Purification of Liver Aldehyde Dehydrogenase by \( p \)-Hydroxyacetophenone-Sepharose Affinity Matrix and the Coelution of Chloramphenicol Acetyl Transferase from the Same Matrix with Recombinantly Expressed Aldehyde Dehydrogenase

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\( p \)-Hydroxyacetophenone was coupled to epoxy-activated Sepharose 6B to generate an affinity chromatographic matrix to purify aldehyde dehydrogenase. Purified beef liver mitochondrial aldehyde dehydrogenase specifically bound to the support and could be eluted with \( p \)-hydroxyacetophenone. A post-ammonium sulfate (30–55%) fraction of bovine liver was applied to the affinity gel column and aldehyde dehydrogenase was effectively purified, although not to complete homogeneity, indicating the potential selectivity of the matrix. Both beef liver cytosolic and mitochondrial aldehyde dehydrogenase bound to the column. A post-Cibacron blue Sepharose 6B affinity-fractionated liver mitochondrial aldehyde dehydrogenase was purified to complete homogeneity by \( p \)-hydroxyacetophenone-Sepharose, thus eliminating the need for the isoelectric focusing step often employed. \( p \)-Hydroxyacetophenone was found to be a competitive inhibitor against propionaldehyde and noncompetitive against NAD. \textit{Escherichia coli} lysates of recombinantly expressed aldehyde dehydrogenase were purified from \textit{E. coli} lysates with one major 25-kDa protein contaminant also binding to the column, as detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis. The 25-kDa contaminant was found to be chloramphenicol acetyl transferase from sequence analysis and binding studies.

Aldehyde dehydrogenase (aldehyde:NAD oxidoreductase, E.C. 1.2.1.3; ALDH) catalyzes the oxidation of aldehydes to their corresponding carboxylic acids (1). The enzyme is typically purified by a procedure involving ion-exchange chromatography followed by isoelectric focusing (2) or affinity chromatography using a NAD-like column such as AMP– (3) or Cibacron blue Sepharose (4). A homogeneous preparation of the enzyme was obtained from many different mammalian sources and tissues using these basic procedures (2,3,5–9). We found, however, that it was not a convenient method for isolating genetically engineered recombinant forms of the enzyme produced by site-directed mutagenesis and expressed in \textit{Escherichia coli} (unpublished observations). Some of the mutants that we generated possessed charge differences which necessitated changing the pH of the buffers used in the ion-exchange chromatography steps. Hence it was desirable to find an alternative method for purifying the enzyme.

Recently it was reported that \( \alpha \)-cyano-4-hydroxycinnamic acid, coupled to Sepharose through an epoxy linkage, became a specific affinity ligand for rat liver mitochondrial ALDH (10). This was an unexpected result as the compound was not one typically associated with the type of reagents which bind to ALDH. The reagent was...
reported to be an uncompetitive inhibitor of the enzyme with respect to aldehyde.

We reported that parasubstituted acetophenones were competitive inhibitors of horse liver ALDH and that the p-methoxy compound had a \( K_i \) of 7 \( \mu M \), which was as low as that of chloral hydrate (5 \( \mu M \)), a compound with the lowest reported \( K_i \) (11). It was suggested, though not proven, that the acetophenones were good inhibitors because the active site nucleophile of the enzyme, presumed to be a cysteine (4,12,13), could form a thiohemiacetal linkage with the ketone. Irrespective of the mode of binding of acetophenones to the enzyme, it appeared that an immobilized acetophenone might be a good affinity ligand for the purification of ALDH. To test this hypothesis, p-hydroxyacetophenone was covalently attached to epoxy-activated Sepharose. The ability of the immobilized acetophenone to function as an affinity ligand for ALDH was tested with the native enzyme as well as the recombinantly expressed enzymes produced by site-directed mutagenesis.

While attempting to purify the recombinantly expressed enzyme in E. coli, we found that a low-molecular-mass protein bound to the column and could be eluted by p-hydroxyacetophenone. In this paper we show that this protein is chloramphenicol acetyl transferase (acetyl-CoA-chloramphenicol acetyl transferase, E.C. 2.3.1.28) by using a combination of N-terminal sequencing, enzyme activity, and binding authentic chloramphenicol acetyl transferase to the affinity column and eluting it with p-hydroxyacetophenone.

MATERIALS AND METHODS

Pharmalyte, Cibacron blue Sepharose CL-6B, and epoxy-activated Sepharose 6B were from Pharmacia LKB Biotechnology, Inc. Alkaline-phosphatase conjugate anti-rabbit IgG, isoelectric focusing standards, and SDS–PAGE molecular weight markers were from BioRad Laboratories. p-Hydroxyacetophenone was from Aldrich Chemical Co. Chloramphenicol acetyl transferase and chloramphenicol were from Sigma Chemical Co. Chemicals for the cloning of the cDNA coding for rat mitochondrial ALDH and for its expression in E. coli strain BL21(DE3)pLysS cells were as described (14). All other chemicals, from various vendors, were reagent grade or better.

Synthesis of affinity resin. The coupling of the ligand (p-hydroxyacetophenone) to the matrix (epoxy-activated Sepharose 6B) was essentially based on previously reported procedures (10,15). The Sepharose was resuspended in water (1 g/ml) and allowed to swell for 1 h at room temperature. The suspension was suction dried on a sintered glass funnel and washed with 0.1 M aqueous NaOH solution. The resin was then added to 0.5 M p-hydroxyacetophenone solution in 0.1 M NaOH. After the pH was adjusted to 11.0, the suspension was incubated at 45°C for 15 h in a water shaker bath. The product was then cooled to room temperature, placed on a sintered glass funnel, and washed with 10 vol of deionized water to remove unreacted ligand. This was followed by successive washes with 0.1 M sodium bicarbonate buffer, pH 8.0, containing 0.5 M NaCl; 0.1 M sodium acetate buffer, pH 4.0, containing 0.5 M NaCl; again with the first buffer, and then with deionized water. The product was placed in 100 mM ethanolamine, pH 8.0, and gently mixed overnight at room temperature to block the unreacted epoxy groups. The gel was finally washed with deionized water; resuspended in 20 mM sodium phosphate buffer, pH 7.4, containing 50 mM NaCl; and stored at 4°C in the presence of 0.02% sodium azide.

Affinity chromatography. The affinity gel (1 ml) was packed in (1.7 \( \times \) 0.86 cm) disposable polypropylene columns in the equilibration buffer (20 mM sodium phosphate, pH 7.4, containing 50 mM sodium chloride, 2 mM benzamidine, 1 mM EDTA, 0.1 mM dithiothreitol). Samples were loaded on the column at flow rates of less than 0.5 ml/min. After the column was washed with at least 4 column vol of the buffer to remove the unbound proteins, the enzyme was eluted with 10 mM p-hydroxyacetophenone in the same buffer and collected in 0.5-ml fractions. Before further use, the column was washed with 1.0 M NaCl in the elution buffer, equilibrated in the equilibrium buffer for immediate application, or stored in the same buffer in presence of 0.02% sodium azide in the cold. Aliquots of the affinity-fractionated samples were used for SDS–PAGE analysis, enzyme activity, immunoblotting, and isoelectric focusing.

Preparation of aldehyde dehydrogenase from beef liver. Beef liver mitochondrial ALDH was prepared from fresh liver as described (16). During the purification, a sample of the post-ammonium sulfate (30–55%) fraction and a sample eluted from the Cibracon blue Sepharose affinity column step were subjected to affinity chromatography on the p-hydroxyacetophenone column.

Preparation of recombinantly expressed rat liver mitochondrial ALDH from E. coli cells. The cloning of the cDNA coding for rat mitochondrial ALDH in pT7-7 plasmid, and its expression in BL21(DE3)pLysS E. coli cells, was previously described (14). Various mutants of ALDH were prepared by the method of Kunkel et al. (17) using the Bio-Rad Mutagene kit. The detailed preparation and characterization of the mutants will be described in other publications. For chromatographic separation purposes, the rat mitochondrial ALDH harboring pT7-7 plasmid-transformed cells were grown overnight at 37°C in 10 ml of LB medium (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter at pH 7.5) in the presence of the antibiotics ampicillin (25 \( \mu g/\))
ml) and chloramphenicol (10 μg/ml). A seed culture, or 100 μl of the overnight culture, was used to inoculate a fresh 10 ml of the media and the cells were incubated at 37°C until the absorbance at 600 nm reached 0.4. The cells were then induced by the addition of β-thiogalactopyranoside to a final concentration of 0.4 mM. Following overnight incubation at 37°C, the cells were harvested by low-speed centrifugation and the pellet either stored at -70°C or processed immediately. To prepare supernatants of the lysates, the pellet was lysed in 10 mM sodium phosphate buffer, pH 7.4, containing 50 mM NaCl, 2 mM benzamidine, 1 mM EDTA, 0.1 mM dithiothreitol, and 0.1% Triton X-100. The suspension was briefly vortexed in the same buffer and allowed to sit at room temperature for 10 min. The DNA in the lysate was digested by the addition of 1.5 μg/ml of DNase followed by incubation at 37°C for 15 min. After cooling on ice, the sample was clarified by centrifugation and the supernatant applied to the affinity gel. Alternatively, the cells were lysed by French pressure cells and the supernatants treated similarly.

**Enzyme assay and kinetic analysis.** ALDH activity measurements were performed as previously described (2) using a thermostated filter fluorometer at 25°C. To 1.0 ml of 100 mM sodium pyrophosphate buffer, pH 9.0, containing 500 μM NAD was added an aliquot of the enzyme solution and the fluorometer was zeroed. The reaction was initiated by the addition of 10 μM of the substrate propionaldehyde. The change in fluorescence due to the formation of NADH was recorded as a function of time. The inhibition of propionaldehyde oxidation was studied as a function of different concentrations of p-hydroxycetophenone in 100 mM sodium phosphate buffer, pH 7.5, containing 500 μM NAD. Similarly, to test whether p-hydroxycetophenone was an inhibitor against NAD, the reduction of NAD in the presence and absence of p-hydroxycetophenone was studied in the same buffer but containing 20 μM propionaldehyde and varying concentrations of the enzyme. Chloramphenicol acetyl transferase was assayed as described (19).

**Slab-gel isoelectric focusing.** Agarose (1%) isoelectric focusing was performed on a Pharmacia flat-bed electrophoresis apparatus (FBE-3000) for 3 h at 10°C based on the manufacturer's procedure. The Pharmalyte ampholyte used was in the pH range of 4–6.5. Focused samples were detected with Coomassie blue protein staining, activity staining, or immunoblotting (14,16). To detect the presence of ALDH by activity staining of isoelectric-focused samples, the focused gel was immersed in 25 ml of 100 mM sodium pyrophosphate buffer, pH 9.0, containing 22.5 mg of nitroblue tetrazolium, 25 mg of NAD, and 1.0 mg of phenazine methosulfate in a sealed, light-tight container. Following the addition of propionaldehyde to 100 μM, the contents were mixed and placed at 37°C for 1 to 3 h. The lanes containing active ALDH are detected as colored bands. After the desired intensity of color was achieved, the reaction was terminated by placing the gel in 7% acetic acid for several hours. The positions of ALDH bands observed by activity staining were further verified by Western blotting, described below, or by Coomassie blue staining. The Coomassie blue staining of focused gels was performed as follows. After focusing, the gels were immediately placed in 5% sulfosalicylic acid and 10% trichloroacetic acid for 30 min. The gels were then incubated in two lots of 35% ethanol and 10% acetic acid for 15 min. The gels were then dried and stained with 0.2% Coomassie blue for 10 min.

**Western blot analysis.** Immunoblotting was performed as described (14). For blotting, the proteins were first subjected to 10% SDS-PAGE and then electrophoretically transferred to a nitrocellulose membrane. Following the transfer, the blots were immersed in Tris-buffered saline (10 mM Tris·HCl buffer, pH 8.0, plus 150 mM NaCl) containing 0.15% Tween 20 and then rinsed briefly in the buffer. The nonspecific binding sites on the membrane were blocked by incubation in 1% bovine serum albumin in the same buffer for 0.5 h. To bind the primary antibody, the blocking solution was replaced with the same buffer containing an appropriate dilution of the antibody and the incubation continued for 2.0 h. This was followed by three 10-min washes of the buffer to remove unbound antibody. Following incubation with the secondary antibody for another 2.0 h, the unbound immunoglobin was removed by three 10-min washes of the buffer and one 10-min wash with alkaline phosphatase buffer (100 mM Tris·HCl buffer, pH 9.5, containing 100 mM NaCl and 5 mM MgCl₂). Antibody binding was detected by incubation of the membrane with the alkaline phosphatase buffer containing 2.20 and 2.48 mg of 5-bromo-4-chloro-3-indolyl phosphate p-toluidine and nitroblue tetrazolium chloride for 10 min. The color development was quenched by washing the membrane in water. All the washings and the incubations were done at room temperature. Three different primary antibodies, one against horse liver mitochondrial ALDH, another against horse liver cytosolic ALDH (20), and a third against rat liver mitochondrial ALDH (21), were used. Alkaline phosphatase conjugate was used as the secondary antibody.

**Protein sequencing.** N-terminal protein sequencing was performed by the Purdue University Biochemistry Department Protein Sequencing Facility on samples electrophoretically transferred from SDS gels to a membrane as we previously described (14).
RESULTS

Given the fact that acetophenones were strong inhibitors of ALDH (11), our purpose was to synthesize an affinity matrix that could effectively reduce the number of chromatographic steps; typically ion-exchange chromatography on DEAE- and carboxymethylcellulose, followed by affinity chromatography on blue Sepharose and 5-AMP Sepharose and isoelectric focusing, previously employed in the purification of the enzyme. The development of a highly selective affinity matrix becomes more significant, especially when one must analyze a large number of recombinantly generated mutants as in our case. Thus, to test if immobilized p-hydroxyacetophenone would act as an affinity ligand for ALDH, a solution of pure beef liver mitochondrial ALDH was applied to a column of the affinity matrix. The enzyme bound to the column and could be eluted by p-hydroxyacetophenone. The fractions having catalytic activity after the ligand was removed by dialysis or dilution were subject to SDS-PAGE and only one band of protein was observed (data not shown). To determine if the ligand would separate the enzyme from other liver proteins, a post-ammonium sulfate fraction (30–55%) of crude beef liver was applied to the column. Though the enzyme was not eluted by p-hydroxyacetophenone in a pure state, it was the major component that bound to the column and was displaced by the ligand (Fig. 1).

Crude beef liver extracts, like those from all other mammalian livers, possesses both cytosolic and mitochondrial forms of ALDH (16). The enzyme that was eluted from the column with p-hydroxyacetophenone was subjected to isoelectric focusing and Western blotting in order to determine if both class I (cytosolic) and class II (mitochondrial) isozymes of ALDH bound to the column. It was previously reported that the pl of these differed (16) and that antibodies prepared against a mitochondrial isozyme would cross react with the cytosolic form while the converse did not occur (20). Both anticytosolic and anti-mitochondrial ALDH antibodies recognized the protein which was eluted from the column (Fig. 2), implying the presence of both isozymes in the eluate. To further substantiate this observation, the pl of the proteins was determined. Enzyme activity was found at both pH 6 and 5, corresponding to the mitochondrial and cytosolic enzymes, respectively (Fig. 3).

From the post-ammonium sulfate fraction it was possible to obtain an ALDH preparation, estimated to be greater than 75% pure. If, however, a partially purified beef mitochondrial ALDH was used (post-Cibacon blue Sepharose affinity step), a pure enzyme could be obtained (Fig. 4). Thus, the p-hydroxyacetophenone column is as effective in purifying liver ALDH as is preparative isoelectric focusing.

Previously, it was shown that many parasubstituted acetophenones were competitive inhibitors against aldehydes when horse liver ALDH was tested (11). The ability of p-hydroxyacetophenone to inhibit the bee liver enzyme was examined and, as expected, the compound was a competitive inhibitor against propionaldehyde (K_i = 50 μM; Fig. 5A). When NAD was the variable substrate, p-hydroxyacetophenone was found not to be a competitive inhibitor, as shown by the double-reciprocal plot (Fig. 5B). Treating the compound as a noncompetitive inhibitor allowed for an estimation of a K_i of 70 μM against NAD.

We recently showed that it was possible to express ALDH in E. coli and to isolate and purify the enzyme using essentially the same procedure used to purify the enzyme from liver (14). Less than 1% of the lysate protein was the recombinantly expressed ALDH. One of the objectives of the research was to develop an affinity agent that would allow us to isolate recombinant ALDHs. To test the ability of p-hydroxyacetophenone to purify expressed ALDH, a lysate from transformed E. coli was applied to the column. The SDS–PAGE analysis of the fractions collected from the affinity column...
Identification of ALDH isozymes by Western blotting. Aliquots from the p-hydroxyacetophenone-eluted samples (Fig. 1) were separated on SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane which was incubated with polyclonal antibodies against horse liver cytosolic ALDH, to probe for the presence of beef liver cytosolic enzyme (lane 1 and 2), and polyclonal antibodies against rat mitochondrial ALDH, to probe for beef mitochondrial ALDH (lanes 3 and 4). The molecular weight markers stained with Coomassie blue (lane 5) are as described in the legend to Fig. 1. The results indicated that both isozymes were present and bound to the affinity support.

Greater than 90% of the recombinant expressed ALDH could be recovered from the affinity column. Though the specific activity of the expressed enzyme was not determined, that from the beef liver preparation was. The enzyme had essentially the same specific activity as the most highly purified enzyme that we obtained using isoelectric focusing as the final step. Though the specific activity of the beef liver mitochondrial enzyme isolated by the two techniques was the same, it was possible for us to crystallize only the enzyme obtained from the affinity column (23). Apparently some ampholines which retarded crystal growth remained bound to the enzyme.

Site-directed mutagenesis was employed to produce different mutant forms of ALDH as listed in Table 1.
FIG. 5. \( p \)-Hydroxyacetophenone inhibition of beef liver mitochondrial aldehyde dehydrogenase. (A) Dixon plot of the inhibition of propionaldehyde oxidation by various concentrations of \( p \)-hydroxyacetophenone in the presence of 500 nM NAD. The substrate concentration was (■) 0.42 FM and (□) 1.2 FM. The \( K_s \) for propionaldehyde was 0.42 nM. (B) Lineweaver-Burk plot of the reduction of NAD (100 nM) in the presence of 50 nM (O) and 100 nM (□) \( p \)-hydroxyacetophenone and in the presence of 20 nM substrate. The data points represent the average of at least two determinations. Data points were fit using a least-squares graphing program.

FIG. 6. SDS-PAGE analysis of \( p \)-hydroxyacetophenone-Sepharose affinity gel-fractionated \( E. \) coli lysates harboring recombinant ALDH. A pellet obtained by centrifugation of ALDH-expressing cells was lysed as described under Materials and Methods. The supernatant was applied to \( p \)-hydroxyacetophenone-Sepharose affinity gel and the chromatography developed. Aliquots from the flowthrough, buffer wash, and specifically eluted fractions were loaded on a 10% SDS–PA gel. The protein bands were detected by Coomassie blue staining. Aliquots were from lane 1, flowthrough; lanes 2 and 3, buffer wash; lanes 4, 5, and 6, ligand-eluted fractions. Lane 7 shows an aliquot of an authentic chloramphenicol acetyl transferase sample applied to the affinity gel and then chromatographed in the same manner as the lysates. Lane 8 represents molecular weight markers. The 55-kDa band clearly seen in lanes 4 and 5 was due to ALDH, as verified by Western blotting. It could be estimated that there was ca. 1000-fold purification of ALDH by the affinity chromatography step.
DISCUSSION

Acetophenone derivatives now have been used for many purposes with liver aldehyde dehydrogenases. They were found to be good substrate competitive inhibitors, and in vivo administration of the compounds caused an increase in blood acetaldehyde following ethanol administration (11), suggesting that ALDH was inhibited by their presence. Ternary complexes formed between enzyme, NAD, and p-methoxyacetophenone allowed for the determination of the stoichiometry of coenzyme binding (28). Recently α-bromoacetophenone has been employed to label an active site component of ALDH (12).

Results presented in this study show that p-hydroxyacetophenone is an effective affinity ligand for isolating ALDH from either liver extracts or E. coli lysates. It was unexpected to have found that p-hydroxyacetophenone had specificity primarily toward ALDH. Thus both α-cyanocinnamic acid (10) and acetophenone appear to be very good affinity ligands for purifying mammalian ALDHs.

Our finding that p-hydroxyacetophenone will interact with the enzyme in the absence of NAD leads us to question the mechanism of its binding to the enzyme. It has been proposed that the enzyme functions with preferred ordered binding (29) and that the presence of NAD increases the nucleophilicity of the active site cysteine (30). If this were the case, one would not have predicted that the compound would have been so effective at binding ALDH in the absence of NAD nor would it have bound so well to the C302A inactive mutant. Independent of the mechanism of binding, however, a new affinity ligand that allows for the rapid isolation of ALDH from crude tissue extracts or from E. coli lysate has been developed and could prove useful for the purification of various recombinant ALDHs. It was totally unexpected to have found that chloramphenicol acetyl transferase also bound to the column.

The E. coli chloramphenicol acetyl transferase gene is widely used as a reporter gene for eukaryotic transformation systems (31). Chloramphenicol acetyl transferase has been purified by conventional chromatography (19,32) and subsequently by affinity chromatography (33) using ε-amino chloramphenicol attached to a Sepharose resin.

Chloramphenicol acetyl transferase, a well characterized enzyme, confers chloramphenicol resistance to many genera of bacteria by catalyzing the acetyl transfer from acetyl-CoA to the 3-hydroxyl of chloramphenicol (34–36). All known variants of the enzyme are highly homologous and are reported to have a subunit molecular weight of 25 kDa (37). The three-dimensional structure of chloramphenicol acetyl transferase has been determined by X-ray crystallography (38,39).

It has been suggested that the mechanism of chloramphenicol acetyl transferase catalysis involves the imidazole of His-195 functioning as a general base to abstract a proton from the 3-hydroxyl of chloramphenicol to promote the nucleophilic attack at the acetyl-CoA thioester (40,41). It is not apparent how the acetophe-

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Note: To test whether recombinantly expressed ALDH, the wild type as well as the mutants, would bind to the affinity matrix, supernatants from the E. coli lysates were applied to the affinity gel and aliquots of the fractions collected loaded on SDS-PAGE. The protein bands in the gels were electrophoretically transferred to nitrocellulose membrane and probed with anti-ALDH antibodies as described under Materials and Methods. The results indicate that the wild-type enzyme, the active mutants, and the inactive mutants bound to the affinity matrix. The activities of the individual samples were assayed in separate experiments.

The preliminary characterization of the A and W mutants has been described (34). The E487K and H235A mutants will be described elsewhere (manuscripts submitted for publication).
none ketone ligand would interact with the enzyme. Perhaps some hydrophobic interactions are involved since chloramphenicol and acetyl-CoA binding sites in chloramphenicol acetyl transferase are reported to be in hydrophobic regions of the enzyme (38). The three-dimensional structure of ALDH has not been solved, though some data are beginning to emerge (42,23). There does not appear to be any regions of sequence homology between ALDH and chloramphenicol acetyl transferase when tested by computer alignment. Thus, it is not possible to probe for structural similarities between ALDH and chloramphenicol acetyl transferase, which could explain why the acetophenone derivative appears to have high affinity toward only these two enzymes.

REFERENCES


