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DERIVATIVES OF CINNAMIC ACID INTERACT WITH THE NUCLEOTIDE BINDING SITE OF MITOCHONDRIAL ALDEHYDE DEHYDROGENASE

EFFECTS ON THE DEHYDROGENASE REACTION AND STIMULATION OF ESTERASE ACTIVITY BY NUCLEOTIDES

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Abstract—A wide variety of cinnamic acid derivatives are inhibitors of the low K_m mitochondrial aldehyde dehydrogenase. Two of the most potent inhibitors are α-cyano-3,4-dihydroxythiocinnamamide (K_i 0.6 μM) and α-cyano-3,4,5-trihydroxycinnamonitrile (K_i 2.6 μM). With propionaldehyde as substrate the inhibition by these compounds was competitive with respect to NAD^+. α-Fluorocinnamate was a much less effective inhibitor of the enzyme, with mixed behaviour towards NAD^+, but with a major competitive component. These cinnamic acid derivatives were ineffective as inhibitors of the aldehyde dehydrogenase-catalysed hydrolysis of *p*-nitrophenyl acetate, but inhibited the ability of NAD^+ and NADH to activate this activity. Inhibition of the stimulation of esterase activity was competitive with respect to NAD^+ and NADH, and the derived K_i values were the same as for inhibition of dehydrogenase activity. NAD^+, but not acetaldehyde, could elute the low K_m aldehyde dehydrogenase from α-cyanocinnamate-Sepharose, to which the enzyme binds specifically (Poole RC and Halestrap AP, *Biochem J* **259**: 105–110, 1989). The cinnamic acid derivatives have little effect on lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase or a high K_m aldehyde dehydrogenase present in rat liver mitochondria. It is concluded that some cinnamic acid derivatives are potent inhibitors of the low K_m aldehyde dehydrogenase, by competing with NAD^+/NADH for binding to the enzyme. They are much less effective as inhibitors of other NAD^+-dependent dehydrogenases.

Aldehyde dehydrogenase (EC 1.2.1.3) catalyses the oxidation of ethanol-derived acetaldehyde to acetate in the liver. There are a large number of isoenzymes with different subcellular locations, specificity and K_m values for the aldehyde substrates [1–3]. Most of the isoenzymes have K_m values for acetaldehyde in the millimolar range, and are believed to be involved in the metabolism of other biologically important aldehydes rather than playing a major role in alcohol metabolism [3, 4]. However, mitochondria contain a low K_m isoenzyme (ALDH2; K_m for acetaldehyde approx. 1 μM) which is thought to be the major isoenzyme involved in ethanol metabolism [5]. This isoenzyme has been purified from a number of sources [6–10], and the complete amino acid sequence of both the mature enzyme and its precursor deduced from the cDNA sequence [11–13]. A mutation resulting in a glutamate to lysine substitution at position 487 causes expression of an inactive form of the low K_m mitochondrial aldehyde dehydrogenase in approx. 50% of orientals, and increased susceptibility to alcohol intoxication [14–16].

We have shown recently that the low K_m aldehyde dehydrogenase (ALDH2) from rat liver mitochondria could be purified by affinity chromatography on α-cyanocinnamate-Sepharose [9]; high K_m isoenzymes did not bind to this affinity matrix. An explanation for this finding was provided when α-cyanocinnamate, α-fluorocinnamate and some derivatives of α-cyanocinnamide were shown to be inhibitors of the low K_m mitochondrial isoenzyme [9, 17]. Inhibitors of aldehyde dehydrogenase are used clinically as alcohol aversive drugs. These include disulphiram ("antabuse") and cyanamide. However, in both cases the mechanism by which these compounds inhibit the low K_m mitochondrial aldehyde dehydrogenase is unclear [18–20]. In view of the inhibition observed with cinnamate derivatives, and the potential use of isoenzyme-specific reversible inhibitors of aldehyde dehydrogenase as alcohol-aversive drugs, further investigation of the interaction of these compounds with the enzyme was warranted. In this paper we present the results of a more detailed investigation of the mechanism by which cinnamic acid derivatives inhibit aldehyde dehydrogenase. Our data indicate that derivatives of cinnamic acid act as inhibitory analogues of NAD^+/NAD for the low K_m mitochondrial aldehyde dehydrogenase. They are much less effective as inhibitors of other NAD^+-linked dehydrogenases.

MATERIALS AND METHODS

Materials

All chemicals and biochemicals were obtained from the sources given previously [9], with the following additions. α-Cyano-3,4-dihydroxythiocin-

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namamide (RG50864), α-cyano-3,4,5-trihydroxycinnamonitrile, α-cyano-3-ethoxy-4-hydroxy-5-phenylthiomethyl-cinnamamide (ST638), α-cyano-4-hydroxy-3,5-diisopropylcinnamamide (ST271) and 3-(3,4-dimethoxyphenyl)-2-(4-pyridyl)-propenitrile (RG13022), synthesized using published procedures [21–23], were generously provided by Dr A. T. Hudson of The Wellcome Research Laboratories (Beckenham, U.K.). Propionaldehyde was obtained from the Sigma Chemical Co. (Poole, U.K.). α-Fluorocinnamate was obtained from the Aldrich Chemical Co. (Gillingham, U.K.).

Methods

Purification of aldehyde dehydrogenase. α-Cyanocinnamate-Sepharose CL-4B was prepared and used to purify the mitochondrial low K_m aldehyde dehydrogenase from Wistar rats exactly as described previously [9]. A 20 mL column of α-cyanocinnamate-Sepharose was used routinely for these preparations, and could bind approx. 1.5 mg of the low K_m enzyme. The purified enzyme was dialysed against 50 mM NaCl/20 mM 4-morpholinepropanesulphonic acid (Mops)/2 mM benzamidine/1 mM EDTA/0.1 mM dithiothreitol, adjusted to pH 7.4 with NaOH, to remove α-cyano-4-hydroxycinnamate, and stored at −20° with the addition of 30% (v/v) glycerol.

An α-cyano-4-hydroxycinnamate-Sepharose column, prepared as described by Bolli *et al.* [24], was used to purify the high K_m mitochondrial aldehyde dehydrogenase. With this matrix, α-cyano-4-hydroxycinnamate is coupled to a long spacer arm at the 3-position on the phenyl ring, leaving the phenolic hydroxyl group unchanged. This column binds both isoenzymes of aldehyde dehydrogenase, and a number of other contaminants [25]. Thus, to enrich the high K_m isoenzyme, the flow-through fractions from α-cyanocinnamate-Sepharose (devoid of low K_m activity) were applied to the α-cyano-4-hydroxycinnamate-Sepharose column. Chromatography was performed in buffer containing 50 mM NaCl/20 mM Mops/2 mM benzamidine/1 mM EDTA/0.1 mM dithiothreitol, adjusted to pH 7.4 with NaOH, and the high K_m aldehyde dehydrogenase eluted by the addition of 2 mM α-cyano-4-hydroxycinnamate. The high K_m aldehyde dehydrogenase comprised approx. 30% of the protein in the final preparation.

Enzyme assay: dehydrogenase and esterase activity. Assays were performed at pH values of 7.4 and 9.0, at a temperature of 30°. The buffers were 100 mM NaCl/20 mM Mops/0.5 mM EDTA, pH 7.4, or 100 mM sodium pyrophosphate, pH 9.0.

For assay of the dehydrogenase activity, initial rates of NAD^+ reduction were determined spectrophotometrically at 340 nm in a Pye-Unicam SP8-100 split-beam spectrophotometer, with acetaldehyde, propionaldehyde or D/L-glyceraldehyde as substrates. D/L-Glyceraldehyde was prepared daily and incubated for 1–2 hr at 37° before use. Such racemic mixtures are less prone to dimerization than the optically pure forms. When glyceraldehyde oxidation was measured at pH 9.0 the change in absorbance was read against a reference cuvette, containing all additions but no enzyme, since a significant rate of increase in A_{340} was observed in the absence of aldehyde dehydrogenase under these conditions (see below). Inhibition by all cinnamic acid derivatives used in kinetic studies was found to be freely reversible. For this purpose gel filtration was used to remove inhibitors from samples that had previously been assayed for aldehyde dehydrogenase activity, and then the eluted enzyme was re-assayed under the same conditions.

Esterase activity of the low K_m aldehyde dehydrogenase was determined at pH 7.4 using *p*-nitrophenyl acetate as substrate. *p*-Nitrophenyl acetate was added from stock solutions in acetone. The release of *p*-nitrophenol was monitored at 400 nm, using an absorption coefficient corrected for pH; $\varepsilon(\text{mM}) = 12.5$ at pH 7.4 [26]. A saturating (100 μM) concentration of *p*-nitrophenyl acetate was used in all experiments. When effects of inhibitors on the ability of NAD^+ and NADH to activate the esterase activity were investigated, buffer containing aldehyde dehydrogenase, *p*-nitrophenyl acetate ± inhibitors was present in both reference and sample cuvettes, and various concentrations of NAD^+/NADH in the sample cuvette of the split beam spectrophotometer. This allowed direct measurement of the activation of esterase activity by nucleotides.

Analytical procedures. Protein was assayed by the dye-binding method of Bradford [27], using bovine serum albumin as standard. The purity of enzyme preparations was judged using SDS–PAGE as described by Laemmli [28].

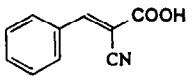
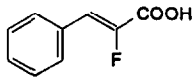
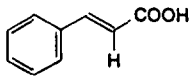
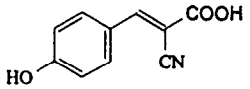
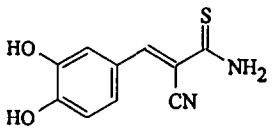
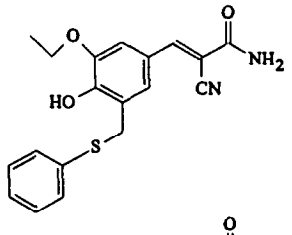
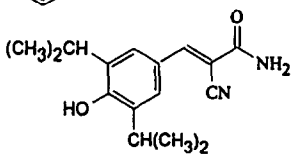
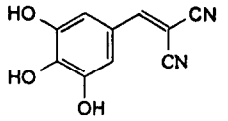
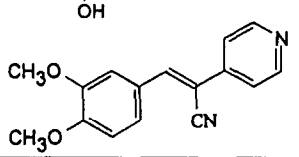
Analysis of kinetic data. Kinetic data were fitted to the equations for mixed, competitive, non-competitive, and uncompetitive inhibition by non-linear least-squares regression analysis. To assess the model of inhibition which the data fitted best, F values were calculated for comparisons of competitive, non-competitive and uncompetitive with mixed inhibition. The more simple model was accepted if the fit was statistically as good (at a 5% probability level) as that to the mixed model.

RESULTS AND DISCUSSION

Inhibition by cinnamic acid derivatives: structure–function relationships

Before studying the kinetics of inhibition of aldehyde dehydrogenase by cinnamic acid derivatives in detail, we investigated the ability of a series of such compounds to inhibit the enzyme. Preliminary studies suggested that some cinnamamide derivatives, originally developed as tyrosine kinase inhibitors [29, 30], are effective inhibitors of mitochondrial aldehyde dehydrogenase [17]. In Table 1 the results of experiments with a range of such compounds are summarized. D/L-Glyceraldehyde was used as substrate in these experiments since high concentrations of NAD^+ and D/L-glyceraldehyde could be used without compromising inhibitory potency (see below), and this allowed measurements to be made with high initial rates. Results are given as apparent K_i values under these conditions, which were derived by fitting the data to an equation which assumes noncompetitive inhibition, as shown in Table 1. A number of features are apparent from these data. Firstly, acids, amide and thioamide

Table 1. Inhibition of the low K_m mitochondrial aldehyde dehydrogenase by derivatives of cinnamic acid

Inhibitor	Structure	$K_{i(app)}$ (μM)
Cinnamic acid		860 ± 58
α-Cyanocinnamate		54 ± 3.2
α-Fluorocinnamate		78 ± 9.3
α-Cyano-4-hydroxycinnamate		>50*
α-Cyano-3,4-dihydroxythiocinnamamide (RG50864)		2.83 ± 0.61
α-Cyano-3-ethoxy-4-hydroxy-5-phenylthiomethylcinnamamide (ST638)		1.52 ± 0.072
α-Cyano-4-hydroxy-3,5-diisopropylcinnamamide (ST271)		>50*
α-Cyano-3,4,5-trihydroxycinnamonitrile		5.68 ± 1.02
3-(3,4-Dimethoxyphenyl)-2-(4-pyridyl)-propenitrile (RG13022)		>50*

* High A_{340} prevented accurate determination of K_i.

Activity of purified low K_m aldehyde dehydrogenase was measured at pH 7.4 in the presence of 0.5 mM NAD^+ and 1 mM D/L-glyceraldehyde. Rates were measured with at least six concentrations of inhibitor and were fitted to the following equation by non-linear least-squares regression analysis to give apparent K_i (±SE) for inhibition:

$$V = V_0/(1 + I/K_{i(app)}),$$

where V is the observed rate and V_0 is the rate in the absence of inhibitor.

derivatives of the basic phenylpropene unit were all inhibitors, although apparent K_i values span a 500-fold range. Cinnamic acid was a poor inhibitor, but substitution with an electron withdrawing group, as in α-cyanocinnamate and α-fluorocinnamate, resulted in a 10-fold decrease in $K_{i(app)}$. However, if the carboxyl group of α-cyanocinnamate derivatives was replaced by an amide, thioamide, or an additional nitrile, then some of the resultant compounds were considerably more potent as inhibitors of the enzyme. Whilst the most potent inhibitors had one or more phenolic hydroxyl groups, it is unclear whether this is an important determinant of inhibitory potency, since α-cyano-4-hydroxycinnamate was no more effective than α-cyanocinnamate. Furthermore, it is difficult to explain the difference in apparent K_i between compounds α-cyano-3-ethoxy-4-hydroxy-5-phenylthiomethyl-

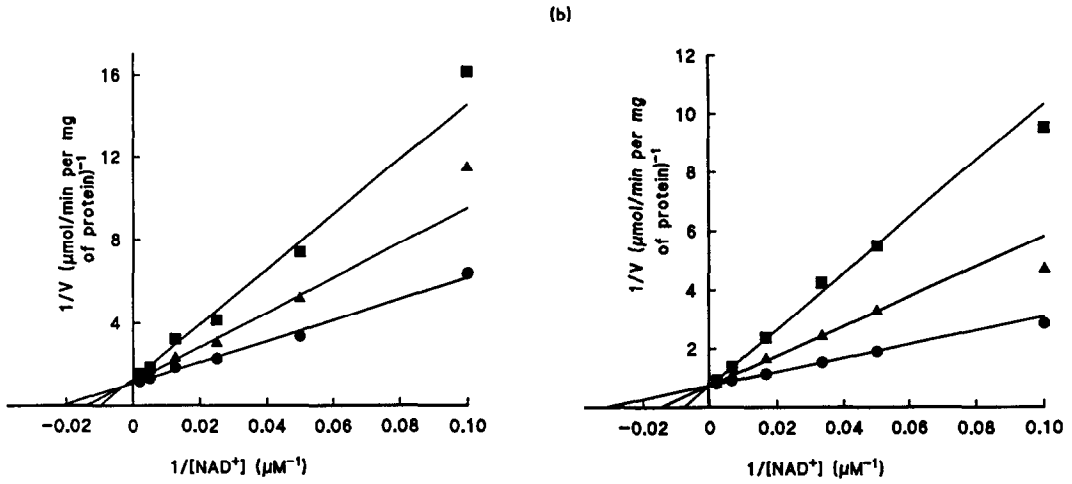


Fig. 1. Kinetics of inhibition of mitochondrial aldehyde dehydrogenase by cinnamic acid derivatives. Initial rates of oxidation of 100 μM propionaldehyde by purified low K_m mitochondrial aldehyde dehydrogenase were measured as described in Materials and Methods. Incubations were performed in the absence of inhibitor (●) or the presence of (a) 100 μM (▲) and 250 μM (■) α-fluorocinnamate; (b) 3 μM (▲) and 8 μM (■) α-cyano-3,4,5-trihydroxycinnamonitrile. The data are presented as Lineweaver–Burk plots; the lines drawn through the data are derived from the fit given by non-linear least-square regression analysis using the equations for mixed inhibition (a) and competitive inhibition (b). Further details are given in Materials and Methods and Table 2.

cinnamamide and α-cyano-4-hydroxy-3,5-diisopropylcinnamamide. On the basis of these results we selected an acid (α-fluorocinnamate), a thioamide (α-cyano-3,4-dihydroxythiocinnamamide) and a compound with a double nitrile substitution (α-cyano-3,4,5-trihydroxycinnamonitrile) for more detailed study.

Kinetics of inhibition of aldehyde dehydrogenase by α-fluorocinnamate and related compounds

In order to investigate the mechanism by which α-cyanocinnamate and related compounds inhibit aldehyde dehydrogenase, the steady-state kinetics of inhibition were investigated in greater detail. In these studies we used propionaldehyde as the substrate, since the kinetic mechanism has been well defined for this substrate. For oxidation of propionaldehyde the enzyme has a compulsory binding order, with NAD^+ as the leading substrate [4, 31]. A step associated with NADH dissociation from the enzyme is believed to be rate limiting, at least for the cytoplasmic enzyme [32, 33]. α-Fluorocinnamate was used since, unlike α-cyanocinnamate, it is unreactive with thiol groups [34]. Such an interaction could complicate kinetic analysis of the inhibition, since aldehyde dehydrogenases are readily inhibited by thiol reagents [8, 35–37]. Experiments were performed at both the physio-

Table 2. Kinetic parameters for inhibition, with respect to NAD^+, of propionaldehyde oxidation by the low K_m aldehyde dehydrogenase

Inhibitor	pH	K_{i1} (μM)	K_{i2} (μM)	Type of inhibition
α-Fluorocinnamate	7.4	146 ± 45	830 ± 154	Mixed-competitive
		265 ± 44	1489 ± 224	Mixed-competitive
α-Fluorocinnamate	9.0	128 ± 65	962 ± 489	Mixed-competitive
		157 ± 31	1263 ± 457	Mixed-competitive
α-Cyano-3,4-dihydroxythiocinnamamide	9.0	0.67 ± 0.049	56 ± 38	Competitive (K_i 0.63 ± 0.037 μM)
α-Cyano-3,4,5-trihydroxycinnamonitrile	9.0	2.44 ± 0.25	>10,000	Competitive (K_i 2.62 ± 0.19 μM)

Measurement of dehydrogenase activity, and subsequent analysis of kinetic data were performed as described in Materials and Methods. The fixed concentration of propionaldehyde was 0.1 mM. The competitive (K_{i1}) and uncompetitive (K_{i2}) inhibition constants (±SE) were derived for individual experiments from a fit to the equation for mixed inhibition. Where the fit was statistically as good using a more simple model (see Materials and Methods), the single K_i value for this model is indicated in parentheses.

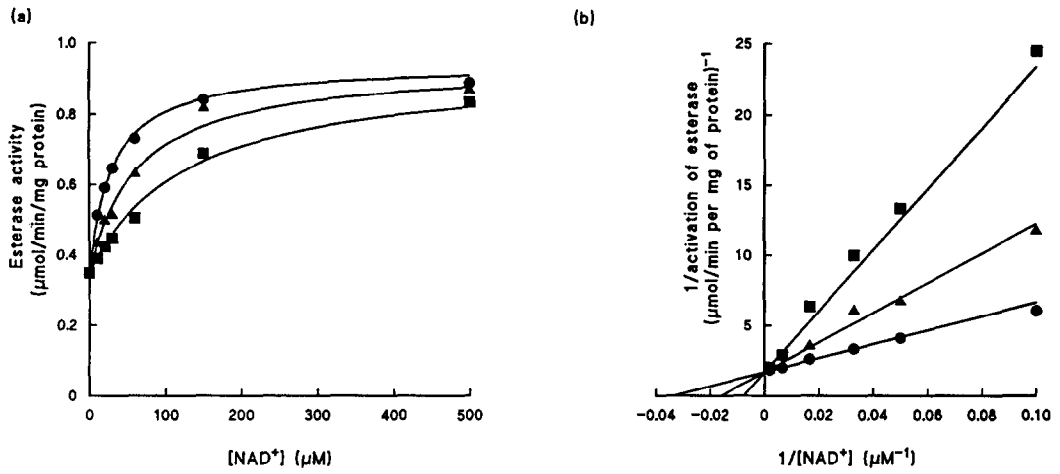


Fig. 2. α-Cyano-3,4,5-trihydroxycinnamonitrile inhibits stimulation, by NAD^+, of aldehyde dehydrogenase-catalysed hydrolysis of *p*-nitrophenyl acetate. Initial rates of hydrolysis of *p*-nitrophenyl acetate catalysed by purified low K_m mitochondrial aldehyde dehydrogenase were measured as described in Materials and Methods. No spontaneous (non-enzymic) hydrolysis of the ester could be detected within the time scale of these assays. In (a) the rates of ester hydrolysis are shown in the absence of NAD^+, and upon increasing the concentration of this nucleotide in the absence (●) and presence of 2 μM (▲) and 6 μM (■) α-cyano-3,4,5-trihydroxycinnamonitrile. Since α-cyano-3,4,5-trihydroxycinnamonitrile had no effect on ester hydrolysis in the absence of NAD^+, the stimulation of ester hydrolysis by NAD^+ was measured directly in a split beam spectrophotometer with both cells containing enzyme, substrate and inhibitor as indicated, but with only the sample cuvette containing NAD^+ at the concentrations shown. In panel (a) this direct measurement of stimulation was added to the rate in the absence of NAD^+ to give the total rate of ester hydrolysis, whilst in (b) a Lineweaver–Burk representation of the stimulation above the control rate is shown. The lines drawn through the data in both panels represent the best fit of the data to a model in which α-cyano-3,4,5-trihydroxycinnamonitrile is a competitive inhibitor of the NAD^+ stimulation of esterase activity. The derived kinetic parameters for this and other experiments are given in Table 3.

logical pH of 7.4 and at pH 9.0, which is frequently used for the assay of the enzyme, because of its alkaline pH optimum [7]. The results of typical experiments with cinnamic acid derivatives are shown in Fig. 1, and the derived kinetic parameters for inhibition by α-fluorocinnamate, α-cyano-3,4-dihydroxythiocinnamamide and α-cyano-3,4,5-trihydroxycinnamonitrile are summarized in Table 2.

Inhibition by α-fluorocinnamate was mixed with respect to NAD^+. This mixed inhibition, however, did contain a major competitive component. The same pattern of inhibition and similar K_i values were obtained at both pH 7.4 and pH 9.0 with this substrate. Using propionaldehyde as substrate, α-cyano-3,4-dihydroxythiocinnamamide and α-cyano-3,4,5-trihydroxycinnamonitrile were found to be purely competitive inhibitors with respect to NAD^+. The K_i values derived for inhibition by the latter compounds were also much lower (50–200-fold) than for α-fluorocinnamate.

Effect of α-fluorocinnamate and related compounds on the esterase activity of aldehyde dehydrogenase

Aldehyde dehydrogenases, in addition to their ability to oxidize aldehydes, can catalyse hydrolysis of esters such as *p*-nitrophenyl acetate [38–40]. There is good evidence for the formation of the same covalent intermediate (at cysteine-302) during both aldehyde oxidation and ester hydrolysis [41], although the "active sites" for these two activities may not be strictly identical. In contrast with the dehydrogenase activity, the esterase activity is independent of nucleotides, but both NAD^+ and NADH are capable of stimulating the enzyme-catalysed hydrolysis of *p*-nitrophenyl acetate [38–40]. Thus, if derivatives of cinnamic acid exert their inhibitory effects by competing with nucleotides, they might be expected either to mimic the effects of NAD^+/NADH on the esterase activity, or to inhibit the stimulation of *p*-nitrophenyl acetate hydrolysis caused by these nucleotides. In contrast with dehydrogenase activity, α-fluorocinnamate, α-cyano-3,4-dihydroxythiocinnamamide and α-cyano-3,4,5-trihydroxycinnamonitrile did not cause inhibition of the hydrolysis of *p*-nitrophenol acetate catalysed by the low K_m aldehyde dehydrogenase. In fact a very small stimulation was sometimes observed with α-fluorocinnamate and α-cyano-3,4-dihydroxythiocinnamamide. However, these compounds did inhibit NAD^+-dependent activation of esterase activity. Typical results of such an experiment are shown in Fig. 2, and the derived kinetic parameters for the different inhibitors are summarized in Table 3. The K_a value for NAD^+ (29.3 ± 0.26 μM; mean ± SEM for three experiments) was similar to the K_m for NAD^+ (31.3 ± 5.8 μM; mean ± SEM for the four experiments in Table 2 with 100 μM propionaldehyde as

Table 3. Kinetic parameters for inhibition, by cinnamic acid derivatives, of the effects of NAD^+ and NADH on the esterase activity of mitochondrial aldehyde dehydrogenase

Nucleotide	Inhibitor	$K_{a(\text{nucleotide})}$ (μM)	K_{i1} (μM)	K_{i2} (μM)	Type of inhibition
NAD^+	α-Fluorocinnamate	28.8 ± 2.54	143 ± 21.6	7790 ± 10,270	Competitive (K_i 134 ± 16 μM)
NADH	α-Fluorocinnamate	1.90 ± 0.14	179 ± 28	1814 ± 360	Mixed-competitive
NAD^+	α-Cyano-3,4-dihydroxythiocinnamamide	29.4 ± 2.33	0.83 ± 0.15	343 ± 3726	Competitive (K_i 0.83 ± 0.11 μM)
NAD^+	α-Cyano-3,4,5- trihydroxycinnamonitrile	29.7 ± 2.93	1.79 ± 0.25	>10,000	Competitive (K_i 1.79 ± 0.24 μM)

The stimulation of esterase activity of aldehyde dehydrogenase by nucleotides was measured as described in Materials and Methods and the legend to Fig. 2. Derived kinetic parameters for inhibition of the effects of nucleotides on this activity are given ± SE for the fit to the equation for mixed inhibition, and for the fit to competitive inhibition where indicated (as in Table 2).

substrate at pH 9.0). Furthermore, the data obtained for the esterase activity indicate that the inhibition is competitive with NAD^+. α-Fluorocinnamate also inhibited activation of the esterase by NADH, again in a manner that was largely competitive. Inspection of Table 3, and comparison with the parameters for inhibition of the dehydrogenase activity (Table 2), reveal that the K_i values are essentially the same.

Elution of the low K_m *aldehyde dehydrogenase from* α*-cyanocinnamate-Sepharose*

In view of the proposed interaction of derivatives of cinnamic acid with the nucleotide binding site on aldehyde dehydrogenase, it was of interest to determine whether substrates (NAD^+ or acetaldehyde) could elute the enzyme when specifically bound to α-cyanocinnamate-Sepharose. In Fig. 3a we show that acetaldehyde (100 μM) caused little elution of aldehyde dehydrogenase from the column, but that subsequent addition of NAD^+ was capable of doing so. α-Cyano-4-hydroxycinnamate, added after NAD^+, caused no further elution. Elution by NAD^+ was as effective in the absence of acetaldehyde (Fig. 3b). These observations provide further evidence that the inhibitors bind at or near the nucleotide binding site on the enzyme.

Inhibition of aldehyde dehydrogenase with glyceraldehyde as substrate

The results described above imply that derivatives of cinnamic acid interact with the nucleotide binding site on aldehyde dehydrogenase. However, it was also of interest to determine the kinetics of inhibition with respect to the aldehyde substrate. Propionaldehyde, like most aldehyde substrates, has a K_m close to 1 μM [4, 6, 7, 10], which makes it difficult to determine accurately inhibition kinetics with respect to this substrate. However, we found that in the presence of 0.5 mM NAD^+, 30 μM α-cyano-3,4-dihydroxythiocinnamamide caused approx. 70% inhibition at both 10 and 100 μM propionaldehyde. No significant substrate activation over this range of propionaldehyde concentrations was observed. For more detailed studies of inhibition kinetics with respect to the aldehyde substrate, we used D/L-glyceraldehyde as substrate [9, 38] (Table 4). Whilst the V_{max} with D/L-glyceraldehyde as substrate is 2–3-fold higher than for acetaldehyde and propionaldehyde, k_{cat}/K_m is approx. 100-fold lower than for these substrates. There are few detailed studies of the reaction mechanism in the presence of this atypical substrate, and some difference in the results obtained [38, 42]. Sidhu and Blair [42] have presented evidence that glyceraldehyde can bind to the free enzyme and that random binding of nucleotide and aldehyde can operate for oxidation of this substrate. At pH 9.0 inhibition by α-fluorocinnamate with respect to D/L-glyceraldehyde had an uncompetitive component (at 0.5 mM NAD^+). This is similar to the inhibition by α-cyanocinnamate, but in this case the inhibition appeared purely uncompetitive under these conditions [9]. Inhibition of glyceraldehyde oxidation at pH 9.5 by the NAD^+ analogues adenine and adenosine is also uncompetitive with respect to the aldehyde [42]. However, when we used NAD^+ at a

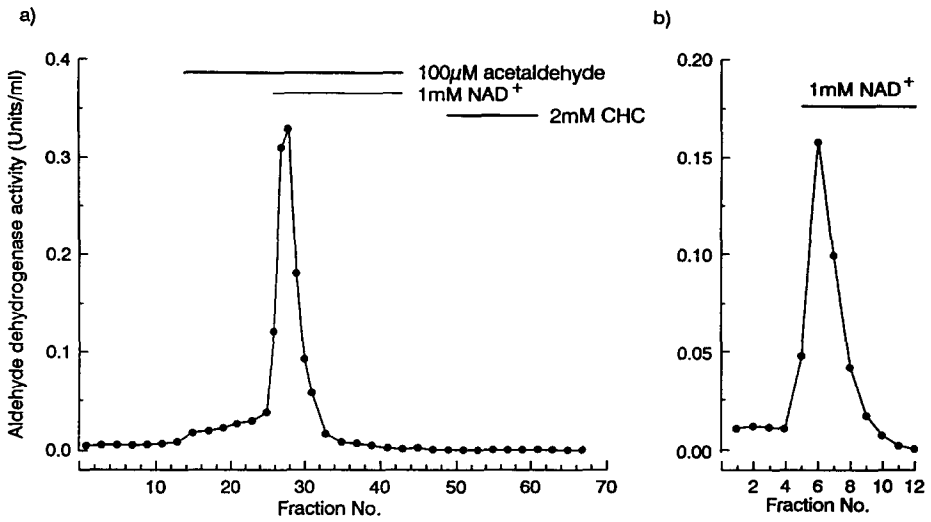


Fig. 3. Elution of the low K_m mitochondrial aldehyde dehydrogenase from α-cyanocinnamate-Sepharose by NAD^+. In (a), an 18 mL column of α-cyanocinnamate-Sepharose was loaded with approx. 120 mg of rat liver mitochondrial matrix protein and washed free of non-bound protein. Fractions of 2 mL were then collected during elution with 100 μM acetaldehyde, 1 mM NAD^+ and 2 mM α-cyano-4-hydroxycinnamate (CHC). These compounds were present in the eluted fractions as indicated by the solid lines in the top part of the figure. The activity of the low K_m aldehyde dehydrogenase in these fractions is shown, where 1 unit represents the amount of enzyme that catalyses the reduction of 1 μmol NAD^+/min at 30°, at pH 9.0 in the presence of 100 μM acetaldehyde and 1 mM NAD^+. A similar experiment is shown in (b), in this case eluting with 1 mM NAD^+ in the absence of acetaldehyde. In this experiment an α-cyanocinnamate-Sepharose column with a lower binding capacity for aldehyde dehydrogenase was used than for the experiment shown in panel (a).

higher fixed concentration of 2 mM, the inhibition became essentially noncompetitive with respect to glyceraldehyde, the same pattern that was observed at pH 7.4 (Table 4). Under no circumstances was there any evidence for competition between α-fluorocinnamate and this aldehyde substrate.

The kinetics of inhibition with respect to NAD^+, using D/L-glyceraldehyde as substrate, were surprising considering the substantial evidence for an interaction at the nucleotide binding site. At pH 9.0 inhibition by α-fluorocinnamate was essentially noncompetitive whilst at pH 7.4 the inhibition was mixed with a major uncompetitive component. Thus, there is considerable discrepancy between the kinetics of inhibition with respect to NAD^+ in the presence of propionaldehyde and D/L-glyceraldehyde. All of the other evidence presented here, using three independent approaches, points to binding of cinnamic acid derivatives within the nucleotide binding site. It is likely that the data with glyceraldehyde reflect important differences between oxidation of this and the better characterized substrates, propionaldehyde and acetaldehyde. The absence of competitive interaction between α-fluorocinnamate and NAD^+ with glyceraldehyde as substrate remains difficult to explain. One factor which may contribute to these observations would be the non-enzymic reaction between NAD^+ and D/L-glyceraldehyde to produce adducts, particularly at high pH. These adducts have spectra similar to that of NADH [43]. With glyceraldehyde as substrate, at pH 9.0 but not pH 7.4, a non-enzymic rate of increase in A_{340} was observed (see Materials and Methods), which presumably was a reflection of the formation of such adducts. The glyceraldehyde–NAD^+ adduct might then interact with the enzyme and/or inhibition and result in complexities in the kinetics of inhibition at the higher pH.

Effects of cinnamic acid derivatives on the high K_m *mitochondrial aldehyde dehydrogenase and other dehydrogenases*

It was found that an alternative α-cyano-4-hydroxycinnamate-Sepharose column with a long spacer arm [24] also bound the low K_m aldehyde dehydrogenase. Some other contaminants were present, but the most notable feature was the presence of high K_m aldehyde dehydrogenase activity (approx. 50% of total aldehyde dehydrogenase activity) in the eluate. This observation suggested that cyanocinnamate derivatives may inhibit the high K_m enzyme, and that the isoenzyme-specificity of the affinity purification on α-cyanocinnamate-Sepharose was due to steric difficulties in the binding of the high K_m isoenzyme to the matrix.

To investigate this possibility further, inhibition of the high K_m mitochondrial aldehyde dehydrogenase by cyanocinnamate derivatives was investigated. The enzyme was partially purified by taking the flow-through fractions from the α-cyanocinnamate-Sepharose column and applying those to an α-cyano-4-hydroxycinnamate-Sepharose column. The dialysed α-cyano-4-hydroxycinnamate eluate was used in these experiments. At pH 9.0, a

Table 4. Kinetic parameters for inhibition of the low K_m aldehyde dehydrogenase catalysed oxidation of D/L-glyceraldehyde by derivatives of cinnamic acid

Inhibitor	Varied substrate	Other substrate	pH	K_{i1} (μM)	K_{i2} (μM)	Type of inhibition
α-Fluorocinnamate	NAD^+	D/L-Glyceraldehyde	7.4	335 ± 247	45 ± 4.3	Mixed-uncompetitive
				159 ± 60	71 ± 9.0	Mixed-uncompetitive
α-Fluorocinnamate	NAD^+	D/L-Glyceraldehyde	9.0	137 ± 104	88 ± 18	
				107 ± 31	212 ± 32	Noncompetitive (K_i 177 ± 14 μM)
α-Fluorocinnamate	D/L-Glyceraldehyde	NAD^+ (2.0 mM)	7.4	32 ± 8.1	41.5 ± 6.0	Noncompetitive (K_i 38 ± 2.6 μM)
				66 ± 11	90 ± 13	Noncompetitive (K_i 79 ± 4.1 μM)
α-Fluorocinnamate	D/L-Glyceraldehyde	NAD^+ (0.5 mM)	9.0	483 ± 144	142 ± 12	Mixed-uncompetitive
α-Fluorocinnamate	D/L-Glyceraldehyde	NAD^+ (2.0 mM)	9.0	230 ± 49.7	161 ± 10	Noncompetitive (K_i 174 ± 7 μM)
				316 ± 69	263 ± 22	Noncompetitive (K_i 278 ± 12 μM)

Measurement of dehydrogenase activity and subsequent analysis of kinetic data were performed as described in Materials and Methods. The fixed concentration of D/L-glyceraldehyde was 4 mM. The fixed concentration of NAD^+ was as indicated. Data are presented as in Table 2.

K_m (±SE) value of 1230 ± 130 μM was determined for acetaldehyde in the presence of 1 mM NAD^+, confirming that this preparation contained a high K_m isoenzyme of aldehyde dehydrogenase. In the presence of 10 mM acetaldehyde a K_m (±SE) for NAD^+ of 45 ± 7.8 μM was determined. These values are similar to those for an isoenzyme of aldehyde dehydrogenase purified from rat liver mitochondria by Senior and Tsai [44]. In the presence of low (20 μM) NAD^+ and 10 mM acetaldehyde, 50% inhibition of this enzyme required approx. 2.5 mM α-fluorocinnamate and approx. 150 μM α-cyano-3,4-dihydroxythiocinnamamide, suggesting that these compounds are considerably less effective as inhibitors of this isoenzyme than the low K_m enzyme. In view of the poor inhibition of the high K_m mitochondrial aldehyde dehydrogenase by these compounds, this process was not investigated any further.

The observation that derivatives of cinnamic acid bind to mitochondrial aldehyde dehydrogenase in competition with NAD^+ raises the possibility that they could inhibit other dehydrogenases. Thus, we investigated their effect on L-lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase. The mechanism of glyceraldehyde-3-phosphate dehydrogenase has been considered to be similar to that of aldehyde dehydrogenase [4, 38]. As might have been expected on the basis of the lack of binding of these enzymes to α-cyanocinnamate-Sepharose, cinnamic acid derivatives were much less effective as inhibitors of these enzymes than of the low K_m aldehyde dehydrogenase. For lactate dehydrogenase, α-fluorocinnamate was a weak competitive inhibitor with respect to NADH (K_i approx. 2.5 mM). Similarly, this compound was a weak inhibitor (K_i approx. 3 mM) with respect to NAD^+ for glyceraldehyde-3-phosphate dehydrogenase. The high absorbance of α-cyano-3,4-dihydroxythiocinnamamide precluded estimation of the K_i for this compound, but this value was in excess of 0.1 mM for both enzymes (>100-fold higher than for the low K_m aldehyde dehydrogenase).

General conclusions

On the basis of three separate lines of evidence, the data presented here indicate that derivatives of cinnamic acid bind at or near the nucleotide binding site on the low K_m mitochondrial aldehyde dehydrogenase. Firstly, they cause inhibition of propionaldehyde oxidation catalysed by the enzyme, in a manner that in most cases is essentially competitive with NAD^+. Secondly, they also inhibit the ability of NAD^+ to stimulate hydrolysis of *p*-nitrophenyl acetate catalysed by the enzyme, whilst having no effect on the hydrolysis of this ester in the absence of nucleotides. Recent work has shown that modification of the cytoplasmic isoenzyme with dicyclohexylcarbodiimide also causes inhibition of dehydrogenase activity without affecting the steady-state hydrolysis of esters [45]. However, in this case the modification was not within the nucleotide binding site. Presumably cinnamic acid derivatives can bind in some part of the nucleotide binding site, but without inducing the conformational change that is presumed to stimulate the esterase activity. Finally,

NAD^+ can elute aldehyde dehydrogenase when bound to α-cyanocinnamate-Sepharose. The finding that aldehyde dehydrogenase can bind to this affinity matrix in the absence of substrates indicates that, like NAD^+ (but unlike acetaldehyde and propionaldehyde), the inhibitors bind to the free enzyme. When considered in the light of these observations, the apparent absence of competitive kinetics between NAD^+ and α-fluorocinnamate when glyceraldehyde was used as substrate suggests that caution should be exercised when extrapolating results obtained with this aldehyde to oxidation of other substrates.

The competition between binding of cinnamic acid derivatives and nucleotides means that high concentrations of the inhibitors would be required to inhibit the enzyme at the high (approx. 1 mM; [46]) concentration of NAD^+ occurring physiologically in the liver. At such high concentrations other processes are inhibited by compounds of this type [17]. Hence, they are perhaps unlikely to be of use as alcohol aversive drugs. However, direct studies of inhibition of the enzyme *in vivo* will be required to resolve this issue; these experiments would need to be performed *in situ* since the inhibition is freely reversible.

Some of the derivatives may prove useful as tools to investigate the nucleotide binding site on this enzyme, especially α-cyano-3,4-dihydroxythiocinnamamide and α-cyano-3,4,5-trihydroxycinnamonitrile, which bind with high affinity. Future work may allow more direct binding studies using fluorimetry to measure the effects of these inhibitors on the binding of NADH to the enzyme. A partial explanation for the apparent selectivity of these compounds for this enzyme over other NAD^+-linked dehydrogenases may be the modification of the normal nucleotide-binding consensus sequence (G-X-G-X-X-G) found in the aldehyde dehydrogenases (G-X-G-X-X-X-G; [47]).

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