Double-Ternary Complex Affinity Chromatography: Preparation of Alcohol Dehydrogenases†

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ABSTRACT: A general affinity chromatographic method for alcohol dehydrogenase purification has been developed by employing immobilized 4-substituted pyrazole derivatives that isolate the enzyme through formation of a specific ternary complex. Sepharose 4B is activated with 300 mg of cyanogen bromide/ml of packed gel and coupled to 4-[3-(*N*-6-aminocaproyl)aminopropyl]pyrazole. From crude liver extracts in 50 mM phosphate–0.37 mM nicotinamide adenine dinucleotide, pH 7.5, alcohol dehydrogenase is optimally bound at a capacity of 4–5 mg of enzyme/ml of gel. Addition of ethanol, propanol, or butanol, 500 mM, results in the formation of a second ternary complex, which allows the elution of bound enzyme in high yield and purity. This double-ternary complex affinity chromatography has been applied successfully to human, horse, rat, and rabbit liver extracts to isolate the respective homogeneous alcohol dehydrogenases.

Alcohol dehydrogenases have been identified in most phyla and species, including bacteria, yeasts, plants, insects, fish, and mammals (Brändén et al., 1975), but the enzyme has been purified in only relatively few instances. Horse liver and yeast alcohol dehydrogenases have been crystallized and have been used in studies of structure–function relationships (Brändén et al., 1973; Jörnvall, 1973; Li and Vallee, 1965), enzyme kinetics (Sund and Theorell, 1963), and the role of zinc in biological systems (Drum and Vallee, 1970; Vallee and Wacker, 1970). Some genetic studies of *Drosophila* and maize alcohol dehydrogenase (Scandalios, 1969; Ursprung and Leone, 1965), and, to a lesser extent, human liver alcohol dehydrogenase (Smith et al., 1973) have been performed, utilizing activity measurements of partially purified enzymes; in these instances, little or no structural information exists. Moreover, detailed information on other alcohol dehydrogenases has not been reported. As a consequence, knowledge of the evolution of alcohol dehydrogenases remains quite fragmentary. This may be attributed largely to the fact that procedures for purification have varied for each species from which the enzyme has been isolated. Therefore, genetic studies of alcohol dehydrogenases and their biological function are dependent on a general method of isolation and purification of the enzyme in a yield sufficient for physicochemical and enzymological characterization.

We here describe an affinity chromatographic procedure employing an immobilized 4-substituted pyrazole derivative, a competitive inhibitor of ethanol oxidation. So far this procedure has been employed to isolate and purify large quantities of alcohol dehydrogenases from human, horse, rabbit, and rat livers, but it appears to be a general technique for such purposes, applicable to isolation of the enzyme from many, if not all, species, as well as from different organs of the same species.

Materials and Methods

Liver alcohol dehydrogenases are assayed at 25 ± 0.2 °C with a Gilford Model 240 or a Unicam SP 800 spectrophotometer equipped with a Heathkit Model IR-18M recorder. The absorbance at 340 nm is recorded after addition of 25 μl. of enzyme to 3 ml of reaction mixture, containing 100 μmol of ethanol, 7.6 μmol of NAD^+,[1] and 283 μmol of glycine, pH 10.0, for the human enzyme, or 150 μmol of pyrophosphate, pH 8.8, for the other dehydrogenases. One unit of activity, U, is equal to ΔA_{340}/min of 1.0. Specific activity is defined as U/mg. For human liver alcohol dehydrogenase, protein concentration is determined using a molar absorptivity at 280 nm of $5.11 \times 10^4\ M^{-1}\ cm^{-1}$ (Lange et al., 1976).

NAD^+ and NADH (yeast) were obtained from Sigma Chemical Co. Solutions were prepared daily and kept at 0 °C. Reagents for syntheses were products of Aldrich Chemical Co. All pH determinations were made with a Radiometer PHM 63 meter equipped with a GK 2321c electrode.

Synthesis of 4-(3-aminopropyl)pyrazole·dihydrochloride. 2-Ethoxy-3-tetrahydropyrancarboxaldehyde diethyl acetal is prepared in 89% yield by the method of Copenhaver (1951). Dihydropyran (47 g, 0.56 mol) is added dropwise over a 2-h period to triethyl orthoformate (240 g, 1.62 mol) and BF_3·Et_2O (0.3 ml). The reaction vessel is cooled periodically to maintain the temperature below 40 °C. After stirring overnight at 25 °C, excess triethyl orthoformate is removed on a Rotovac and the product is distilled to give 106 g of clear, colorless liquid, bp 60–65 °C at 0.2 mm Hg. The NMR spectrum is as follows: δ = 1.2(t), 1.8(t), 3.5(q), 4.2(d), and 4.3(d) ppm.

4-(3-Hydroxypropyl)pyrazole is prepared according to the method of Jones and Mann (1953) by adding the acetal (34.5 g, 0.158 mol) in 25 ml of ethanol to hydrazine·dihydrochloride (19.0 g, 0.18 mol) in 50 ml of water. The temperature rises 3 °C, a white precipitate forms, and the supernatant becomes yellow. After heating overnight at 40–50 °C, the pH of the now homogeneous solution is adjusted to 12 with NaOH and volatile material is removed on a Rotovac. The residue is extracted with ethanol (3 × 100 ml) and the solvent is removed on a Rotovac, yielding a yellow oil which is then distilled. The product, bp 141–143 °C at 0.25 mm Hg, is isolated in 70% yield and has an NMR spectrum consistent with the desired product: δ = 1.9 (multiplet), 2.7(t), 3.7(t), 7.6(s) and 8.2(s) ppm.

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4-(3-Chloropropyl)pyrazole·hydrochloride is prepared by adding the alcohol (9.0 g, 0.0715 mol) to 35 ml of thionyl chloride, holding the temperature at 60 °C for 5 min, and removing the excess thionyl chloride at 70 °C under vacuum to leave a crystalline, yellow residue. This is then recrystallized from ethanol–ethyl ether to give a white solid, 7.7 g, mp 113–114 °C (dried in vacuo).

4-(3-Phthalimidopropyl)pyrazole is prepared by adding sodium bicarbonate (17.7 g, 0.21 mol) at 25 °C to a solution containing 4-(3-chloropropyl)pyrazole·hydrochloride (28.5 g, 0.198 mol) and potassium phthalimide (38.8 g, 0.21 mol) in 200 ml of dimethylformamide. The mixture is refluxed for 30 min, cooled, filtered, and added to 500 ml of water to give a white precipitate, mp 123–126 °C. This is recrystallized from water to give a white solid, mp 133–135 °C, in 80% yield.

4-(3-Aminopropyl)pyrazole·dihydrochloride is prepared in 75% yield by refluxing the phthalimide intermediate (8.0 g, 0.0314 mol) in 100 ml of 6 N HCl overnight. The clear solution is cooled to 4 °C, phthalic acid is removed by filtration, and the filtrate is evaporated to dryness. The residue is recrystallized from ethanol–ethyl ether to give 4.4 g of a white solid, mp 188–190 °C (lit. 188–190 °C).

Synthesis of 4-[3-(N-6-Aminocaproyl)aminopropyl]-pyrazole[1] *and Analogous Compounds.* Carbobenzoxy 6-aminocaproate is prepared by a conventional Schotten–Baumann procedure. Its infrared spectrum in CCl_4 shows a carbonyl absorption at 1720 cm^{-1} and a broad band at 3100–3400 cm^{-1}. This compound, or carbobenzoxyglycine (Cyclo Chemical Co.) is coupled to 4-(3-aminopropyl)pyrazole by the succinimide ester method of Anderson et al. (1964) by stirring 5.1 mmol of the N-blocked acid with equimolar quantities of *N*-hydroxysuccinimide and *N,N*-dicyclohexylcarbodiimide in 100 ml of dry dioxane/dimethoxyethane (10/1) overnight at 4 °C. The reaction mixture is filtered into 50 ml of water containing sodium bicarbonate (2.1 g, 20.4 mmol) and 4-(3-aminopropyl)pyrazole·dihydrochloride (1.0 g, 5.1 mmol). After 5 h, the reaction mixture is acidified and extracted with chloroform. It is then made basic with sodium hydroxide and extracted with chloroform (3 × 200 ml). The latter, combined extracts are dried over anhydrous magnesium sulfate and the solvent is removed on a Rotovac to give an 80% yield of a white powder, recrystallized from water–ethanol, mp 127–128 °C and 137–138 °C for the caproyl and acetyl derivatives, respectively. The infrared spectrum in Nujol shows two absorptions at 1630 and 1690 cm^{-1}, which in chloroform shift to 1660 and 1715 cm^{-1}, consistent with the presence of two hydrogen-bonded, secondary amides. The elemental analysis of the caproyl derivative is 64.37% C, 7.87% H, and 15.10% N (theoretical for $C_{20}H_{28}N_4O_3$: 64.49% C, 7.58% H and 15.08% N). The NMR spectrum is also consistent with the desired structure for the caproyl derivative: δ = 1.4–2.6 (multiplet), 3.1(t), 5.1(s), 7.4(s), 7.6(s) and 7.9(t) ppm.

The carbobenzoxy blocking group is removed by catalytic hydrogenation at atmospheric pressure in methanol over palladium on charcoal. After filtration and solvent removal, a clear viscous liquid remains. Its infrared spectrum in Nujol and chloroform shows the absence of the carbobenzoxy carbonyl bands at 1690 and 1715 cm^{-1}, respectively. The NMR spectrum is similar to that observed prior to hydrogenation, except that the aromatic region shows the presence of only the pyrazole ring protons.

The ligand with the longest spacer arm is prepared by coupling 6-(4-nitrobenzoyl)aminocaproic acid (easily available from 4-nitrobenzoyl azide and 6-aminocaproic acid) to 4-(3-aminopropyl)pyrazole by the method of Anderson et al. (1974). Catalytic hydrogenation over palladium on charcoal in methanol–1% acetic acid gives the 4-amino derivative, which is then coupled directly to Sepharose.

Affinity Resin Preparation. Sepharose 4B (Pharmacia), 35 ml, is washed extensively with distilled water and then activated with either 180 or 300 mg of cyanogen bromide/ml of gel in a total volume of 70 ml (Cuatrecasas, 1970). During activation, the pH is maintained between 10.5 and 11.0 by the addition of 4 N sodium hydroxide, and the temperature is held at about 20 °C by the addition of ice. When the release of protons ceases, the gel is quickly transferred to a Büchner funnel and washed with 1000 ml of 0.1 M sodium bicarbonate, pH 9.5, at 4 °C over a 90-s period. A known volume of the activated gel, gently dried by suction, is added to an equal volume of 0.1 M sodium bicarbonate, pH 9.5, containing CapGapp or other ligands at desired concentrations. The gel is gently agitated at 4 °C over a 48-h period, and then washed with 1000 ml of 1 M sodium chloride followed by 200 ml of 0.05 M sodium phosphate, pH 7.5. A 0.01% sodium azide solution is added to all stored gels to prevent microbial growth.

Liver Extracts. Human livers were obtained at autopsy and verified to be free of disease by microscopic examination; all other livers were received frozen from Pel-Freez, Inc. (St. Louis, Mo.). The tissue is fragmented in a meat grinder at 4 °C and stirred with 2 l. of water/kg of wet tissue for 2 h. After screening to remove large pieces of debris, the crude extract is applied to DEAE-cellulose in a Büchner funnel (10 × 15 cm) equilibrated with 0.01 M Tris-Cl, pH 7.9. The extract is aspirated through the bed under vacuum, and the resulting straw-colored solution is prepared for affinity chromatography by adding phosphate buffer and NAD^+ to give a final concentration of 50 mM phosphate–0.37 mM NAD^+ at pH 7.5. At this stage, enzyme activity varies from 1.0 to 4.0 U/ml.

Evaluation of Affinity Resins. A crude extract of human liver (vide supra) is applied to a column (0.9 × 20 cm) to be evaluated at a flow rate of 1 ml/min until the effluent activity equals the affluent activity. The column is then washed with equilibrating buffer until the absorbance at 280 nm in the effluent approaches zero. The enzyme is eluted by the addition of ethanol, 500 mM, to the buffer. Binding capacity is defined as the amount of enzyme eluted from a saturated column per ml of wet gel. Total enzymatic activity recovered is always 90–100% of that bound.

Characterization of Compounds. Melting points were obtained using a Hoover capillary melting point apparatus (Arthur Thomas Co.) and are reported uncorrected. Infrared spectra were measured with a Beckman IR-20, and a Varian A-60 spectrometer was used for NMR spectra (deuterated dimethyl sulfoxide as solvent). Elemental analyses were performed by PAR, Inc., Gainesville, Fla.

Results

Inhibition of Human Liver Alcohol Dehydrogenase by CapGapp. The suitability of CapGapp as an affinant was first evaluated kinetically by examining its interaction in solution with human liver alcohol dehydrogenase. Experiments performed at a fixed, saturating concentration of NAD^+, 25 mM, with ligand concentration varied from 0 to 8.3 μM, demonstrate an apparent increase in K_M, while k_{cat} for ethanol oxi-

[1] Abbreviations used are: CapGapp, 4-[3-(*N*-6-aminocaproyl)aminopropyl]pyrazole; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; NMR, nuclear magnetic resonance.

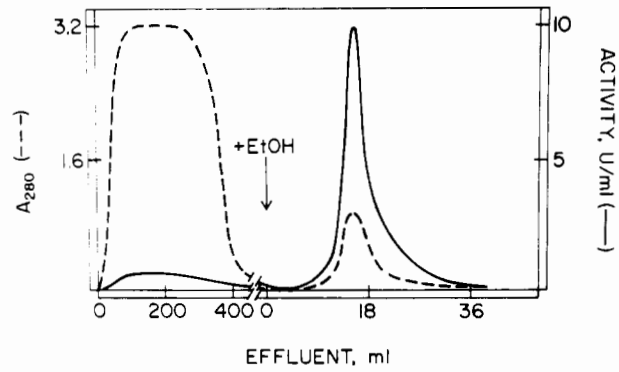

FIGURE 1: Affinity chromatography of human liver alcohol dehydrogenase. A crude extract, 1.2 U/ml, was applied to Sepharose-CapGapp, 0.9 × 20 cm, equilibrated with 50 mM phosphate-0.37 mM NAD^+, pH 7.5, at a flow rate of 1 ml/min. The column was then washed with 20 column volumes of equilibrating buffer and, finally, ethanol, 500 mM, was added, indicated by the arrow.

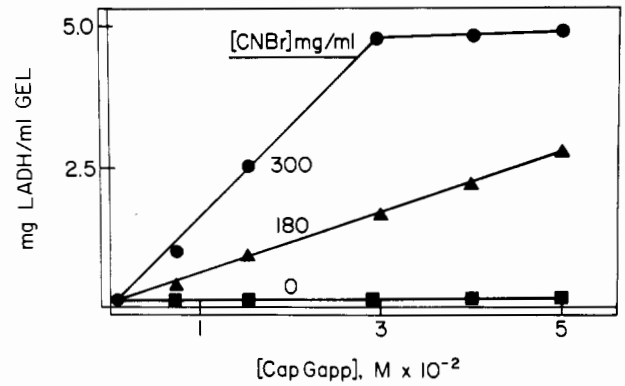

FIGURE 2. The effect of CapGapp concentration in the reaction mixture on the capacity of the substituted Sepharose to reversibly bind human liver alcohol dehydrogenase. One series of resins was prepared by activation of Sepharose with 300 mg of cyanogen bromide/ml of gel (●) and the other with 180 mg/ml of gel (▲). In the control (■) cyanogen bromide was omitted.

TABLE I: Effect of Ligand Length on Binding Capacity.[a]

Sepharose-Ligand	Length (Å)	Binding Capacity (mg/ml)
$NH(CH_2)_3$—Pz	6	0.50
$NHCH_2CONH(CH_2)_3$—Pz	10	0.40
$NH(CH_2)_5CONH(CH_2)_3$—Pz	16	4.85
$NHC_6H_4CONH(CH_2)_5$-$CNH(CH_2)_3$—Pz	27	4.66

[a] Each resin was prepared by mixing equal volumes of Sepharose 4B activated with 300 mg of cyanogen bromide/ml and 100 mM ligand in bicarbonate, pH 9.5. Lengths were calculated based on an extended conformation of the ligand. Binding capacity was determined as described under Methods. Pz represents a 4-substituted pyrazole derivative.

dation remains constant. Varying the concentration of CapGapp while holding ethanol concentration constant results in progressively increasing inhibition which does not reach a plateau. This behavior, typical of inhibitors competitive with ethanol, is apparent when the data are plotted according to Lineweaver and Burk, or Dixon. The inhibition constant, K_i, is 1 μM; hence, this ligand seems an ideal affinant for human liver alcohol dehydrogenase.

Affinity Chromatography of Human Liver Alcohol Dehydrogenase. When a crude human liver extract, 50 mM in phosphate and 0.37 mM in NAD^+, pH 7.5, is applied to a column of Sepharose-CapGapp (0.9 × 20 cm) at a flow rate of 1 ml/min, all of the alcohol dehydrogenase activity in the first 32 ml is bound (Figure 1). Subsequently, activity in the column effluent increases linearly, until constant. After washing the column with 20 volumes of 50 mM phosphate-0.37 mM NAD^+, pH 7.5, bound enzyme is eluted by the addition of ethanol, 500 mM (Figure 1, arrow). Approximately 65% of the enzymatic activity present initially, and all of that which is bound, is eluted in a bolus with a 270-fold increase of specific activity.

Ligand Length. To assess the effect of the distance between the supporting matrix and the ligand on binding capacity, ligand-spacer ensembles of various lengths were synthesized and coupled to Sepharose 4B under identical conditions. While a distance of 6 or 10 Å between matrix and ligand results in very low binding capacities, 0.50 and 0.40 mg/ml, respectively (Table I, lines 1 and 2), a further increase by 6 Å raises it almost tenfold to 4.85 mg/ml (line 3). An additional increment of 11 Å maintains this capacity but does not increase it further (line 4). Thus, a spacer arm resulting minimally in a distance of 10-16 Å is required for high binding capacities. For subsequent work the 16 Å arm is employed.

Ligand Concentration. Sepharose 4B was activated with either 180 or 300 mg of cyanogen bromide/ml of packed gel. An aliquot of each activated resin was added to varying concentrations of ligand in sodium bicarbonate, pH 9.5, and after completion of the reaction, the binding capacity of each gel was determined (Figure 2). At any given ligand concentration, the capacity of the gels activated by 300 mg of cyanogen bromide/ml of gel is greater than that of those activated by 180 mg. For the former, a plateau is reached when the ligand concentration exceeds 29 mM. Above this, the binding capacity does not increase appreciably. Sepharose 4B activated with 300 mg of cyanogen bromide/ml of gel and reacted with 29 mM ^{14}C-labeled ligand contains 6.45 μmol of ligand/ml of gel. Human liver alcohol dehydrogenase does not bind to Sepharose that is not activated with cyanogen bromide (Figure 2, bottom curve).

This resin has been employed to optimize binding and elution conditions, isolate alcohol dehydrogenases on a preparative scale, and to study column specificity and the mechanism of binding.

Binding Conditions. The effect of NAD^+ concentration on the binding capacity was examined by adding varying amounts of coenzyme to the equilibrating buffer and sample. Binding capacity increases linearly over a concentration range from 10^{-6} to 10^{-4} M NAD^+ (Figure 3). Above 10^{-4} M, however, a plateau is reached and the capacity does not increase further, even at 10^{-2} M. For additional studies, 3.7×10^{-4} M NAD^+ was chosen to achieve maximal capacity at minimal cost.

The effect of pH on binding capacity was determined by equilibrating the column at pH values from 6.0 to 9.5 (Figure 3). Phosphate, Tris, and glycine buffers were employed at constant ionic strength; no buffer effect is observed in the pH overlap regions. Above pH 8.0, capacity of the gel falls rapidly to 1.28 mg/ml as pH is increased. However, from pH 7.5 to 6.0 binding capacity is constant at 4.85 mg/ml. An operating pH of 7.5 was chosen.

The addition of salt to the equilibrating buffer and sample results in a nearly linear decrement of binding capacity. However, even at 1.0 M NaCl, binding capacity is 0.7 mg/ml. Therefore, phosphate, 50 mM, was used to prevent nonspecific protein binding without affecting specific binding of alcohol

TABLE II: Purification of Human Liver Alcohol Dehydrogenase.[a]

	Vol (ml)	Total Protein (mg)	Act. (U/ml)	Sp Act. (U/mg)	Yield (%)	Time (h)
Crude extract	950	100 000	2.2	0.021	100	2.5
DEAE-cellulose	950	10 000	2.2	0.21	100	0.5
Affinity chromatography	245	195	4.5	5.7	65	3.0

[a] Human liver, 606 g (wet weight), was ground into 1200 ml of cold H_2O and the mixture was stirred at 4 °C for 2 h. The strained crude extract was passed through DEAE-cellulose (10 × 15 cm) equilibrated with 0.01 M Tris-Cl, pH 7.9, and the resultant effluent was chromatographed over CapGapp-Sepharose. See text for other conditions.

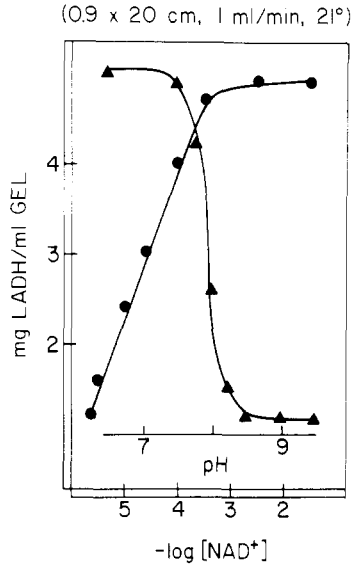

FIGURE 3. The effect of NAD^+ concentration (●) and pH (▲) on the binding capacity of the affinity resin activated with 300 mg of cyanogen bromide/ml of gel and reacted with 29 mM CapGapp. For the former, the pH was 7.5 in 50 mM phosphate, 21 °C, and for the latter, NAD^+ concentration was 0.37 mM. Other conditions are as described in the text.

dehydrogenase.

Elution Conditions. The removal of NAD^+ from the buffer allows dissociation of the ternary complex, and alcohol dehydrogenase activity elutes from a column of affinity resin in a slowly rising peak with a long trailing shoulder. Removal of coenzyme is, therefore, not satisfactory for elution on a preparative scale. However, the addition to the buffer of ethanol, 1-propanol, or 1-butanol, all 500 mM, elutes 100% of the bound enzyme in one or two fractions of 6 ml each. On elution with an ethanol gradient, alcohol dehydrogenase activity begins to elute at 40 mM ethanol and is complete at 300 mM, resulting in a broad, featureless chromatogram. Effective elution is also accomplished at lower concentrations of the longer chain alcohols: 1-butanol, 5 mM, elutes 100% of the bound enzyme in four or five fractions of 6 ml each. To minimize the amount of coenzyme present in the fraction containing pure alcohol dehydrogenase (Lange et al., 1976), elution in preparative work is best achieved by simultaneous addition of alcohol to *and* removal of NAD^+ from the buffer.

Mechanism of Binding. In the *absence* of NAD^+, alcohol dehydrogenase activity from crude extracts is not bound, but 5–10% of the material absorbing at 280 nm is bound to the column, suggesting that NAD^+ is required for specific binding. To exclude the possibility that the ligand arm and not the pyrazole ring is the NAD^+-dependent site, the column and sample were equilibrated with NAD^+, 0.37 mM, and pyrazole, 10 mM, and the sample was applied subsequently.

Neither alcohol dehydrogenase activity nor material absorbing at 280 nm is bound, suggesting that the NAD^+-dependent site is specific for the pyrazole ring. Moreover, pyrazole, 100 mM, elutes 80% of alcohol dehydrogenase activity. However, the specific activity of the resultant enzyme is less than that obtained by ethanol elution. This effect may reflect an affinity of other hepatic proteins for pyrazole, even in the absence of NAD^+. Thus, when NAD^+ is absent, 5–10% of the protein in the crude extract is ound and then eluted by addition of pyrazole. Protein does not bind when the extract is first equilibrated with NAD^+ and pyrazole, further supporting this view. The presence of other proteins has not been problematic when ethanol elution is used.

Preparative-Scale Isolation of Human Liver Alcohol Dehydrogenase. Under the conditions described, human liver alcohol dehydrogenase was isolated and purified from 606 g of human liver (Table II). After grinding and extraction, followed by passage through DEAE-cellulose, the crude extract solution is applied to 80 ml of affinity resin in a 150-ml Büchner funnel (6.5-cm diameter) at a flow rate of 5–10 ml/min. The resin is then washed with 800 ml of equilibrating buffer and enzyme is eluted with ethanol addition, 500 mM, and NAD^+ removal. After dialysis against 50 mM phosphate, pH 7.5, 195 mg of homogeneous enzyme is obtained in 6 working hours, excluding dialysis, a fraction of the time required for other methods, and in much greater purity than that previously attained (Lange et al., 1976).

Other Alcohol Dehydrogenases. Livers from several other species were used to evaluate the affinity chromatographic procedure as a general means to isolate alcohol dehydrogenases from crude extracts. Crude extracts are passed through DEAE-cellulose in a batchwise fashion and the resulting effluent is chromatographed under conditions optimal for human liver alcohol dehydrogenase. Alcohol dehydrogenase from horse, rat, and rabbit liver are isolated and purified in large quantities, with increases of specific activity of 88, 69, and 78%, respectively. Importantly, sodium dodecyl sulfate disc gel electrophoresis (Weber and Osborn, 1969) demonstrates the existence of only a single band migrating with a molecular weight of 41 500 for horse, rat, and rabbit liver alcohol dehydrogenases.

Discussion

The detailed biochemical properties and functions of alcohol dehydrogenases from horse liver and yeast have been studied for nearly 40 years. Their isolation and purification, while much improved over original procedures (Negelein and Wolff, 1937; Bonnichsen and Wassén, 1948), are still cumbersome and time consuming (Racker, 1955; Bonnichsen and Brink, 1955). Definitive information about homogeneous preparations of alcohol dehydrogenases from other sources is sparse, though alcohol dehydrogenase activity has been identified in most phyla and species (Sund and Theorell, 1963; Brändén et al.,

1975). The absence of procedures for rapid purification of large quantities of enzyme from crude extracts has thwarted the study of such enzymes.

Similar considerations pertain to our long-standing efforts to examine human liver alcohol dehydrogenase (von Wartburg et al., 1964; Blair and Vallee, 1966), its purification and biological properties. The role, if any, of this enzyme in human physiology and pathology, e.g., alcoholism and cirrhosis, is unknown, in large measure due to the technical difficulties described. The present studies were undertaken to overcome these obstacles.

Alcohol dehydrogenase abundant in both horse liver and yeast is purified with relative ease from both sources; further, it tends to crystallize from crude extracts (Bonnichsen and Brink, 1955; Racker, 1955). Typical procedures involve aqueous extraction from ground liver or dried yeast, heat treatment, and repeated precipitations with ethanol, acetone, or ammonium sulfate, followed by crystallization from concentrated enzyme solutions. The isolation of other alcohol dehydrogenases has proven more problematical. Thus, human liver alcohol dehydrogenase has been purified partially by ammonium sulfate precipitation of crude extracts, DEAE- and CM-cellulose chromatography (Blair and Vallee, 1966). Similarly, rat liver alcohol dehydrogenase is isolated by ammonium sulfate precipitation, DEAE-Sephadex, Sephadex G-100, and sulfoethyl-Sephadex chromatography (Arslanian et al., 1971). In all cases, the yields vary from 10 to 40% and at least 6–8 days are required.

Affinity chromatography results in potentially higher yields during short periods if a suitable affinity resin for alcohol dehydrogenases can be devised. Immobilized coenzyme derivatives provide one approach to the solution of this problem. Initial attempts to immobilize NAD^+ or adenosine monophosphate employed diazotization reactions (Lowe and Dean, 1974), periodate oxidation of the vicinal ribose hydroxyl groups (Lamed et al., 1973), and coupling with carbodiimides (Larsson and Mosbach, 1971; Lowe et al., 1973). However, these resins are generally of low binding capacity, ionic interactions, as the basis for affinity, have not always been excluded, and, most importantly, the site and mode of ligand attachment have not been established. Derivatives of NAD^+, such as N^6-[*N*-(6-aminohexyl)]acetamide, have been reported to serve as efficient affinants (Lindberg et al., 1973), and, in elegant studies, Kaplan et al. (1974) and Andersson et al. (1974) used the corresponding adenosine monophosphate compound to isolate lactate and alcohol dehydrogenase, respectively, from crude extracts.

Work in this area has advanced to isolation of a number of enzymes, but the use of general ligands has practical limitations for this work. Thus, such coenzyme columns will adsorb the large number of dehydrogenases and/or kinases in crude extracts, thereby substantially lowering the specificity of the procedure which can be increased again by careful adjustment of operating conditions. Also, such columns favor the isolation from crude extracts of the enzymes with the most favorable binding constants, correspondingly reducing the binding of other dehydrogenases and kinases, often the one which is sought. Moreover, such binding priorities likely vary widely from species to species in response to environmental factors. Use of a general ligand for a specific purpose is, thus, seriously limited when isolating a single enzyme in large quantities from multiple sources.

Theorell et al. (1969) found that pyrazole is a specific, potent inhibitor of horse liver alcohol dehydrogenase, competitive with ethanol with a K_i of 0.2 μM. Pyrazole binds to the enzyme–NAD binary complex to form an abortive ternary complex consistent with the ordered bi-bi reaction mechanism. Similarly, 4-methyl-, iodo-, or bromopyrazole strongly inhibit horse liver alcohol dehydrogenase with a K_i of 0.08, 0.02, or 0.02 μM, respectively. In contrast, 1-methyl- and 3(5)-methylpyrazole are much poorer inhibitors, their K_i being 17 and 0.13 mM, respectively. Thus, substitution in the 4-position enhances inhibition, while substitution in any other position markedly weakens it. Preliminary studies on human liver alcohol dehydrogenase have established a similar pattern of inhibition (Li and Theorell, 1969).

These results suggest that 4-substituted pyrazole derivatives might serve as effective ligands for affinity chromatography of alcohol dehydrogenases. Accordingly, derivatives containing functional groups amenable to coupling to solid supports have been synthesized and these still inhibit human liver alcohol dehydrogenase. Like pyrazole, they compete with ethanol binding with K_i of the order of μM and, therefore, are ideal for use as affinants.

Pyrazole derivatives of this type, coupled directly to Sepharose, provide excellent affinity resins for a number of alcohol dehydrogenases, greatly simplifying the isolation of these enzymes. After passage through DEAE-cellulose, crude material can be applied to the affinity resin in a Büchner funnel at flow rates of 20–30 ml/min without decrements of either yield or purity. Resins have been reused many times in the course of a year and cleaned periodically with 4 M urea and 100 mM pyrazole.

In the present study, the ligand and spacer arm have been synthesized en bloc and the ensemble coupled directly to Sepharose, subjecting tlhe resin to only one reaction. The alternative method of first coupling the spacer arm, e.g., 6-aminocaproic acid, to activated Sepharose is undesirable; subsequent linkage of the pyrazole ligand can be achieved, but free carboxyl groups from uncoupled spacer arms always remain, reducing the yield of effective ligand and yielding a resin with ion-exchange properties. It was found that such gels offer lower capacities and less pure enzyme compared to the other resins prepared in one step, in accord with similar observations in the literature (Cuatrecasas, 1972).

Studies of binding and elution conditions demonstrate that human liver alcohol dehydrogenase from crude extracts interacts with the immobilized ligand according to patterns predicted from inhibition studies in solution. Enzyme binding capacity is linearly dependent, over a wide range, on NAD^+ concentrations (Figure 3), in keeping with the obligate prior binding of coenzyme in the ordered bi-bi mechanism. Once, however, the NAD^+ concentration reaches 3×10^{-4} M or tenfold above K_M (Lange et al., 1976) binding capacity reaches a maximum. Addition of pyrazole to the crude extract prevents binding; its addition to buffer elutes bound enzyme, which can also be eluted with ethanol, 1-propanol, or 1-butanol. These results clearly show that alcohol dehydrogenase binds reversibly to the immobilized pyrazole resins through elements of true affinity, as expected on the basis of inhibition studies.

The procedure then involves the formation of two ternary complexes and, thus, constitutes double ternary complex affinity chromatography. The binding mechanism is ordered where NAD^+ binds to form a binary enzyme–NAD complex. Enzyme binds to the resin by forming the ternary complex, enzyme–NAD–pyrazole. Addition of ethanol results in the formation of the secornd ternary complex, enzyme–NAD–ethanol, and bound enzyme is eluted as ethanol competes with and displaces pyrazole.

Several advantages are apparent. The dual selection process

based on enzyme function imparts high specificity to the method. Thus, even though pyrazole may bind to other proteins, only alcohol dehydrogenase seems to form complexes with both pyrazole and ethanol and, hence, a large degree of purification is achieved. Secondly, binding capacities of nearly 5 mg/ml from crude extracts are attained at high flow rates. Moreover, alcohol dehydrogenase can be purified from multiple species without regard to the presence in crude extracts of other dehydrogenases or kinases, all of which would bind to a general ligand such as NAD^+ serving as an affinant.

The number of enzymes that form ternary or even quaternary complexes with specific substrates or inhibitors appears to be quite large. In addition, among the dehydrogenases and kinases studied, ordered reaction mechanisms in which the general ligand binds before the specific ligand appear common. Immobilization of the specific ligand would seem to offer great promise in the design of high capacity, specific bioadsorbents for many of these enzymes, in addition to those reported here.

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