Inhibition of dimeric dihydrodiol dehydrogenases of rabbit and pig lens by ascorbic acid

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The dehydrogenase activity of dimeric dihydrodiol dehydrogenases (DD) purified from pig and rabbit lenses was inhibited by either L-ascorbic acid or its epimer, isoascorbic acid, at pH 7.5. Isoascorbate [IC_{50} (concn. giving 50% inhibition) = 0.043 mM for the pig enzyme; IC_{50} = 0.13 mM for the rabbit enzyme] was a more potent inhibitor than ascorbate (IC_{50} values 0.45 and 0.90 mM respectively), but 1 mM-dehydroascorbate gave less than 30% inhibition. Glucose, glucuronate, gulono- γ -lactone, glutathione and dithiothreitol did not inhibit the enzyme activity. The inhibition by isoascorbate and ascorbate was instantaneous and reversible, and their inhibitory potency was decreased by addition of ascorbate oxidase. In the reverse reaction, isoascorbate and ascorbate gave low IC_{50} values of 0.013 and 0.10 mM respectively for the pig enzyme and 0.025 and 0.25 mM for the rabbit enzyme. The inhibition patterns by the two compounds were competitive with respect to dihydrodiols of naphthalene and benzene and uncompetitive with respect to NADP⁺, but those in the reverse reaction were uncompetitive with respect to both carbonyl substrate and NADPH. The steady-state kinetic measurements in the forward and reverse reactions by the pig enzyme were consistent with an ordered Bi Bi mechanism, in which NADP⁺ binds to the enzyme first and NADPH leaves last. The results indicate that ascorbate and its epimer directly bind to an enzyme:NADP⁺ binary complex as dead-end inhibitors. Thus ascorbate may be an important modulator of DD in the lens.

INTRODUCTION

Naphthalene is cataractogenic in man [1], and cataract has been produced experimentally in rabbits by feeding large doses of naphthalene [2,3]. The cataractogenicity of the drug has been postulated to be mediated via its catechol metabolite, 1,2-dihydroxynaphthalene, which is autoxidized to reactive quinone and H₂O₂ [3,4], and dihydrodiol dehydrogenase (DD), which converts naphthalenedihydrodiol into the catechol metabolite, has been thought to play a crucial role in cataract formation in the rabbit [5]. The hypothesis is further supported by our preceding report that lens has the highest content of DD activity of extra-hepatic tissues of the rabbit and contains large amounts of dimeric DD as a specific protein for this tissue [6]. The dimeric DD of rabbit lens catalyses the reduction of α -dicarbonyl compounds and some aldehydes as well as the oxidation of *trans*-dihydrodiols of naphthalene and benzene, and is immunologically similar to dimeric DD in pig lens [7].

It has been reported that the appearance of naphthalene cataract is variable, depending on the rabbits used [8]. Furthermore, there is an increased concentration of ascorbic acid in lenses of naphthalene-fed rabbits, in which cataractous changes are not severe [9]. During the search for intra-lenticular components which affect the activity of lens dimeric DD, we found that ascorbic acid and its epimer inhibited the enzyme activity. Here we describe the mechanism of inhibition of dimeric DD by ascorbic acid and its epimer. The inhibition appears to result from selective binding of the two compounds to a kinetic enzyme species in the enzyme reaction which follows an ordered Bi Bi mechanism.

EXPERIMENTAL

Materials

L-Ascorbic acid, L-ascorbic acid 2-phosphate and 3-

nitrobenzaldehyde were obtained from Wako Pure Chemical Industries (Osaka, Japan), NADP(H) and ascorbate oxidase from Oriental Yeast Co. (Tokyo, Japan), 3-nitrobenzyl alcohol and camphorquinone from Tokyo Kasei Industries (Tokyo, Japan), and D-isoascorbic acid and Cibacron Blue F3GA from Nacalai Tesque Inc. (Kyoto, Japan). Dehydroascorbic acid was purchased from Fluka Chemie A.G. (Buchs, Switzerland). Cibacron Blue F3GA was purified by paper chromatography as described previously [10]. Other chemicals used were specified in the preceding paper [6]. Lens dimeric DDs of the rabbit [6] and pig [7] were purified as described.

Enzyme assay

Dehydrogenase and reductase activities were determined as described in the preceding paper [6]. The standard assay mixture for dehydrogenase activity contained 0.1 M-potassium phosphate, pH 7.5, 0.25 mM-NADP⁺, 1.8 mM-naphthalenedihydrodiol and enzyme in a total volume of 2.0 ml, and that for reductase activity consisted of the phosphate buffer, 0.1 mM-NADPH, 0.5 mM-camphorquinone and enzyme. The concentrations of cofactor, substrate and inhibitor are given below. Controls contained all reactants except substrate. The reaction was initiated by addition of enzyme. One unit of enzyme activity was defined as that amount catalysing oxidation or formation of 1 μ mol of NADPH per minute at 25 °C.

The concentrations of NADP⁺ and NADPH were calculated from the absorbances at 260 nm and 340 nm respectively. The preparation of cofactors and substrate and all assays contributing data to one experimental set were performed on the same day. The solutions of ascorbic acid and its derivatives, neutralized to about pH 6.8 with NaOH and kept in ice, were also freshly prepared from each experiment.

Abbreviation used: DD, dihydrodiol dehydrogenase.

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Fig. 1. Inhibition of dimeric DDs by ascorbate and its derivatives

The inhibition (%) of naphthalenedihydrodiol dehydrogenase (a) and camphorquinone reductase (b) activities of DDs from pig lens (●, ▲, ■) and rabbit lens (○, △) by ascorbate (●, ○), isoascorbate (▲, △) and dehydroascorbate (■) was determined at pH 7.5, and is shown as a function of the inhibitor concentration.

Data analysis

Initial velocities in the asymptotic region which follow Michaelis kinetics were fitted to eqn. (1) [11]:

$$v = \frac{V_{AB}}{AB + K_a B + K_b A + K_{ia} K_b} \quad (1)$$

by using a computer program for least-squares linear regression [12], and results are presented as double-reciprocal forms. Kinetic studies in the presence of inhibitors were carried out in an identical manner.

RESULTS AND DISCUSSION

DD inhibition by ascorbate and its derivatives

When the effects of several organic components of the lens on the dehydrogenase activity of pig lens DD were examined at a physiological pH of 7.5, ascorbic acid was found to be inhibitory to the enzyme, but inositol, glucose, sorbitol, lactate and glutathione at 1 mM concentrations did not influence the activity at all. The IC_{50} values (concn. giving 50% inhibition) for ascorbate were 0.45 mM for naphthalenedihydrodiol oxidation and 0.13 mM for camphorquinone reduction (Fig. 1). Isoascorbate inhibited the enzyme more potently than did ascorbate, but dehydroascorbate showed weak inhibition on the enzyme and 2 mM-ascorbic acid 2-phosphate did not inhibit. Isoascorbate and ascorbate also inhibited the oxidoreductase activities of rabbit lens DD, although the IC_{50} values for the inhibition of rabbit lens DD were slightly higher than those obtained with pig lens DD. The enzyme activities of both the pig and rabbit enzymes were not inhibited by 1 mM concentrations of

metabolic intermediates of ascorbic acid biosynthesis, such as D-glucuronate, D-glucurono- γ -lactone and L-gulono- γ -lactone, and by 1 mM concentrations of other reductants, such as dithiothreitol, cysteine and 2-mercaptoethanol. It should be noted that ascorbate, isoascorbate and dehydroascorbate did not serve as substrates of the two enzymes. These data suggest that the α -keto-lactone ring with an enediol structure is the important part of the ascorbate for DD inhibition.

Reversibility of DD inhibition by ascorbate and its epimer

Catalase [13] and glucose-6-phosphate dehydrogenase [14] have been reported to be inactivated in a time-dependent manner by incubating with ascorbate, in which free radicals generated from the autoxidation of ascorbate are responsible for the inactivation of catalase. To test whether the free radicals are the obligatory intermediates in the inhibition process of DD, we incubated pig lens DD with ascorbate or isoascorbate before assay of the enzyme activity (Table 1). Almost all the dehydrogenase and reductase activities of the enzyme were recovered by diluting the enzyme-inhibitor mixture and retained for 30 min after the incubation, compared with the rapid loss of the activities in the mixture of the enzyme and *p*-chloromercuribenzenesulphonate, which has been described as a potent inhibitor for dimeric DDs [6,7]. The result indicates that ascorbate and isoascorbate behave as instantaneous and reversible inhibitors, and suggests that formation of the free radicals is not an important process for the DD inhibition by ascorbate and its epimer. This was further confirmed by the experiments with ascorbate oxidase (Fig. 2). When ascorbate oxidase was added to the reaction mixtures which had been initiated in the presence of ascorbate or isoascorbate, the inhibition by the inhibitors rapidly

Table 1. Effect of incubation of dimeric DD with ascorbate, isoascorbate and *p*-chloromercuribenzenesulphonate (PCMBs) on the enzyme activity

The pig lens enzyme (0.1 mg/ml of 50 mM-potassium phosphate, pH 7.0) was incubated in the presence of the inhibitor at 25 °C, and then 40 μ l portions of the mixture were assayed for the residual enzyme activity. The NADP⁺-linked dehydrogenase (D) and NADPH-linked reductase (R) activities were assayed with 1.8 mM-naphthalenedihydrodiol and 0.5 mM-camphorquinone, respectively, as substrates. The values are shown as the activity relative to the respective activities in the control incubation mixtures without the inhibitor, in which the enzyme activity was not changed up to 30 min.

Addition	Activity (%)					
	Incubation time ...		0 min			
			D	R	D	R
0.4 mM-Ascorbate			98	99	98	98
0.4 mM-Isoascorbate			85	57	86	57
0.1 mM-PCMBs			89	88	13	10
0.1 mM-PCMBs + 0.1 mM-NADPH			99	99	87	90
					67	65

disappeared. Since ascorbate oxidase converts ascorbate and isoascorbate into their oxidized forms [15], the inhibitory potency of dehydroisoascorbate may be much weaker than that of its reduced form. On the other hand, the inhibition of dehydroascorbate gradually increased after the addition of dithiothreitol, which reduced dehydroascorbic acid to ascorbic acid [16]. Thus ascorbate and isoascorbate themselves interact directly with dimeric DDs.

The mechanism of inhibition by ascorbate is also different from that for human leucocyte 3-hydroxy-3-methylglutaryl-CoA reductase, in which a free-radical intermediate, monodehydroascorbate, has been identified as the active inhibitory species [17]. Ascorbate inhibits β -N-acetylhexosaminidase [18], ferredoxin-NADP⁺ reductase [19] and glyoxalase I [20], which are also inhibited by sugar derivatives with a lactone ring, α -keto acids and aromatic reductones such as catechol, respectively. In

Fig. 2. Effects of ascorbate oxidase and dithiothreitol on the inhibitory potency of ascorbate and its derivatives

Naphthalenedihydrodiol dehydrogenase (a) and camphorquinone reductase (b) activities of the pig lens DD were first assayed in the absence (1) or presence of ascorbate (2), isoascorbate (3) or dehydroascorbate (4). Ascorbate oxidase (AO) (10 μ l of 100 units) or 0.2 M-dithiothreitol (DTT) were added to the reaction mixture approx 1.0 min after the initiation of the reaction. The concentrations of ascorbate, isoascorbate and dehydroascorbate were 0.5, 0.05 and 0.5 mM respectively, for the dehydrogenase activity, and the respective concentrations were 0.1, 0.02 and 0.1 mM for the reductase activity.

contrast with the broad inhibitor spectra of the enzymes, dimeric DDs were selectively inhibited by ascorbate and isoascorbate, but not by glucurono- γ -lactone, gulono- γ -lactone (as described above), 2 mM-pyruvate and 2 mM-catechol.

pH-dependency of DD inhibition by ascorbate and isoascorbate

The inhibitory potency of ascorbate and isoascorbate on the camphorquinone reductase activity of pig and rabbit DDs was

Table 2. Inhibition patterns and constants of ascorbate, isoascorbate, naphthalenedihydrodiol and camphorquinone

The dehydrogenase activity was determined with 0.25 mM NADP⁺ as a cofactor and 0.09–0.9 mM-naphthalenedihydrodiol, and the reductase activity was assayed with 0.1 mM-NADPH and 0.01–0.2-mM camphorquinone. K_{is} (slope effect) and K_{ii} (intercept effect) values were determined from replots of the slopes and intercepts, respectively, of double-reciprocal plots with the inhibitors, and are expressed as means of duplicate analyses.

DD source	Inhibitor	Varied substrate	Inhibition pattern	Inhibition constant	
				K_{is} (μ M)	K_{ii} (μ M)
Pig DD	Ascorbate	Naphthalenedihydrodiol	Competitive	60	–
	Isoascorbate	Naphthalenedihydrodiol	Competitive	11	–
	Ascorbate	Camphorquinone	Uncompetitive	–	70
	Isoascorbate	Camphorquinone	Uncompetitive	–	8
	Camphorquinone	Naphthalenedihydrodiol	Non-competitive	470	220
	Naphthalenedihydrodiol	Camphorquinone	Non-competitive	630	410
Rabbit DD	Ascorbate	Naphthalenedihydrodiol	Competitive	200	–
	Isoascorbate	Naphthalenedihydrodiol	Competitive	17	–
	Ascorbate	Camphorquinone	Uncompetitive	–	181
	Isoascorbate	Camphorquinone	Uncompetitive	–	20

profoundly pH-dependent. Ascorbate and isoascorbate inhibited the two enzymes similarly and maximally at a pH range of 7–8, and their inhibitions decreased at lower and higher pH ranges than 7–8. Since ascorbic acid has two pK_a values, 4.17 and 11.57 [21], its anionic form, in which one hydroxy group of the enediol structure is ionized, may be required for DD inhibition.

Kinetics of DD inhibition by ascorbate and its epimer

The kinetic patterns obtained from dead-end inhibitor studies with ascorbate and isoascorbate are summarized in Table 2. The patterns of two inhibitors were competitive with respect to naphthalenedihydrodiol in the forward reaction by dimeric DDs of pig and rabbit lenses, whereas those were uncompetitive with respect to camphorquinone in the reverse reaction. Since our preliminary NADPH-titration experiments indicate that there is one active site per subunit of pig lens DD (A. Hara & T. Harada, unpublished work), the results of the dead-end inhibition studies seem to suggest that dimeric DD has two distinct active sites: one site catalyses naphthalenedihydrodiol dehydrogenation and another catalyses camphorquinone reduction. However, camphorquinone inhibited the dehydrogenase activity non-competitively versus naphthalenedihydrodiol, and, conversely, naphthalenedihydrodiol inhibited the reductase activity non-competitively versus camphorquinone. In addition, preincubation of the enzyme with *p*-chloromercuribenzenesulphonate resulted in inactivation of both the dehydrogenase and reductase activities, and inactivation by the thiol-blocking agent was prevented by addition of NADPH (Table 1). Therefore, dimeric DD may catalyse naphthalene oxidation and camphorquinone reduction at the same catalytic site.

Kinetic mechanism of the DD reaction

Initial-velocity measurement. It is necessary to know the kinetic mechanism of dehydrogenase and reductase reactions of dimeric DD in order to explore the mechanism of inhibition by ascorbate and isoascorbate. We therefore examined the steady-state kinetic mechanism of pig lens DD in the forward reaction with benzenedihydrodiol, which is more stable than naphthalenedihydrodiol, and in the reverse reaction with 3-nitrobenzaldehyde, because its reduced product, 3-nitrobenzyl alcohol, is commercially available. The double-reciprocal plots of initial rate versus concentration of the varied substrate, benzenedihydrodiol, at fixed levels of NADP^+ , yielded a series of intersecting lines (Fig. 3a). Similar patterns of initial velocity were observed when the varied substrate was NADP^+ . The slopes and intercepts for

Fig. 3. Initial-velocity patterns of benzenedihydrodiol dehydrogenation and 3-nitrobenzaldehyde reduction by pig lens DD

(a) Reciprocal plots of the dehydrogenase activity with NADP^+ as the varied substrate. Benzenedihydrodiol (BD) concentrations were 0.20 mM ($\square$), 0.25 mM ($\blacktriangle$), 0.33 mM ($\triangle$), 0.50 mM ($\bullet$) and 1.0 mM ($\circ$). (b) Reciprocal plots of the reductase activity with NADPH as the varied substrate. 3-Nitrobenzaldehyde (NBA) concentrations were 0.20 mM ($\square$), 0.25 mM ($\blacktriangle$), 0.33 mM ($\triangle$), 0.50 mM ($\bullet$) and 1.0 mM ($\circ$). Re-plots of the slopes ($\bullet$) and intercepts ($\circ$) are shown in the insets. Velocities (v) of the dehydrogenases and reductase are expressed as m-units/ml and units/ml respectively.

each experiment were re-plotted against the reciprocal of the fixed substrate concentration, and were linear in each case. These results are consistent with a reaction mechanism which proceeds in a sequential manner. Initial-velocity studies carried out in a

Table 3. Product inhibition patterns and constants in the reduction of 3-nitrobenzaldehyde by pig lens DD

The reductase activity was assayed with 5–80 μM -NADPH or 0.2–1.0 mM-3-nitrobenzaldehyde as the varied substrate. The constants were determined and are expressed as described in Table 2.

Product	Varied substrate	Fixed substrate	Inhibition pattern	Inhibition constant	
				K_{is} (mM)	K_{ii} (mM)
NADP^+	NADPH	0.2 mM-Nitrobenzaldehyde	Competitive	0.007	—
	NADPH	1 mM-Nitrobenzaldehyde	Competitive	0.003	—
	Nitrobenzaldehyde	4 μM -NADPH	Noncompetitive	0.009	0.025
	Nitrobenzaldehyde	100 μM -NADPH	Non-competitive	0.15	0.18
3-Nitrobenzyl alcohol	NADPH	0.2 mM-Nitrobenzaldehyde	Non-competitive	5.2	16
	NADPH	1 mM-Nitrobenzaldehyde	Uncompetitive	—	2.0
	Nitrobenzaldehyde	4 μM -NADPH	Non-competitive	1.3	6.6
	Nitrobenzaldehyde	100 μM -NADPH	Non-competitive	1.9	2.8

Table 4. Inhibition patterns and constants of dead-end inhibitors

The dehydrogenase activity was assayed with 0.25 mM-NADP⁺ or 1.8 mM-benzene dihydrodiol as a fixed substrate, and the reductase activity was with 0.1 mM-NADPH or 1 mM-3-nitrobenzaldehyde. The ranges of concentration of varied substrates were $(1-10) \times K_m$ for the cofactors and $(0.25-5) \times K_m$ for the substrates. The inhibition constants were determined as described in Table 2.

Inhibitor	Varied substrate	Inhibition pattern	Inhibition constant	
			K_{is} (μ M)	K_{it} (μ M)
Cibacron Blue	NADP ⁺	Competitive	5.6	—
	Benzene dihydrodiol	Non-competitive	43	51
Ascorbate	NADP ⁺	Uncompetitive	—	127
	Benzene dihydrodiol	Competitive	69	—
Ascorbate	NADPH	Uncompetitive	—	65
	3-Nitrobenzaldehyde	Uncompetitive	—	82
Isoascorbate	NADP ⁺	Uncompetitive	—	44
	Benzene dihydrodiol	Competitive	7.3	—
	NADPH	Uncompetitive	—	10
	3-Nitrobenzaldehyde	Uncompetitive	—	12

similar manner with 3-nitrobenzaldehyde and NADPH (Fig. 3b) indicate that a sequential mechanism is also operating in the reverse reaction. The kinetic constants for substrates and cofactors, K_a , K_b , K_{ia} and V in eqn. (1), were $2.6 \pm 0.2 \mu$ M, 0.24 ± 0.03 mM, $12.8 \pm 1.9 \mu$ M and 0.56 ± 0.06 unit/mg of protein respectively in the forward reaction, and $4.7 \pm 0.2 \mu$ M, 0.26 ± 0.1 mM, $4.0 \pm 0.3 \mu$ M and 4.96 ± 0.05 units/mg of protein in the reverse reaction.

Inhibitions by products and dead-end inhibitors. Product-inhibition studies of the reverse reaction were carried out to determine the substrate binding order. A summary of the studies of product inhibition is shown in Table 3. From these data, the kinetic mechanism for pig lens DD appears most likely to be an ordered Bi Bi mechanism, in which NADPH binds first and NADP⁺ leaves last. On the other hand, product-inhibition studies of the forward reaction were impossible, because the direct oxidized product, an unstable ketol form of benzenedihydrodiol, is not available. Therefore, dead-end inhibition studies with Cibacron Blue, ascorbate and isoascorbate were performed in the forward reaction (Table 4). Cibacron Blue, which is known to bind to many oxidoreductases as a nucleotide analogue [22], inhibited the pig DD competitively with respect to NADP⁺ and non-competitively with respect to benzenedihydrodiol. The inhibitions by ascorbate and isoascorbate were uncompetitive with respect to NADP⁺ and competitive with respect to benzenedihydrodiol, as observed with naphthalenedihydrodiol as the substrate (Table 2). The inhibition patterns by the dead-end inhibitors are consistent with the ordered addition of cofactor followed by dihydrodiol substrate. When the patterns of inhibition by ascorbate and isoascorbate were examined in the reverse reaction, the two compounds inhibited the enzyme uncompetitively with respect to both 3-nitrobenzaldehyde and NADPH. This, together with the inhibition patterns in the forward reaction, indicates that ascorbate and isoascorbate selectively bind to the enzyme-NADP⁺ binary complex in the reaction mechanism.

Significance of DD inhibition by ascorbate in the lens

Ascorbate is not uniformly distributed in human and animal tissues. Lens is one of the tissues which contain relatively large amounts of the vitamin: its concentrations in lenses of the rabbit and pig are about 0.7 and 1.2 mM respectively [23]. The concentration of ascorbate in the lens seems to prevent the con-

tribution of DD in the formation of naphthalene-induced cataract. However, since the inhibition of DD by ascorbate was competitive with respect to dihydrodiol substrate, the enzyme can exert its dehydrogenase activity when the intra-lenticular concentration of naphthalenedihydrodiol is elevated by administration of naphthalene. In fact, large doses of naphthalene have been administered to produce cataract, although the great variation in the rabbit lens's susceptibility to the drug has been noted [2,3,8,9]. The contents of DD activity, which is mainly due to dimeric DD in rabbit lens [6], varied from 1.3 to 2.2 units/g wet wt. of lens ($n = 10$), and ascorbate concentrations in this animal lens have been reported to range from 0.6 to 1.7 mM [9,24]. Therefore, in addition to the concentration of naphthalenedihydrodiol in the lens, the difference in ratio of DD content to ascorbate concentration in lenses of individual rabbits may be an important reason why the appearance of cataract is so variable in this animal.

The inhibition of DD by ascorbate would suggest an important regulatory system for certain processes in lens other than drug metabolism, but the physiological substrates of the enzyme are little known. Nakayama *et al.* [25] have recently reported that dimeric DDs of pig tissues efficiently reduce methylglyoxal to lactaldehyde at pH 7.5. Rabbit lens DD also showed a relatively low K_m value of 0.3 mM and moderate V_{max} value of 3.2 units/mg for methylglyoxal. Since methylglyoxal has been shown to be synthesized from dihydroxyacetone phosphate, glyceraldehyde 3-phosphate, acetone and threonine in mammals [26], and to act as a regulator of cell growth by inhibiting the proliferation of a wide range of cells, including healthy mammalian cells and tumour cells [27], its metabolizing enzymes are important to control the concentration of the 2-oxoaldehyde in a cell. As glyoxalase I, one of the methylglyoxal-metabolizing enzymes, has been reported to be inhibited by ascorbate [20], the inhibition of DD by ascorbate may be one of the regulatory systems of the action of methylglyoxal. If methylglyoxal would also inhibit the differentiation of lens epithelial cells, DD and ascorbate might act as regulators of lens growth of the rabbit and pig.

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