BIOORGANIC CHEMISTRY **26,** 75–90 (1998)
ARTICLE NO. BH981080

Covalent Modification Reactions Involving Cytosolic Aldehyde Dehydrogenase and a Variety of Resorufin Derivatives

Trevor M. Kitson* and Kathryn E. Kitson†

**Institute of Fundamental Sciences and †Institute for Food, Health and Human Nutrition, Massey University, Palmerston North, New Zealand*

Received May 6, 1997

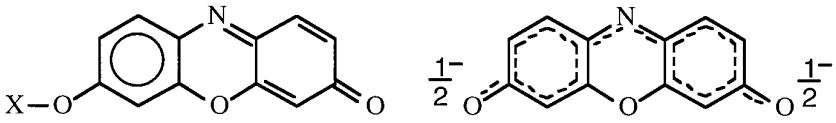
The bromoacetates of resorufin and of *p*-nitrophenol act simultaneously as very rapidly hydrolyzed substrates and as potent irreversible inactivators of cytosolic aldehyde dehydrogenase. Following inactivation, the resorufin or *p*-nitrophenoxide moiety is released fairly quickly by hydrolysis from the modified enzyme. Resorufin dimethylcarbamate and resorufin methanesulphonate inactivate aldehyde dehydrogenase in an ''oxidative addition'' reaction that is consistent with the involvement of the enzyme's catalytically essential cysteine residue with the quinonimine ring of the resorufin structure. The resulting covalently modified enzyme is yellow-orange and the redox properties of the colored label can be conveniently observed. Resorufin ethyl ether also slowly inactivates aldehyde dehydrogenase, but the resulting labeled enzyme is a surprising pink-violet color at pH values of about 5 to 10. The expected yellow-orange derivative is obtained at pH less than 4 or upon denaturation of the protein. The possible chemical identity of the species responsible for the pink-violet color is discussed. The resorufin anion binds noncovalently to aldehyde dehydrogenase apparently at the cofactor binding site, as it may be displaced by NAD^+ or NADH.

INTRODUCTION

For a number of years we have been interested in the esterase activity of cytosolic aldehyde dehydrogenase. We have utilized a variety of alternative substrates to the traditional *p*-nitrophenyl acetate, and we have designed and investigated substrate analogues that act as inactivators and ''reporter group'' reagents (*1–5*). Recently our attention has focused on the use of resorufin acetate (*6*, *7*); this substrate gives rise to a much more intensely colored product than does *p*-nitrophenyl acetate and also shows some interesting novel kinetic effects (for example its rate of hydrolysis is dramatically accelerated by NAD^+ or NADH). We thought it would be of interest to investigate resorufin bromoacetate as a comparison, and this work constitutes part of the present paper. Resorufin bromoacetate has the potential of being a very reactive substrate and also of being a potent inactivator of aldehyde dehydrogenase (like other compounds with a bromoacetyl group; *8*, *9*).

p-Nitrophenyl dimethylcarbamate has been used as an active site-directed irreversible inactivator of cytosolic aldehyde dehydrogenase, enabling Cys-302 to be confirmed as the enzyme's active site nucleophile (*3*) and suggesting the use of a closely related cyclic compound as a reagent that would label the active site with a colored reporter group (*5*). As a second part of the present paper we present a

0045-2068/98 $25.00



SCHEME 1. Structure of the resorufin compounds referred to in this work. Resorufin bromoacetate, X = $BrCH_2CO$; resorufin dimethylcarbamate, X = $(CH_3)_2NCO$; resorufin methanesulphonate, X = CH_3SO_2; resorufin ethyl ether, X = CH_3CH_2. The structure shown on the right is the resorufin anion with its symmetrically delocalised electronic arrangement.

study of the action of resorufin dimethylcarbamate on the enzyme. We anticipated it would behave similarly to its *p*-nitrophenyl analogue (but more sensitively in a spectrophotometric sense) and were intrigued to find that it reacts in an interestingly different manner, involving attack on the resorufin moiety itself rather than on the sidechain, as discussed below. This observation prompted our investigation of other derivatives of resorufin (as reported in the remainder of this paper), one of which, namely resorufin ethyl ether, was found to react with the enzyme in a surprising way, giving a striking pink-violet derivative. The structures of the compounds referred to in this work are given in Scheme 1.

MATERIALS AND METHODS

Cytosolic aldehyde dehydrogenase was isolated from sheep liver as previously described (*4*). Resorufin acetate was prepared as before (*10*); resorufin dimethylcarbamate, resorufin methanesulphonate, and resorufin ethyl ether were prepared as described in the accompanying paper (*11*). *p*-Nitrophenyl bromoacetate was prepared as before (*8*). Resorufin bromoacetate was prepared as follows. The sodium salt of resorufin (Aldrich Chemical Co.) (235 mg) was suspended in acetone (100 ml); bromoacetyl bromide (75 μl) was added and the mixture was stirred at room temperature overnight. After filtration, the deep yellow solution was evaporated to give an orange solid, which was stored at 4°C protected against atmospheric moisture. $C_{14}H_8BrNO_4$ confirmed by mass spectrometry (m/z = 332.963112 [^{79}Br], 334.961388 [^{81}Br]).

All assays of enzyme activity and all UV/visible spectra were recorded using a Varian Cary 1 spectrophotometer. Modification of aldehyde dehydrogenase by *p*-nitrophenyl dimethylcarbamate, resorufin dimethylcarbamate, resorufin methanesulphonate, or resorufin ethyl ether was carried out at pH 7.4 and 25°C, adding the modifier in a small volume of acetonitrile. The progress of modification was monitored directly by UV/visible spectroscopy or by taking small samples of the reaction mixture for assay of enzyme activity. After reaction, modified enzyme was isolated at room temperature by passing the reaction mixture (3–5 ml) down a gel filtration column (Biogel P-6; 25 × 0.08 cm), eluting with 10 mM sodium phosphate buffer, pH 7.4. Modified enzyme was denatured with perchloric acid and redissolved in urea solution as previously described (*5*).

TABLE 1
Inactivation of Aldehyde Dehydrogenase by Bromoacetates

Inactivator	Extent of inactivation (%)	
	Dehydrogenase	Esterase
Resorufin bromoacetate (10 μM)	97.5	94.6
p-Nitrophenyl bromoacetate (20 μM)	95.7	97.2

Note. The enzyme (0.52 μM) and modifier were incubated at 25°C in 50 mM sodium phosphate, pH 7.0, for 5 min before substrates were added to assay remaining activity. The dehydrogenase assay used NAD^+ (1 mM) and acetaldehyde (1 mM); the esterase activity was measured using resorufin acetate (50 μM).

Modification of aldehyde dehydrogenase by *p*-nitrophenyl bromoacetate or resorufin bromoacetate was carried out as follows. Three aliquots (67 μl) of modifier solution in acetonitrile and three aliquots (0.3 ml) of ice-cold enzyme solution in 50 mM sodium phosphate, pH 7.4, were added alternately over a period of less than 1 min to 1.8 ml of ice-cold 0.2 M sodium phosphate buffer, pH 7.0, with stirring. The total concentrations of enzyme and modifier were 19–22 μM and 0.2 mM, respectively. Modified enzyme was then isolated as rapidly as possible by gel filtration (as described above), eluting with 20 mM sodium phosphate buffer, pH 7.0, at 4°C. In the case of resorufin bromoacetate, the initial protein-containing eluate was immediately subjected to a second identical gel filtration procedure. Control experiments were carried out in an identical way except the modifier was first completely hydrolyzed by warming in the buffer; after cooling the resulting solution on ice, the enzyme was then added.

RESULTS AND DISCUSSION

Reaction of Aldehyde Dehydrogenase with Resorufin Bromoacetate and p-Nitrophenyl Bromoacetate

The results in Table 1 show that compounds with a bromoacetate functionality are rapid potent inactivators of both the dehydrogenase and the esterase activities of cystosolic aldehyde dehydrogenase, confirming the preliminary study with *p*-nitrophenyl bromoacetate (*8*). Figure 1a shows that when *p*-nitrophenyl bromoacetate (20 μM) is added to enzyme already in the presence of resorufin acetate (25 μM), the inactivator competes successfully against the substrate resulting in substantial loss of activity within a minute or so. (In this experiment, any release of *p*-nitrophenoxide, λ_{max} = 399 nm, would not interfere with the monitoring of resorufin liberation at 571 nm.) It is obvious therefore that bromoacetates (like various substituted bromoacetamides; *8*) are capable of reacting with aldehyde dehydrogenase (presumably at its catalytically essential Cys-302 residue) according

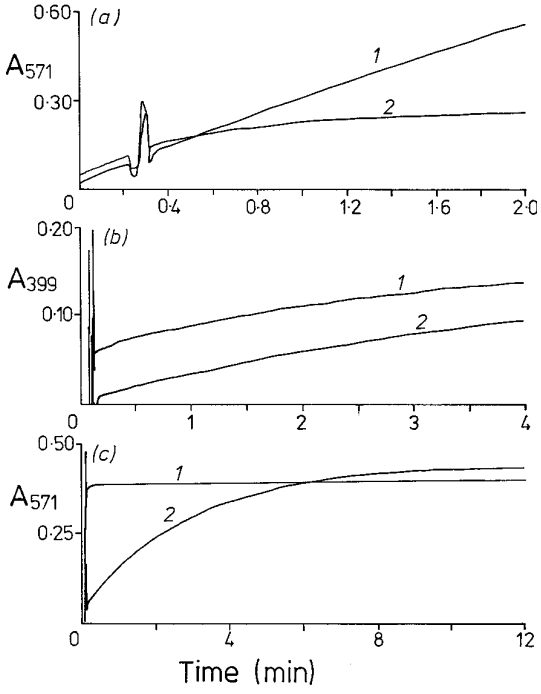
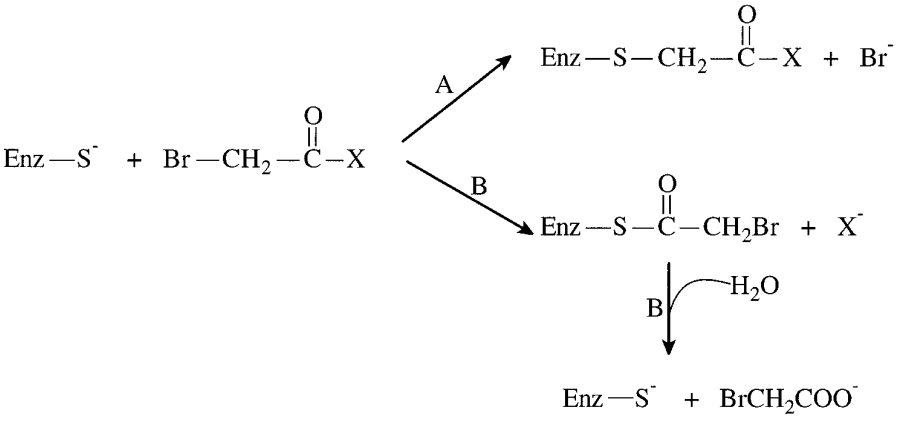


FIG. 1. Reaction of aldehyde dehydrogenase with bromoacetate compounds. In (a), trace 1 shows the hydrolysis of resorufin acetate (25 μM) catalyzed by aldehyde dehydrogenase (0.26 μM) in 50 mM sodium phosphate buffer, pH 7.0, at 25°C. Trace 2 shows the effect of adding *p*-nitrophenyl bromoacetate (20 μM) at the point shown by the irregularity in the curve. (At the equivalent point in trace 1, the same volume of solvent but without modifier was added.) In (b), trace 1 shows the release of *p*-nitrophenoxide when *p*-nitrophenyl bromoacetate (20 μM) is added to enzyme (0.52 μM) in 50 mM sodium phosphate buffer, pH 7.0, at 25°C. Trace 2 is the corresponding control in the absence of enzyme. In (c), trace 1 shows the release of resorufin anion when resorufin bromoacetate (10 μM) is added to enzyme (1.56 μM) in the same buffer; trace 2 is the corresponding control in the absence of enzyme.

to pathway A in Scheme 2. However, we were interested in whether any reaction can simultaneously occur according to pathway B (that is, the standard acylation-deacylation pathway of esterase action).

The trace labeled 1 in Fig. 1b shows the production of *p*-nitrophenoxide when *p*-nitrophenyl bromoacetate (20 μM) is added to aldehyde dehydrogenase (0.52 μM); similarly trace 1 in Fig. 1c represents the release of the resorufin anion when resorufin bromoacetate (10 μM) is added to enzyme (1.56 μM). In each case the trace labeled 2 is the corresponding control in the absence of enzyme showing the fairly rapid spontaneous hydrolysis of these reactive esters. It is clear from these experiments that there is a very fast enzyme-catalyzed hydrolysis of the esters giving rise to a large jump in absorbance within the time of adding the substrate and closing the spectrophotometer's cell compartment. At the same time, as already discussed, the enzyme is being inactivated; thus in Fig. 1b, the jump in absorbance



SCHEME 2. Possible modes of reaction of aldehyde dehydrogenase with the bromoacetates used in this work. (X = the resorufin or *p*-nitrophenoxide moiety.)

is then followed by a slower increase in absorbance due to spontaneous hydrolysis of the remaining ester after inactivation of the enzyme. In Fig. 1c, with a sixfold larger ratio of enzyme to ester, almost all the ester is hydrolyzed within the time of mixing. These results clearly show that both pathways A and B in Scheme 2 are indeed operative and that the precise outcome depends on the initial concentrations of enzyme and bromoacetate. If the enzyme concentration is relatively low, then shortly after the addition we have a mixture of inactive enzyme, some hydrolysis product (X^- in Scheme 2), and some unreacted excess ester; on the other hand, if the enzyme concentration is high enough, then shortly after mixing we would end up with some hydrolysis product, some inactive enzyme and some unaffected enzyme, and no ester left. The fact that the limiting absorbance of trace 1 in Fig. 1c is less than the level attained by spontaneous hydrolysis of the control resorufin bromoacetate is consistent with the idea that under these conditions some of the resorufin is "locked away" in the form of the product of pathway A in Scheme 2, albeit temporarily as we will see shortly.

We isolated aldehyde dehydrogenase labeled according to pathway A by reacting enzyme with resorufin bromoacetate or *p*-nitrophenyl bromoacetate as described under Materials and Methods. Control experiments consisted of allowing the bromoacetate completely to hydrolyze before adding enzyme and otherwise proceeding in an identical manner. With both the reaction mixture and the control in the case of resorufin bromoacetate the initial gel filtration eluate was immediately applied to a second identical gel filtration column in an attempt to improve the separation of noncovalently bound resorufin anion (see below), produced by either enzyme-catalyzed or spontaneous hydrolysis. Figure 2a shows that over a period of 1–2 h after isolation of aldehyde dehydrogenase modified by resorufin bromoacetate, there is a gradual release of the resorufin anion from the protein, consistent with the occurrence of the reaction shown in Scheme 3. A similar release of initially

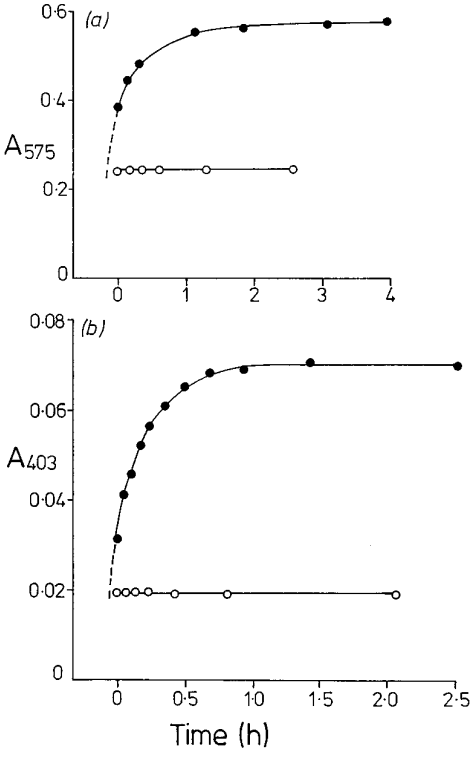


FIG. 2. Release of label from aldehyde dehydrogenase after modification by bromoacetate compounds. (a) Aldehyde dehydrogenase was modified by resorufin bromoacetate (as described under Materials and Methods) and the labeled protein was isolated by two passages through a gel filtration column. The release of the resorufin anion was then monitored at 25°C in 20 mM sodium phosphate buffer, pH 7.0 (●). The dashed part of the curve represents what is assumed to happen in the few minutes between completion of the gel filtration at 4°C and commencement of the spectrophotometric observation at 25°C. Also shown is the result of the control experiment in which the resorufin bromoacetate was allowed to hydrolyze before the addition of enzyme (○). (b) Enzyme was modified by *p*-nitrophenyl bromoacetate (as described under Materials and Methods) and the labeled protein was isolated by a single passage through a gel filtration column. The release of *p*-nitrophenoxide was then monitored at 25°C in 20 mM sodium phosphate buffer, pH 7.0 (●). Also shown is the result of the control experiment in which the *p*-nitrophenyl bromoacetate was allowed to hydrolyze before the addition of enzyme (○).

$$\mathrm{Enz{-}S{-}CH_2{-}\overset{\overset{O}{\|}}{C}{-}X + H_2O \longrightarrow Enz{-}S{-}CH_2{-}COO^- + X^- + 2H^+}$$

SCHEME 3. X^- = resorufin anion or *p*-nitrophenoxide.

"cryptic" label is seen over a period of 1 h in the case of enzyme modified by *p*-nitrophenyl bromoacetate (Fig. 2b).

The fact that the label produced as a result of pathway A (Scheme 2) is susceptible to hydrolysis establishes that it is not buried in a binding site on the enzyme that is inaccessible to water. As a comparison, a solution of resorufin acetate was prepared that would give a final value of A_{571} when totally hydrolyzed of 0.30 (similar to that due to the released label in Fig. 2a); when this was allowed spontaneously to hydrolyze under the same conditions as used in the experiment of Fig. 2a (i.e., 20 mM phosphate buffer, pH 7.0, 25°C), a value of A_{571} of only 0.0125 was attained within 1 h. In other words, the rate of hydrolysis of the label furnished to the enzyme by resorufin bromoacetate is over 20-fold faster than the spontaneous rate of resorufin acetate hydrolysis. This may mean that the same enzymic residues that catalyze the hydrolysis of the thioester intermediate in the esterase reaction (pathway B, Scheme 2) can also catalyze the hydrolysis of the ester group of the label, albeit much less efficiently of course than in the case of the "natural" acyl-enzyme.

Binding of the Resorufin Anion to Aldehyde Dehydrogenase

The control experiments in Fig. 2 show that gel filtration is unsuccessful in completely separating either the resorufin anion or *p*-nitrophenoxide from aldehyde dehydrogenase. The binding of the resorufin anion is particularly striking as even after two passages through Biogel P-6 a considerable amount of this highly colored species clings tenaciously to the enzyme.

Free resorufin anion has a sharp absorbance peak that is maximal at 571 nm, whereas in the presence of a high concentration of aldehyde dehydrogenase, the peak is more rounded with λ_{max} = 575 nm (see trace 1 in Fig. 3a). We observed, however, that on the addition of NAD^+ the absorbance spectrum reverts to that expected for the free resorufin anion, as shown in trace 2, Fig. 3a, suggesting that NAD^+ causes displacement of resorufin from its binding site on the enzyme. This suggestion is confirmed by the results shown in Fig. 3b; passing a mixture of enzyme and resorufin anion through a gel filtration column in the presence of NAD^+ results in much less resorufin travelling with the protein fraction (trace 2) than in the absence of NAD^+ (trace 1). Similarly, as shown in Fig. 3c, gel filtration of an initial mixture of aldehyde dehydrogenase, NADH, and resorufin anion results in an enzyme fraction that contains some bound NADH (as shown by the absorbance in the region of 340 nm) but less bound resorufin anion than in the control NADH-free experiment. Under the conditions of routine assay of aldehyde dehydrogenase (which include 1 mM NAD^+ and 1 mM acetaldehyde), the presence of the resorufin anion (10 μM) has no detectable effect. However, with much lower NAD^+ concentrations, the resorufin anion is a competitive inhibitor with K_i approximately 30 μM (data not shown).

The simplest explanation for the results here is that the resorufin anion binds in the nucleotide-binding site of aldehyde dehydrogenase. It is well known that bulky aromatic negatively charged dye molecules bind strongly to the coenzyme site of various dehydrogenases and this phenomenon is widely used in affinity chromatogra-

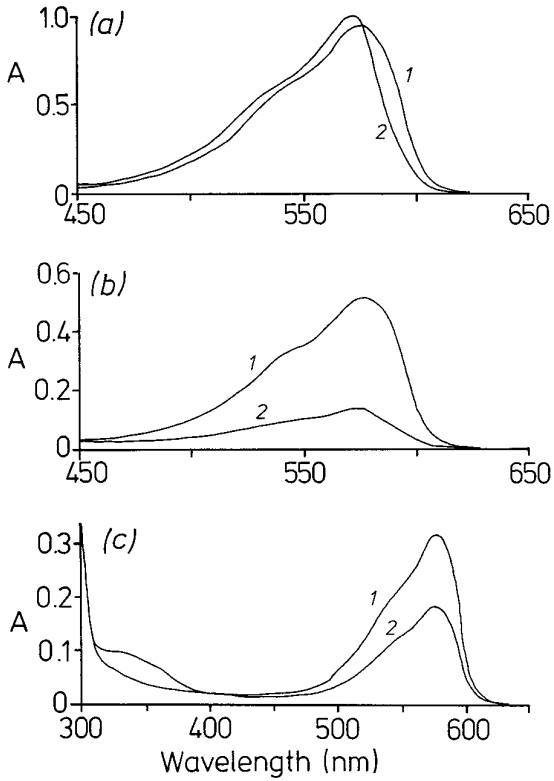


FIG. 3. Binding of the resorufin anion to aldehyde dehydrogenase. (a) Trace 1 shows the spectrum of resorufin (14 μM) in the presence of enzyme (8 μM) in 50 mM sodium phosphate buffer, pH 7.4. Trace 2 shows the spectrum of the same solution following the addition of a small amount of NAD^+ (approx. 2 mg to 3 ml). (b) A mixture of enzyme (19 μM) and resorufin (0.1 mM) in 20 mM sodium phosphate buffer, pH 7.0, was passed through a gel filtration column at 4°C (eluting with the same buffer) and the protein-containing eluate gave the spectrum shown as trace 1. Trace 2 is the corresponding result found when the original mixture and the elution buffer both contained 0.5 mM NAD^+. (c) A mixture of enzyme (12.5 μM) and resorufin (25 μM) in 50 mM sodium phosphate buffer, pH 7.4, was passed through a gel filtration column at 4°C eluting with 10 mM sodium phosphate buffer, pH 7.4; the protein-containing eluate gave the spectrum shown as trace 1. Trace 2 is the corresponding result found when the original mixture (but not the elution buffer) contained 25 μM NADH.

phy (*12*, *13*). The present observations have implications for past and future experiments involving aldehyde dehydrogenase and resorufin compounds. In all assays of esterase activity (with resorufin acetate, for example; *7*), it is presumably the activity of the enzyme–resorufin anion complex that is actually being monitored, at least after the initial production of a significant concentration of the anion. In experiments involving the dehydrogenase activity, competitive inhibition by the resorufin anion is a potential complication unless the NAD^+ concentration is saturating.