Biochem. J. (1989) 264, 403–407 (Printed in Great Britain)

Isolation from pig lens of two proteins with dihydrodiol dehydrogenase and aldehyde reductase activities

Akira HARA, Tatsuhiro HARADA, Makoto NAKAGAWA, Kazuya MATSUURA, Toshihiro NAKAYAMA and Hideo SAWADA*

Department of Biochemistry, Gifu Pharmaceutical University, Mitahora-higashi, Gifu 502, Japan

Dimeric and monomeric proteins containing dihydrodiol dehydrogenase and aldehyde reductase activities were purified from pig lens. The dimeric enzyme of M_r 65000 specifically oxidized the *trans*-dihydrodiols of naphthalene and benzene with $NADP^+$ as a strict cofactor, and reduced α-diketones, aromatic aldehydes and glyceraldehyde with NADPH as a cofactor. The monomeric enzyme of M_r 35000, although identical with aldose reductase, oxidized the *trans*-dihydrodiol of naphthalene at a pH optimum of 7.6. These results suggest that the two enzymes are involved in the pathogenesis of naphthalene cataract.

INTRODUCTION

Naphthalene is an important industrial chemical that is found in cigarette smoke [1], and its administration results in cataract formation [2,3] as well as injury to lung [4] and kidney [5]. The damage to the eye has been suggested to be due to the enzymic formation of 1,2-dihydroxynaphthalene and its autoxidation to 1,2-naphthoquinone, which reacts with amino acids, glutathione and lens protein [2,3,6]. A dehydrogenase activity that converts 1,2-dihydro-1,2-dihydroxynaphthalene (naphthalene dihydrodiol) into 1,2-dihydroxynaphthalene has been detected in the tissues of rabbit eye including lens and is thought to be identical with dihydrodiol dehydrogenase (EC 1.3.1.20) [2], which was first identified in rabbit liver [7]. However, the naphthalene dihydrodiol dehydrogenase in the eye has not hitherto been further characterized.

Recently, two dihydrodiol dehydrogenases in guinea-pig testis have been suggested to be aldose reductase (EC 1.1.1.21) and aldehyde reductase (EC 1.1.1.2) [8]. Lens contains aldose reductase, which has been postulated to be involved in the pathogenesis of diabetic cataracts [9]. In addition, aldehyde reductase has been purified from pig lens [10]. In order to clarify whether the oxidation of naphthalene dihydrodiol in lens is catalysed by aldose reductase, aldehyde reductase and/or a distinct dihydrodiol dehydrogenase, we co-purified naphthalene dihydrodiol dehydrogenase and aldehyde reductase from pig lens, and found that lens contains at least two proteins with both dihydrodiol dehydrogenase and aldehyde reductase activities. The present paper reports the purification and properties of the two lens proteins.

EXPERIMENTAL

Materials

Pig lenses were obtained from a slaughterhouse and stored at −20 °C. *cis*-1,2-Dihydrobenzene-1,2-diol (benzene dihydrodiol) was obtained from Fluka Chemie. *trans*-Benzene dihydrodiol and naphthalene dihydrodiol were synthesized as described by Platt & Oesch [11,12].

Enzyme assay

Dehydrogenase and reductase activities were determined spectrophotometrically or fluorimetrically with 1.8 mM-naphthalene dihydrodiol and 10 mM-DL-glyceraldehyde respectively as a substrate as previously described [8]. Reactions were initiated by addition of the enzyme solution and specific activities are expressed as units (μmol of NADPH produced or oxidized/min) per mg of protein at 25 °C. Protein concentration was determined by the method of Bradford [13], with bovine serum albumin as a standard.

Enzyme purification

The purification of lens dihydrodiol dehydrogenase and aldehyde reductase was carried out at 4 °C. All buffers contained 5 mM-2-mercaptoethanol. Pig lens (22 g) was homogenized in 100 ml of buffer A (20 mM-potassium phosphate buffer, pH 7.5, containing 0.5 mM-EDTA and 0.15 M-KCl). After centrifugation at 12000 *g* for 10 min, the supernatant was fractionated by the addition of solid $(NH_4)_2SO_4$. The 35–65%-saturated-$(NH_4)_2SO_4$ precipitate was collected by centrifugation at 10000 *g* for 15 min, dissolved in 30 ml of buffer A and then passed through a 5 cm × 100 cm Sephadex G-100 column in buffer A. The enzyme activities were resolved into two distinct peaks, F1 and F2 (Fig. 1). The F1 and F2 fractions were separately concentrated by ultrafiltration on an Amicon YM-10 membrane, dialysed against buffer B (10 mM-Tris/HCl buffer, pH 7.5, containing 0.5 mM-EDTA) and then applied to 1.2 cm × 20 cm Matrex Red A (Amicon) columns equilibrated with buffer B. After the columns had each been washed with 50 ml of buffer B containing 10% (v/v) glycerol, the two enzymes were eluted with this buffer plus 0.5 mM-$NADP^+$. The two enzyme fractions were separately concentrated by ultrafiltration, dialysed against 10 mM-Tris/HCl buffer, pH 8.0, containing 0.5 mM-EDTA and then applied to 1.3 cm × 10 cm DEAE-Sephacel columns equilibrated with the same buffer. The two enzymes were eluted with a linear 0–0.2 M-NaCl gradient in the buffer. The two enzyme

* To whom correspondence should be addressed.

fractions were separately concentrated by ultrafiltration, dialysed against buffer B without EDTA, and then applied to 1.3 cm × 5 cm HA-Ultrogel (LKB) columns equilibrated with the same buffer. The enzymes were eluted with linear 0–50 mM-potassium phosphate gradients in the buffer. The F1 and F2 fractions were separately concentrated by ultrafiltration and stored at 4 °C.

Other methods

Isoelectric focusing on a 7.5% (w/v) polyacrylamide disc gel [14] or SDS/polyacrylamide-gel electrophoresis on a 10% (w/v) slab gel [15] was carried out as described in the references cited. Dehydrogenase and reductase activities in the gels were stained with 1 mM-naphthalene dihydrodiol and 10 mM-DL-glyceraldehyde as the respective substrates as described previously [16], and protein in the gel was stained with Coomassie Brilliant Blue R-250. The M_r of the native enzyme was estimated by gel filtration on a Sephadex G-100 column in buffer A, and that of the denatured enzyme by SDS/polyacrylamide-gel electrophoresis standardized by the use of M_r markers.

RESULTS

Sephadex G-100 filtration of the 35–65%-saturated-$(NH_4)_2SO_4$ fraction of lens extract revealed the existence of two enzymes with both dihydrodiol dehydrogenase and aldehyde reductase activities; fraction F1 (M_r 65000) exhibited high dihydrodiol dehydrogenase activity, whereas fraction F2 (M_r 33000) had high aldehyde reductase activity (Fig. 1). The dihydrodiol dehydrogenase and aldehyde reductase activities of the two enzymes were not separated from each other; some minor peaks with the two enzyme activities appeared on DEAE-Sephacel chromatography of fraction F2. The ratios of the two activities were essentially constant at the subsequent purification steps of fractions F1 and F2 (Table 1). Both dihydrodiol dehydrogenase and aldehyde reductase activities of the purified F1 enzyme were stained at the same pH of 7.0 on gel focusing, and those of the

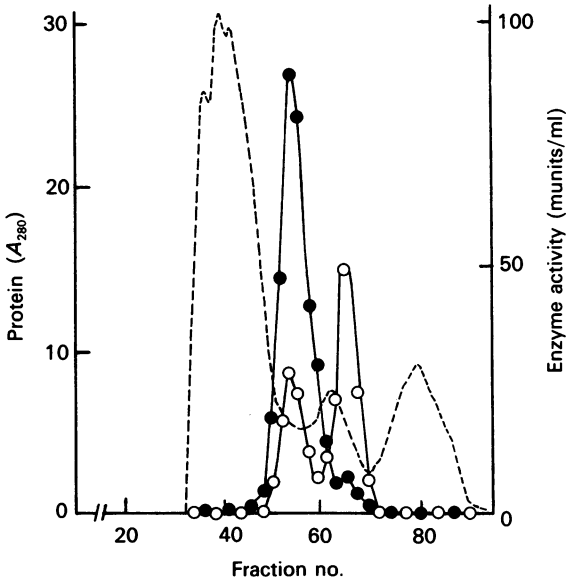

Fig. 1. Gel-filtration pattern of lens dihydrodiol dehydrogenase and aldehyde reductase activities on a Sephadex G-100 column

The 35–65%-saturated-$(NH_4)_2SO_4$ fraction of the lens extract was applied on to the column, and the fractions (10 ml) were analysed for protein at 280 nm (----) and for activities of dihydrodiol dehydrogenase (●—●) and aldehyde reductase (○—○). Fractions 51–59 (F1) and 61–70 (F2) were collected.

Table 1. Purification from lens of two proteins with dihydrodiol dehydrogenase and aldehyde reductase activities

For details of the purification procedure see the Experimental section. The dehydrogenase activity was assayed in 0.1 M-glycine/NaOH buffer, pH 10.0, containing 0.25 mM-$NADP^+$ and 1.8 mM-naphthalene dihydrodiol, and the reductase activity in 0.1 M-potassium phosphate buffer, pH 6.0, containing 80 μM-NADPH and 10 mM-DL-glyceraldehyde.

Step	Protein (mg)	Dihydrodiol dehydrogenase Activity (munits/mg)	Dihydrodiol dehydrogenase Yield (%)	Aldehyde reductase Activity (munits/mg)	Aldehyde reductase Yield (%)	Dehydrogenase/reductase activity ratio
$(NH_4)_2SO_4$ fractionation	3170	2.8	100	1.9	100	1.5
Sephadex G-100 chromatography						
F1	302	19.8	66	6.3	31	3.1
F2	182	3.2	6	17.2	52	0.19
Matrex Red A chromatography						
F1	1.6	2430	43	937	25	2.6
F2	2.3	122	3	956	37	0.13
DEAE-Sephacel chromatography						
F1	0.6	5450	36	1820	17	3.0
F2	2.0	95	2	950	32	0.10
HA-Ultrogel chromatography						
F1	0.3	8460	29	2620	12	3.2
F2	0.8	100	0.9	1240	16	0.08

F2 enzyme at pH 5.0. These results indicate that both enzymes are capable of catalysing dihydrodiol oxidation and glyceraldehyde reduction. SDS/polyacrylamide-gel electrophoresis of enzymes F1 and F2 gave single protein bands of M_r 39000 and 35000 respectively (Fig. 2), indicating that the F1 enzyme is dimeric whereas the F2 enzyme is monomeric.

Table 2 compares the substrate-specificities of the two enzymes. The F1 enzyme specifically oxidized *trans*-dihydrodiols of naphthalene and benzene with $NADP^+$ as a strict cofactor, with optimum activity around pH 10.2. The K_m and $V_{max.}$ values for naphthalene dihydrodiol determined at pH 7.5 were 0.31 mM and 6.8 units/mg respectively. On the other hand, the F2 enzyme showed high dehydrogenase activity towards naphthalene dihydrodiol with both $NADP^+$ and NAD^+ as cofactors at a pH optimum of 7.6, but also slowly oxidized *trans*- and *cis*-benzene dihydrodiols and some alcohols. The two enzymes were inactive towards 50 μM-hydroxy steroids such as 3α-hydroxy-5α-androstan-17-one, 17β-hydroxy-5β-androstan-3-one and 5β-androsterone-3α,17β-diol, and 1–10 mM other alcohols such as ethanol, indan-1-ol and acenaphthen-1-ol.

In the reverse reaction, the F1 enzyme reduced vicinal diketones, aromatic aldehydes and glyceraldehydes in the presence of NADPH and showed a broad pH optimum at 6.0–8.0, whereas the F2 enzyme displayed high reductase activity towards aldoses as well as the substrates for the F1 enzyme in the presence of either NADPH or NADH, and its pH optimum was 5.8. The two enzymes did not reduce 0.1 mM-menadione and 1 mM aromatic ketones such as 4-nitroacetophenone and 4-benzoylpyridine.

The F1 enzyme was sensitive only to thiol-blocking reagents, whereas the F2 enzyme was greatly inhibited by aldose reductase inhibitors such as quercitrin, sorbinil, indomethacin and hexoestrol (Table 3). The F2 enzyme was not inhibited by the aldehyde reductase inhibitors diphenic acid and valproate [10], implying that the F2 preparation was not contaminated with aldehyde reductase that might be separated from the F2 enzyme as a minor activity peak in the purification. The F1 enzyme was inhibited by high concentrations of SO_4^{2-} ions, which in contrast activated the F2 enzyme. Conversely, NaCl and pyrophosphate at high concentrations stimulated the activity of the F1 enzyme but were inhibitory to the F2 enzyme.

DISCUSSION

Recent studies on dihydrodiol dehydrogenase have shown that the enzyme appears in multiple forms in the

Table 2. Substrate-specificities of lens dihydrodiol dehydrogenases

The $NADP^+$-dependent dehydrogenase activity of enzymes F1 and F2 was assayed in 0.1 M-glycine/NaOH buffer, pH 10.0, and 0.1 M-potassium phosphate buffer, pH 7.5, respectively, and the respective specific activities of enzymes F1 and F2 were 8.5 and 0.5 units/mg. The NADPH-dependent reductase activity was assayed in 0.1 M-potassium phosphate buffer, pH 7.5, for the F1 enzyme and in the phosphate buffer, pH 6.0, for the F2 enzyme. The relative activities and apparent K_m values for $NAD(P)^+$ were determined with 1.8 mM-naphthalene dihydrodiol, and those for NAD(P)H with 0.5 mM-camphorquinone. The values are means for two determinations. Abbreviation: N.D., no activity detected.

		F1 enzyme		F2 enzyme	
Substrate	Concn. (mM)	Relative activity (%)	K_m (mM)	Relative activity (%)	K_m (mM)
Dehydrogenation					
Naphthalene dihydrodiol	1.8	100	0.14	100	8.7
trans-Benzene dihydrodiol	1.8	63	0.54	4	3.3
Benzyl alcohol	1.0	N.D.	–	6	2.8
cis-Benzene dihydrodiol	1.8	N.D.	–	2	–
Butan-1-ol	5.0	N.D.	–	4	–
Glycerol	5.0	N.D.	–	2	–
$NADP^+$	0.25	100	0.003	100	0.002
NAD^+	2.5	N.D.	–	325	0.30
Reduction					
Camphorquinone	0.5	136	0.23	118	0.02
Acenaphthenequinone	0.1	100	0.024	48	–
3-Nitrobenzaldehyde	1.0	75	0.47	175	0.01
Butane-2,3-dione	1.0	46	1.1	178	0.22
Pyridine-3-aldehyde	1.0	37	6.6	186	0.003
4-Nitrobenzaldehyde	1.0	13	–	198	0.006
D-Glyceraldehyde	10	30	1.3	227	0.04
Succinic semialdehyde	1.0	N.D.	–	116	0.04
D-Glucuronate	10	N.D.	–	125	–
D-Xylose	10	N.D.	–	111	–
D-Galactose	10	N.D.	–	47	–
D-Glucose	10	N.D.	–	32	–
NADPH	0.08	136	0.005	118	0.003
NADH	0.12	N.D.	–	61	–

Table 3. Effects of various compounds on lens dihydrodiol dehydrogenases

The naphthalene dihydrodiol dehydrogenase activity was assayed in 0.1 M-glycine/NaOH buffer, pH 10.0, for the F1 enzyme and in 0.1 M-potassium phosphate buffer, pH 7.5, for the F2 enzyme, except that the effects of Li_2SO_4 and sodium pyrophosphate were determined in 0.1 M-Tris/HCl buffer, pH 7.5, for the F2 enzyme. The values represent the means ± S.D. for three or four determinations.

Compound	Concn. (mM)	Relative activity (%) F1 enzyme	Relative activity (%) F2 enzyme
Quercitrin	0.01	84±16	11±7
Sorbinil	0.01	99±1	45±15
Hexoestrol	0.05	97±3	40±12
Indomethacin	0.05	75±24	43±5
p-Chloromercuribenzoic acid	0.1	3±1	36±6
p-Chloromercuribenzenesulphonate	0.1	6±4	34±11
Diphenic acid	0.1	91±8	99±1
Cyclopentane-1,1-diacetic acid	1.0	99±1	62±24
Barbital	1.0	96±5	74±6
Li_2SO_4	300	26±8	153±21
$(NH_4)_2SO_4$	300	37±6	138±8
Sodium pyrophosphate	200	139±4	96±4
NaCl	300	159±15	35±6
Others*		100	100

* 0.05 mM-Medroxyprogesterone acetate, 0.1 mM-dexamethazone, 1 mM-valproate, 1 mM-1,10-phenanthroline and 5 mM-pyrazole.

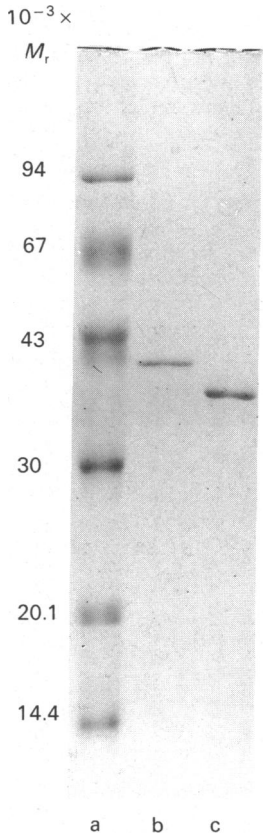

Fig. 2. SDS/polyacrylamide-gel electrophoresis of lens dihydrodiol dehydrogenases

The samples were as follows: track a, M_r standards; track b, F1 enzyme; track c, F2 enzyme.

liver of several animal species; these multiple forms have been identified as genetically distinct enzymes such as 3α-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase isoenzymes and aldehyde reductase [17–20]. Dihydrodiol dehydrogenases, distinct from the hepatic enzymes in substrate-specificity and structure, have been isolated from guinea-pig testis [8] and monkey kidney [21]. Together with these studies, the existence of two distinct dihydrodiol dehydrogenases in lens indicates that multiplicity is a feature common to the enzyme in various mammalian tissues and that different enzymes act as dihydrodiol dehydrogenase depending on the tissue.

The properties of the dimeric lens dihydrodiol dehydrogenase, F1, are clearly different from those of the monomeric enzymes in liver [17–20] and testis [8]. The F1 enzyme resembles the monkey kidney enzyme [21] in its dimeric structure and substrate-specificity, but it was not sensitive to the inhibitors of the monkey kidney enzyme such as quercitrin and indomethacin [21]. The lens enzyme reduced α-diketones, aromatic aldehydes and glyceraldehyde, but not D-glucuronate, aromatic ketones and succinic semialdehyde, which suggests that it is not aldehyde reductase, carbonyl reductase or succinic semialdehyde reductase [22]. The F1 enzyme can also be differentiated from high-M_r aldehyde reductase in human liver, placenta and kidney [23,24]. The human enzyme is a heterodimer that can reduce D-glucose and is inhibited by sorbinil and NaCl, whereas the F1 enzyme was a homodimer, inactive towards D-glucose, and resistant to the inhibitors. It has been reported that pig lens contains three pyridine-3-aldehyde reductase activities, two of which are monomeric aldose reductase and aldehyde reductase, but the nature of the third reductase has not been examined [10]. Since the F1 enzyme differs from the

monomeric reductases in many respects, it may correspond to the third reductase of pig lens [10]. Although the significance of the aldehyde reductase activity of the F1 enzyme remains unknown, its high reactivity towards naphthalene dihydrodiol even at the physiological pH of 7.5 indicates that in lens the dimeric enzyme is the predominant enzyme form for the oxidation of naphthalene dihydrodiol, which ultimately leads to cataract formation by naphthalene administration [2,3].

The structure, substrate-specificity, cofactor-specificity, inhibitor-sensitivity and activation by SO_4^{2-} ions of the F2 enzyme, the primary aldehyde reductase in lens, are similar to those of aldose reductase [10,22,25,26], which indicates that this enzyme is aldose reductase, although further studies with specific antibodies against aldose reductase are required. *trans*-Benzene dihydrodiol was a poor substrate for the F2 enzyme, as was observed with the testicular aldose reductase [8], but the enzyme readily oxidized naphthalene dihydrodiol with both $NADP^+$ and NAD^+ as cofactors at pH 7.5. The results not only support the concept that aldose reductase is responsible for the multiplicity of dihydrodiol dehydrogenase in mammalian tissues [8], but also suggest that it may be involved in the metabolism of naphthalene when the concentration of the *trans*-dihydrodiol metabolite becomes high in a tissue.

REFERENCES

1. Schmeltz, I., Tosk, J. & Hoffmann, D. (1976) Anal. Chem. **48**, 645–650
2. van Heyningen, R. & Pirie, A. (1967) Biochem. J. **102**, 842–852
3. van Heyningen, R. (1976) Invest. Ophthalmol. **15**, 685–697
4. Mahvi, D., Bank, H. & Harley, R. (1977) Am. J. Pathol. **86**, 559–571
5. O'Brien, A. F., Smith, L. & Cohen, G. M. (1982) Chem.-Biol. Interact. **55**, 109–122
6. Rees, J. R. & Pirie, A. (1967) Biochem. J. **102**, 853–863
7. Ayengar, P. K., Hayaishi, O., Nakajima, M. & Tomida, I. (1959) Biochim. Biophys. Acta **33**, 111–119
8. Matsuura, K., Hara, A., Nakayama, T., Nakagawa, M. & Sawada, H. (1987) Biochim. Biophys. Acta **912**, 270–277
9. Kinoshita, J. H. (1974) Invest. Ophthalmol. **13**, 713–724
10. Branlant, G. (1982) Eur. J. Biochem. **129**, 99–104
11. Platt, K. L. & Oesch, F. (1977) Synthesis **7**, 449–450
12. Platt, K. L. & Oesch, F. (1983) J. Org. Chem. **48**, 265–268
13. Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
14. Hara, A., Deyashiki, Y., Nakagawa, M., Nakayama, T. & Sawada, H. (1982) J. Biochem. (Tokyo) **92**, 1753–1762
15. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
16. Sawada, H., Hara, A., Hayashibara, M. & Nakayama, T. (1979) J. Biochem. (Tokyo) **86**, 883–892
17. Wörner, W. & Oesch, F. (1984) FEBS Lett. **170**, 263–267
18. Penning, T. M., Mukharji, I., Barrows, S. & Talalay, P. (1984) Biochem. J. **222**, 601–611
19. Hara, A., Hasebe, K., Hayashibara, M., Matsuura, K., Nakayama, T. & Sawada, H. (1986) Biochem. Pharmacol. **35**, 4005–4012
20. Sawada, H., Hara, A., Nakayama, T., Nakagawa, M., Inoue, Y., Hasebe, K. & Zhang, Y. P. (1988) Biochem. Pharmacol. **37**, 453–458
21. Hara, A., Mouri, K. & Sawada, H. (1987) Biochem. Biophys. Res. Commun. **145**, 1260–1266
22. Cromlish, J. A. & Flynn, T. G. (1985) J. Neurochem. **44**, 1485–1493
23. Das, B. & Srivastava, S. K. (1985) Biochim. Biophys. Acta **840**, 324–333
24. Srivastava, S. K., Ansari, N. H., Hair, G. A. & Das, B. (1984) Biochim. Biophys. Acta **800**, 220–227
25. Wermuth, B., Burgisser, H., Bohren, K. & von Wartburg, J. P. (1982) Eur. J. Biochem. **127**, 279–284
26. Griffin, B. W. & McNatt, L. G. (1986) Arch. Biochem. Biophys. **246**, 75–81

Received 3 April 1989/19 June 1989; accepted 27 June 1989