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Allosteric Inhibition of Human Liver Aldehyde Dehydrogenase by the Isoflavone Prunetin

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ABSTRACT. Isoflavonoid derivatives including prunetin (4′,5-dihydroxy-7-methoxyisoflavone) were shown to be potent inhibitors of human aldehyde dehydrogenases (Keung W-M and Vallee BL, *Proc Natl Acad Sci USA* **90:** 1247–1251, 1993). The inhibition reaction was reinvestigated using recombinantly expressed human aldehyde dehydrogenases. The kinetic analyses showed that prunetin inhibits competitively against both NAD and propionaldehyde with the mitochondrial and cytoplasmic enzymes. The K_i value for the mitochondrial enzyme was much lower than for the cytoplasmic enzyme. A mixed pattern of inhibition was obtained with the mitochondrial enzyme in the presence of Mg^{2+}. Only one mole of prunetin binds per mole of tetrameric mitochondrial enzyme, which remains unaltered in the presence of Mg^{2+}. Prunetin did not displace NADH from the enzyme-NADH complex. Propionaldehyde did not reverse the loss of fluorescence obtained due to enzyme-prunetin complex formation, indicating that prunetin may not be interacting at the substrate site. The esterase activity of the mitochondrial enzyme was also inhibited by prunetin in a competitive manner. The replacement of lysine 192 by glutamine resulted in a mutant with a 20% k_{cat} and a 100-fold increase in the K_m for NAD compared with the native enzyme. However, the K_i value of prunetin against NAD was similar to that observed with the native enzyme. Prunetin, even at a very high concentration, was not an inhibitor of alcohol and malate dehydrogenase. It was concluded that prunetin may act as an allosteric inhibitor of aldehyde dehydrogenase. BIOCHEM PHARMACOL 53;4:471–478, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. aldehyde dehydrogenase; prunetin; isoflavone; allosteric; kinetics

ALDH† exists in several forms and is distributed in different subcellular locations including the mitochondria (ALDH2) and the cytoplasm (ALDH1). It was found that the mitochondrial enzyme is the one largely responsible for the oxidation of acetaldehyde during the metabolism of ethanol [1, 2]. Several irreversible inhibitors of liver ALDH have been described [3, 4]. Disulfiram and cyanamide are perhaps the best known, each being used as a drug to deter people from abusive alcohol consumption. Chloral hydrate is a potent reversible inhibitor (K_i *ca.* 5 μM), but its therapeutic use is limited by the fact that the compound has psychotropic effects as does its metabolite, trichloroethanol [5].

Recently, a new class of *in vitro* inhibitors that are isoflavonoid derivatives have been discovered. Keung and Vallee [6] showed that not only are compounds of this family inhibitors of human ALDH but they also possess selectivity in that they are better inhibitors against the mitochondrial enzyme than against the cytosolic isozyme. Since mitochondria is the subcellular location of acetaldehyde oxidation [1, 7, 8], there was a possibility that these compounds could be of therapeutic value. The compounds are found in a plant (*Radix puerariae*) that was reported to be used in Oriental medicine as a deterrent of abusive alcohol consumption [6]. Some isoflavone components were not inhibitors of ALDH, whereas others were found to be inhibitors of alcohol dehydrogenase [6].

The human cytosol and mitochondrial isozymes share 70% sequence identity, and the K_m for acetaldehyde is 10-fold lower for the mitochondrial enzyme [9, 10]. It was reported that the isoflavones were competitive inhibitors against acetaldehyde but uncompetitive against NAD [6].

Keung and Vallee [6] reported that the derivative with the lowest K_i was daidzin, a compound with an O-glucose attached to carbon 7 of the isoflavone ring. A compound with nearly as low a K_i was prunetin, which has an O-methyl at carbon 7. The authors tried to assess the ability of daidzin to inhibit acetaldehyde metabolism in hamsters but reported that no inhibition was seen, in spite of their report that the compound with its O-glucose moiety was found in liver mitochondria [11]. No *in vivo* data for other derivatives have been reported in the literature.

We undertook a detailed analysis of the inhibitory effects of prunetin on recombinantly expressed human liver ALDHs. Here we report results obtained at pH 7.4 that are

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† *Abbreviations:* ALDH, aldehyde dehydrogenase; ALDH1, cytoplasmic aldehyde dehydrogenase; ALDH2, mitochondrial aldehyde dehydrogenase; DACA, 4-dimethylamino cinnamaldehyde; HAP, *p*-hydroxyacetophenone; and K192Q, lysine is replaced by glutamine in ALDH2 at position 192.

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different from those of Keung and Vallee, who did their assays at pH 9.5 [6]. We found that prunetin inhibited competitively against both NAD and propionaldehyde. To be a competitive inhibitor the compound does not have to actually bind to the substrate binding site but only to the same form of the enzyme that binds them. The enzyme underwent ordered binding with NAD being the leading substrate, hence the inhibition patterns suggest that both the free enzyme and the enzyme-NAD complex could bind the inhibitor. Alternatively, prunetin could bind to a unique site on the enzyme.

MATERIALS AND METHODS

Materials

Propionaldehyde, *p*-nitrophenyl acetate, and DACA were obtained from the Aldrich Chemical Co. (Milwaukee, WI); alcohol dehydrogenase, DEAE-cellulose, NAD, and NADH were from the Sigma Chemical Co. (St. Louis, MO); prunetin was from the Indofine Chemical Co. (Somerville, NJ), and malate dehydrogenase from the Boehringer Mannheim Corp. (Indianapolis, IN). All other reagents and chemicals were of the highest purity grade commercially available.

Enzyme Preparation

The cDNAs for human ALDH1 and ALDH2 enzymes in pT 7-7 plasmid were expressed in *Escherichia coli* BL-21 (DE3) PLysS cells [11, 12]. The lysine 192 mutant of ALDH2 was constructed by site-directed mutagenesis using a method employed earlier in our laboratory [12, 13]. The synthetic oligonucleotide primer used was 5′-CTCAGC-TACCTGCATCACAACCAC-3′, where the lysine codon at the 192 position (underlined) was replaced by glutamine. The mutation was verified by sequencing, using synthetic oligonucleotide primer [11, 14]. After growing the cells containing native and mutant enzyme in 2xYT medium, the cells were lysed using a French pressure cell, and the crude enzyme was isolated after precipitating the DNA with 0.5% protamine sulfate. This crude sample was dialyzed at 4° for 3–4 hr against 10 mM sodium phosphate (pH 7.4) containing 1 mM EDTA and 0.025% 2-mercaptoethanol.

The purification of the ALDH enzymes was carried out with column chromatographic procedures using a DEAE-cellulose and a HAP-affinity column [15]. Fractions containing only ALDH enzyme, as judged by SDS-PAGE, were pooled and stored in 50% glycerol at −20°. Before use, the enzyme was dialyzed against the 0.1 M sodium phosphate (pH 7.4) containing 0.05% dithiothreitol overnight to remove the glycerol, which interfered with the enzyme activity measurements.

ALDH Activity Measurements

The activity of ALDH was measured with an Aminco filter fluorometer in 0.1 M sodium phosphate (pH 7.4) at 25° [16, 17]. The assay for mitochondrial enzyme was carried out in the presence of 0.8 mM NAD and 14 μM propionaldehyde. The activity of the cytoplasmic enzyme was determined similarly using 0.8 mM NAD and 140 μM propionaldehyde. The total reaction volume was 1 mL.

Esterase activity was determined in 0.1 M sodium phosphate (pH 7.4) containing 500 μM *p*-nitrophenyl acetate with a Gilford spectrophotometer [13, 17]. *p*-Nitrophenyl acetate, being insoluble in aqueous buffer, was dissolved in 1% acetone, which did not have any effect on the enzyme activity. This reaction was monitored at 400 nm and was corrected for the small increase in absorption at this wavelength due to the hydrolysis of *p*-nitrophenyl acetate in the absence of enzyme.

Inhibition Kinetics of ALDH

Prunetin was first dissolved in DMSO or acetonitrile and then diluted in 0.1 M sodium phosphate (pH 7.4). Different concentrations of propionaldehyde (<14 μM) and NAD (<1 mM) were used to determine the kinetics of inhibition of native ALDH in the presence of prunetin. To determine the K_i for prunetin with the K192Q mutant, the concentration of NAD was varied from 0.3 to 7 mM. Several concentrations of prunetin (1–6 μM) were employed with variable concentrations of *p*-nitrophenyl acetate to determine the inhibition of esterase activity.

The inhibition of malate and alcohol dehydrogenase by prunetin was determined at subsaturating concentrations of NAD and substrates. DMSO itself was found to be an inhibitor of alcohol dehydrogenase; therefore, prunetin was dissolved in acetonitrile.

Binding of Prunetin to ALDH2

The decrease in fluorescence of the enzyme in the presence of prunetin was monitored with a Hitachi spectrofluorometer (model F-2000). There was some slow decay of fluorescence intensity when only enzyme was kept in the light path. This decrease was subtracted from the decrease in fluorescence in the presence of prunetin. The number of binding sites of prunetin per tetrameric enzyme was calculated using the following equation [18]:

$$\frac{[EL]}{[E_T]} = n - K_d \frac{[EL]}{[E_T][L]}$$

where $[EL]/[E_T]$ is the moles of prunetin bound per mole of the enzyme; n is the number of interacting sites present in the enzyme; K_d is the dissociation constant for prunetin; and $[L]$ is the concentration of the prunetin added to the enzyme solution.

Effect of Prunetin on NADH Binding

Fluorescence emission of free and bound NADH was measured at 450 nm with excitation at 340 nm [13, 16] using

the Hitachi spectrofluorometer (model F-2000). The addition of NADH was corrected for dilution by adding an equal volume of buffer (0.1 M sodium phosphate, pH 7.4). The concentration of the NADH solution prepared was determined spectrophotometrically at 340 nm using an extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}. Increasing concentrations of prunetin were added to the E-NADH complex, and the change in the fluorescence was recorded. A control titration without prunetin was carried out to correct the fluorescence readings due to the dilution of the E-NADH complex.

Effect of Propionaldehyde on Prunetin Binding to ALDH2

Variable concentrations of propionaldehyde (0–60 μM) were added to the enzyme-bound prunetin, and the fluorescence changes were monitored on the Hitachi spectrofluorometer. Equal volumes of buffer were also used in the absence of propionaldehyde to serve as the control reaction.

Elution of ALDH2 from Affinity Columns Using Prunetin

HAP- and AMP-affinity columns were used for the purification of ALDH [13, 15, 16, 19]. A small amount (0.5 mL) of affinity resin was equilibrated with several volumes of 10 mM sodium phosphate (pH 7.4) containing 0.1 mM dithiothreitol, 1 mM EDTA, and 50 mM sodium chloride. The enzyme (200 μL) was loaded on each column and was kept for 30 min before washing with 2 mL of equilibration buffer. Prunetin (10 mM) was used for the elution of the enzyme from the HAP- and AMP-columns. The enzyme was also eluted by HAP (10 mM) and NAD (10 mM) from HAP- and AMP-columns, respectively. The eluted samples were analyzed for the activity as described above.

RESULTS

Expression and Purification of ALDHs

The recombinantly expressed mitochondrial native and K192Q enzymes were purified to homogeneity using methods described previously [11, 12, 17]. The purified fractions were examined after DEAE-cellulose and HAP-affinity chromatography by SDS–PAGE. A single band of protein with a subunit molecular weight of 55,000 was obtained. The fractions containing the protein were also analyzed by western blotting to confirm the presence of the enzyme. The recombinant cytoplasmic isozyme was also expressed and purified following the same procedure as that used for mitochondrial enzyme.

Kinetics of ALDH Inhibition in the Presence of Prunetin

The effect of prunetin on the ALDH1 and ALDH2 enzyme activity was examined. Analyses of the inhibition pattern by Lineweaver-Burk double-reciprocal plots showed that prunetin was a competitive inhibitor against both NAD and propionaldehyde for ALDH1 and ALDH2 enzymes (Fig. 1). Although prunetin appeared to be competitive against NAD, complete recovery of inhibition could not be obtained even at a very high concentration of NAD. The K_i value of prunetin against DACA, a hydrophobic substrate, was also similar to that obtained against propionaldehyde (Table 1). It was found that prunetin was a better inhibitor for the mitochondrial than for the cytoplasmic enzyme, consistent with previous results obtained by Keung and Vallee [6].

Effect of Prunetin on ALDH2 in the Presence of Mg^{2+}

The dehydrogenase activity of mammalian ALDH2 increased by 2-fold in the presence of Mg^{2+} [12, 16, 20, 21]. The inhibition of ALDH2 enzyme by prunetin was investigated in the presence of Mg^{2+}. It was found that a greater inhibition was obtained compared with that observed in the absence of Mg^{2+}. Double-reciprocal plots of the data showed that the kinetic properties of the inhibition reaction were altered such that a mixed pattern of inhibition, and not competitive, was obtained (Fig. 1).

Quantitative Analysis of Prunetin Binding with ALDH2

The quenching of intrinsic fluorescence of ALDH2 in the presence of prunetin was monitored at 350 nm. The fluorescence decreased with increasing concentrations of prunetin, but above 4 μM essentially no further decrease in fluorescence of the enzyme was observed (Fig. 2). The presence of Mg^{2+} led to a greater decrease in the fluorescence of the enzyme-prunetin complex compared with that observed in the absence of Mg^{2+}. It was found that only one mole of prunetin was bound per mole of functional enzyme in both the absence and presence of Mg^{2+} (Fig. 3, A and B). A dissociation constant of about 0.6 μM was calculated from the plots.

Effect of Prunetin on NADH and Propionaldehyde Binding to ALDH2

The binding of NADH to the enzyme was determined by measuring the fluorescence enhancement that occurs due to E-NADH complex formation [13, 17, 22]. It was found that no displacement of NADH takes place upon the addition of prunetin (Fig. 4). Only a small decrease in the fluorescence was observed; this might be due to the formation of a ternary complex (E-NADH-prunetin). If prunetin had displaced NADH from the E-NADH binary complex, then the final fluorescence obtained should have been the same as the fluorescence of the free NADH. This suggests that prunetin might not interact with the nucleotide binding of ALDH2. To elucidate whether or not prunetin binds to a substrate binding site, the fluorescence change of the enzyme-prunetin complex was monitored by the addition of

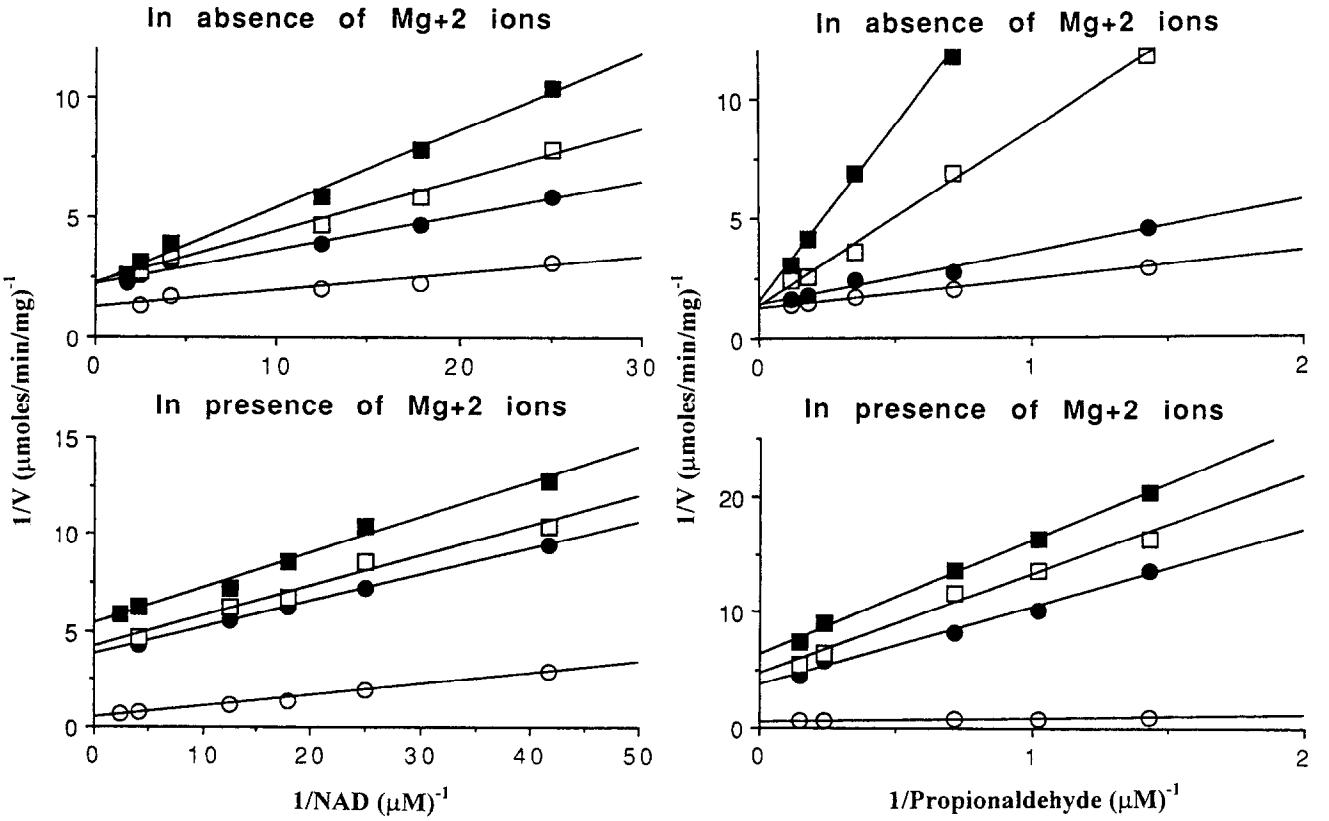

FIG. 1. Kinetic analysis of aldehyde dehydrogenase inhibition by prunetin in the absence and presence of Mg^{2+}. The inhibition of enzyme activity in the presence of 0.0 μM (○), 8.8 μM (●) 17.6 μM (□) and 35.2 μM (■) prunetin was determined. Double-reciprocal plots are shown for variable NAD and propionaldehyde concentrations with fixed concentrations of propionaldehyde (14 μM) and NAD (1 mM), respectively, in the absence and presence of Mg^{2+}. The activity was measured in 0.1 M sodium phosphate (pH 7.4) at 25°. Each assay point was performed at least in triplicate and averaged. The standard deviations were less than ±10%.

propionaldehyde (data not shown). This was based on the fact that the fluorescence of free enzyme did not change when propionaldehyde alone was added. No reversal of fluorescence was found, indicating that prunetin was not interacting at the propionaldehyde binding site. It can be concluded, then, that prunetin does not bind to either the NAD or the propionaldehyde binding regions, but may interact with an independent, presumably allosteric, binding site.

TABLE 1. Kinetic properties of recombinantly expressed human liver mitochondrial, cytoplasmic, and mitochondrial mutant (K192Q) aldehyde dehydrogenases

Kinetic constants	Mitochondrial	Cytoplasmic	K192Q
K_m(NAD)	30	8	3600
K_m(Prop)	0.7	4.6	3.5
K_m(DACA)	0.1	ND*	ND
K_i(NAD)	6.2	127	2.0
K_i(Prop)	0.45	42	ND
K_i(DACA)	0.85	ND	ND

The dehydrogenase activity of aldehyde dehydrogenase was measured in 0.1 M sodium phosphate (pH 7.4) at 25°. Kinetic constants were determined by varying NAD and substrate (Prop, propionaldehyde; DACA, 4-dimethylamino cinnamaldehyde) concentrations in the presence and absence of different concentrations of prunetin. All the constants are expressed in terms of μM.

*ND = not determined.

Elution of ALDH2 from Affinity Columns by Prunetin

HAP has been found to be a competitive inhibitor against propionaldehyde but noncompetitive against NAD with

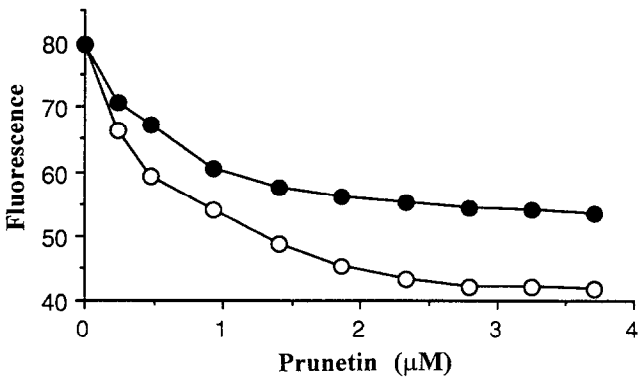

FIG. 2. Effect of prunetin binding on the fluorescence of ALDH2 in the absence and presence of Mg^{2+}. Prunetin (0–4 μM) was added to the enzyme (0.1 μM), and the decrease in fluorescence in the absence (●) and presence (○) of Mg^{2+} was monitored at 350 nm, when excited at 280 nm in 0.1 M sodium phosphate (pH 7.4) at 25°.

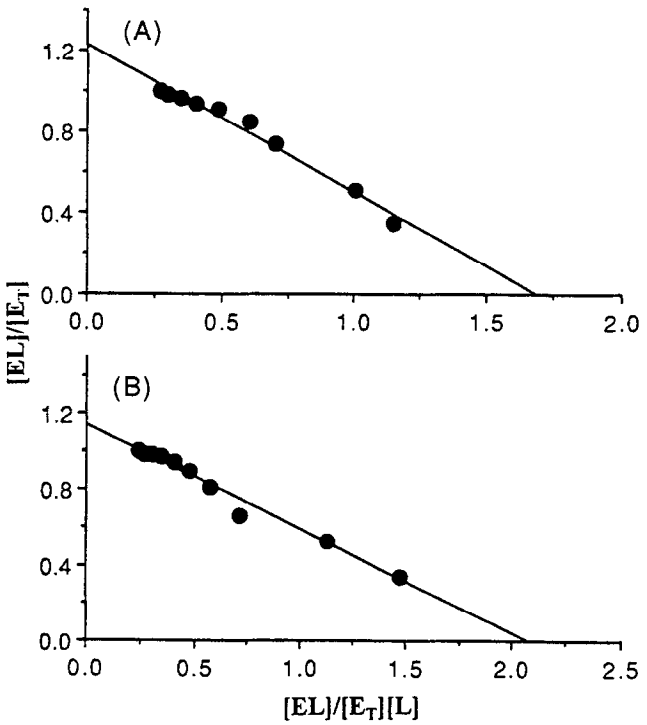

FIG. 3. Stoichiometry of prunetin binding to ALDH2. The number of moles of prunetin bound per mole of tetrameric enzyme in the absence (A) and presence (B) of Mg^{2+} was plotted using the Scatchard equation, as described in Materials and Methods.

beef liver ALDH2 [15]. In the present study, it was found that HAP also inhibits noncompetitively against NAD with human ALDH2 (data not shown). We have demonstrated previously that a HAP-column binds ALDH efficiently [15] and the enzyme can be eluted by HAP itself. In this study, we found that if prunetin was used instead of HAP, the enzyme was eluted from the HAP-column. ALDH also binds to an AMP-column and can be eluted by NAD [13, 19]. It was found that prunetin could also elute the enzyme from the AMP-column. These results confirm that prunetin binds to the free enzyme.

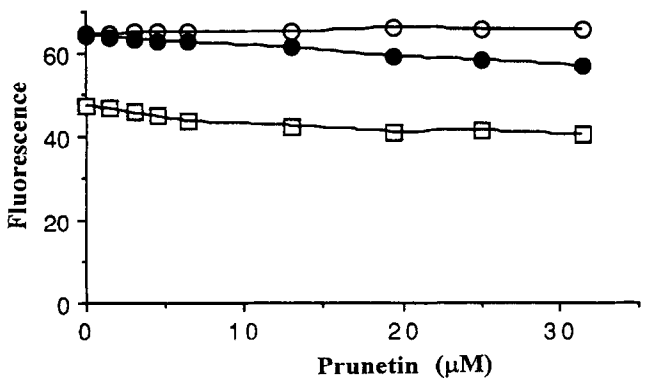

FIG. 4. Effect of prunetin on the binding of NADH to ALDH2. Prunetin was added to the E-NADH complex (●) and free NADH (□) in 0.1 M sodium phosphate (pH 7.4) at 25°. To show that the decrease in the fluorescence in the presence of prunetin was not due to a dilution effect, an equal volume of buffer without prunetin was added to the E-NADH complex (○).

Esterase Activity of ALDH2 in the Presence of Prunetin

ALDH can hydrolyze esters [23–26] as was found for glyceraldehyde 3-phosphate dehydrogenase. The esterase activity was found to be enhanced several-fold in the presence of NAD. Since it was possible to elute the enzyme from the affinity columns, we also determined whether or not prunetin binding to the free enzyme could affect the esterase activity. The kinetic analysis of the esterase inhibition reaction showed that prunetin was a competitive inhibitor against nitrophenyl acetate. Even in the presence of NAD, prunetin inhibited the esterase activity. The inhibition constants obtained are shown in Table 2. Thus, the loss of esterase activity supported the fact that prunetin was capable of interacting with the free enzyme.

Determination of the Inhibition Constant for K192Q Enzyme

Replacement of the conserved lysine at position 192 with glutamine results in more than a 100-fold increase in the K_m for NAD [27]. The mutant allowed us to test whether or not prunetin binds poorly to an enzyme with impaired NAD binding. Several concentrations of prunetin were employed to determine the inhibition constant for the mutant enzyme. It was found that the K_i for prunetin inhibition was still very low, 2 μM (Table 1). This K_i value was similar to what was obtained for the native enzyme form, suggesting that prunetin might not be interacting directly at the NAD binding region.

Effect of Prunetin on Other Dehydrogenases

The effect of prunetin on malate dehydrogenase and alcohol dehydrogenase was also determined (data not shown). No inhibition was found with alcohol and malate dehydrogenase even when the concentration of prunetin was raised

TABLE 2. Esterase activity of human liver recombinant mitochondrial aldehyde dehydrogenase

Kinetic constants	μM
K_m(NPA)	6.8
K(NAD)	2.2
K_i(prunetin)	0.7
K_i(prunetin, NAD)	2.2

Esterase acticity was determined in 0.1 M sodium phosphate (pH 7.4) with nitrophenyl acetate (NPA) as a substrate. The value for K_m(NPA) was obtained with varied concentrations of NPA; K(NAD) value was obtained with varied concentrations of NAD and a fixed concentration of NPA (500 μM); K_i(prunetin) was obtained with varied concentrations of NPA and prunetin; K_i(prunetin, NAD) was obtained with varied concentrations of NAD and prunetin with a fixed concentration of NPA (500 μM).

to 60 μM. A significant loss of ALDH2 activity was obtained at a very low concentration of prunetin (1 μM). Thus, prunetin is not a general dehydrogenase inhibitor and must interact with a unique site in ALDH.

DISCUSSION

Since the discovery of ALDH, several chemical modifiers have been used either to explore the catalytic mechanism or to test them as a drug against abusive alcohol consumption in people. A few drugs given to deter people from abusive drinking are actually covalent inhibitors of the enzyme. The two most common ones are disulfiram and cyanamide. It has been shown that disulfiram is a general sulfhydryl modifying reagent that reacts with many other enzymes [28]. It was discovered that isoflavones, including prunetin, may be used as a good substitute for the drugs that are now in use to prevent alcohol abuse [6]. This was based on the observation that roots of the *Radix puerariae* plant, which contains isoflavonoids, were reported to be used to prevent alcohol abuse in the Chinese population for a long period of time. We report here the in-depth study of prunetin, an isoflavone derivative that was shown by Keung and Vallee [6] to be a potent inhibitor of ALDH.

Kinetic data obtained in the present study with ALDH showed that prunetin appeared to be a competitive inhibitor against both NAD and propionaldehyde. Two models can be proposed to explain how a compound could be a competitive inhibitor against both substrates in a two-substrate enzyme reaction. First, if the enzyme binds substrates in a random order, then the inhibition could be competitive against both substrates if the inhibitor binds to either substrate site. Alternatively, the inhibitor could bind to a unique site, affecting the enzyme, such that it appears to compete against either substrate. No evidence has been presented to show that aldehyde binds to the free ALDH2 when its concentration is near the K_m value. In the esterase reaction, though, substrate binds to the enzyme in the absence of coenzymes [23].

The successful elution of ALDH2 from both HAP- and AMP-affinity columns suggested that prunetin is capable of binding to the free enzyme. The inhibition of the esterase activity further confirmed that prunetin can bind to the free enzyme. Another inhibitor, triiodothyroxine, was found to elute the enzyme from the AMP- but not from the HAP-column.* This inhibitor was competitive against NAD, but noncompetitive against propionaldehyde.

The kinetic studies showed that prunetin inhibition against NAD for ALDH2 enzyme was not recovered completely even at a very high concentration of NAD. This incomplete protection against inhibition might be due to the formation of an E-NAD-prunetin ternary complex. To test whether or not a ternary complex was formed, NADH, rather than NAD, was used since its binding to the enzyme could easily be monitored fluorometrically. The enhanced fluorescence due to the binary E-NADH complex formation showed a small decrease in the presence of prunetin. However, it was not possible to displace NADH completely from the enzyme, suggesting that prunetin does not replace NADH, but forms a ternary complex (E-NADH-prunetin). This could be the reason for the incomplete recovery of enzyme activity even at a very high concentration of NAD, as shown in Fig. 1. The formation of a ternary complex also suggests that both prunetin and NAD may not be interacting at the same site. Another example to support the hypothesis that different sites of NAD and prunetin exist on the enzyme was obtained by examining the inhibition of the K192Q mutant by prunetin. Even though the binding of NAD to K192Q was impaired significantly, the prunetin binding affinity was similar to that of the native enzyme. These results support the notion that prunetin does not interact directly with the NAD binding sites. Furthermore, the fact that there was no reversal of the prunetin-quenched enzyme fluorescence after the addition of a high concentration of propionaldehyde indicates that propionaldehyde does not displace prunetin. Therefore, we conclude that prunetin does not bind at the NAD or the propionaldehyde binding site but may interact with an independent, allosteric, binding site.

It was shown that the rate of the aldehyde dehydrogenase reaction depends on the nature of the substrate used [23]. Recently, Klyosov [29] reported that the K_m for aliphatic substrates decreases as the chain length increases with both ALDH1 and ALDH2. We observed that the binding of prunetin remained essentially unaffected when either propionaldehyde or DACA was used as a substrate in the dehydrogenase reaction. Moreover, a similar K_i value for prunetin was obtained when nitrophenyl acetate was used as the substrate in the esterase reaction. These data are indicative of an unaltered binding property of prunetin, irrespective of the nature of the substrate, which further supports the notion that prunetin does not interact at the substrate binding site.

A stoichiometry of only one mole of prunetin binding per mole of ALDH2 was obtained and not 2 or 4, as could be expected for a tetrameric enzyme that exhibits half-of-the-site reactivity [16, 30]. This precluded prunetin from binding randomly at both the substrate and NAD binding sites. Although a greater decrease in fluorescence of the enzyme-prunetin complex was observed in the presence of Mg^{2+}, the stoichiometry did not change, suggesting the formation of an E-prunetin-Mg^{2+} complex. This was unlike what was found when measuring coenzyme binding, where Mg^{2+} increased the stoichiometry of NAD and NADH from 2 to 4 moles per mole of tetrameric ALDH2 enzyme [16]. Finding that only one prunetin molecule binds to a tetrameric enzyme suggests that it might interact in-between the subunits and not with any one of them.

If one predicts that prunetin binds to an allosteric site, then it is possible that a structural change in the enzyme

* Zhou J and Weiner H, unpublished observations.

may result in differential effects on the inhibition of the enzyme. Several studies have shown that addition of Mg^{2+} increases the ALDH2 enzyme activity by 2-fold [12, 16, 21]. Although the mechanism of the 2-fold increase in the activity is not clear, this has been attributed to structural alterations in the enzyme [16]. The kinetics of inhibition by prunetin in the presence of Mg^{2+} showed a mixed pattern of inhibition, while competitive inhibition was found in the absence of Mg^{2+}. This alteration in the inhibition pattern could be a result of a different interaction of prunetin with the E–Mg^{2+} complex.

Prunetin did not inhibit alcohol dehydrogenase and malate dehydrogenase. This is further proof that prunetin is binding to a unique site in ALDH and not to the NAD binding site present in all dehydrogenases. Finding that prunetin may be an allosteric modulator does not prove that the enzyme *in vivo* is subjected to allosteric inhibition or activation. It does open the question as to whether or not there is some *in vivo* regulation. The subunit molecular weight of ALDH is much larger than that of most NAD-dependent dehydrogenases: 55 kDa compared with 30–40 kDa. Isoflavones do not resemble many cellular components; thus, it is not possible to predict what could be the natural modulator, if it exists. The selective inhibition of ALDH by prunetin might be an advantage for this compound to be used for pharmaceutical purposes. However, Keung *et al.* [11] recently reported that daidzin, another isoflavone, might not be an *in vivo* inhibitor of ALDH2 in the Syrian golden hamster.

Drugs that are uncompetitive inhibitors of an enzyme have an advantage over those that mimic the active site and are competitive with substrate. For example, chloral hydrate is a competitive inhibitor of ALDH. It would slow down the rate of an aldehyde oxidation only when the aldehyde was present at low concentrations. As the aldehyde concentration increased, it would displace the competitive inhibitor from the enzyme. A non- or uncompetitive inhibitor such as prunetin would function independently of the concentration of substrate. In conclusion, prunetin, having properties of an allosteric inhibitor, could prove to be of pharmaceutical importance not only as an Antabuse-like compound, but also may be useful in slowing down the metabolism of the cytotoxic aldehyde intermediate formed from cyclophosphamides in neoplastic cells [31].

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