Involvement of two basic residues (Lys-270 and Arg-276) of human liver 3 α -hydroxysteroid dehydrogenase in NADP(H) binding and activation by sulphobromophthalein: site-directed mutagenesis and kinetic analysis

Kazuya MATSUURA, Yoshiyuki TAMADA, Kumiko SATO, Harunori IWASA, Gunpei MIWA, Yoshihiro DEYASHIKI and Akira HARA*

Biochemistry Laboratory, Mitahora-higashi, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502, Japan

A human liver 3 α -hydroxysteroid dehydrogenase isoenzyme, a member of the aldo-keto reductase family, shows a marked preference for NADP(H) over NAD(H), and is activated by sulphobromophthalein, which increases the K_m values for both NADP(H) and substrates. Here we report kinetic alterations in binding of the coenzymes and the activator to the enzyme caused by site-directed mutagenesis of Lys-270 and Arg-276, which are strictly conserved among the aldo-keto reductase family of enzymes. The mutated enzymes, K270M and R276M, showed increases in the K_m for NADP⁺ of 22- and 290-fold respectively; the K_m for alcohol substrate and the k_{cat} of the NADP⁺-linked reaction were also elevated, by 9- and 5-fold respectively. No kinetic constant of the NAD⁺-linked reaction was altered by more than 3-fold. Calculation of the free-energy changes showed that the 2'-phosphate group of NADP⁺ contributes 16.3 kJ/mol

(3.9 kcal/mol) of binding energy to its interaction with the wild-type enzyme, and the mutagenesis to K270M and R276M destabilized the binding energy of NADP⁺ by 6.3 and 13.0 kJ/mol (1.5 and 3.1 kcal/mol) respectively. In addition, the mutations attenuated enzyme activation by sulphobromophthalein, which bound to the mutant enzymes as an inhibitor. The inhibition for the R276M mutant was competitive with respect to NADP⁺ and non-competitive with respect to the substrate, whereas that for the K270M mutant was mixed-type, showing activation at coenzyme concentrations greater than $20 \times K_m$. These results suggest that the two basic residues in the 3 α -hydroxysteroid dehydrogenase isoenzyme play crucial roles in binding both the negatively charged 2'-phosphate group of NADP⁺ and the sulphonic groups of sulphobromophthalein.

INTRODUCTION

Human liver contains multiple forms of 3 α -hydroxysteroid dehydrogenase (3HSD; EC 1.1.1.213), which catalyses the oxidation of xenobiotic alicyclic alcohols and *trans*-dihydrodiols of aromatic hydrocarbons, as well as the reversible oxidoreduction of 3 α -hydroxysteroids, with NADP(H) as the preferred coenzyme [1,2]. Previously we isolated two 3HSDs (DD2 and DD4) from human liver [3] and determined their amino acid sequences by cDNA cloning [4] and protein sequencing [5], demonstrating that DD2 and DD4 are identical with hepatic bile-acid-binding protein [6] and chlordecone reductase [7] respectively. These isoenzymes are members of the aldo-keto reductase family [8] and have a high sequence identity of 83 % [4,5], but differ in their substrate specificity for 3 α -hydroxysteroids and their inhibitor sensitivity. DD2 oxidizes the 3 α -hydroxy group of some androstanes and pregnanes, and is sensitive to bile acids, whereas DD4 shows broad substrate specificity for various 3 α -hydroxysteroids, including bile acids, and is inhibited by steroidal anti-inflammatory drugs [3]. In addition, DD4 is uniquely activated by sulphobromophthalein {BSP; 3,3'-[4,5,6,7-tetrabromo-3-oxo-1(3H)-isobenzofuranylidene]bis-(6-hydroxybenzenesulphonic acid) disodium salt}, an agent used for testing liver function [9]. Since DD4 is affected by drugs and also plays a significant role in the reduction of drug ketones in the human liver [10], it is important from a pharmacological point of view to elucidate the binding sites in DD4 for these inhibitory and stimulatory drugs.

The reaction catalysed by DD4 has been shown to follow an ordered sequential mechanism, in which the coenzyme binds first to the enzyme followed by the alcohol substrate [11]. BSP is a

non-essential activator that binds to both the enzyme and its binary complex in this kinetic mechanism, leading to a large increase in the K_m value for NADP(H) [9]. While BSP activates the dehydrogenase activity of DD4 about 4-fold, its analogue lacking the two sulphonic groups shows low activation (1.2-fold), and other phenolphthalein derivatives are inhibitory [9,11]. These observations suggest that the negatively charged sulphonic group(s) interact with positively charged residue(s) in or close to the coenzyme-binding site of the enzyme.

Of the aldo-keto reductase family proteins, aldose reductase has been reported to be activated 1.2–2.0-fold by cacodylate [12] and inorganic sulphate ions [13]. Site-directed mutagenesis of Lys-262 of human aldose reductase to Met results in an increase in the K_m values for both NADPH and the aldehyde substrate, and in the abolition of enzyme activation by sulphate ions and cacodylate [12,14], so that the stimulatory anions are thought to interact with Lys-262 and weaken the binding between this residue and the 2'-phosphate group of NADPH. In addition, X-ray crystallographic studies [12,15] and site-directed mutagenesis [16] of human aldose reductase have shown that Arg-268, together with Lys-262, interacts with the 2'-phosphate of NADPH, although the involvement of the former residue in the activation process remains to be confirmed. These two basic residues are conserved among other members of the aldo-keto reductase family, and the corresponding residues in DD4 are Lys-270 and Arg-276. Since the activation of DD4 by BSP greatly increases the K_m value for NADP(H) [9], these two residues are the most likely candidates to interact with the sulphonic groups of BSP.

Here we report the kinetic characterization of 3HSD isoenzymes in which the residues at positions 270 and 276 were

Abbreviations used: 3HSD, 3 α -hydroxysteroid dehydrogenase; BSP, sulphobromophthalein; WT, wild-type DD4 isoenzyme; K270M, DD4 with Met replacing Lys-270; R276M, DD4 with Met replacing Arg-276; IN, S-(+)-indan-1-ol.

* To whom correspondence should be addressed.

altered by site-directed mutagenesis to Met. The results show that the two basic residues Lys-270 and Arg-276 are required for binding of both NADP(H) and BSP.

EXPERIMENTAL

Materials

Pfu DNA polymerase was obtained from Stratagene, and a Genepure kit for isolation of DNA was from Nippongene. The expression plasmid for wild-type DD4 (WT), pKKDD4, previously constructed in a pKK223-3 vector [11], was used. The sources of other chemicals, DNA-modifying enzymes and *Escherichia coli* cells used in this study are specified elsewhere [3,4,9–11].

Site-directed mutagenesis

To elucidate the roles of Lys-270 and Arg-276 in NADP(H) binding, Met was chosen to replace these two basic residues because the same mutations of the corresponding residues of human aldose reductase have been shown not to cause any gross structural perturbation [14,16]. Two DD4 mutants, K270M and R276M, were generated using the overlap-extension technique [17], using *pfu* DNA polymerase and partially complementary primer pairs. The forward and reverse primers for K270M were GTCCTGGCCATGAGCTACAA and TTGTAGCTCATGGCCAGGAC respectively, and the forward and reverse primers for R276M were AATGAGCAGATGATCAGAGAG and CTCTCTGATCATCTGCTCATT respectively (codons of altered amino acids are underlined). The entire coding regions of the DD4 cDNAs were amplified by PCR using pKKDD4 as a template and two additional primers, EH and HE, which contain the sequences at positions of 22–41 and 4491–4510 respectively around the multiple cloning site of the pKK223-3 vector. Since the DD4 cDNA has two *Eco*RI sites, at position –6 from the initiation codon [11] and position 851 in its coding region [4], a cDNA fragment of 860 bp was obtained from the mutated cDNA by digestion with this restriction enzyme. The cDNA fragment encoding the N-terminal amino acid sequence (residues 1–284) of the enzyme was isolated on agarose-gel electrophoresis followed by use of the Genepure kit. This fragment was then ligated into pKKDD4-L, which had been made by digestion with *Eco*RI and encoded the partial C-terminal amino acid sequence (residues 285–323) of DD4. The complete coding regions of the mutated cDNAs were sequenced as previously described [4], in order to confirm the presence of the desired mutation and to ensure that no other mutation had occurred.

Expression and purification

The wild-type and mutated cDNAs were expressed in *E. coli* (JM109) cells, and the recombinant enzymes were purified from the 12000 g supernatants of the cell homogenates (each 2 litres of culture), as previously described [11].

Characterization of protein samples

The protein concentrations of the crude extract and enzyme preparations during the purification were determined by the Bradford method [18], using BSA as the standard.

Measurements of the molecular mass of the purified enzyme by SDS/PAGE [19] and of its pI value by isoelectric focusing [20], and Western-blot immunoanalysis [21] using the anti-DD4 serum, were carried out as previously described [11].

Enzyme assay and kinetic analysis

The dehydrogenase activity of the recombinant 3HSDs was assayed by recording the rate of change in NAD(P)H fluorescence at 455 nm (excitation at 340 nm). The standard reaction mixture for the measurement of dehydrogenase activity consisted of 0.1 M potassium phosphate buffer, pH 7.4, 1.0 mM NADP⁺, 2.0 mM *S*-(+)-indan-1-ol (IN) and enzyme, in a total volume of 2.0 ml. One unit of enzyme activity was defined as the amount of enzyme catalysing the formation of 1 µmol of NAD(P)H per min at 25 °C.

The kinetic mechanism and constants for the oxidation of IN were analysed according to the method of Cleland [22]. The initial velocities were fitted to the equation:

$$v = V[A][B]/([A][B] + K_m^A[B] + K_m^B[A] + K_i^A K_m^B)$$

where K_i^A is the dissociation constant of substrate A if the reaction proceeds by an ordered Bi Bi mechanism. The kinetic studies in the presence of inhibitors were carried out in a similar manner, and the inhibition constant, K_i , was determined by assuming the appropriate kinetic model for inhibition as described [23]. All kinetic measurements were performed at least three times, and mean values were used for subsequent calculation. All standard errors of fits were less than 15 %, otherwise noted.

Thermal stability study

The enzyme solutions (0.1 mg/ml) were incubated at 50 °C (for WT), 49 °C (for K270M) or 48 °C (for R276M) in 0.1 M potassium phosphate, pH 7.4, containing 0.1 % BSA and 20 % (v/v) glycerol in the absence or presence of NADP⁺ or BSP. At different times, aliquots of 50 µl from each sample were taken and assayed for dehydrogenase activity.

RESULTS AND DISCUSSION

Expression and purification of 3HSD mutants

Three forms of human liver DD4, i.e. K270M, R276M and WT, were expressed in *E. coli*. Affinity chromatography on a Matrex Red A column was used as the final purification step to obtain

Table 1 Kinetic parameters for IN oxidation by WT, K270M and R276M

The constants for the NADP⁺- and NAD⁺-linked activities were estimated by initial-velocity analyses. NADPH and phenolphthalein were competitive inhibitors with respect to NADP⁺ and IN respectively, and their K_i values were determined with 2.5 mM IN and an NADP concentration of $10 \times K_m^{\text{NADP}}$ as the respective fixed substrates. Values are means; S.E.M.s are < 15 %, except where indicated (*15 % < S.E.M. < 26 %).

Parameter	WT	K270M	K270M/WT	R276M	R276M/WT
K_i^{NADP} (µM)	1.2*	16	13	240	200
K_m^{NADP} (µM)	1.7	42	22	490	290
K_m^{IN} (mM)	0.12	1.1*	9.2	1.1	9.2
k_{cat} (s ⁻¹)	0.18	0.93	5.2	1.0	5.7
$k_{\text{cat}}/K_m^{\text{NADP}}$ (s ⁻¹ · mM ⁻¹)	110	22	0.2	2.1	0.02
$k_{\text{cat}}/K_m^{\text{IN}}$ (s ⁻¹ · mM ⁻¹)	1.5	0.72	0.5	0.94	0.6
K_i^{NAD} (mM)	0.81	2.4	3.0	2.8	3.5
K_m^{NAD} (mM)	1.8	5.3	2.9	2.4	1.3
K_m^{IN} (mM)	1.4	2.2	1.6	1.1	0.8
k_{cat} (s ⁻¹)	0.75	0.78	1.0	0.65	0.9
$k_{\text{cat}}/K_m^{\text{NAD}}$ (s ⁻¹ · mM ⁻¹)	0.42	0.15	0.4	0.27	0.6
$k_{\text{cat}}/K_m^{\text{IN}}$ (s ⁻¹ · mM ⁻¹)	0.54	0.35	0.6	0.59	1.0
K_i for NADPH (µM)	0.7*	15	21	50	71
K_i for phenolphthalein (nM)	20	40	2	60	3

Figure 1 Protective effects of NADP⁺ and BSP against heat inactivation of WT and the mutant enzymes

WT (A), K270M (B) and R276M (C) were incubated at 50, 49 and 48 °C respectively, without (△) or with 0.2 mM NADP⁺ (●), 1 mM NADP⁺ (○) or 1 mM BSP (▲).

Table 2 Thermodynamic effects of removing Lys-270 or Arg-276 of human 3HSD isoenzyme DD4

Changes in the binding energy ($\Delta\Delta G_b$, kJ/mol) for removal of the 2'-phosphate group of NADP⁺ by the modification NADP⁺ $\rightarrow$ NAD⁺ and for removal of Lys-270 or Arg-276 by the modification WT $\rightarrow$ K270M or WT $\rightarrow$ R276M were calculated using the equations $\Delta\Delta G_b = -RT \ln(K_{NADP}/K_{NAD})$ and $\Delta\Delta G_b = -RT \ln(K_{WT}/K_{mutant})$ respectively, where K is the dissociation constant for coenzyme binding to either WT or one of the mutant enzymes, R is the gas constant and T is the temperature in K [25]. The change in the activation energy ($\Delta\Delta G_i$, kJ/mol) of the transition state during catalysis by the modified enzyme was calculated from the k_{cat}/K_m^{IN} value, as described in [16]. Values in parentheses are kcal/mol (1 kcal = 4.184 kJ).

Modification	Enzyme or coenzyme	$\Delta\Delta G_b$ (kJ/mol)	$\Delta\Delta G_i$ (kJ/mol)
NADP ⁺ $\rightarrow$ NAD ⁺	WT	16.3 (3.9)	2.55 (0.61)
	K270M	12.6 (3.0)	1.76 (0.42)
	R276M	6.3 (1.5)	1.55 (0.37)
WT $\rightarrow$ K270M	NADP ⁺	6.3 (1.5)	1.76 (0.42)
	NAD ⁺	2.6 (0.63)	1.05 (0.25)
	NADPH	7.5 (1.8)	—
WT $\rightarrow$ R276M	NADP ⁺	13.0 (3.1)	1.17 (0.28)
	NAD ⁺	3.1 (0.73)	−0.25 (−0.06)
	NADPH	10.5 (2.5)	—

Figure 2 Effects of BSP on the IN dehydrogenase activities of WT and the mutant enzymes

The effects of BSP concentration on the activities of WT (○), K270M (△) and R276M (▲) were determined in the presence of a concentration of NADP⁺ of $10 \times K_m^{NADP}$ and 2.5 mM IN (A), or in the presence of 10 mM NAD⁺ and 2.5 mM IN (B).

homogeneous WT, with a yield of 16 mg of protein per 2 litres of culture [11]. While WT was retained on the column on washing with buffer containing 0.1 M NaCl and then eluted with buffer containing both 0.1 M NaCl and 0.5 mM NADP⁺, the two mutant enzymes were gradually eluted by washing with 0.1 M NaCl, owing to their low affinity for the coenzyme as described below. This caused low overall yields of 8 mg and 4 mg respectively for K270M and R276M, but the respective specific activities of 0.64 and 0.54 unit/mg were higher than the value of 0.17 unit/mg for WT. All of the enzymes were apparently homogeneous on SDS/PAGE, and reacted similarly with the antibody against DD4 on Western blot immunoanalysis (results not shown). The two mutant enzymes had the same pI value of 6.7, which was lower than that of WT (pI 7.2) due to the loss of the positive charge of Lys-270 or Arg-276.

Roles of Lys-270 and Arg-276 in NADP(H) binding

K270M and R276M, similarly to WT [11], showed broad substrate specificity for various 3 α -hydroxysteroids, alicyclic alcohols and carbonyl compounds. As the reaction catalysed by WT follows an ordered Bi Bi mechanism, with the coenzyme binding first to the enzyme [11], the double-reciprocal plots of initial velocity versus concentration of NADP⁺ or NAD⁺ at five fixed levels of IN yielded a series of intersecting lines for K270M and R276M (results not shown). NADPH and phenolphthalein gave linear competitive inhibition patterns with respect to NADP⁺ and IN respectively. The kinetic constants are shown in Table 1. Thus the mutations did not affect the kinetic mechanism of the enzyme.

When the kinetic constants for the NADP⁺- and NAD⁺-linked reactions for WT, K270M and R276M were compared, the mutations produced remarkable changes in the kinetic constants for the NADP⁺-linked reaction, but only minor changes in those for the NAD⁺-linked reaction. Although the two mutations increased the K_m^{IN} and k_{cat} values by 9- and 5-fold respectively, more significant changes occurred in the K_m^{NADP} , K_i^{NADP} and K_i values for NADPH. Thus the replacement of Lys-270 or Arg-276

Figure 3 Inhibition of the NADP⁺-linked dehydrogenase activities of R276M (A, C) and K270M (B, D) by BSP

Inhibition was analysed with respect to NADP⁺ in the presence of 2.5 mM IN (A and B), or with respect to IN in the presence of an NADP⁺ concentration of $10 \times K_m^{\text{NADP}}$ (C) or $24 \times K_m^{\text{NADP}}$ (D). BSP concentrations (μM): 0 ($\circ$), 1.25 ($\blacklozenge$), 2.5 ($\triangle$), 5.0 ($\blacktriangle$) and 10 ($\bullet$).

with Met mainly affected NADP(H) binding. The decrease in the affinity for NADP⁺ caused by the mutations was consistent with a small protective effect of the coenzyme against heat inactivation for the mutant enzymes, compared with marked protection for WT (Figure 1).

The thermodynamic effects of removing the 2'-phosphate group of NADP⁺ or of removing Lys-270 and Arg-276 on the coenzyme binding energy were calculated from the K_i^{NADP} and K_i for NADPH, in order to gain insight into the strength and specificity of coenzyme binding to DD4 (Table 2). The change in the binding energy for the removal of the 2'-phosphate group in the presence of both Lys-270 and Arg-276 (i.e. WT) was 16.3 kJ/mol (3.9 kcal/mol), whereas the values were lowered by 12.6 and 6.3 kJ/mol (3.0 and 1.5 kcal/mol) for K270M and R276M respectively. In addition, the change in the binding energy towards NADP(H) on replacing Lys-270 or Arg-276 with Met was much larger than that in the binding energy towards NAD⁺. This indicates that both basic residues contribute to the energy of binding of NADP(H) to WT, probably through electrostatic forces between their positively charged groups and the negatively charged 2'-phosphate of the coenzymes. The larger values for the salt link made by Arg-276 [10.5 and 13.0 kJ/mol (2.5 and 3.1 kcal/mol)] compared with those for Lys-270 [6.3 and 7.5 kJ/mol (1.5 and 1.8 kcal/mol)] suggest that Arg-276 makes a greater contribution to the energy of binding of the coenzymes than does Lys-270.

The roles of Lys-270 and Arg-276 of DD4 are similar to those of the corresponding residues of human aldose reductase, which have been demonstrated by X-ray crystallographic studies

[12,15,24] and site-directed mutagenesis [12,14,16]. The Lys residue of aldose reductase, unlike the Arg residue, has been suggested to play another role in the stabilization of the transition state during catalysis by the enzyme. This was studied by comparing changes in the activation energy for removal of the 2'-phosphate of NADPH and for removal of the Lys or Arg residue in wild-type and mutant aldose reductases [16]. When the changes in the activation energy resulting from the modifications among the WT and mutant enzymes of DD4 were calculated using the equations described for aldose reductase [16], the values for removal of the 2'-phosphate group of NADP⁺ with WT, K270M and R276M were as low as those for removal of Lys-270 or Arg-276 with NAD(P)⁺ as coenzyme (Table 2), suggesting that the two residues of DD4 do not play a significant role in stabilization of the transition state. Thus although residues Lys-270 and Arg-276, which are conserved in members of the aldo-keto reductase family, probably play significant roles in NADP(H) binding, their roles in stabilization of the transition state differ, at least between human aldose reductase and a 3HSD isoenzyme (DD4). This may be due to differences between the two enzymes in other residues that are involved in interactions with the nucleotide coenzyme molecule.

Role of Lys-270 and Arg-276 in activation by BSP

In the presence of an NADP⁺ concentration of $10 \times K_m^{\text{NADP}}$ and 2.5 mM IN, BSP activated WT up to 4-fold, whereas it inhibited K270M and R276M (Figure 2). BSP did not influence the NAD⁺-linked activity of WT, but again was inhibitory towards

the two mutant enzymes. Since the structural requirement for BSP as an activator is the sulphonic group(s) in addition to the hydrophobic tetrabromophthalein ring [9], the abolition of activation by the mutations and by the use of NAD⁺ as the coenzyme, together with the kinetic alteration of NADP⁺-linked activity by the mutations, suggest that the negatively charged sulphonic group(s) of BSP interact electrostatically with the positively charged side-chains of both Lys-270 and Arg-276 in WT, and that the BSP binding causes perturbation of the interaction between the 2'-phosphate group of NADP⁺ and the two residues, which leads to rapid release of the products.

Since BSP inhibited K270M and R276M, its pattern of inhibition towards NADP⁺-linked activity was examined. The inhibition of R276M by BSP was competitive with respect to the coenzyme and non-competitive with respect to IN (Figures 3A and 3C). The K_i for BSP was 1.5 μ M, which is slightly lower than its dissociation constant of 2.5 μ M for WT [9]. In the case of K270M, inhibition by BSP appeared to be competitive with respect to NADP⁺, but BSP acted as an activator at NADP⁺ concentrations of greater than 1.0 mM (Figure 3B). Although in the presence of 0.2 mM NADP⁺ BSP was a mixed-type inhibitor with respect to IN (results not shown), in the presence of 1.0 mM NADP⁺ it showed mixed-type activation, in which the addition of BSP lowered the apparent K_m value for IN (Figure 3D). The tight binding of BSP on K270M and R276M was also indicated by the large protective effect of this compound against heat inactivation of the mutant enzymes compared with that with WT (Figure 1).

These results suggest that BSP binds near the coenzyme-binding site of the enzymes through parts of the molecule other than the sulphonic group(s). The tetrabromophthalein ring of BSP has been suggested to be another structural factor in its binding to DD4 [9]. We speculate that the interactions of the tetrabromophthalein ring and the sulphonic group(s) of BSP with hydrophobic amino acid residues and with the basic residues (Lys-270 or Arg-276) respectively lead to the activated binary complex of the enzyme, but that the loss of the interaction between the sulphonic group(s) of BSP and Lys-270 or Arg-276 might cause a slight alteration in the orientation of this compound with respect to its binding site, which would result in the formation of an inhibitory binary complex. The much lower affinity of NADP(H) than of BSP (200 μ M and 1.5 μ M respectively) may lead R276M to form mainly the dead-end enzyme-BSP complex. K270M was distinct from R276M in that it was activated by BSP at high NADP⁺ concentrations, although the inhibitory mechanism of BSP at low coenzyme concentrations can be thought to be similar to that for R276M. Since the K_i^{NADP} of K270M was lower than that of R276M, it appears that under such high coenzyme concentrations most of the enzyme exists as binary complexes with NADP⁺, and may undergo conformational changes on subsequent binding of BSP, as indicated by the decrease in K_m^{IN} . The difference in the inhibition patterns for K270M and R276M may be due to differences in the contributions of Lys-270 and Arg-276 to NADP(H) binding, as demonstrated in the comparison of the kinetic and thermodynamic properties of WT and the mutant enzymes.

We have shown that Lys-270 and Arg-276 are required for both NADP(H) binding to the DD4 isoenzyme of 3HSD and activation of the enzyme by BSP. However, there have been no previous reports of activation by BSP of other members of the aldo-keto reductase family that contain two basic residues corresponding to Lys-270 and Arg-276. In addition, BSP has been reported to act as an inhibitor of aldose reductase, aldehyde reductase, DD1 and DD2 [9]. The discrepancy in the effects of BSP on DD4 and on the other enzymes may be due to differences in the amino acid residues that interact with this compound. We propose that, as well as the two basic residues, other amino acids of DD4, required for interactions with other parts of BSP, are important for its activation by this compound.

This work was supported by a grant from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

- 1 Takikawa, H., Fumimoto, M., Nishikawa, K. and Yamanaka, M. (1992) *Hepatology* **16**, 365–371
- 2 Hara, A., Taniguchi, H., Nakayama, T. and Sawada, H. (1990) *J. Biochem. (Tokyo)* **108**, 250–254
- 3 Deyashiki, Y., Taniguchi, H., Amano, T., Nakayama, T., Hara, A. and Sawada, H. (1992) *Biochem. J.* **282**, 741–746
- 4 Deyashiki, Y., Ogasawara, A., Nakayama, T., Nakanishi, M., Miyabe, Y., Sato, K. and Hara, A. (1994) *Biochem. J.* **299**, 545–552
- 5 Hara, A., Matsuura, K., Tamada, Y., Sato, K., Miyabe, Y., Deyashiki, Y. and Ishida, N. (1996) *Biochem. J.* **313**, 373–376
- 6 Stolz, A., Hammond, L., Lou, H., Takikawa, H., Ronk, M. and Schively, J. E. (1993) *J. Biol. Chem.* **268**, 545–552
- 7 Winters, C. J., Molowa, D. T. and Guzelian, P. S. (1990) *Biochemistry* **29**, 1080–1087
- 8 Bohren, K. M., Bullock, B., Wermuth, B. and Gabbay, K. H. (1989) *J. Biol. Chem.* **264**, 9547–9551
- 9 Matsuura, K., Tamada, Y., Deyashiki, Y., Miyabe, Y., Nakanishi, M., Ohya, I. and Hara, A. (1996) *Biochem. J.* **313**, 179–184
- 10 Ohara, H., Miyabe, Y., Deyashiki, Y., Matsuura, K. and Hara, A. (1995) *Biochem. Pharmacol.* **50**, 221–227
- 11 Deyashiki, Y., Tamada, Y., Miyabe, Y., Nakanishi, M., Matsuura, K. and Hara, A. (1995) *J. Biochem. (Tokyo)* **118**, 285–290
- 12 Harrison, D. H., Bohren, K. M., Ringe, D., Petsko, G. A. and Gabbay, K. H. (1994) *Biochemistry* **33**, 2011–2020
- 13 Hyman, S. and Kinoshita, J. H. (1965) *J. Biol. Chem.* **240**, 877–882
- 14 Bohren, K. M., Page, J. L., Shankar, R., Henry, S. P. and Gabbay, K. H. (1991) *J. Biol. Chem.* **266**, 24031–24037
- 15 Wilson, D. K., Bohren, K. M., Gabbay, K. H. and Quiocho, F. A. (1992) *Science* **257**, 81–84
- 16 Kubiseski, T. J. and Flynn, G. (1995) *J. Biol. Chem.* **270**, 16911–16917
- 17 Horton, R. M. (1993) in *PCR Protocols* (White, B. A., ed.), pp. 251–261, Humana Press, Clifton, NJ
- 18 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 19 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 20 Hara, A., Deyashiki, Y., Nakagawa, M., Nakayama, T. and Sawada, H. (1982) *J. Biochem. (Tokyo)* **92**, 1753–1762
- 21 Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- 22 Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 104–137
- 23 Cornish-Bowden, A. (1995) in *Fundamentals of Enzyme Kinetics*, pp. 105–108, Portland Press, London
- 24 Kubiseski, T. J., Green, N. C., Borhani, D. W. and Flynn, G. (1994) *J. Biol. Chem.* **269**, 2183–2188
- 25 Sem, D. S. and Kasper, C. B. (1993) *Biochemistry* **32**, 11548–11558