 weeks following the second injection, this injection was repeated. Aliquots of serum samples from ear nicks were screened for antibody formation using enzyme-linked immunosorbant assay [24] and Western blot analysis [25]. Two weeks following the third injection, the rabbits were killed by cardiac puncture and their blood was collected. The serum was centrifuged at 1000 g for 15 min and loaded onto a 1 × 10 cm protein A-Sepharose 4 Fast Flow column (Sigma), previously equilibrated with phosphate-buffered saline (PBS). The column was washed with 4 column volumes of PBS, and the IgG antibodies were eluted with a 0.2 M glycine-HCl buffer, pH 2.5, containing 0.075 M NaCl. Immediately upon elution from the column, the fractions were neutralized with 1.0 M Tris–HCl, pH 10, and concentrated by using an Amicon centricon 10 concentrator.

Immunoblot analysis. The primary antibody used in the study was the purified rabbit polyclonal antibody generated against either human liver DPD [19] or rat liver DPD described above. A 7% SDS–PAGE was performed using both the freshly prepared 100,000 g liver supernatant and the purified liver DPD from human, pig, rat, and cow. The proteins were transferred from the gel to a nitrocellulose filter following the method of Towbin *et al.* [25]. Following incubation overnight at 4° with the primary antibody (diluted 1:2000) in a 120 mM borate-saline solution containing 1% (w/v) bovine serum albumin (BSA), pH 8.5, the nitrocellulose filter was washed with borate-saline containing 0.5% (v/v) Tween 20 and then incubated with a secondary, alkaline phosphatase-labeled goat anti-rabbit antibody. The location of immunoreactive proteins on the nitrocellulose filter was developed in a 0.1 M sodium carbonate buffer (100 mL), pH 9.5, containing 30 mg nitroblue tetrazolium (added as a 1-mL solution dissolved in 70% dimethylformamide) and 15 mg 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (added as a 1-mL solution dissolved in 100% dimethylformamide).

RESULTS

Enzyme purification. In the present study, DPD activity was purified from the cytosol fractions of homogenates of fresh rat liver and frozen pig and cow livers. The purifications were accomplished by a combination of acid precipitation, salt fractionation, dialysis, chromatofocusing, affinity chromatography, and HPLC gel filtration. In typical preparations, the final products had an approximately 2600 to 3700-fold enrichment of enzyme activity, with an overall recovery of 17–43% (Table 1).

Molecular weight determination. Purified enzyme

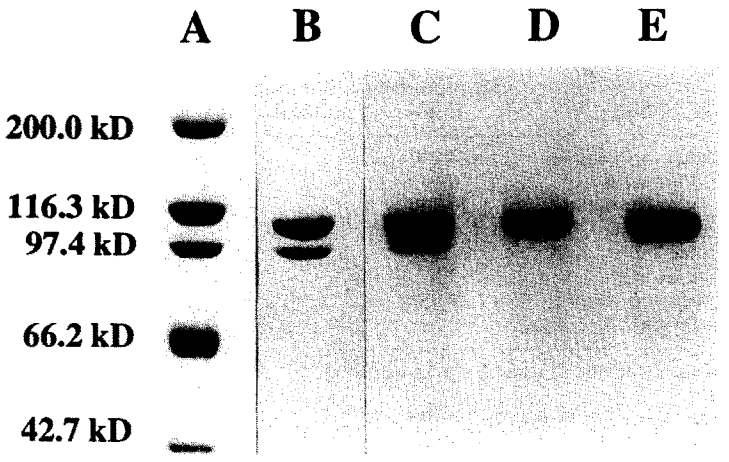

Fig. 1. SDS–PAGE of denatured pure liver DPD from human, pig, rat, and cow. Lane A contains molecular mass markers. Lanes B, C, D and E contain 10 μg of the denatured pure enzyme from human, pig, rat, and cow, respectively, stained by Coomassie brilliant blue R-250.

Table 2. Comparison of liver dihydropyrimidine dehydrogenase from human [19], pig, rat, and cow

Species	Molecular mass (kDa)	Subunits molecular mass (kDa)	pI
Human	210	105	4.60
Pig	204	102	4.80
Rat	210	105	4.85
Cow	216	108	5.25

was homogeneous as judged by HPLC gel filtration on a TSK 250 column (calibrated with known standards) showing a single, symmetrical peak for each purified enzyme (data not shown). The molecular masses of native DPDs from pig, rat, and cow were 204, 210, and 216 (± 5) kDa, respectively. The denatured pig liver DPD resulted in a pattern similar to that of purified human liver DPD, showing two sharp protein bands with apparent molecular masses of 102 and 90 (± 3) kDa on a 7% SDS–polyacrylamide gel (Fig. 1). Under these same conditions, the denatured rat and cow liver DPDs each gave one sharp protein band with apparent molecular masses of 105 and 108 (± 3) kDa, respectively (Fig. 1).

Determination of the isoelectric point of DPD. Elution from the chromatofocusing column demonstrated apparent isoelectric points (pI) of 4.6, 4.8, 4.85, 5.25 (± 0.2) for DPDs from human, pig, rat, and cow, respectively (Table 2). The elution pattern of each purified enzyme was symmetrical, further suggesting that each purified DPD was homogeneous (data not shown).

Amino acid composition. The amino acid composition of carboxymethylated DPDs from human, pig, rat and cow are listed in Table 3. Pig liver DPD had an amino acid composition similar to that of human liver DPD, with fewer residues of Gly, His, and Val and more residues of Thr and Cys. Similarity was also shown between rat liver and cow liver DPD. Compared with the human enzyme, DPDs from the two species have more residues of Asp + Asn, Glu + Gln, Ser, and Thr, and fewer residues of His, Val, Tyr, and Leu. Cow liver DPD has fewer Phe.

Table 3. Amino acid compositions of liver dihydropyrimidine dehydrogenase from human [19], pig, rat and cow

Amino acid	Amount (residues/mol enzyme) from liver of: Human	Pig	Rat	Cow
Asp + Asn	163.5	154.1	200.8	215.8
Glu + Gln	189.8	207.6	213.7	237.2
Ser	108.7	94.8	129.6	122.5
Gly	180.5	153.3	177.5	174.0
His	38.7	27.3	28.0	25.4
Arg	92.6	91.5	92.6	94.8
Thr	130.3	202.2	184.7	222.1
Ala	185.1	181.0	172.9	197.0
Pro	147.3	141.7	141.2	144.2
Tyr	66.0	56.8	49.3	49.2
Val	140.4	118.5	110.7	116.3
Met	51.9	49.0	50.4	39.4
Cys	16.2	22.8	16.6	18.1
Ile	115.7	130.2	114.5	101.7
Leu	199.7	188.2	173.0	157.8
Phe	92.2	90.4	83.7	68.2
Lys	94.3	107.1	101.7	82.0
Trp	ND*	ND	ND	ND

These data represent the mean of three to four separate DPD preparations.
* ND = not determined.

Table 4. Comparison of kinetics for liver dihydropyrimidine dehydrogenase from human [19], pig, rat, and cow

Substrate	Parameter	Liver from Human	Pig	Rat	Cow
Uracil	K_m (μM)	4.9 ± 0.30	5.9 ± 0.43	2.4 ± 0.21	3.9 ± 0.33
	V_{max} (μmol/min/mg)	0.6 ± 0.02	0.7 ± 0.02	0.8 ± 0.02	0.7 ± 0.02
Thymine	K_m (μM)	4.8 ± 0.43	6.8 ± 0.53	2.5 ± 0.21	4.6 ± 0.54
	V_{max} (μmol/min/mg)	0.7 ± 0.04	0.7 ± 0.03	0.6 ± 0.02	0.6 ± 0.05
FUra	K_m (μM)	3.3 ± 0.21	1.1 ± 0.06	8.9 ± 0.55	1.8 ± 0.19
	V_{max} (μmol/min/mg)	0.9 ± 0.06	0.7 ± 0.02	0.9 ± 0.03	1.0 ± 0.03
NADPH	K_m (μM) with uracil	9.6 ± 0.85	11.9 ± 1.01	20.6 ± 1.38	8.9 ± 0.69
	K_m (μM) with thymine	15.8 ± 0.94	16.7 ± 0.76	28.9 ± 1.71	6.8 ± 0.68
	K_m (μM) with 5-fluorouracil	10.1 ± 0.81	8.2 ± 0.83	20.9 ± 0.97	8.5 ± 0.42

Values are means ± SD, N = 3–5.

Kinetic properties. Table 4 summarizes the kinetic studies of purified liver DPD from human, pig, rat, and cow. Using standard assay conditions at pH 7.4 and 37°, in the presence of 200 μM NADPH, FUra was the best substrate for DPDs from human, pig, and cow. For rat DPD, however, the K_m of FUra was approximately 3.5-fold that observed with thymine and uracil and approximately 3-, 4-, and 8-fold that observed with human, cow, and pig DPD, respectively, with statistical significance ($p < 0.05$). With each of the purified DPDs, substrate inhibition was observed for all pyrimidine substrates examined in the present study, at concentrations of 100 μM and above (Fig. 2). In the presence of 20 μM pyrimidine substrate, pig liver DPD had an apparent K_m for NADPH similar to that of human liver DPD. In contrast, apparent K_m values for NADPH with rat DPD were 2-fold greater than those observed with human DPD. Cow DPD had slightly lower K_m values for NADPH. Under the above conditions, no significant inhibition by NADPH was observed until 1000 μM NADPH.

Immunologic characterization. In the present study, a rabbit polyclonal antibody was generated against purified rat liver DPD. Antibodies against human DPD [19] or rat DPD were utilized in immunoblot analysis of the 100,000 *g* liver supernatant from human, pig, rat and cow. After separation on SDS–PAGE, a single protein band for each species was observed (Fig. 3). Pre-immune serum from the same rabbit did not detect any band under the same conditions (data not shown). Both antibodies were also shown to recognize denatured pure liver DPD from each of the four species (Fig. 4).

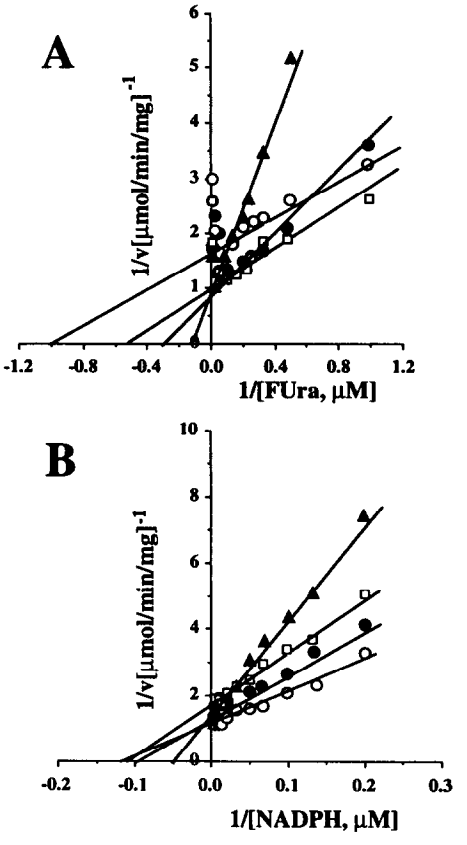

Fig. 2. Kinetic analysis of purified liver DPD from human, pig, rat, and cow. Double-reciprocal plots of reaction velocity versus the concentration of (A) FUra and (B) NADPH. Reaction conditions were described in Materials and Methods. Key: (●) human DPD; (○) pig DPD; (▲) rat DPD; and (□) cow DPD.

DISCUSSION

The present study compared DPD from different mammalian species, i.e. human, pig, rat, and cow, using pure enzymes generated from a similar purification procedure. Study of liver DPD from these four mammalian species revealed not only similarity, e.g. molecular mass and subunits, but also some differences, e.g. pI, amino acid composition, kinetic and immunocharacterization. In addition, a polyclonal antibody has been raised for the first time against rat liver DPD. The availability of pure liver DPD from different species, polyclonal antibodies, and new data on the

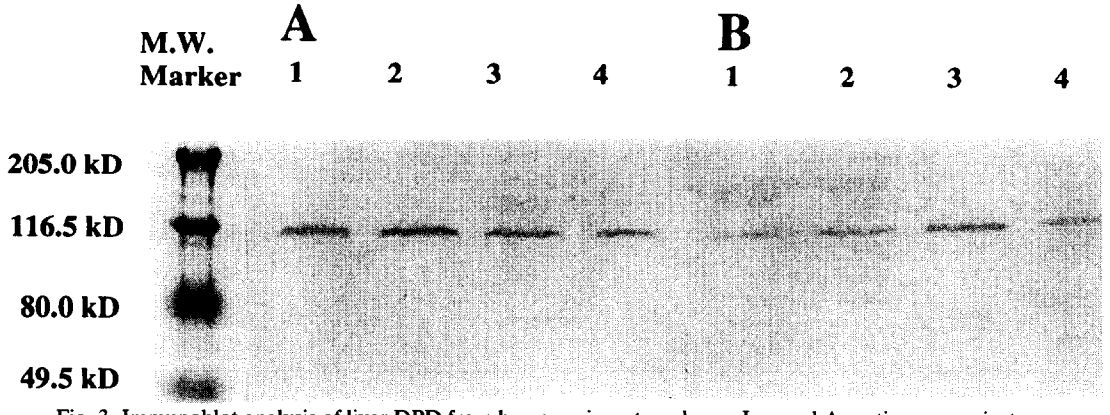

Fig. 3. Immunoblot analysis of liver DPD from human, pig, rat, and cow. In panel A, antiserum against human liver DPD [19] was used as the primary antibody. In panel B, antiserum against rat liver DPD was used as the primary antibody. In both A and B, lanes 1, 2, 3 and 4 contain 200 μg of crude liver cytosol from human, pig, rat, and cow, respectively.

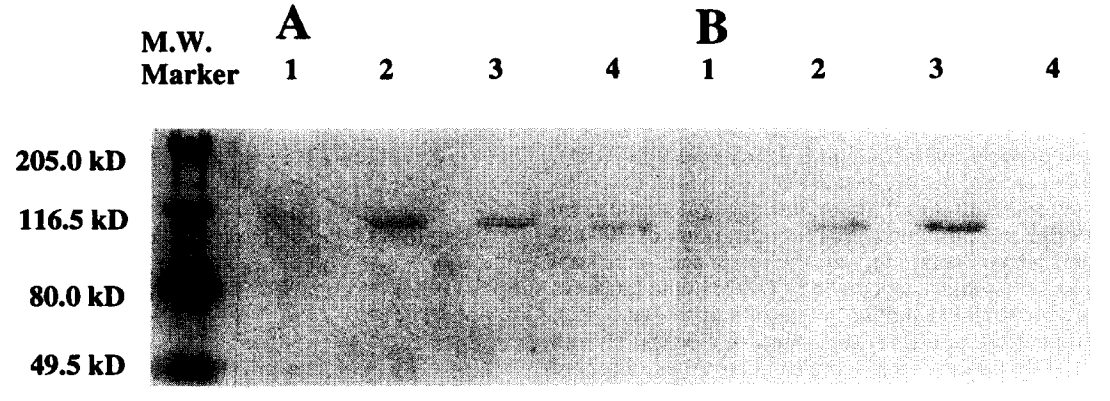

Fig. 4. Immunoblot analysis of denatured pure liver DPD from human, pig, rat, and cow. In panel A, antiserum against human liver DPD [19] was used as the primary antibody. In panel B, antiserum against rat liver DPD was used as the primary antibody. In both A and B, lanes 1, 2, 3 and 4 contain 0.5 μg of denatured pure liver DPD from human, pig, rat, and cow, respectively.

biochemical and immunological characteristics should provide a basis for further biochemical and molecular studies with this enzyme, particularly relevant to extrapolation of data from animal studies to human study.

Using the methods developed in the purification of human liver DPD [19], purification of DPD from rat, pig, and cow to homogeneity was accomplished by a combination of acid precipitation, ammonium sulfate fractionation, chromatofocusing, affinity chromatography, and HPLC gel filtration. When denatured pure pig liver DPD was resolved by 7% SDS–PAGE, two polypeptide bands with molecular masses of 102 and 90 kDa were observed, which is similar to the pattern of purified human DPD on SDS–PAGE [19], suggesting that pig liver DPD consists of two 102-kDa subunits with the 90-kDa polypeptide representing a degradation product. This is based on the following data: (1) under non-denaturing conditions, purified pig liver DPD was eluted by HPLC gel filtration as one symmetrical peak that corresponded to DPD activity; (2) fractions from chromatofocusing, affinity, and HPLC gel filtration columns that had DPD activity were shown on SDS–PAGE to contain the 102- and 90-kDa polypeptides (other fractions without DPD activity did not contain any one of these polypeptides); and (3) immunoblot analysis using the rabbit polyclonal antibodies detected a single 102-kDa protein band in the crude pig liver cytosol, whereas two bands with molecular masses of 102- and 90-kDa were detected in the denatured pure pig liver DPD. It should be noted that other studies have also suggested the same pattern with pig liver DPD on SDS–PAGE [17]. Data from HPLC gel filtration, SDS–PAGE and Western blot analyses demonstrated that rat and cow liver DPD consisted of two identical subunits for each purified enzyme.

Determination of the isoelectric point (pI) of purified enzymes revealed that human liver DPD had a pI (4.6) [19] similar to those of pig and rat liver DPDs (pI 4.8 and 4.85), whereas cow liver DPD had a higher pI (5.25). In this respect, data from the present study were similar to that of a previous study of pig liver DPD (pI 4.65) [17], but different from an earlier study of rat liver DPD (pI 5.25) [15]. The significance and reasons for the differences were not clear but may be due to the methods in determination of pI. Comparison of the amino acid composition of DPD from the four mammalian species demonstrated a significant deviation in composition. In this respect, human liver DPD appears closer to pig liver DPD.

Significant variations in estimated kinetic parameters have been reported [14, 15, 17–19, 26] and may result from several factors, including species differences, varying methods in determination of enzyme activity, or varying degrees of purification. In most of the previous DPD purifications [14–17] and in the kinetic studies [14, 15, 17, 18], enzyme activity was determined by the decrease in NADPH assessed by measuring changes in absorbance at 340 nm. This method is limited in both sensitivity and specificity, particularly in the first several steps of purification where more than one enzyme consumes NADPH. The enzyme activity in the present study was quantitated by measuring specific product formation. Using this HPLC method, kinetic studies have demonstrated similar kinetic properties for the purified liver DPD from human, pig, and cow, with the notable exception being the K_m for NADPH with cow DPD. Compared with the other three species, rat liver DPD had statistically significantly lower K_m values for uracil and thymine and statistically significantly greater K_m values for FUra and NADPH. Although the mechanisms responsible for the species difference are not known, these differences may be important in the selection of an animal model or an *in vitro* system for biochemical studies of this enzyme as well as pharmacologic studies with fluoropyrimidine drugs.

In addition to the development of a polyclonal antibody against human liver DPD, in the present study we generated a polyclonal antibody against purified rat liver DPD. This is particularly important since most studies of DPD have been conducted with rats. Using the two antibodies, Western blot analyses revealed cross-reactivity among the four species, i.e. the two antibodies can react with DPD from all four species although the strength of the reaction signals was different. For the antibody generated with human DPD, the strongest reaction was observed with human liver tissue; for the antibody generated with rat DPD the strongest reaction was observed with rat liver tissue. For both antibodies, a weak reaction was observed with cow liver DPD, suggesting species difference in immunocharacteristics of DPD. The availability of pure enzymes and polyclonal antibodies from different species as well as new data on the biochemical properties of DPD will provide a basis for further biochemical and molecular studies with this enzyme.

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