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INACTIVATION OF YEAST ALCOHOL DEHYDROGENASE BY NITRILOPROPIONAMIDES †

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A series of halonitrilopropionamides have been examined as potential inhibitors of yeast alcohol dehydrogenase. Analogues with a good leaving group on the α-carbon, and a geminal electronegative atom, were found to be initial competitive inhibitors against NAD with inhibition constants as low as 0.6 μM. Incubation of the enzyme with these inhibitors leads to a slow, irreversible inactivation, with an inactivation constant for 2,2-dibromo-3-nitropropionamide of about 100 μM. No protection against inactivation was observed with the substrate ethanol, while the presence of saturating levels of NAD slowed the rate, but not the final extent, of enzyme inactivation. The resulting enzyme-inactivator complex is stable to a range of conditions that are known to denature the enzyme, indicating that a covalent modification of yeast alcohol dehydrogenase has led to the inactivation. A bimodal inactivation model is proposed to account for the observed interactions of these halonitrilopropionamides with yeast alcohol dehydrogenase.

KEY WORDS: Enzyme inactivation, yeast alcohol dehydrogenase, nitrilopropionamides

INTRODUCTION

Yeast alcohol dehydrogenase (EC 1.1.1.1) is a tetrameric enzyme, composed of four identical subunits, with an overall molecular weight of 140,000–150,000.[1] Each subunit contains one tightly bound zinc atom, and the enzyme is specific for NAD as a substrate in the reaction. The primary structure of this enzyme has been determined by several groups, and it has been shown that the N-terminus is a serine residue blocked by an acyl group, while the C-teminus is a lysine residue.[2] This enzyme is most active with ethanol and acetaldehyde as the substrate-product pair, but will also catalyze the oxidization of all primary straight-chain alcohols to their corresponding aldehydes.

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Abbreviations: BCNPA, 2-bromo-2-chloro-3-nitrilopropionamide; DBA, dibromoacetamide; DBAA, dibromoacetic acid; DBAN, dibromoacetonitrile; DBNPA, dibromonitrilopropionamide; DBECA, dibromonitrilopropionate ethyl ester; DBMCA, dibromonitrilopropionate methyl ester; DCECA, dichloronitrilopropionate ethyl ester; DCNPA, dichloronitrilopropionamide; MBECA, monobromonitrilopropionate ethyl ester;MBMCA monobromonitrilopropionate methyl ester; MBNPA, monobromonitrilopropionamide.

With the exception of methanol in the straight-chain alcohol series, the shorter chain length alcohols are better substrates for the yeast enzyme.[3] The enzyme activity is very low with branched-chain and secondary alcohols.

Substrate analogues containing chemically reactive functional groups have been utilized to improve our understanding of the structure of the enzyme active site. Yeast alcohol dehydrogenase has from 8 to 9 free sulfhydryl groups per subunit, and has been shown to be inactivated by thiol reagents and by heavy metal ions.[1] Two of these cysteines have been shown to be sensitive to chemical modification by iodoacetamide[4] or by reaction with butyl isocyanate.[5] One of these residues has been identified by peptide mapping as cysteine-43 in the yeast enzyme.[5] Subsequent treatment of this modified enzyme with a photolabile arylazide led to the identification of cysteine-153 as the second reactive sulfhydryl group.[6] The iodoacetamide or isocyanate inactivated enzymes retain the ability to bind NAD, but not to form a ternary complex with alcohol analogues,[7] implying that these cysteines are located at the alcohol and not the NAD binding site. However, modification of alcohol dehydrogenase with a reactive NAD analogue also inactivates the enzyme by modification of cysteine-43 in the yeast enzyme, and a corresponding cysteine in the mammalian liver enzyme.[8] This cysteine in liver alcohol dehydrogenase has also been shown to be particularly sensitive to reaction with iodoacetate.[9] Subsequent structural characterization of this inactivated enzyme showed a dual mode of inactivation, with carboxymethylation of cysteine-46 and coordination of the iodide ion released in the reaction to the enzyme-bound zinc.[10]

The series of nitrilopropionamides that are examined in this report are an important group of bioactive compounds, the most active of which are currently being utilized as antimicrobial agents. 2,2-Dibromo-3-nitrilopropionamide (DBNPA) has been shown to be a potent fungicide,[11] and has also been used to inhibit the growth of microorganisms in water cooling towers.[12] Chemical and biological decomposition studies have shown that the halonitrilopropionamides are readily degraded, and do not persist in the environment.[13] The detailed mode of activity of this series of compounds has not, however, been thoroughly investigated. A potential site of action of these compounds has now been investigated by examining their interactions with a series of pyridine-linked dehydrogenases. Among this group of oxidation-reduction catalysts, only the yeast and the bacterial alcohol dehydrogenases and, to a much lesser extent isocitrate dehydrogenase, were sensitive to inhibition by this class of compounds. We report here on the mode of interaction of this group of nitrilopropionamides with yeast alcohol dehydrogenase.

MATERIALS AND METHODS

Materials

The enzyme yeast alcohol dehydrogenase was obtained from Sigma Chemical, and was used without further purification. Enzyme solutions were freshly prepared by dissolving the lyophilized powder in 10 mM Hepes buffer (pH 8.0), and aliquots were removed from this stock solution for each assay. Other enzymes were purchased either from Sigma Chemical or Boehringer-Mannheim. Dibromoacetic acid and di-

bromoacetonitrile were purchased from Aldrich. Dibromoacetamide was prepared from dibromoacetic acid by conversion to the acid chloride and subsequent reaction with ammonium chloride. The compounds of the nitrilopropionamide series, including DBNPA (Dow Antimicrobial 7287/8536) and all other inhibitors examined in this study, were obtained from Dow Chemical. Solutions of each of these compounds were freshly prepared in dimethylsulfoxide before each set of experiments. ^{14}C-labeled dibromonitrilopropionamide (DBNPA) was synthesized by Dow Chemical (> 99% purity, specific activity = 3 mCi/mmole). NAD and NADP were purchased from Sigma.

Enzyme Assays

Initial reaction rates were measured spectrophotometrically in 1-cm pathlength quartz cuvettes at 30°C by following the formation of NADH at 340 nm. Assay mixtures for yeast alcohol dehydrogenase consisted of 50 mM Hepes buffer, pH 8.0, 6.8 mM ethanol and 0.1 mM NAD, and the various inhibitors at concentrations ranging from 0.1μM to 2.0 mM depending on the potency of the inhibitor. Assays were conducted in 20% DMSO and 80% water since most of the inhibitors that were examined have very limited solubility in water. The enzyme was determined to be extremely stable, and to have the full catalytic activity observed in aqueous solution, under these assay conditions. Inhibition constants were determined for the compounds examined by varying both the NAD and the inhibitor concentrations, and fitting the measured reaction rates to the equation for competitive inhibition (Equation 1) by a computer program transcribed into BASIC from those written by Cleland.[14]

$$v = \frac{VA}{K_m(1 + I/K_{is}) + A} \tag{1}$$

Incubation Studies

These studies were carried out by preparing incubation mixtures consisting of 20 μl (20%) DMSO, 50 units enzyme (30 μl), various levels of each inhibitor, and Hepes buffer (pH 8.0) to make a total volume of 100 μl. Aliquots of 10 μl were removed at predetermined time intervals, and were added to the standard assay mixture to determine the residual enzyme activity. Control experiments were conducted in the absence of added inhibitors to establish the stability of yeast alcohol dehydrogenase under these incubation conditions. Protection experiments were carried out to determine whether saturating levels (> $10K_m$) of either substrate could protect the enzyme from inactivation. A model for irreversible enzyme inactivation was utilized, which assumes an initial collision complex with the inhibitor that is reversible, followed by slow irreversible inactivation:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E \cdot I \xrightarrow{k_3} E\text{–}I \qquad \text{where } K_i = k_2/k_1 \tag{2}$$

The inactivation constant, K_i, and the rate of inactivation of the enzyme, k_3, were calculated from Equation 3:[15]

$$ln([E]_t - [E\text{–}I]) = \frac{-k_3 t}{1 + K_i/[I]} + ln[E]_t \quad (3)$$

where $[E]_t$ = total enzyme concentration and $[E\text{–}I]$ = concentration of covalently inactivated enzyme.

Spectroscopic Studies

UV-visible spectroscopic studies of the effect of nitrilopropionamides on yeast alcohol dehydrogenase were conducted in 1-cm pathlength cells in a Hewlett Packard model 8452A diode array spectrophotometer. The assay mixtures contained 50 mM Hepes (pH 8.0), 20% DMSO, 0.1 mM DBNPA or its analogues, and 1.2 μM yeast alcohol dehydrogenase in the presence or absence of 0.1 mM NAD. Control experiments were run in the absence of enzyme. The absorption spectrum was examined between 200 nm and 400 nm, and the changes in absorption observed at 242 nm were plotted as a function of time.

Binding Studies

The enzyme was incubated with 30–250 μM ^{14}C-DBNPA for times ranging from several minutes to several hours. The resulting complex was subjected to gel filtration on a Sephadex G-75 column (2.5 cm × 25 cm) that had been equilibrated in 50 mM Hepes buffer, pH 8.0. Fractions were collected and analyzed for protein content (biuret), enzyme activity (standard assay procedure) and radioactivity (liquid scintillation counting on a Beckman model LS-3133T spectrometer).

The rate of formation of the enzyme-DBNPA complex was examined by adding yeast alcohol dehydrogenase (6 μM final concentration) to a solution containing 50 mM Hepes buffer (pH 7.0) and various concentrations of ^{14}C-DBNPA. At fixed time intervals after initiating the reaction by enzyme addition, aliquots were removed and added to a quenching solution containing excess unlabeled DBNPA. These solutions were then filtered in a Centricon 30 filter (Amicon, molecular weight cutoff = 30,000) to determine the amount of ^{14}C-DBNPA that was tightly bound to the enzyme as a function of time. The stability of the resulting DBNPA-enzyme complex was also assessed by examining the effects of pH, temperature, and different protein denaturants on the release of ^{14}C-labeled DBNPA from the complex that had been formed after incubation for periods ranging from 30 minutes to 2 hours. After treatment under various conditions, the complex was filtered in a Centricon 30 filter, washed with buffer, and analyzed as above for protein and radioactivity.

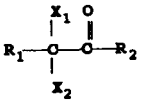
RESULTS

Enzyme Inhibition

The inhibition of yeast alcohol dehydrogenase by nitrilopropionamides was studied by an examination of the effect of DBNPA, and a series of structural analogues, on the initial velocity of the enzyme-catalyzed reaction. DBNPA was found to be a potent

TABLE 1
Structure of Nitrilopropionamide Analogues[a] and Inhibition Constants with Yeast Alcohol Dehydrogenase

Compound	Substituents				Inhibition Constant (μM)[b]
	X_1	X_2	R_1	R_2	
DBNPA	Br	Br	CN	NH_2	0.65 ± 0.15
BCNPA	Br	Cl	CN	NH_2	0.59 ± 0.12
MBNPA	Br	H	CN	NH_2	7.1 ± 1.2
DCNPA	Cl	Cl	CN	NH_2	> 90
DBNPA-Benzocaine[c]	Br	Br	CN	NH-Bnz	0.24 ± 0.03
MBNPA-Benzocaine	Br	H	CN	NH-Bnz	1.54 ± 0.20
MBNPA-Phenyl	Br	H	CN	NH-Ph	3.25 ± 0.88
DBAN	Br	Br	CN	OH	n.i.[d]
DBAA	Br	Br	H	OH	n.i.
DBA	Br	Br	H	NH_2	n.i.
DBECA	Br	Br	CN	OEt	1.29 ± 0.16
DBMCA	Br	Br	CN	OMe	3.52 ± 0.93
MBECA	Br	H	CN	OEt	3.07 ± 0.34
MBMCA	Br	H	CN	OMe	8.0 ± 1.6
DCECA	Cl	Cl	CN	OEt	n.i.

[a] Parent structure: 

[b] competitive inhibition against NAD. Reactions were examined in 50 mM Hepes, pH 8.0, with the addition of 20% DMSO to keep the relatively insoluble inhibitors in solution.

[c] benzocaine = NH_2-phenyl-COOEt.

[d] n.i. = no inhibition observed under these conditions.

inhibitor of the yeast enzyme. The nature of the inhibition by DBNPA, and several of its structural analogues, was determined by running inhibition patterns against each of the substrates. These inhibitors were found to be competitive against NAD, and non-competitive against ethanol. The structures, and the inhibition constants (K_i) that were determined for each of the analogues that have been examined, are reported in Table 1. This series of fifteen compounds includes analogues in which either the nitrile or amide groups of the parent compound are absent, and compounds in which one or both of the bromo groups have been replaced by a chloro group or a hydrogen. The potency of inhibition of yeast alcohol dehydrogenase for this series of compounds ranges from analogues that have no effect on enzyme activity at concentrations up to 2 mM to compounds that are effective inhibitors at submicromolar levels.

From these inhibition studies, it appears that this group of nitrilopropionamides are interacting at the pyridine nucleotide binding site of yeast alcohol dehydrogenase. These results suggest that this class of compounds may be capable of functioning as general inhibitors of reactions catalyzed by pyridine-linked dehydrogenases. However, the strong inhibition that has been observed with yeast alcohol dehydrogenase was found to be quite specific. The alcohol dehydrogenase obtained from mammalian liver is not inhibited to an appreciable extent by any members of this group of compounds. An alcohol dehydrogenase from the thermophilic bacteria *Thermoanaerobium brockii* is inhibited in the micromolar range by DBNPA, and is substantially inhibited by 10 μM DBNPA-benzocaine. Several other pyridine-linked dehydrogenases were also surveyed. DBNPA was observed to be either a weak inhibitor, or a non-inhibitor, of glucose 6-phosphate dehydrogenase (either NAD or NADP), glutamate dehydrogenase (either NAD or NADP), and dihyroorotate dehydrogenase (NAD-linked). Fairly strong inhibition was observed, however, at micromolar levels with NADP-specific isocitrate dehydrogenase. The specificity of inhibition was similar to that observed with yeast alcohol dehydrogenase. DBNPA ($K_i = 12\mu M$) and the benzocaine analogue ($K_i = 9\mu M$) were found to be the strongest inhibitors of isocitrate dehydrogenase, and the removal of one of the bromides dramatically decreases the potency of inhibition. The inhibition constants that were determined for these compounds were a factor of 20 to 40 higher than the corresponding values observed with yeast alcohol dehydrogenase.

Enzyme Inactivation

Incubation studies were conducted to determine if the enzyme inhibition that has been observed with this series of nitrilopropionamides is freely reversible. Incubation experiments were carried out with yeast alcohol dehydrogenase at pH 8.0, as described in Methods, with some of the more potent inhibitors of the series of DBNPA analogues. The results obtained with DBNPA, at concentrations ranging from 20 μM to 100 μM, are shown in Figure 1. When yeast alcohol dehydrogenase is incubated with 100 μM DBNPA, 90% of the enzyme activity is lost within the first 30 seconds, as compared to a 10% loss in enzyme activity at 20 μM DBNPA within the same period of time. Control experiments run in the absence of DBNPA showed no loss of enzyme activity under these incubation conditions in the absence of added nitrilopropionamides. The inactivation constant, calculated from the slopes of the family of lines in Figure 1, was determined to be 104±24 μM. Substrate protection studies were conducted at saturating levels of either NAD or ethanol. No protection of enzyme inactivation was seen with ethanol concentrations as high as 34 mM. NAD (at 0.5 mM) afforded partial protection of alcohol dehydrogense, with the rate of inactivation at 20 μM DBNPA decreasing to 0.033 min^{-1}, as compared to 0.050 min^{-1} in the absence of NAD. However, in longer term incubation experiments, the extent of inactivation of yeast alcohol dehydrogenase was still complete, even in the presence of 0.5 mM NAD.

A similar series of inactivation curves was observed in incubation studies of DBNPA-benzocaine with alcohol dehydrogenase. At 80 μM DBNPA-benzocaine, a 90% loss

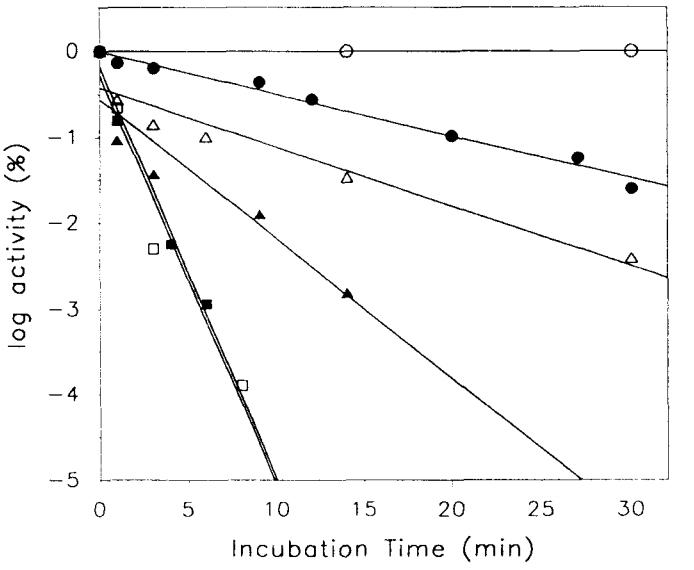


FIGURE 1 Inactivation of yeast alcohol dehydrogenase by 2,2-dibromo-3-nitrilopropionamide. The enzyme was incubated in 50 mM Hepes, pH 8.0, with DBNPA at: (○) 0 μM; (●) 20 μM; (△) 30 μM; (▲) 40 μM; (□) 50 μM; and (■) 100 μM. Aliquots were removed at the indicated incubation times and enzyme activities were measured in a standard assay mixture.

in enzyme activity was observed within the first 30 seconds. These results show that the rate of inactivation of yeast alcohol dehydrogenase by DBNPA-benzocaine is even more rapid than with DBNPA. The inactivation with the benzocaine analogue appears to be biphasic, with an initial rapid phase followed by a slower inactivation event (data not shown). There is also some evidence of biphasic behavior in the inactivation of yeast alcohol dehydrogenase by DBNPA at intermediate concentrations (Figure 1). Similarly, irreversible inactivation of the bacterial alcohol dehydrogenase, and protection by NADP but not by 2-propanol, was observed with DBNPA-benzocaine (data not shown).

UV-Visible Spectroscopic Studies

The effect of DBNPA binding on the structure of yeast alcohol dehydrogenase was monitored by examining the UV-visible spectrum of the enzyme. Slow formation of a peak at 242 nm was observed following the addition of 100 μM DBNPA to an assay mixture containing yeast alcohol dehydrogenase. The observed change in absorbance was essentially complete after about three hours (Figure 2). Control experiments run in the absence of either enzyme or DBNPA did not show the formation of this peak.

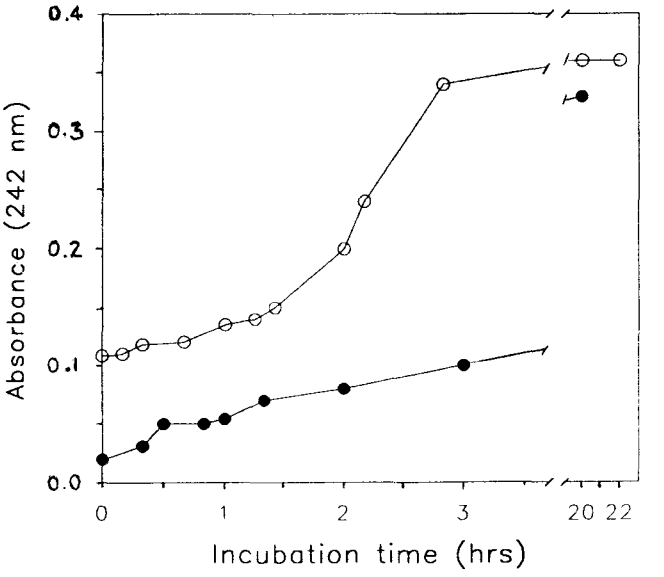


FIGURE 2 UV-visible spectral changes of yeast alcohol dehydrogenase at 242 nM upon incubation of the enzyme with 100 μM DBNPA, in the absence (○) and in the presence (●) of 100 μM NAD.

Examination of the spectral changes of this reaction mixture in the presence of 100 μM NAD showed a decrease in the rate of peak formation at 242 nm, although the same final absorbance value was eventually achieved (Figure 2). This is consistent with the previous kinetic observations that addition of NAD only slowed the rate of enzyme inactivation, but did not affect the final level of enzyme activity. Similar experiments were also carried out for several analogues of DBNPA. The rate of inactivation of yeast alcohol dehydrogenase by MBNPA has been shown by incubation studies to be considerably slower than that measured with DBNPA. Consistent with this observation, a slower rate of appearance of the absorbance at 242 nm was seen during the initial incubation period with MBNPA, as compared to that with DBNPA. Interaction of the benzocaine analogue of DBNPA with the enzyme gave a rate of appearance of the peak at 242 nm that is comparable to that observed with DBNPA (results not shown).

Kinetics of Formation of the Enzyme-DBNPA Complex

The rate of formation of the enzyme-DBNPA complex was also examined by binding studies with various concentrations of ^{14}C-labeled DBNPA, as described in Methods. An example of the results of these studies, with DBNPA at a total concentration of 30 μM, is shown in Figure 3. At this concentration of inactivator approximately 0.4–0.5 moles of DBNPA were bound per mole of enzyme subunits, with a half-time for

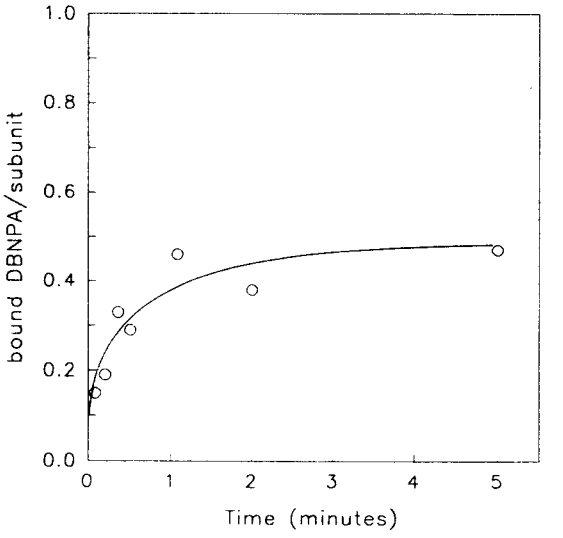


FIGURE 3 Rate of Formation of the Enzyme-DBNPA Complex. Yeast alcohol dehydrogenase (6 μM) was incubated in a solution containing 50 mM Hepes (pH 7) and 25 μM ^{14}C-DBNPA. Aliquots were removed at various times, quenched with excess (150 μM) unlabeled DBNPA, and then filtered to allow determination of the amount of bound DBNPA by scintillation counting.

binding of about 30 seconds. For a first order process, this gives a value of 0.3 min^{-1} for the rate of formation of the tight enzyme-DBNPA complex.

Inactivation of alcohol dehydrogenase by DBNPA may involve attack by an enzyme nucleophile on this compound, with subsequent release of bromide. In an attempt to confirm this mechanism of inactivation, and to follow the kinetics of bromide release, the inactivation of yeast alcohol dehydrogenase by DBNPA was examined in the presence of a bromide electrode. The electrode response (in millivolts) was monitored as a function of incubation time after addition of enzyme to a buffered solution containing 100 μM DBNPA. A reproducible electrode response was observed over a period of 1 to 2 minutes after the addition of an aliquot (6.8 μM) of enzyme. Addition of subsequent enzyme aliquots resulted in further electrode responses. However, the limits of detectability of the bromide electrode precluded a determination of the rate of bromide release, or an accurate assessment of the total concentration of bromide produced.

Stability of the Enzyme-DBNPA Complex

The stability of the complex formed between DBNPA and yeast alcohol dehydrogenase was examined by gel filtration studies. The enzyme was incubated with ^{14}C-labeled DBNPA for various times. The incubation mixture was then applied to a

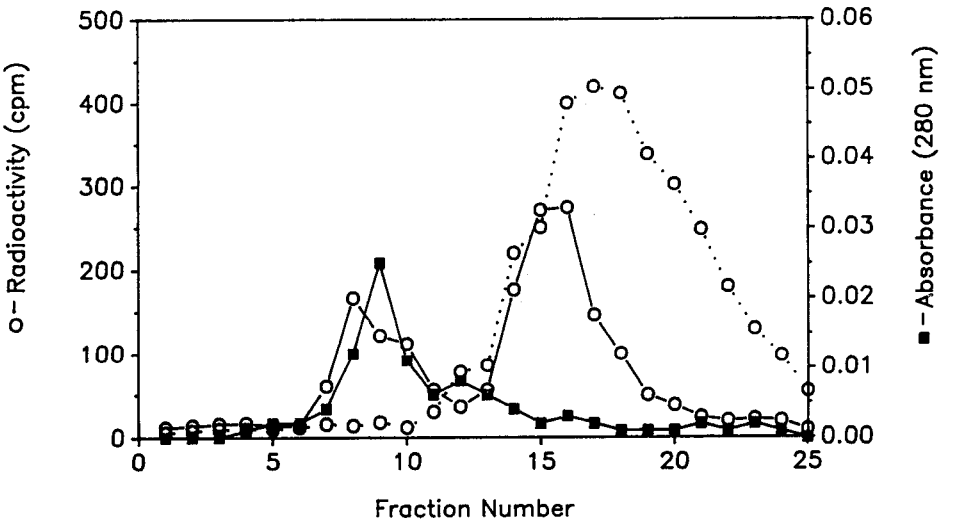


FIGURE 4 Binding of DBNPA to alcohol dehydrogenase examined by gel filtration chromatography. Enzyme and ^{14}C-DBNPA were incubated, and then chromatographed in a Sephadex G-75 gel filtration column. This incubation mixture (o—o) led to two peaks containing radiolabeled material. The leading peak was shown to contain the enzyme by measuring the absorbance at 280 nm (■—■) of a control solution of yeast alcohol dehydrogenase. The second peak of the incubation mixture co-chromatographs with a control sample (o · · · o) of ^{14}C-DBNPA.

Sephadex G-75 column and eluted with buffer at pH 8. A typical elution profile, for a mixture incubated for 30 minutes, is shown in Figure 4. Two radioactive peaks were observed for this mixture, one corresponding to the peak observed for free DBNPA and one co-eluting with the alcohol dehydrogenase peak.

The observed loss of enzyme activity was shown to be directly correlated to the extent of DBNPA binding. When incubated with 30 μM DBNPA, approximately 0.4 moles of inactivator were bound per mole of enzyme subunits, and the enzyme retained about 67% residual activity. Incubation at higher DBNPA levels resulted in complete loss of enzyme activity. The final stoichiometry of the enzyme DBNPA complex was determined, by measurement of ^{14}C-DBNPA binding, to be about 1.2 moles of DBNPA bound per mole of subunits of yeast alcohol dehydrogenase.

The long-term stability of the complex between DBNPA and yeast alcohol dehydrogenase was examined across a range of conditions. The enzyme-DBNPA complex is quite stable in the neutral pH range. After removal of free DBNPA by Amicon filtration, and incubation for times ranging from 30 minutes to 20 hours, the number of bound DBNPA molecules per subunit in the complex was found to be in the range of 1.0 to 1.3. Exhaustive dialysis for five additional days resulted in an average of a 10% loss in bound DBNPA from the complex. The DBNPA-enzyme complex is also fairly stable to a variety of conditions that are known to perturb the native structure of yeast alcohol dehydrogenase. Treatment of the enzyme with 3.3 M urea or 4.3 M guanidine-HCl did not affect the amount of DBNPA bound (Table 2). Incubation in 0.6 M HCl

TABLE 2
Effect of Denaturants on the Enzyme-DBNPA Complex[a]

Treatment	Exp.	DBNPA released (cpm)	DBNPA bound (nmoles)	Avg. DBNPA bound per subunit
Neutral	1	3050	6.14	1.00 ± .09
(pH 7)	2	3124	6.42	
	3	2730	5.42	
Acid	1	3240	6.39	1.07 ± .01
(0.6M)	2	3250	6.40	
	3	3250	6.40	
Base	1	7722	4.96	0.82 ± .02
(0.6M)	2	8270	4.77	
	3	7524	5.02	
Urea	1	2540	6.60	1.09 ± .01
(3.3M)	2	2890	6.48	
	3	2660	6.56	
Guanidine HCl	1	3040	6.26	1.04 ± .01
(4.3M)	2	3170	6.21	
	3	2990	6.27	

[a] [enzyme] = 6.1 μM, [DBNPA] = 250 μM; incubation under each set of conditions for 2.5 h at 4°C

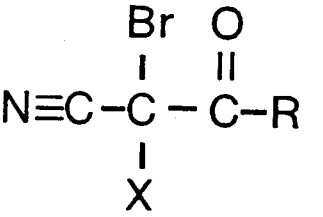
for 2.5 hours also had no effect on DBNPA binding to yeast alcohol dehydrogenase. Treatment with 0.6 M NaOH did result in a small, but reproducible release of bound DBNPA (Table 2).

DISCUSSION

Enzyme Binding Specificity

The series of nitrilopropionamide analogues that have been examined permits a determination of the structural features that are important for binding to, and inhibition of, yeast alcohol dehydrogenase by this family of antimicrobial compounds. Removal of the nitrile group (DBA) or changing the amide to a carboxylate (DBAN) yields structural analogues that do not inhibit the enzyme. Conversion of the α-dibromo substituents to α-dichloro substantially decreases the affinity of this compound for yeast alcohol dehydrogenase, while removal of only one bromide (MBNPA) has a less

dramatic effect on the observed inhibition constant. The methyl and ethyl esters of these analogues are only slightly poorer inhibitors than the corresponding amides. Aromatic amides are also good inhibitors of yeast alcohol dehydrogenase, with the N-benzocaine analogue being the most potent inhibitor that was examined. In general, it appears that both the nitrile and either an amide or an ester function group are required for tight binding to the enzyme. A good leaving group on the α-carbon (such as bromide), and a geminal electronegative atom (bromide or chloride), are also required, leading to the following general structural requirements for strong enzyme inhibition:



where X is an electronegative atom and R can be an amine, an alcohol, or hydroxide.

These nitrilopropionamides bear some structural resemblance to the aldehyde products of the enzyme-catalyzed reaction. However, surprisingly, the compounds in this family that inhibit yeast alcohol dehydrogenase were found to be initial competitive inhibitors against the pyridine nucleotide substrate NAD. A bacterial alcohol dehydrogenase is also inhibited by this family of compounds, however, several other pyridine-linked dehydrogenases that were examined, including liver alcohol dehydrogenase and glutamate dehydrogenase, and bacterial glucose 6-phosphate dehydrogenase, did not show sensitivity to this group of compounds. This observation implies the presence of some specific interactions between this class of inhibitors and the NAD binding site of alcohol dehydrogenase. Mammalian isocitrate dehydrogenase was also inhibited by these nitrilopropionamides, with the same pattern of relationships between inhibition constant and inhibitor structure that was observed with yeast alcohol dehydrogenase. The most potent inhibitors of yeast alcohol dehydrogenase have also been observed to be potent antimicrobial agents. For example, DBNPA has been shown to inhibit the growth of a wide range of bacteria, yeast, and fungi when present in the growth media in the 50–100 ppm concentration range.[16] These results suggest that an alcohol dehydrogenase in these organisms may be one of the targets of antimicrobial action.

Nature of the Enzyme-Inhibitor Complex

Incubation of the enzyme with radiolabeled DBNPA resulted in inactivation of yeast alcohol dehydrogenase with a stoichiometric incorporation of DBNPA (Table 2). The resulting complex was found to be stable at low pH, with no detectable release of the radiolabeled inactivator, and also stable to high salt conditions that are known to denature the enzyme. These results indicate that inactivation of the enzyme by nitrilopropionamides leads to covalent modification of a group, or groups, ostensibly

at the NAD binding site of yeast alcohol dehydrogenase. A partial, slow release of the labeled DBNPA was detected at high pH, indicating some lability of the covalently modified enzyme complex under these conditions.

Inactivation of the enzyme resulted in the formation of a chromophore with an absorbance maximum at 242 nm. This new chromophore is associated with the protein, and its rate of appearance is dependent on the structure and the concentration of the inhibitor added. The rate of development of this chromophore is slowed in the presence of the substrate NAD. Thus, the appearance of this chromophore appears to correlate with the formation of the covalent enzyme-inhibitor complex.

Mechanism of Inactivation

The two pyridine-linked dehydrogenases that are sensitive to inhibition by nitrilopropionamides, alcohol dehydrogenase and isocitrate dehydrogenase, both require zinc for catalytic activity. The interaction of nitrilopropionamides with yeast alcohol dehydrogenase involves several steps. There is an initial rapid, and reversible inhibition of the enzyme at low concentrations of nitrilopropionamides. Incubation of the enzyme with these compounds leads to the formation of a stable, substoichiometric complex. The half time for complex formation with DBNPA is less than one minute, and is even more rapid with DBNPA-benzocaine. Continued incubation leads to the slow development (with a half-time of many minutes to hours) of a stoichiometric enzyme-inactivator complex that is stable under a range of denaturing conditions.

The mechanism of inactivation may involve attack at carbon-2 of the nitrilopropionamides. The presence of four electron withdrawing substituents makes this center somewhat electron deficient, and susceptible to attack by an enzyme nucleophile. Release of bromide would lead to a stable enzyme adduct, and the appearance of bromide in the incubation mixture has been confirmed by bromide electrode studies. Alternatively, there is evidence for a nucleophilic displacement on bromide which could lead to the generation of a carbanion at carbon-2 with a two electron reduction occurring by either a step-wise or a concerted mechanism.[17] Additional interactions with yeast alcohol dehydrogenase could involve the binding of the released bromide at the enzyme-bound zinc. This proposed mechanism is analogous to the inactivation of liver alcohol dehydrogenase by iodoacetate, which has been proposed to occur by a bimodal mechanism, with acylation of an active site cysteine and subsequent binding of the released iodide ion to the enzyme-bound zinc atom.[10] The nature and location of the enzyme nucleophile, and the detailed steps that are involved in the inactivation of yeast alcohol dehydrogenase, remain to be elucidated.

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