Activation of human liver 3α-hydroxysteroid dehydrogenase by sulphobromophthalein

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Human liver contains at least two isoenzymes (DD2 and DD4) of 3α-hydroxysteroid/dihydrodiol dehydrogenase. The NADP(H)-linked oxidoreductase activities of DD4 were activated more than 4-fold by sulphobromophthalein at concentrations above 20 μM and under physiological pH conditions. Sulphobromophthalein did not stimulate the activities of DD2 and human liver aldehyde reductase, which are functionally and/or structurally related to DD4. No stimulatory effect on the activity of DD4 was observed with other organic anions such as Indocyanine Green, haematin and Rose Bengal. The binding of sulphobromophthalein to DD4 was instantaneous and reversible, and was detected by fluorescence and ultrafiltration assays. The activation by sulphobromophthalein decreased the activation energy in the dehydrogenation reaction for the enzyme, and increased both $k_{cat.}$ and K_m values for the coenzymes and substrates. Kinetic analyses with respect to concentrations of $NADP^+$ and (*S*)-(+)-indan-1-ol indicated that sulphobromophthalein was a non-essential activator of mixed type showing a dissociation constant of 2.6 μM. Thus, the human 3α-hydroxysteroid dehydrogenase isoenzyme has a binding site specific to sulphobromophthalein, and the hepatic metabolism mediated by this isoenzyme may be influenced when this drug is administered.

INTRODUCTION

3α-Hydroxysteroid dehydrogenase (3HSD; EC 1.1.1.213) from rat and human liver is a multifunctional enzyme with an M_r of around 36000. It catalyses the reversible oxidoreduction of various 3α-hydroxysteroids and prostaglandins, the oxidation of *trans*-dihydrodiols of aromatic hydrocarbons and alicyclic alcohols, and the reduction of xenobiotic carbonyl compounds [1–4]. In addition, rat liver 3HSD has the ability to bind bile acids, which indicates that this enzyme is involved in the intracellular transport of bile acids [5,6]. On the other hand, human liver 3HSD exists in multiple forms, none of which has been reported to bind bile acids [4]. A bile acid-binding protein isolated from human liver exhibits dihydrodiol dehydrogenase (EC 1.3.1.20) activity for *trans*-dihydrodiols of aromatic hydrocarbons and alicyclic alcohols, but not 3HSD activity for bile acids [7,8].

The cloning of cDNAs for hepatic 3HSDs of rat [9] and man [10] has demonstrated that the rat and human enzymes have similar amino acid sequences and are members of the aldo-keto reductase family. We previously characterized structural and functional properties of two dihydrodiol dehydrogenases (DD2 and DD4) of human liver [11,12]. DD2 has low 3HSD activity for limited androgens and progestins, whereas DD4 exhibits high 3HSD activity for various steroids, including bile acids, which resembles the substrate specificity for rat liver 3HSD [1]. The sequences of cDNAs for DD2 and DD4 are virtually identical to those for human liver bile acid-binding protein [8] and 3HSD [10] respectively. DD2 and DD4 share 82% identity between their deduced amino acid sequences, and also show about 63% sequence identity with rat liver 3HSD [9].

Recently, we have established the bacterial overexpression system of recombinant DD4 (rDD4), which has almost the same catalytic properties as the native enzyme, and elucidated the kinetic mechanisms of the reaction catalysed by the enzyme and of inhibition by phenolphthalein, a potent competitive inhibitor with respect to the substrate [13]. During the course of further biochemical characterization of DD4, we found that sulphobromophthalein (BSP; Figure 1), an agent for testing liver function, specifically and significantly activated both rDD4 and

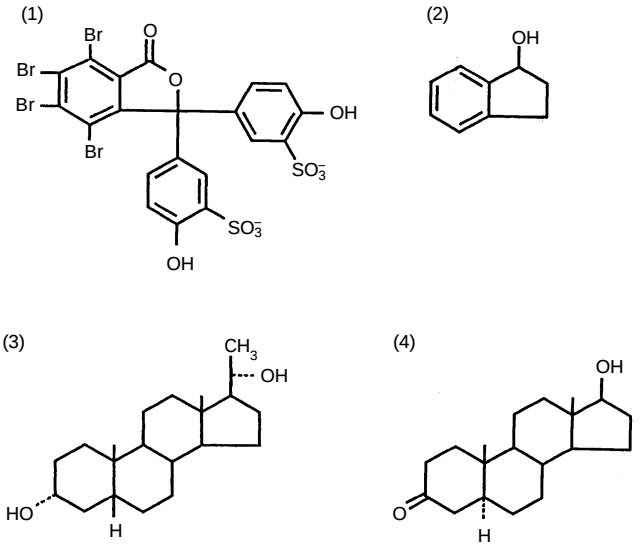

Figure 1 Structures of BSP (1), (*S*)-indan-1-ol (2), 5β-pregnane-3α,20α-diol (3) and 5α-dihydrotestosterone (4; 5α-androstan-17β-ol-3-one)

Abbreviations: 3HSD, 3α-hydroxysteroid dehydrogenase; rDD4, recombinant DD4; BSP, sulphobromophthalein; Ex, excitation wavelength; Em, emission wavelength.

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native DD4 of the human liver aldo-keto reductases. In this study, we have characterized the activation mechanism of DD4, and show that the enzyme has a binding site specific to this drug.

EXPERIMENTAL

Materials

(*S*)-(+)-Indan-1-ol was purchased from Fluka Chemie AB (Buchs, Switzerland); 4,5,6,7-tetrabromophenolphthalein was from Tokyo Chemical Industries (Tokyo, Japan); NADP(H) were from Oriental Yeast (Tokyo, Japan); and Indocyanine Green and steroids were from Sigma Chemicals. Other chemicals were obtained from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries (Osaka, Japan). Four multiple forms (DD1–DD4) of dihydrodiol dehydrogenase were purified from human liver [11,14], and rDD4 was expressed in *Escherichia coli* and purified to homogeneity as described [13].

Enzyme assay and kinetic analysis

Dehydrogenase and reductase activities of rDD4 and the other enzymes were assayed spectrophotometrically or fluorometrically by recording the reduction and oxidation, respectively, of NADPH as described [11,13]. The standard assay for the dehydrogenase activity was performed in 2.0 ml of 0.1 M potassium phosphate, pH 7.4, containing 0.25 mM $NADP^+$, 2 mM (*S*)-indan-1-ol and enzyme, and that for the reductase activity was in 2.0 ml of 0.1 M potassium phosphate, pH 7.4, containing 0.1 mM NADPH, 2 mM pyridine-3-aldehyde and enzyme. The reaction was initiated by the addition of enzyme. Haematin was dissolved in 10 mM NaOH to form 0.5 mM stock solutions just before use. One unit of the enzyme activity was defined as the amount catalysing the reduction or oxidation of 1 μmol of NADPH/min at 25 °C.

Initial velocities in the asymptotic region which follow Michaelis kinetics were fitted to an equation for a sequential bireactant system, according to the method of Cleland [15], by using a computer program for least-squares linear regression. Kinetic studies in the presence of BSP were carried out in an identical manner. For determining activation energy for the (*S*)-indan-1-ol dehydrogenation catalysed by the enzyme, the assay was performed at various temperatures (16–44 °C) under the standard assay conditions, and the data were analysed according to the Arrhenius plot [16].

Fluorometric analysis

The effect of BSP on the intrinsic protein fluorescence [excitation wavelength (Ex) 280 nm; emission wavelength (Em) 330 nm] of rDD4 and NADPH fluorescence (Ex 340 nm; Em 450 nm) in the presence of rDD4 was measured at 25 °C in 1.0 ml of 0.1 M potassium phosphate buffer, pH 7.4. The fluorescence intensities of the protein and NADPH were corrected for the inner filter effect of BSP with glycyl-L-tryptophan and NADPH respectively, which showed the same fluorescence intensities as those of the enzyme and enzyme–NADPH complex measured.

Ultrafiltration assay

The binding of BSP to rDD4 was directly assessed by determining the unbound fraction of BSP at 25 °C. A spin microconcentrator with an M_r cut-off of 10000 (Microcon 10; Amicon, Danvers, MA, U.S.A.) was used to separate the free from the enzyme-bound BSP. A volume of 0.4 ml of binding assay mixture (15 μM rDD4 and 10 μM BSP in 0.1 M potassium phosphate, pH 7.4) was filtered by centrifugation at 13000 ***g*** for 15 min. The filtrate (0.2 ml) was diluted 5-fold with 0.1 M NaOH, and the concentration of BSP was determined at 580 nm.

RESULTS

Activation of BSP

When the effects of phenolphthalein derivatives on the $NADP^+$-linked (*S*)-indan-1-ol dehydrogenase and NADPH-linked pyridine-3-aldehyde reductase activities of recombinant DD4 were examined at pH 7.4, BSP was found to stimulate the enzyme activity in a dose-dependent manner (Figure 2). The stimulation percentage of the reductase activity was higher than that of the dehydrogenase activity, but stimulation percentages of both activities slightly decreased at BSP concentrations above 35 μM. Although phenolphthalein derivatives without bromo groups on the phthalein ring or sulphonic groups on the phenol rings are inhibitory to the dehydrogenase activity [13], 4,5,6,7-tetrabromophenolphthalein showed weak stimulation with a maximum of 20 % at concentrations above 10 μM. The enzyme

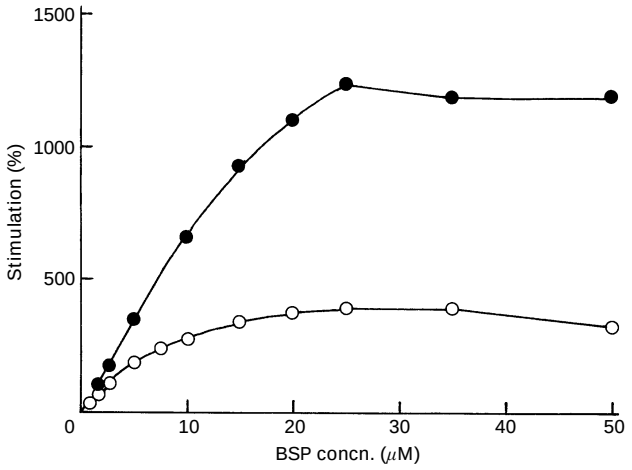


Figure 2 Effects of BSP on (*S*)-indan-1-ol dehydrogenase and pyridine-3-aldehyde reductase activities of rDD4

The stimulation percentage [$(v - v_0)/v_0 \times 100$] of the dehydrogenase (○) and reductase (●) activities was plotted versus BSP concentration. v and v_0 represent velocities in the absence and presence, respectively, of BSP.

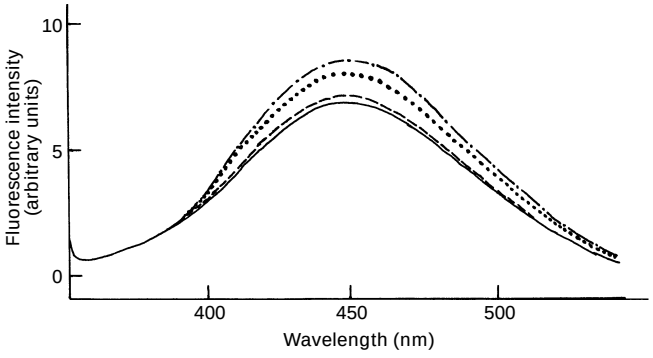


Figure 3 Effect of BSP on fluorescence of NADPH complexed with rDD4

BSP was added to the mixture of 1 μM NADPH and 1 μM rDD4, and the emission spectra were measured at an excitation wavelength of 340 nm. BSP concentrations: 0 μM (–·–), 1 μM (...), 5 μM (- - -) and 10 μM (—).

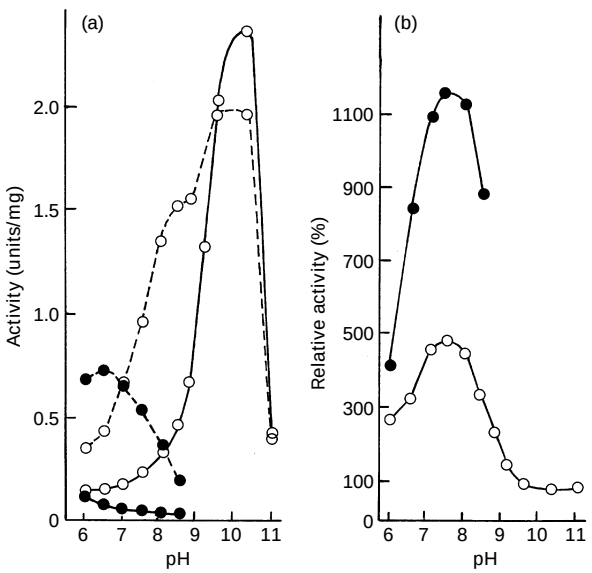


Figure 4 Effect of BSP on the pH dependency of dehydrogenase and reductase activities of rDD4

(**a**) pH–activity profiles. The activities of (*S*)-indan-1-ol dehydrogenase (○) and pyridine-3-aldehyde reductase (●) were assayed in the absence (—) and presence (- - -) of 20 μM BSP in buffers (pH 6.0–11.0) which were prepared by mixing 0.1 M solutions of H_3PO_4 and K_3PO_4. (**b**) pH dependency of the stimulation by BSP.

activity was not activated by organic anions, such as Rose Bengal, haematin, Indocyanine Green and amaranth, at concentrations up to 50 μM, at which Rose Bengal and haematin instead showed high inhibition (the concentrations required for 50% inhibition were 4 μM and 15 μM respectively).

BSP also activated the dehydrogenase activity of native DD4 from human liver to a similar extent to that observed with rDD4 (results not shown), but not the activities of other dihydrodiol dehydrogenases of human liver. BSP (20 μM) did not affect the dehydrogenase activity of DD1, and inhibited by 25% the activity of DD2 and by 77% the reductase activity of DD3 (this enzyme is identified as aldehyde reductase [14]). Thus, the activation by BSP was specific to DD4 of the human liver 3HSDs and/or dihydrodiol dehydrogenases.

BSP binding assays

The reversibility of the activation of rDD4 by BSP was examined with BSA, which is known to bind this drug. When the albumin (0.2%) was added to reaction mixture which had been initiated in the presence of 20 μM BSP, the (*S*)-indan-1-ol dehydrogenase activity rapidly decreased to the level of the non-activated enzyme. On the other hand, BSP quickly activated the enzyme activity when it was added to the reaction mixture without the activator. A similar result was obtained in the pyridine-3-aldehyde reductase activity of the enzyme. The results indicate that binding of BSP to the enzyme is instantaneous and reversible.

The binding of BSP to the enzyme was also detected by fluorescence and ultrafiltration assays. The addition of less than 1 μM BSP slightly quenched the intrinsic protein fluorescence of rDD4 (results not shown), but titration by BSP could not be carried out because of its high inner filter effect. On the other hand, such an inner filter effect was not significant in the analysis of NADPH fluorescence. The fluorescence of NADPH shifted its emission maximum from 456 nm to 445 nm by the formation of the enzyme–NADPH complex [13], and further addition of BSP decreased the fluorescence in a dose-dependent manner (Figure 3). In the ultrafiltration assay of a mixture of rDD4 and BSP, the fraction of unbound BSP was 0.68 ± 0.09 ($n = 3$).

Effect of BSP on the pH dependency of the enzyme activity

Activation by BSP resulted in a marked effect on the pH-dependent profiles of the dehydrogenase and reductase activities of rDD4. While the non-activated enzyme showed a sharp pH optimum for the dehydrogenase activity around 10.0, the pH–activity profile in the presence of BSP was broad and shifted to a more neutral pH (Figure 4a). No apparent pH optimum was

Table 1 Effect of BSP on the kinetic constants for alcohols and oxo compounds

The activity was assayed at pH 7.4 in the presence 20 μM BSP as an activator. The $k_{cat.}$ values were calculated from the $V_{max.}$ values for the substrates, assuming that the molecular mass of the enzyme is 36 kDa. The values in parentheses are the constants determined in the absence of BSP [13]. The structures of the representative steroid substrates are shown in Figure 1.

Substrate	K_m (μM)	$k_{cat.}$ (min^{-1})	$k_{cat.}/K_m$ ($min^{-1} \cdot \mu M^{-1}$)
Oxidation of alcohols			
(*S*)-(+)-Indan-1-ol	522 ± 60	49 ± 4	0.09
	(146)	(6.1)	(0.04)
5α-Androstan-3α-ol-17-one	10 ± 2	38 ± 7	3.8
	(0.5)	(2.6)	(5.2)
5α-Androstane-3α,17β-diol	14 ± 1	62 ± 9	4.4
	(0.8)	(5.2)	(6.5)
5β-Pregnane-3α,20α-diol	2.0 ± 0.5	17 ± 5	8.5
	(0.2)	(1.2)	(6.0)
Reduction of oxo compounds			
Pyridine-3-aldehyde	495 ± 35	30 ± 1	0.06
	(358)	(2.5)	(0.007)
5α-Dihydrotestosterone	1.2 ± 0.3	26 ± 3	22
	(0.4)	(1.6)	(4.0)
5β-Androstane-3,17-dione	0.4 ± 0.1	27 ± 3	68
	(0.3)	(1.7)	(5.7)
5β-Pregnane-3,20-dione	0.6 ± 0.1	32 ± 1	53
	(0.4)	(1.3)	(3.3)

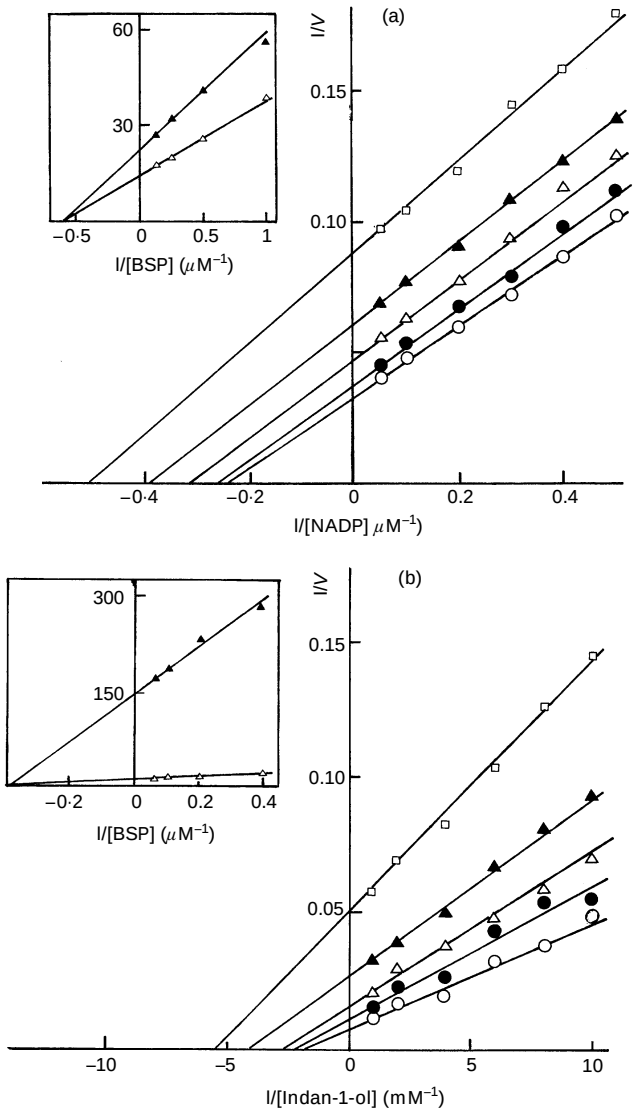


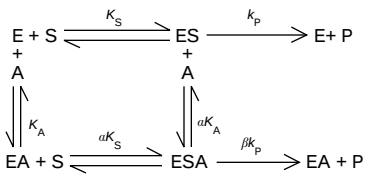
Figure 5 Effect of BSP on the dehydrogenase activity as a function of concentration of NADPH or (*S*)-indan-1-ol

(**a**) Double-reciprocal plots of velocity versus NADPH concentration in the presence of a fixed concentration of (*S*)-indan-1-ol (2 mM). BSP concentrations: 0 μM (□), 1 μM (▲), 2 μM (△), 4 μM (●) and 8 μM (○). (**b**) Double-reciprocal plots of velocity versus (*S*)-indan-1-ol concentration in the presence of a fixed concentration of NADPH (0.25 mM). BSP concentrations: 0 μM (□), 2.5 μM (▲), 5.0 μM (△), 10 μM (●) and 15 μM (○). Velocity is expressed in m-units/ml. Replots of the 1/change in slope (▲) or intercept (△) against 1/[BSP] are shown in the inset.

observed for the reductase activity of the non-activated enzyme above pH 6.0, whereas the activity assayed in the presence of BSP showed a pH optimum around 6.5. Thus, the stimulation of the dehydrogenase and reductase activities by BSP was most significant around pH 7.5 (Figure 4b), but the elevating or lowering of the pH values decreased the stimulation percentage and slight inhibition was observed at alkaline ranges higher than 9.5.

Effect of BSP on kinetic properties of 3HSD

Apparent activation energy for (*S*)-indan-1-ol oxidation by rDD4



Scheme 1 Scheme for non-essential activation

Abbreviations: E, enzyme; S, substrate; A, activator; P, product.

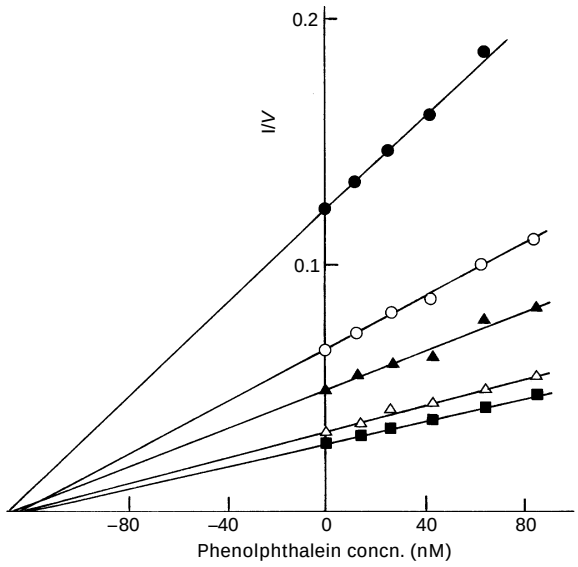


Figure 6 Dixon plot of velocity versus phenolphthalein concentration at different fixed concentrations of BSP

The dehydrogenase activity was determined with 2 mM (*S*)-indan-1-ol as the substrate. BSP concentrations: 0 μM (○), 2 μM (●), 4 μM (▲), 10 μM (△) and 20 μM (■). Velocity is expressed in m-units/ml.

was calculated to be 52.8 kJ/mol, which is similar to the value reported with native DD4 [11]. The value of 25.7 kJ/mol was obtained with the enzyme activated by 20 μM BSP.

The addition of BSP to the reaction mixture led to increases in both K_m and $k_{cat.}$ values for the substrates (Table 1), compared with those reported in the absence of BSP [13]. The catalytic efficiency ($k_{cat.}/K_m$) for the alcohol substrates was slightly affected on activation, whereas that for the carbonyl substrates was elevated greatly. The K_m values for $NADP^+$ and NADPH (1.8 μM and 1.0 μM respectively) were also increased to 5.4 μM and 2.7 μM, respectively, by the addition of BSP.

The dependence of dehydrogenase activity on $NADP^+$ was examined at different fixed concentrations of BSP in the presence of 2 mM (*S*)-indan-1-ol. A double-reciprocal plot of the activity versus $NADP^+$ concentrations at increasing concentrations of BSP resulted in a family of linear plots intersecting below the $1/[NADP^+]$ axis (Figure 5a). A replot of the reciprocal of change in slope or intercept of the primary reciprocal plot data versus 1/[BSP] was linear. Similar results were obtained in the kinetic analysis of the activity versus (*S*)-indan-1-ol concentrations at different fixed concentrations of BSP in the saturating concentration of $NADP^+$ (Figure 5b). The results are consistent with a non-essential activation system (Scheme 1), in which the kinetic constants can be determined [17]. The values of K_A (dissociation

constant for BSP), α and β were calculated to be 2.6 μM, 4.2 and 5.9, respectively, from the data in Figure 5(a), and the respective values were 8.2 μM, 27 and 69 from the data in Figure 5(b).

Phenolphthalein has been shown to be a competitive inhibitor which binds to the DD4–NADPH complex [13]. Since BSP is an analogue of phenolphthalein, the kinetics of inhibition by phenolphthalein in the presence of BSP was examined as described by Segel [17]. The Dixon plots of $1/v$ versus phenolphthalein concentration at different fixed concentrations of BSP were linear, and the lines intersected near the [I] axis (Figure 6).

DISCUSSION

We have demonstrated here that BSP exerts a stimulatory effect on DD4, an isoenzyme of human liver 3HSD. BSP (4,5,6,7-tetrabromo-3′,3″-disulphophenolphthalein) is a derivative of phenolphthalein. Our previous study with inhibitory phenolphthalein derivatives suggested that the non-substituted phthalein and phenol rings of the phenolphthalein molecule are important for its binding to the enzyme–$NADP^+$ complex as a competitive inhibitor [13]. The present kinetic analyses and binding assays indicated that BSP reversibly binds to both the apoenzyme and enzyme–coenzyme complex. Of the compounds related to BSP, 4,5,6,7-tetrabromophenolphthalein showed a weak activation, but organic anions other than BSP did not stimulate the enzyme activity. This, together with the previous structural requisites of phenolphthalein derivatives for the inhibitor [13], suggests that the tetrabromophthalein part and sulphonated phenol rings of the BSP molecule may be necessary to interact with the activator site. The pK_a of BSP is about 8.6 [18]. Above that pH most of the BSP molecules are in the open (with respect to the phthalein ring), quinoid, deprotonated form, whereas below that pH the closed, protonated, lactonic form of BSP increases. Since the stimulation percentage by BSP was maximal at pH 7.5 and decreased to a half of the percentage around pH 8.6 in the alkaline pH range, the latter form of BSP appears to interact directly with the enzyme.

The activation by BSP was of mixed-type with respect to $NADP^+$ and (*S*)-indan-1-ol, which classifies this phenomenon as a case of a non-essential activator [17]. The linear replots of $1/\Delta$slope and $1/\Delta$intercept of the primary reciprocal plots versus 1/[BSP] provide convincing kinetic evidence for binding of one molecule of BSP to the activator site of the enzyme but not to the substrate. The K_A value for BSP calculated from the data with respect to $NADP^+$ was lower than the value from the data with respect to (*S*)-indan-1-ol. Since the dehydrogenation reaction catalysed by DD4 follows an ordered Bi Bi mechanism with the coenzyme binding first [13], the former value implies the dissociation constant for the activator to the enzyme, whereas the latter value includes constants for BSP binding to the apoenzyme and the enzyme–$NADP^+$ complex. Therefore, BSP may bind more rapidly to the apoenzyme than to the enzyme–$NADP^+$ complex.

DD4 is a member of the aldo-keto reductase family [11]. Since DD4 conserves functional amino acid residues for catalysis and coenzyme-binding that have been identified or suggested by site-directed mutagenesis and crystallographic studies of rat liver 3HSD [19,20] and human aldose reductase [21,22], the catalytic mechanism and orientation of the coenzyme in the binding site of DD4 are probably the same as those proposed for the rat and human oxidoreductases [19–22]. The activation by BSP may be a unique property of DD4. Indeed, BSP did not activate human liver DD2 and aldehyde reductase (DD3), which show more than 56 % identity with DD4 [11], and the activities of rat liver 3HSD and mouse liver oestradiol 17β-dehydrogenase, which share more than 63 % identity with DD4 [9,23], were not enhanced by BSP (K. Matsuura and A. Hara, unpublished work). Such high activation by organic anion(s) has not been reported for other members of the aldo-keto reductase family, except that human aldose reductase is activated 1.2- to 1.3-fold by cacodylate and citrate (at pH 8.0), as well as by the known inorganic sulphate and phosphate ions [24]. For the activation mechanism proposed for aldose reductase [24,25], the anions interact with Lys-262 that forms a salt link with the 2′-phosphate of the adenosine ribose of NADPH, and the interaction weakens the binding of the coenzyme and results in an increase in turnover. A similar activation mechanism can be expected for the activation of DD4 by BSP with two negatively charged sulphonic groups, because DD4 conserves the lysine residue corresponding to Lys-262 of aldose reductase and the K_m values for NADPH and $NADP^+$ increased on the activation. However, the fact that BSP activated the reductase activity of DD4 more highly than the dehydrogenase activity could not be explained by the simple interaction of the activator with the lysine residue. The binding of BSP may evoke some conformational changes in the enzyme molecule. This was suggested by the observations that the addition of BSP causes lowering of the activation energy of the reaction by DD4, quenching of the fluorescence of NADPH complexed with the enzyme, and shift of the pH–activity profiles of the enzyme in the forward and backward reactions.

BSP is one of the best agents for testing liver function. The drug is efficiently incorporated into hepatic cells via membrane-associated transporters [26], and excreted into the bile duct. In addition, some hepatic soluble proteins, such as glutathione S-transferase and Z-protein [27–31], have been proposed to be involved in the intracellular transport of organic anions. The K_A value of DD4 for BSP is comparable with the dissociation constants of the organic anion-binding proteins of human liver cytosol [29,31]. The high affinity for BSP suggests a non-enzymic function, i.e. that this 3HSD isoenzyme acts as a BSP-specific binding protein in human liver cytosol. On the other hand, the activation of DD4 by BSP is in contrast to the inhibition of glutathione S-transferases by this organic anion [32]. It is possible that the activity of DD4 is also modulated by administered BSP, which may result in impairment of the hepatic metabolism of steroids, xenobiotics and prostaglandins mediated by this enzyme. Especially, the catalytic efficiency for carbonyl substrates was enhanced more than 6-fold by BSP. Since DD4 has been shown to play an important role in hepatic reductive metabolism of several drug ketones administered therapeutically [33], simultaneous administration of BSP and the drug ketones may influence the pharmacological potency of the drug ketones.

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