Identification of hepatic Z-protein in a marine elasmobranch, *Platyrrhinoides triseriata*

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Previous studies were unable to identify Z-protein in elasmobranch liver with bromosulphophthalein as ligand. By using 8-anilino-naphthalene-1-sulphonate and Rose Bengal as ligands, however, we demonstrated in hepatic cytosol from *Platyrrhinoides triseriata* an organic-anion-binding protein with gel-filtration characteristics identical with those of rat Z-protein. By comparison with pooled rat Z-protein, *Pl. triseriata* Z-protein had slightly lower affinity for 8-anilino-naphthalene-1-sulphonate and Rose Bengal, greatly decreased binding affinity for bromosulphophthalein and no binding activity for oleic acid or squalene. The *Pl. triseriata* Z-protein binding site was less hydrophobic than that of rat Z-protein. This observation may explain the differences in binding characteristics between the Z-proteins of these species.

Cytosolic proteins in liver are known to bind organic anions and are thus thought to function in hepatic transport. Two major classes of organic-anion-binding proteins have been described, the so-called Y- (ligandin) and Z-proteins (Levi *et al.*, 1969). Little is known about the phylogeny of these proteins. We recently characterized ligandin-like proteins in the liver of a marine elasmobranch, *Platyrrhinoides triseriata* (Sugiyama *et al.*, 1981). We presently report the extension of these studies to Z-protein.

Z-protein has been shown to bind selectively fatty acids (Mishkin *et al.*, 1972; Ockner *et al.*, 1972) and squalene (Dempsey *et al.*, 1981) in mammals. The only studies of Z-protein in non-mammalian species have employed bromosulphophthalein as ligand for identification of organic-anion-binding activity. Levine *et al.* (1971) and others (Boyer *et al.*, 1976*a,b*) were thus unable to identify Y- and Z-proteins in elasmobranchs. However, we have previously demonstrated that Y-protein in the shark has a very low affinity for bromosulphophthalein but readily binds other organic anions. Therefore we examined the binding characteristics of cytoplasmic proteins in the shark by using several different

organic anions with the hope of identifying a protein similar to Z-protein.

Materials and methods

Materials

ANS and bilirubin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and bromosulphophthalein and Rose Bengal were from J. T. Baker Chemical Co. (Philipsburg, NJ, U.S.A.) and Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) respectively. [¹⁴C]Oleic acid (sp. radioactivity 58 Ci/mol) and [³H]squalene (sp. radioactivity 3.9 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.).

Animals and preparation of liver cytosol

The livers of six male Sprague–Dawley rats and six *Pl. triseriata*, the latter kindly supplied by Pacific Marine Biologists (Culver City, CA, U.S.A.), were removed, and 33% cytosol was prepared as described previously (Sugiyama *et al.*, 1981). Non-esterified fatty acid concentration in the cytosol was measured colorimetrically (Mikac-Devic *et al.*, 1973).

Determination of binding activities of gel-filtration eluates

Gel filtration was performed on a 100 cm × 2.5 cm column of Sephadex G-75 (superfine grade; Pharmacia, Uppsala, Sweden) at 4°C, with 10 mM-sodium phosphate, pH 7.4 (buffer A), as elution

Abbreviation used: ANS, 8-anilino-naphthalene-1-sulphonate.

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buffer. Binding of various organic anions to the eluted fractions (2.5 ml) was determined at the following ligand concentrations as previously described (Sugiyama *et al.*, 1981): bromosulphophthalein, $0.5 \mu\text{M}$, by equilibrium dialysis (Sugiyama *et al.*, 1979); Rose Bengal, $1.15 \mu\text{M}$, and bilirubin, $1.5 \mu\text{M}$, by spectrophotometric methods (Tipping *et al.*, 1976; Sugiyama *et al.*, 1978); ANS, $20 \mu\text{M}$, by a fluorimetric technique (Sugiyama *et al.*, 1978, 1980). Binding activity in pooled fractions was determined over a range of ligand concentrations. Results were expressed in a Scatchard (1949) plot, and the dissociation constant for the high-affinity binding site was obtained by non-linear least-squares analysis by using the equation:

$$C_b = \frac{n \cdot P \cdot C_f}{K_d + C_f}$$

where C_b is bound concentration, C_f is free concentration, K_d is the dissociation constant, P is protein concentration and n is binding capacity.

Binding of [^{14}C]oleic acid and [^3H]squalene was measured by gel filtration. Cytosol (6 ml) was mixed with $0.3 \mu\text{Ci}$ of oleic acid or squalene at 4°C , shaken and left to stand for 30 min before chromatography on a column of Sephadex G-75 (superfine grade) as described above. Column fractions were assayed for A_{280} and radioactivity.

Determination of dissociation constants by inhibition of ANS binding

Competitive binding studies were performed by the inhibition of ANS binding (decreased fluorescence). To each of cuvettes containing 2 ml mixtures of $50 \mu\text{g}$ of Z-protein/ml, $2 \mu\text{M}$ -ANS and buffer A

Fig. 1. Comparison of the binding of [^{14}C]oleic acid to cytosol proteins from rat (a) and *Pl. triseriata* (b) liver [^{14}C]Oleic acid (5.2 nmol , sp. radioactivity 58 Ci/mol) was added to 6 ml of *Pl. triseriata* cytosol or rat cytosol prepared from a 33% (w/v) homogenate. The mixtures were chromatographed on a $2.5 \text{ cm} \times 108 \text{ cm}$ column of Sephadex G-75 (superfine grade) at a flow rate of 12–16 ml/h. A_{280} (●) and radioactivity (○) of each fraction (2.5 ml) were measured.

was added a maximum of 30 μ l of the individual competitor in 2 or 5 μ l portions. The solution was mixed after each addition, and the fluorescence was measured at 480 nm during excitation at 400 nm. Calculation of dissociation constants was performed as described previously (Sugiyama *et al.*, 1978).

Results and discussion

The binding of [14 C]oleic acid to liver supernatant proteins obtained from rat and *Pl. triseriata* is depicted in Fig. 1. In rat, oleic acid binding was observed in four peaks associated with the void volume, albumin fraction, Y-protein fraction and Z-protein fraction. As previously reported (Ockner *et al.*, 1972), the Z-protein peak predominated. In *Pl. triseriata*, oleic acid binding was associated only with the void-volume fraction, and no binding was detected in the Z-protein fraction. Similar results

were found with squalene (not shown). To exclude the possibility that saturation of Z-protein with endogenous fatty acids prevented additional binding of exogenous ligands, we measured fatty acid concentrations in liver cytosol of both *Pl. triseriata* and rat and observed no differences (1.53 ± 0.26 compared with 1.76 ± 0.15 mM respectively). The Z-protein content of *Pl. triseriata* was 40% of that in rats (per g of liver), a difference not expected to mask Z-protein binding of fatty acid through saturation.

Binding of organic anions to individual column fractions in the region of Z-protein is shown in Fig. 2. In the rat, binding peaks for all the organic anions appeared in the same position. In *Pl. triseriata*, no binding peak for bromosulphophthalein and bilirubin was detected, as reported previously for other elasmobranchs (Levine *et al.*, 1971; Boyer *et al.*, 1976a,b). However, peaks of ANS and Rose Bengal binding were observed. Binding activities for ANS

Fig. 2. Comparison of organic anion binding to the Z-protein fractions from gel filtration of rat (a) and *Pl. triseriata* (b) cytosol

The conditions of gel filtration of liver cytosol (6 ml) from rat and *Pl. triseriata* on a column of Sephadex G-75 (superfine grade) were the same as in Fig. 1. A_{280} and organic-anion-binding activities of each eluate fraction in the Z-protein region (fractions 140–160) were determined as described in the text. Units of fluorescence (excitation 400 nm, emission 480 nm) are arbitrary.

and Rose Bengal in peak fractions for the Z-protein region of *Pl. triseriata* cytosol were approx. 15% and 50% respectively of those in rat.

Pooled fractions of Z-protein prepared by gel filtration of cytosol from both species were used for characterization of binding, as summarized in Table 1. The ratio of binding affinity of *Pl. triseriata* Z-protein over rat Z-protein for Rose Bengal and ANS was approx. 0.33 and 0.13, but the ratio of binding affinity for bromosulphophthalein was only 0.03. To obtain the fluorescence quantum yield of ANS bound to Z-proteins, we performed fluorimetric titration of a fixed concentration of ANS with increments of the pooled Z-protein fractions, and plotted the reciprocal of fluorescence intensity on the y-axis against the reciprocal of protein concentration of the x-axis (Fig. 3). Fluorescence intensities derived from the reciprocal values of the y-intercepts correspond to the quantum yields of bound ANS and were 40 units and 10 units in rat Z-protein and *Pl. triseriata* Z-protein respectively. Oleic acid inhibited the fluorescence of ANS-rat Z-protein complex without altering quantum yield, indicating competition between the two ligands for binding. However, oleic acid did not inhibit the fluorescence of ANS-*Pl. triseriata* Z-protein complex, directly confirming that *Pl. triseriata* Z-protein does not bind oleic acid.

The fluorescence quantum yield of a protein-bound probe reflects the hydrophobicity of the ligand-binding site of a protein (Turner & Brandt, 1968; Sugiyama *et al.*, 1978). Thus our findings suggest that the *Pl. triseriata* Z-protein binding site is less hydrophobic than that of the rat. It is possible that this difference accounts for the differences in specificities and affinities of *Pl. triseriata* Z-protein binding.

Since organic anions are known to decrease the

fluorescence of ANS bound to rat Z-protein by competing with ANS for a binding site (Sugiyama *et al.*, 1978, 1980), it is assumed that diminished fluorescence of ANS binding to *Pl. triseriata*

Fig. 3. Fluorimetric titration of ANS with pooled Z-protein fractions of rat and *Pl. triseriata*

A 2.0 ml solution of ANS ($2\mu\text{M}$) was titrated with a solution containing both ANS ($2\mu\text{M}$) and concentrated Z-protein fraction (2mg/ml) so that the ANS concentration remained constant ($2\mu\text{M}$) and the protein concentration increased. After each addition, the solution was stirred and the fluorescence was measured at 480nm , with excitation at 400nm . Symbols: ●, rat Z-protein fraction; ○, rat Z-protein fraction with $1\mu\text{M}$ -oleic acid; ▲, *Pl. triseriata* Z-protein fraction. The data are represented as a double-reciprocal plot. Extrapolation to the y-axis gave the inverse of the quantum yield.

Table 1. Dissociation constants and binding capacities for rat and *Pl. triseriata* pooled Z-protein fractions
For experimental details see the text. Abbreviation: BSP, bromosulphophthalein.

	Dissociation constant (μM)				
	ANS	Bilirubin	BSP	Rose Bengal	Oleic acid
Rat Z-proteins	2.2*	0.35†	0.35‡ (0.33§)	0.046† (0.05§)	(0.2§)
<i>Pl. triseriata</i> Z-protein	17*	Not detectable†	17‡ (7§)	0.15† (0.33§)	(Not detectable)
	Binding capacity ($\mu\text{mol/g}$ of protein)				
	ANS	Bilirubin	BSP	Rose Bengal	
Rat Z-protein	24	21	27	33	
<i>Pl. triseriata</i> Z-protein	24	—	72	26	

* Measured by the fluorescence change of ANS.

† Measured by the spectrophotometric method.

‡ Measured by equilibrium dialysis.

§ Determined by the competitive technique with ANS fluorescence quenching.

Fig. 4. Competition for ANS binding to pooled Z-protein by organic anions

The decrease of fluorescence intensity of bound ANS by various other ligands was measured at a constant concentration of ANS ($2\mu\text{M}$) and Z-protein fraction ($50\mu\text{g/ml}$) of rat (a) and *Pl. triseriata* (b). Ligands used were oleic acid (Δ), bromosulphophthalein ($\circ$) and Rose Bengal ($\bullet$). The lines are theoretical curves, calculated as described previously (Sugiyama *et al.*, 1978), by using K_a values of 2.2 and $17\mu\text{M}$ for rat and *Pl. triseriata* respectively.

Z-protein by other organic anions also represents competition for binding. Inhibition constants obtained from inhibition curves (Fig. 4) are listed in Table 1. Good agreement between this and other methods for determining binding was obtained.

Previous work with bromosulphophthalein or bilirubin binding to gel-filtration fractions of liver cytosol suggests that Y- and Z-protein fractions,

initially described in rats, were absent from marine elasmobranchs. We recently purified the major glutathione S-transferase from the Y-protein fraction of *Pl. triseriata* and demonstrated organic-anion-binding activity comparable with that observed with rat Y-protein with ANS and Rose Bengal as ligands (Sugiyama *et al.*, 1981). We have extended this work to Z-protein and have demonstrated binding of ANS and Rose Bengal to proteins of similar molecular weight in *Pl. triseriata* and rat. The absence of fatty acid binding and the markedly lower affinity for bromosulphophthalein of *Pl. triseriata* Z-protein may result from diminished hydrophobicity of its binding site as compared with that of the rat.

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